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Effects of Fermented Cottonseed Meal Substitution for Fish Meal on Intestinal Enzymatic Activity, Inflammatory and Physical-Barrier-Related Gene Expression, and Intestinal Microflora of Juvenile Golden Pompano (*Trachinotus ovatus*)

Zhanzhan Wang [1,2], Shuling Liao [3], Jun Wang [1,4], Yun Wang [1,4], Zhong Huang [4,5], Wei Yu [4,5], Xiaolin Huang [4,5], Heizhao Lin [4,5], Maoyan Luo [3], Zhenyan Cheng [2,*] and Chuanpeng Zhou [1,4,*]

1 Key Laboratory of Aquatic Product Processing, Ministry of Agriculture and Rural Affairs, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510300, China; wangyun@scsfri.ac.cn (Y.W.)
2 School of Fisheries, Tianjin Agricultural University, Tianjin 300384, China
3 School of Life Science, Guangzhou University, Guangzhou 510006, China
4 Key Laboratory of Efficient Utilization and Processing of Marine Fishery Resources of Hainan Province, Sanya 572426, China
5 Shenzhen Base of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shenzhen 518121, China
* Correspondence: chengzhenyan2005@126.com (Z.C.); chpzhou@163.com (C.Z.)

Abstract: The present study was conducted to investigate the effects of dietary fermented cottonseed meal (FCSM) substitution for fish meal on intestinal enzymatic activity, inflammatory and physical-barrier-related gene expression, and intestinal microflora of juvenile golden pompano. The 375 golden pompanos were divided into 15 groups of 25 fish each, with three replicates for each experimental group. The fish were fed five experimental diets (0 (FM), 12.5% (CSM12.5), 25% (CSM25), 50% (CSM50), and 100% (CSM100) substitution levels) for 8 weeks. The fish were reared and fed the experimental diets under a natural-day light cycle. Compared with the control group, the activities of AMY (amylase) enzymes in the CSM12.5 group and all other groups were elevated ($p < 0.05$). The CSM25 group exhibited a considerable up-regulation of *IL-10* (Interleukin-10) expression relative to the FM group ($p < 0.05$). With an increase in dietary FM substitution with FCSM from 0 to 25%, the relative expressions of *NF-κB* (Nuclear factor kappa-B), *IL-1β* (Interleukin-1 beta), and IL-8 (Interleukin-8) were down-regulated. In this study, the relative expressions of ZO-1 (zonula occluden-1) and *Occludin* were up-regulated, and those of *Claudin-3* and *Claudin-15* significantly up-regulated, when the FCSM substitution ratio was 25%. The results of high-throughput sequencing of the intestinal microflora showed that ACE indices the lowest in the CSM25 group, which was significantly different from those in the CSM100 group ($p < 0.05$). The CSM50 group had the highest Shannon and Simpson indices and the highest community diversity. In addition, replacing a high percentage of fish meal with FCSM can negatively affect the intestinal flora of fish. In this study, the 25% substitution ratio improved nutrient absorption, reduced intestinal inflammation, improved intestinal physical barrier damage, did not affect intestinal microecology, and had no adverse effects on fish. However, substitution of a high proportion of FM with FCSM negatively affects the intestinal microflora and nutrient absorption capacity of fish.

Keywords: fermented cottonseed meal; substitution; intestinal enzymatic activity; gene expression; intestinal microflora

Key Contribution: A. Appropriate substitution ratio could improve nutrient absorption, reduce intestinal inflammation, and improve intestinal physical barrier damage while not affecting intestinal microecology. B. Replacing a high percentage of fish meal with fermented cottonseed meal can negatively affect the intestinal flora of fish.

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1. Introduction

Golden pompano (*Trachinotus ovatus*) is a valuable edible fish with thornless flesh, a tender texture, and a delicious taste, with the unique aroma of the trevally family. It is distributed in the Atlantic, Indian, and Pacific Oceans' tropical and subtropical seas. In China, the coasts of Guangdong, Guangxi, Hainan, and Fujian are main growing regions [1]. The demand for protein raw materials (e.g., fishmeal or soybean meal) is rising as aquaculture production increases. Import policies have a significant impact on the aquaculture industry, since fishmeal and soybean meal are import-dependent. To fulfill the rising domestic demand for protein raw materials, guarantee a continuous supply of aquatic products, and maintain global food security, it is crucial to discover and develop novel, effective, sustainable, and environmentally friendly protein sources.

The ability of the intestines to digest and absorb nutrients is crucial for fish, especially stomachless fish, and is considered to be an important indicator of the overall health of the body [2]. There are large amounts of microflora in the intestines of fish which live in symbiosis with the fish and are known as the normal intestinal flora. Fish with normal intestinal flora can have better digestion and receive essential nutrients which strengthen their natural defenses and immunity [3]. Dysbacteriosis has the potential to trigger enteritis, leading to diminished appetite, stunted growth, and potentially fatal outcomes in fish [4]. An effective and properly controlled intestinal barrier is crucial for safeguarding the organism against food antigens and its indigenous intestinal bacteria [5]. As a result, research on the structure and changes of fish intestinal microflora has recently gained attention in the aquaculture industry.

Cottonseed meal (CSM) serves as a valuable source of protein of superior quality. The utilization of this nutrient-dense resource as animal feed, however, encounters obstacles due to the presence of free gossypol (FG) [6]. FG-containing diets can have negative effects on animal growth, digestive health, and reproduction. Fermentation technology allows for efficient separation of cottonseed phenols in cottonseed meals, raising the crude protein level of the raw material and producing small peptides and growth-promoting factors that enrich the nutrition of cottonseed protein [7]. The application of fermented cottonseed protein in mackerel culture was investigated, and it was found that the survival and weight gain rates of mackerel in the group with fermented cottonseed protein were significantly higher than those in the control group [8]. It was discovered that using 23% FCSM to replace 9% soybean meal and 15% cottonseed meal promoted the growth of grass carp, reduced the feed coefficient, and improved nonspecific immunity [9]. Most current research on fermented cottonseed protein is gathered in livestock and poultry, with little focus on aquatic animals. Based on these findings, this study was conducted to investigate the effects of the replacement of fish meal (FM) with FCSM on the intestinal enzymatic activity, inflammatory and physical-barrier-related gene expression, and intestinal microflora of juvenile *T. ovatus*.

2. Materials and Methods

2.1. Preparation of Experimental Diets

Fish meal, casein, soybean protein concentrate, and soybean meal were added as protein sources, and fish oil and soybean lecithin were added as lipid sources. Five isoproteic and isolipidic experimental diets were formulated with different levels of replacement of FM with FCSM, specifically, 0 (FM), 12.5% (CSM12.5), 25% (CSM25), 50% (CSM50), and 100% (CSM100), respectively; the feed formulations and the contents of various nutrients are shown in Table 1. To eliminate the effect of limiting amino acids, lysine and methionine were added to each group, respectively, so that the amino acid content of each group of feeds was balanced; the amino acid composition of the experimental feeds is shown in Table 2. All the ingredients were ground into powder, sieved through 60 mesh, and thoroughly mixed with oil and water; the 2.5 mm and 3.0 mm diameter long doughs were extruded using a twin screw extruder (F-26, South China University of Technology, Guangzhou, China), cut into pelletized feeds using a pelletizer (G-500, South China Uni-

versity of Technology, Guangzhou, China), and then stored at −20 °C in a refrigerator until used.

Table 1. Formulation and nutrient levels of the experimental diets (% dry matter).

Ingredients	Fermented Cottonseed Meal Substitution Percentage/%				
	FM	**CSM12.5**	**CSM25**	**CSM50**	**CSM100**
Fish meal	40	35	30	20	0
Soy protein concentrate	16	16	16	16	16
Soybean meal	4	4	4	4	4
Fermented cottonseed meal	0	5.1	10.2	20.5	40.9
Corn starch	17.7	16.2	14.7	11.7	5.8
Porcine blood cell protein powder	2	2	2	2	2
Beer yeast powder	2	2	2	2	2
Fish oil	7.3	7.7	8.1	8.9	10.5
Vitamin and mineral premix [1]	1	1	1	1	1
$Ca(H_2PO_4)_2$	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.5	0.5	0.5	0.5	0.5
Lecithin	1	1	1	1	1
Microcrystalline cellulose	7.5	8.4	9.1	10.7	13.8
Betaine	0.5	0.5	0.5	0.5	0.5
Lysine	0	0.1	0.3	0.5	1
Methionine	0	0	0.1	0.2	0.5
Nutrient levels					
Ash	12.08	12.03	12.01	10.35	7.99
Crude protein	43.89	43.65	43.31	43.62	43.49
Crude lipid	11.30	11.54	11.71	11.85	12.02
Crude fiber	7.29	7.83	8.46	10.04	12.93
Nitrogen free extract	24.71	24.95	24.52	24.13	23.57
Free gossypol (mg/kg)	64.7	95.9	88.6	171.0	190.0

[1] Vitamin and mineral premix provided by Shenzhen Jingji Zhinong Times Co., Ltd. (mg kg^{-1} diet). The formulation includes the following amounts of vitamins and minerals per kilogram: vitamin A at a minimum of 450,000 IU, vitamin B1 at a minimum of 1000 mg, vitamin B2 at a minimum of 1000 mg, vitamin B6 at a minimum of 1500 mg, vitamin B12 at a minimum of 5 mg, vitamin K3 at a minimum of 800 mg, inositol at a minimum of 12,000 mg, D-Pantothenic acid at a minimum of 3500 mg, nicotinic acid at a minimum of 2000 mg, folic acid at a minimum of 500 mg, D-Biotin at a minimum of 5 mg, vitamin D3 at a range of 300,000 to 400,000 IU, vitamin E at a minimum of 8000 IU, Na_2SeO_3 at 20 mg, $CuSO_4 \cdot 5H_2O$ at 24 mg, $FeSO_4 \cdot H_2O$ at 266.65 mg, $ZnSO_4 \cdot H_2O$ at 100 mg, $MnSO_4 \cdot H_2O$ at 120 mg, Ca $(IO_3)_2$ at 50 mg, $CoSO_4 \cdot 7H_2O$ at 10 mg, Mg at 20 g, and zeolite at 4380.55 mg.

2.2. Fish and Experimental Conditions

The feeding trial was conducted in a seawater pond at the Shenzhen Base of the South China Sea Fisheries Research Institute of the Chinese Academy of Fishery Sciences (Shenzhen, China). For two weeks, juvenile golden pompanos were acclimated to the experimental system and fed commercial diets (Guangdong Yuequn Biotechnology Co., Ltd., Guangzhou, China). At the outset of the feeding experiment, the fish were fasted for 24 h and then weighed. The fish were randomly assigned into 18 cages, with 25 uniformly sized fish per cage (5.6 ± 0.14 g) for 8 weeks. Experimental fish were fed different experimental diets twice daily, at 6:00 and 18:00, until they appeared to be satiated. During the feeding-trial period, the water temperature was maintained at 28.3–33.3 °C. Dissolved oxygen was higher than 6.0 mg/L. The salinity and ammonia were in the range of 20–25‰ and 0.05–0.1 mg/L, respectively. The photoperiod was the natural-day light cycle throughout the experimental period. The protocols for all fish were approved by the Ethical Committee of the South China Sea Fisheries Research Institute.

Table 2. Proximate amino acid profiles of experimental diets (g/100 g).

Amino Acids	Fermented Cottonseed Meal Substitution Percentage/%				
	FM	**CSM12.5**	**CSM25**	**CSM50**	**CSM100**
Aspartic acid	2.73	2.71	2.72	2.72	2.54
Threonine	1.15	1.14	1.13	1.08	0.95
Serine	1.04	1.07	1.11	1.15	1.10
Glutamic acid	3.92	4.07	4.26	4.47	4.30
Glycine	1.63	1.49	1.44	1.25	0.83
Alanine	1.72	1.72	1.67	1.54	1.28
Cystine	0.24	0.25	0.26	0.26	0.26
Valine	1.42	1.41	1.38	1.34	1.26
Methionine	0.54	0.51	0.57	0.52	0.59
Isoleucine	1.09	1.08	1.04	0.98	0.87
Leucine	2.17	2.17	2.13	2.09	1.88
Tyrosine	0.69	0.68	0.72	0.71	0.72
Phenylalanine	1.13	1.19	1.23	1.30	1.37
Lysine	2.02	1.98	1.98	1.89	1.59
Histidine	0.72	0.74	0.75	0.76	0.74
Arginine	1.69	1.77	1.90	2.19	2.60
Proline	1.44	1.41	1.35	1.30	1.18
Total	25.3	25.4	25.6	25.6	24.1

2.3. Collection of Samples

By the end of the feeding trial, all the fish were deprived of diets for 24 h. Fish that were put under anesthesia were treated with diluted MS-222 (Sigma, St. Louis, MO, USA). Three fish per cage were anesthetized and sampled. The digestive contents of four fish from each cage were collected and were then quickly frozen in liquid nitrogen, followed by storage at −80 °C. To reduce the impact of interindividual differences, intestinal contents from each treatment were mixed for the analysis of intestinal microbiota. Mid-intestines from three fish in each cage were frozen in liquid nitrogen and then stored at −80 °C until total RNA was extracted. Three fish in each cage had a section of their gut frozen in liquid nitrogen, which was subsequently kept at −80 °C until the enzyme activity was determined.

2.4. Measurement and Analysis

2.4.1. Intestinal Enzymes Activities Measurements

Intestinal samples were homogenized in sterilized physiological saline (0.86%, pH = 7.4; 1:9, *w/v*) by a handheld homogenizer. Then the samples were centrifuged for 15 min (2000 r/min, 4 °C) and the supernatant removed for the quantification of chymotrypsin, lipase, and α-amylase using commercial kits (Beijing Huaying Biotechnology Research Institute, Beijing, China).

2.4.2. Free Cotton Phenol Content Measurements

The free cotton phenol content analysis of diets was determined according to the standard methods of the American Oil Chemists Society (AOCS 2009; method Ba 7b-96) [10]. All samples were analyzed with an 8453 ultraviolet-visible spectrophotometer (Agilent Technologies Co., Ltd., Qingdao, China).

2.4.3. Quantitative Real-Time PCR

Total RNA was extracted from intestinal tissues using the Animal Total RNA Isolation Kit (FOREGENE Co., Ltd., Chengdu, China), and the integrity and quality of the RNA were detected using the NanoDropOne Micro Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The cDNA was obtained by reverse transcription using the PrimeScriptTM RT reagent kit with a gDNA Eraser kit (Takara, Kusatsu City, Japan). The reaction conditions: 37 °C for 15 min, 85 °C for 5 s, and 4 °C. The obtained cDNA was stored at −20 °C. The primer sequences of the target gene and the internal reference gene β-Actin for real-time

fluorescence quantitative PCR are shown in Table 3. All primer pairs required for real-time fluorescence quantitative PCR were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Real-time fluorescence quantitative PCR was performed on the Quant Studio Dx PCR instrument (ABI, Foster City, CA, USA). The expression of these genes was quantified by the $2^{-\Delta\Delta CT}$ method [11].

Table 3. The primers for real-time fluorescence quantification PCR.

Gene	Sequence	Reference
β-Actin-qF	TACGAGCTGCCTGACGGACA	[12]
β-Actin-qR	GGCTGTGATCTCCTTCTGCA	
il-1β-qF	CGGACTCGAACGTGGTCACATTC	[12]
il-1β-qR	AATATGGAAGGCAACCGTGCTCAG	
IL-8-qF	TGCATCACCACGGTGAAAAA	[12]
IL-8-qR	GCATCAGGGTCCAGACAAATC	
TNF-α-qF	CGCAATCGTAAAGAGTCCCA	[12]
TNF-α-qR	AAGTCACAGTCGGCGAAATG	
il-10-qF	CTCCAGACAGAAGACTCCAGCA	[12]
il-10-qR	GGAATCCCTCCACAAAACGAC	
NF-κB-qF	TGCGACAAAGTCCAGAAAGAT	[12]
NF-κB -qR	CTGAGGGTGGTAGGTGAAGGG	
ZO-1-qF	TTTGTGGCAGGAGTTCT	[12]
ZO-1-qR	TTCTTGTTGGGGATGAT	
Occludin-qF	TACGCCTACAAGACCCGCA	[12]
Occludin-qR	CACCGCTCTCTCTGATAAA	
Claudin-3-qF	CTCCTCTGCTGCTCCTGTCC	[12]
Claudin-3-qR	CGTAGTCTTTCCTTTCTAACCCTG	
Claudin-15-qF	AAGGTATGAAATAGGAGAAGGGC	[12]
Claudin-15-qR	TGGTTTGATAAGGCAGAGGGTA	

2.4.4. Gut Microflora

The microbial DNA extraction was performed using Hi Pure Soil DNA Kits (Magen, Guangzhou, China). The 16S rDNA V4 region of the ribosomal RNA gene was amplified using the polymerase chain reaction (PCR) technique. The primers used for this amplification were Arch519 (CAGCMGCCGCGGTAA) and Arch915R (GTGCTCCCCCGCCAATTCCT). In a duplicate 50 μL combination, the components included 5 μL of 10 × KOD buffer, 5 μL of 2.5 mM dNTPs, 1.5 μL of each primer (5 μM), 1 μL of KOD Polymerase, and 100 ng of template DNA for PCR reactions. The AxyPrep DNA Gel Extraction Kit, manufactured by Axygen Biosciences in Union City, CA, U.S., was employed to extract amplicons from 2% agarose gels. The ABI Step One Plus Real-Time PCR System, manufactured by Life Technologies in Foster City, USA, was employed for the purpose of quantification. In accordance with established techniques, the purified amplicons were combined in equimolar proportions and subjected to paired-end sequencing (2 × 250) using an Illumina platform. The raw data underwent splicing and filtering processes in order to create a refined dataset. The construction of operational taxonomic units was carried out, and the final feature table and feature sequences were created using the Divisive Amplicon Denoising Algorithm method.

2.5. Statistical Analysis

The experimental data were presented as mean ± SEM. Statistical analysis was performed using SPSS 26.0 software (IBM Corporation, Somers, NY, USA) for Windows. Before performing analysis of variance (ANOVA), the normality and homogeneity of experimental data were tested using the Kolmogorov–Smirnov test and Levene's test, respectively. After passing the test, the experimental data were subjected to a one-way analysis of variance. When there were significant differences, the group means were further compared with Duncan's multiple-range test, and a probability of $p < 0.05$ was considered significant.

3. Results

3.1. Intestinal Enzymatic Activity of T. ovatus

The effects of dietary fish meal substitution by fermented cottonseed meal on the intestinal enzymatic activity of golden pompano are shown in Table 4. The LPS content levels of the FM, CSM12.5, and CSM25 groups were not significantly different ($p > 0.05$), with the highest content being found in the CSM12.5 group. There was a tendency towards an increase in chymotrypsin activity in the intestine of golden pompano at a substitution rate of 25%, which was significantly lower in the CSM12.5 and CSM50 groups ($p < 0.05$). As the substitution level of a fermented cottonseed meal increased, the AMY activity in the substitution group was significantly higher than that in the FM group ($p < 0.05$), and the highest activity of AMY in the intestine of golden pompano was observed when the substitution rate reached 25%.

Table 4. Effects of fish meal substitution by fermented cottonseed meal on an index of intestinal digestive enzymes of *T. ovatus*.

Enzyme	Fermented Cottonseed Meal Substitution Percentage/%				
	FM	CSM12.5	CSM25	CSM50	CSM100
LPS (U/mg)	26.26 ± 0.66 ^{a}	26.3 ± 1.43 ^{a}	26.02 ± 1.76 ^{a}	21.53 ± 0.32 ^{b}	21.2 ± 1.25 ^{b}
Chymotrypsin (U/mg)	49.92 ± 2.17 ^{ab}	42.23 ± 0.93 ^{c}	51.69 ± 3.99 ^{a}	43.03 ± 0.3 ^{c}	46.24 ± 1.59 ^{bc}
AMY(U/mg)	236.33 ± 6.45 ^{d}	290.11 ± 3.63 ^{c}	405.72 ± 10.24 ^{a}	304 ± 6.84 ^{b}	290.22 ± 3.98 ^{c}

LPS: Lipase; AMY: Amylase. Data are expressed as means ± SEM ($n = 3$). Means with different superscripts are significantly different ($p < 0.05$).

3.2. Intestinal Immune-Related Gene Expression of T. ovatus

The gene expressions of the intestinal NF-κB-related signaling pathway in golden pompano after ingestion of different levels of experimental diets are shown in Figure 1. Compared with FM and CSM25, the expression of *IL-1β* and *TNF-α* of the fish in the group fed with CSM12.5, CSM50, and CSM100 was notably increased ($p < 0.05$). Compared with the FM group, the relative expression of the IL-8 gene in the intestine of golden pompano was not significantly different between the FM, CSM25, and CSM50 groups ($p > 0.05$), with the highest expression being found in the CSM100 group, which was significantly different from the other groups ($p < 0.05$). The expression of *IL-10* of the fish in the CSM12.5 dietary group was significantly upregulated ($p < 0.05$). The relative expression of the *NF-κB* gene was significantly lower in the intestine of golden pompano at different levels in the FCSM substitution group compared to the FM group ($p < 0.05$), with the highest expression found in the CSM100 group.

3.3. Intestinal Physical-Barrier-Related Gene Expression of T. ovatus

The expression of physical-barrier-related genes in golden pompano after ingestion of different levels of FCSM are shown in Figure 2. The relative expression of the *ZO-1* gene was lowest in the CSM50 group, which was lower than the FM group ($p < 0.05$), while the relative expression was significantly higher in the CSM12.5 and CSM100 groups. The relative expression of the *Occludin* gene was highest in the CSM100 group, and the levels of the CSM12.5 and CSM100 groups were significantly higher than that of the FM group ($p < 0.05$); additionally, there was no significant difference between the CSM25 and FM groups ($p > 0.05$). The relative expression of the *Claudin-3* gene was significantly affected by the substitution of FM by FCSM ($p < 0.05$), with the highest relative expression found in the CSM25 group. When the level of FCSM substitution was 50%, the relative expression of the *Claudin-15* gene in the intestine of golden pompano was significantly ($p < 0.05$) lower than in all other groups. Meanwhile, the CSM12.5 group, CSM25 group, and CSM100 group were significantly higher than the FM group ($p < 0.05$).

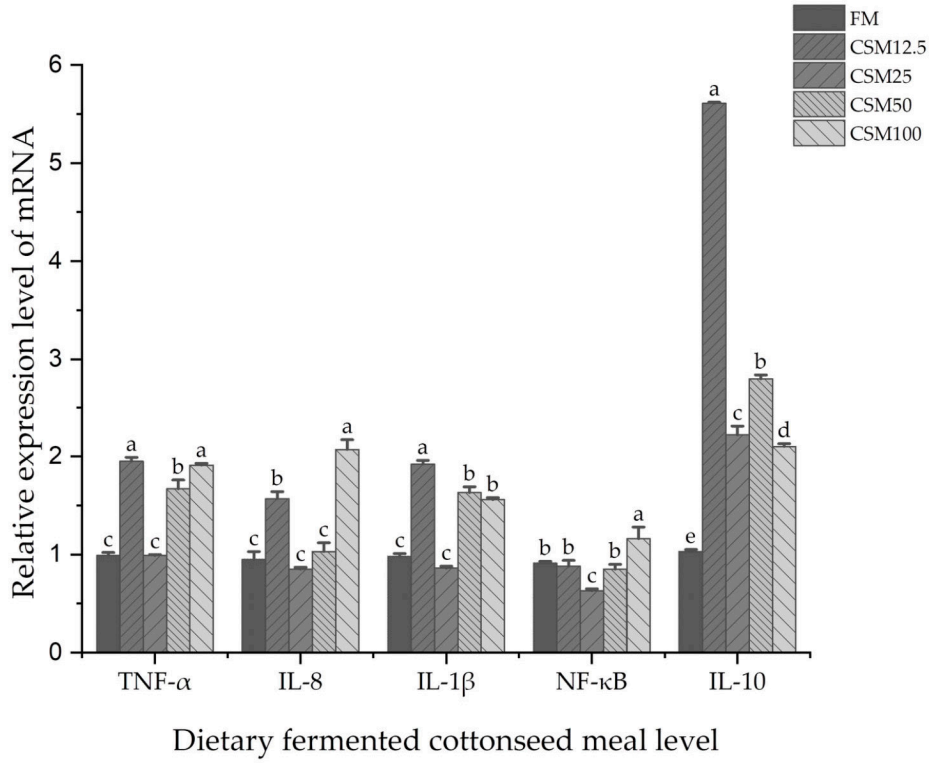

Figure 1. Effect of fermented cottonseed meal substitution for fish meal on immune-related gene expression of *T. ovatus*. Data are expressed as means ± SEM (n = 3). Means with different superscripts are significantly different ($p < 0.05$).

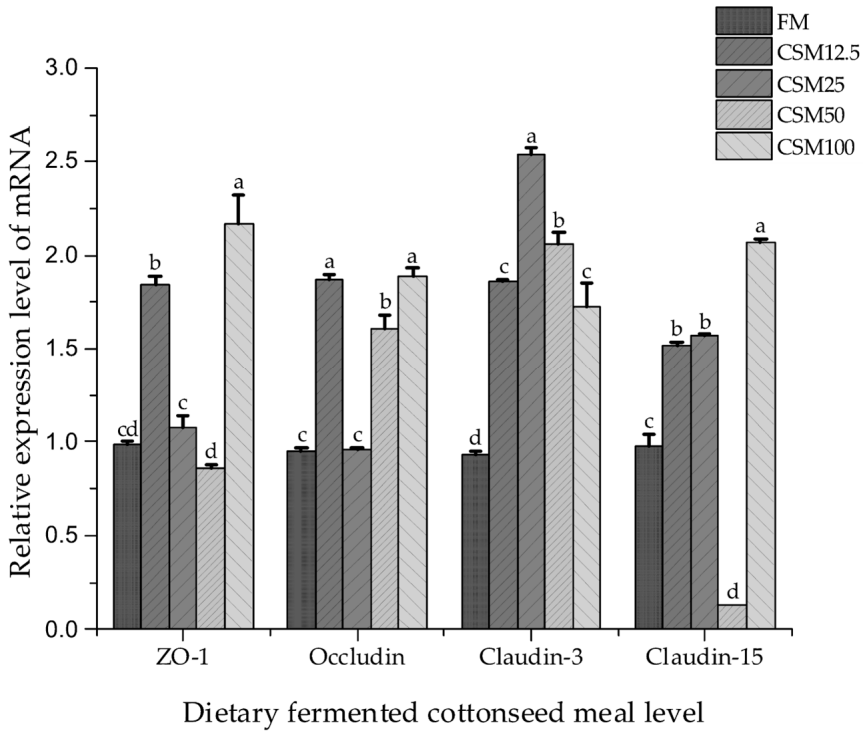

Figure 2. Effects of fish meal substitution by a fermented cottonseed meal on the barrier-related gene expression. Data are expressed as means ± SEM (n = 3). Means with different superscripts are significantly different ($p < 0.05$).

3.4. Intestinal Microbiota

3.4.1. Analysis of Microbial OTUs and Alpha Diversity of Intestinal Flora

The curve of intestinal microflora dilution (Figure 3) of juvenile golden pompano in different treatment groups tends to be flat, with sequencing coverage ≥ 99.96%. This indicates that the sequencing depth of the intestinal microflora of each group of golden pompano is reasonable and can cover the vast majority of species in the sample. A grand number of 1,549,208 sequencing reads of good quality were acquired.

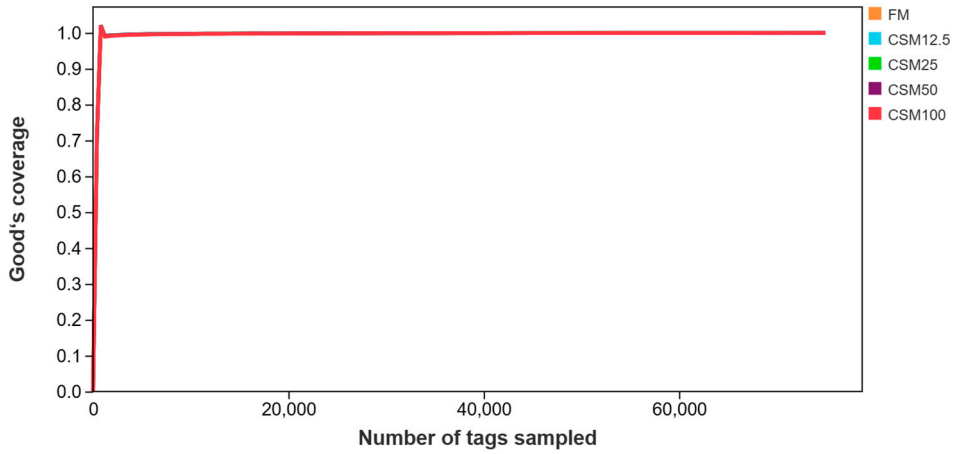

Figure 3. Effects of fish meal substitution by a fermented cottonseed meal on the dilution curve of the intestinal flora of *T. ovatus*.

From the Venus plot of OTUs of golden pompano in different treatment groups (Figure 4), the total number of OTUs between groups was 71. The alpha diversity metrics were computed by analyzing the rarefaction curves at the operational taxonomic units (OTUs) level for each experimental meal. Table 5 displays the Chao1, ACE, Shannon, and Simpson indexes. The CSM100 group had the highest ACE indices and the largest community richness, which was not significantly different from the other groups ($p > 0.05$). Compared with the remaining groups, the CSM25 group had the lowest ACE and Chao1 indices and the lowest community richness, while the CSM100 group had the highest ACE and Chao1 indices and the highest community richness. The CSM50 group had the highest Shannon index and Simpson index, and the highest community diversity. The Shannon index and Simpson index in the CSM50 group differed significantly from those of the other groups ($p < 0.05$) (Table 5).

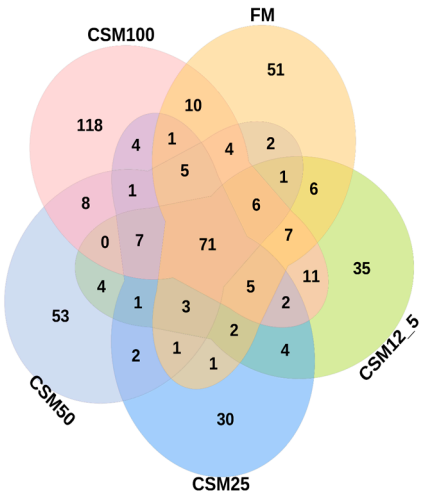

Figure 4. Venus map of OTUs of the intestinal flora of *T. ovatus* in different treatment groups.

Table 5. Diversity statistics of intestinal samples of juvenile *T. ovatus* in different treatment groups.

Item	Group				
	FM	**CSM12.5**	**CSM25**	**CSM50**	**CSM100**
OTU	166.7 ± 11.09	158.25 ± 8.06	139.25 ± 7.85	154.75 ± 27.92	195.00 ± 59.57
Ace	189.67 ± 15.13	176.73 ± 1.76	161.73 ± 14.21	165.01 ± 9.89	191.77 ± 22.51
Chao1	183.38 ± 18.21	169.74 ± 3.1	157.15 ± 9.73	156.37 ± 9.01	187.16 ± 21.13
Shannon	1.86 ± 0.08 ^{bc}	1.68 ± 0.11 ^{c}	2.34 ± 0.29 ^{ab}	2.71 ± 0.26 ^{a}	1.88 ± 0.29 ^{bc}
Simpson	0.61 ± 0.03 ^{b}	0.57 ± 0.06 ^{b}	0.7 ± 0.1 ^{ab}	0.78 ± 0.05 ^{a}	0.64 ± 0.06 ^{ab}

ACE: The ACE index is utilized as a means to approximate the abundance of operational taxonomic units (OTUs) within a given ecological community. It has been observed that higher ACE values exhibit a positive correlation with the overall diversity and richness of the microbial community. Chao1: The number of OTU in the community was estimated by Chao1 algorithm, and the value of Chao1 was positively correlated with the total number of species. Shannon: The index considers both the abundance and evenness of the community, and there is a positive correlation between its value and the richness and evenness of the community. Simpson: The index was used to estimate microbial diversity in samples, and the values were negatively correlated with community diversity. Data are expressed as means ± SEM (n = 4). Means with different superscripts are significantly different ($p < 0.05$). ^{a,b,c} Means (±SEM) values within the row unlike superscript letters were significantly different ($p < 0.05$).

3.4.2. Analysis of Intestinal Flora Composition and Relative Abundance

The analysis of the intestinal contents of juvenile golden pompano encompassed multiple taxonomic levels, specifically, phylum, order, family, and genus. Phylum and genus were chosen as representative taxonomic levels for this study.

As shown in Figure 5, at the phylum level, Proteobacteria and Firmicutes formed the core microflora. Proteobacteria were the most abundant phyla in the control group and CSM25 group, accounting for 49.4% and 52.16%, respectively. Firmicutes was the most abundant phylum in CSM12.5, CSM50, and CSM100, accounting for 61.46%, 51.71%, and 52.83%, respectively. There were no significant differences among the five groups, except for Fusobacteria and Planctomycetes ($p > 0.05$) (Table 6).

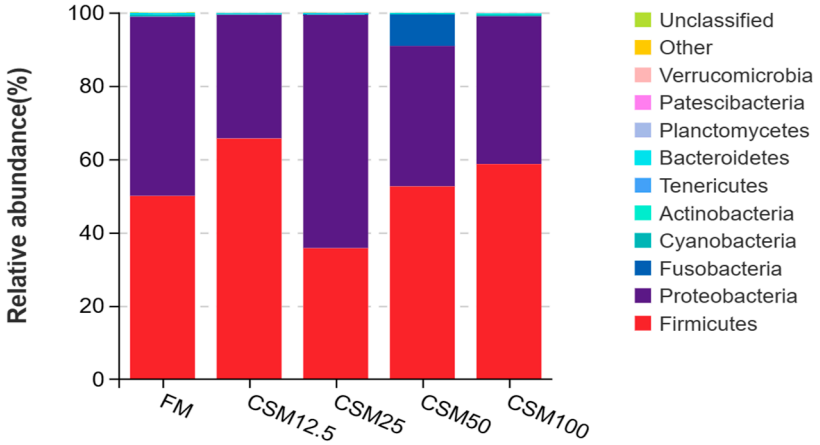

Figure 5. Distribution of the top 10 microbial phylum levels in the intestinal contents of *T. ovatus* in different treatment groups.

As shown in Figure 6, at the genus level, Photobacterium and Paraclostridium formed the core microflora. The dominant genera in the intestinal flora of *T. ovatus* were Photobacterium and Paraclostridium, which accounted for 52.5% and 44.86% of the total species, respectively. Photobacterium is the most abundant genus in the FM group and CSM25. There were significant differences among the five groups, except for Lysinibacillus, Bacillus, and Aerococcus ($p < 0.05$) (Table 7). The Fusobacteria levels of CSM100 were significantly lower than those of all other groups ($p < 0.05$). There was no significant difference in Planctomycetes levels for CSM12.5, CSM25 and CSM50 ($p > 0.05$), and the FM group and CSM100 were significantly higher than all other groups ($p < 0.05$).

Table 6. Distribution of the top 10 microbial phylum levels in the intestinal contents of *T. ovatus* in different treatment groups.

Phylum	Group				
	FM	**CSM12.5**	**CSM25**	**CSM50**	**CSM100**
Firmicutes	49.36 ± 6.73	61.46 ± 30.38	47.14 ± 23.64	51.71 ± 14.17	52.83 ± 13.50
Proteobacteria	49.40 ± 6.44	37.84 ± 30.28	52.16 ± 23.64	41.16 ± 12.34	43.82 ± 10.15
Fusobacteria	0.11 ± 0.03 ^{a}	0.10 ± 0.01 ^{a}	0.10 ± 0.04 ^{a}	0.07 ± 0.04 ^{a}	0.01 ± 0.00 ^{ab}
Cyanobacteria	0.45 ± 0.13	0.19 ± 0.02	0.20 ± 0.02	0.26 ± 0.12	0.40 ± 0.38
Actinobacteria	0.22 ± 0.08	0.14 ± 0.02	0.16 ± 0.02	0.21 ± 0.13	0.30 ± 0.29
Tenericutes	0.17 ± 0.09	0.14 ± 0.10	0.11 ± 0.03	0.09 ± 0.07	0.29 ± 0.42
Planctomycetes	0.05 ± 0.03 ^{b}	0.03 ± 0.01 ^{ab}	0.01 ± 0.00 ^{ab}	0.02 ± 0.02 ^{ab}	0.06 ± 0.02 ^{a}
Bacteroidetes	0.10 ± 0.06	0.02 ± 0.00	0.04 ± 0.02	0.03 ± 0.02	0.10 ± 0.13
Patescibacteria	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.02	0.13 ± 0.17
Verrucomicrobia	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	0.09 ± 0.14

Data are expressed as means ± SEM (n = 4). Means with different superscripts are significantly different ($p < 0.05$).

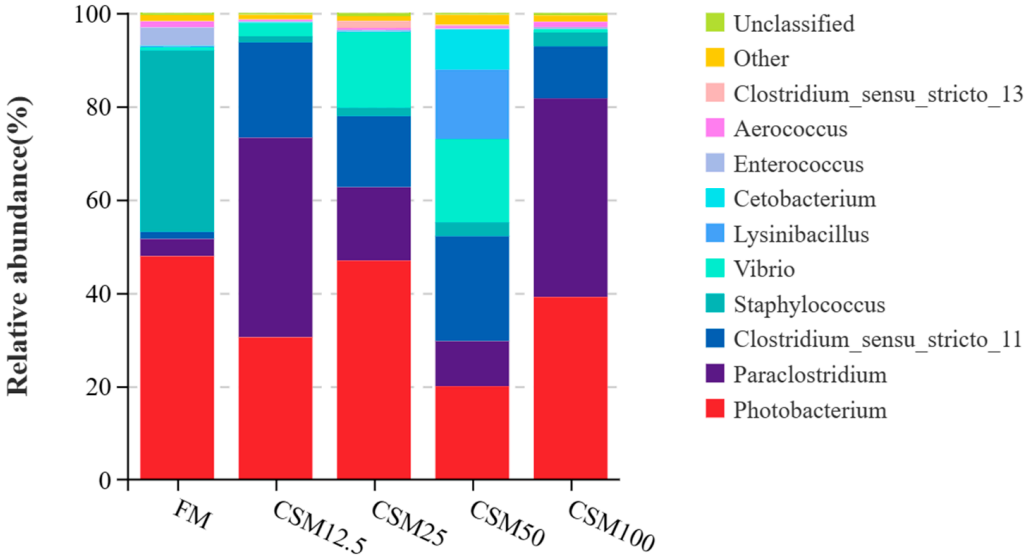

Figure 6. Distribution of the top 10 microbial genus levels in the intestinal contents of *T. ovatus* in different treatment groups.

Table 7. Distribution of the top 10 microbial genus levels in the intestinal contents of juvenile *T. ovatus* in different treatment groups.

Genus	Group				
	FM	**CSM12.5**	**CSM25**	**CSM50**	**CSM100**
Photobacterium	48.34 ± 6.37 ^{a}	41.44 ± 24.84 ^{ab}	39.41 ± 18.01 ^{ab}	21.91 ± 3.84 ^{b}	42.26 ± 9.75 ^{ab}
Paraclostridium	4.07 ± 1.85 ^{b}	53.97 ± 1.43 ^{a}	17.58 ± 3.88 ^{ab}	13.16 ± 8.79 ^{ab}	32.97 ± 19.24 ^{ab}
Clostridium_sensu_stricto_11	2.16 ± 1.42 ^{b}	29.93 ± 10.68 ^{ab}	31.67 ± 20.52 ^{a}	19.98 ± 10.36 ^{ab}	8.58 ± 9.41 ^{ab}
Staphylococcus	34.36 ± 10.65 ^{a}	1.44 ± 0.77 ^{b}	1.88 ± 1.48 ^{b}	3.87 ± 2.35 ^{b}	5.54 ± 5.45 ^{b}
Vibrio	0.71 ± 0.13 ^{ab}	4.70 ± 4.47 ^{ab}	12.28 ± 8.86 ^{ab}	18.62 ± 11.27 ^{a}	0.63 ± 0.30 ^{b}
Lysinibacillus	0.23 ± 0.04	0.30 ± 0.32	0.21 ± 0.03	0.15 ± 0.12	0.02 ± 0.01
Enterococcus	3.92 ± 1.66 ^{a}	0.11 ± 0.05 ^{b}	0.13 ± 0.03 ^{b}	0.23 ± 0.09 ^{b}	0.48 ± 0.29 ^{b}
Bacillus	0.20 ± 0.05	0.13 ± 0.07	0.19 ± 0.08	0.33 ± 0.31	0.14 ± 0.23
Cetobacterium	0.11 ± 0.03 ^{a}	0.10 ± 0.0 ^{a}	0.10 ± 0.04 ^{a}	0.07 ± 0.04 ^{a}	0.01 ± 0.00 ^{b}
Aerococcus	0.41 ± 0.20	0.05 ± 0.03	0.02 ± 0.01	0.74 ± 0.37	1.58 ± 0.79

Data are expressed as means ± SEM (n = 4). Means with different superscripts are significantly different ($p < 0.05$).
^{a,b} Means (±SEM) values within the row unlike superscript letters were significantly different ($p < 0.05$).

3.4.3. Analysis of β Diversity of Intestinal Flora

Non-measured multidimensional analysis (NMDS) is a commonly used β diversity analysis method. As the basis for community structure research, NMDS analysis is often used to compare the differences between different ecosystems and reflect the heterogeneity of biological species due to the environment. The points in the graph represent the sample, and the distance between the points reflects the degree of sample difference; the closer the distance, the higher the similarity. Points in the same circle represent no significant difference between the samples, and points in the non-intersection circle represent significant differences between the samples. As can be seen from Figure 7, the FM group intersects with the CSM12.5 group, but does not intersect with any other groups. The CSM25 group intersects with the CSM12.5 group and the CSM50 group and does not intersect with any other groups. The CSM50 group intersects with the CSM25 group and does not intersect with any other groups. The CSM100 group intersects with the CSM12.5 group but does not intersect with any other groups. The CSM12.5 group is not intersected with the CSM50 group and is intersected with all other groups. This means that there is no significant difference between the FM group and the CSM12.5 group ($p > 0.05$), while there is a significant difference with all other groups ($p < 0.05$). There is no significant difference between the CSM25 group and the CSM12.5 group or the CSM50 group ($p > 0.05$), and there is a significant difference with all other groups ($p < 0.05$). There is no significant difference between the CSM50 group and the CSM25 group ($p > 0.05$), and the former is significantly different from each of the other groups ($p < 0.05$); there is no significant difference between the CSM100 group and the CSM12.5 group ($p > 0.05$), and the former is significantly different from each of the other groups ($p < 0.05$). There is a significant difference between the CSM12.5 group and the CSM50 group ($p < 0.05$), and the former is not significantly different from any of the other groups ($p > 0.05$). There was an intersection between the CSM25 group and the CSM50 group, indicating that when the amount of FCSM in the feed was 25%, there was no significant difference in the intestinal microbial composition than with the addition of 50% in *T. ovatus* ($p > 0.05$). There was an intersection among the CSM25 group, the CSM12.5 group, and the CSM100 group, indicating that when the amount of FCSM in the feed was 25%, there was no significant difference in the intestinal microbial composition than with the addition of 12.5% and 100% in *T. ovatus* ($p > 0.05$).

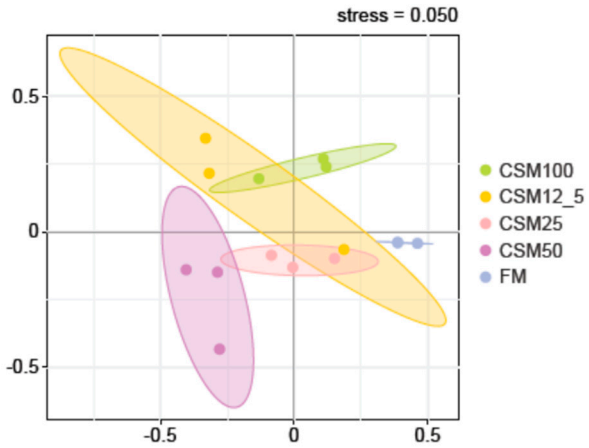

Figure 7. NMDS analysis at the OTU level of *T. ovatus* in different treatment groups.

4. Discussion

Fish meal (FM) is widely utilized as a protein source in commercial aquafeeds due to its well-balanced composition of essential amino acids, vitamins, and minerals, and its palatability [10]. In recent years, the increasing demand for animal nutrition has led to a search for sustainable protein sources of both plant and animal origin, due to the limited availability of fishmeal [13]. Cottonseed meal is digested relatively well by fish and

crustaceans [14]. When markets are favorable, CSM is an economical alternative protein source for use in aquatic animal feeds. Furthermore, it is generally highly palatable to most aquatic animals [14].

The intestinal tracts of animals serve as complex microbial ecosystems that harbor dynamic communities of microorganisms. These microorganisms play various roles, such as absorbing nutrients, improving energy production, and maintaining immune homeostasis [15]. The process of digestion is related to the activity of digestive enzymes, including chymotrypsin and α-amylase. These enzymes play a crucial role in breaking down nutrients, as they facilitate the digestion process [16]. In the present study, the intestinal amylase content levels in the CSM12.5 and CSM25 groups were higher than that in the FM group. The chymotrypsin and AMY activity levels in the intestine were higher than in the FM group when the substitution rate reached 25%. AMY activity reflects the absorption and utilization of nutrients by fish [17]. Protease is an important proteolytic enzyme in fish intestine, and its activity can reflect the ability of fish to decompose feed protein [18]. These findings indicated that 25% substitution of FCSM for FM boosted the activity of digestive enzymes and improved nutrient uptake, which in turn improved growth performance of *T. ovatus*. In this study, when 50–100% of FM was replaced by FCSM, the activity levels of LPS and chymotrypsin were lower than those of the control group. This may be due to the lower digestion and absorption ability of FCSM as compared to fish meal. A similar phenomenon was also observed in a previous study by Sillago sihama Forsskál [19]. Research findings indicate that the American redfish species (*Sciaenops ocellatus*) has demonstrated proficient ability in digesting cottonseed meal [20].

The significant function of pro- and anti-inflammatory cytokines in the maintenance of tissue and immunological homeostasis has been well established [21]. The pro-inflammatory cytokines, which include *TNF-α* and *IL-8*, have a variety of effects that cause inflammation [22]. Interleukin-1β (*IL-1β*) plays a crucial role in both the onset and amplification of the inflammatory response, which ultimately leads to intestinal damage [23]. Inflammatory cytokine *IL-8* is a component of the immune response, and disruption of the balance of these cytokines is key to the pathophysiology of inflammatory bowel disease [24]. In epithelial tissues, *NF-κB* signaling is crucial for preserving immunological homeostasis. Thus, *NF-κB* appears to exhibit two sides in chronic inflammation: on the one hand, *NF-κB* activation increases and continues to induce inflammation and tissue damage, but on the other hand, inhibition of *NF-κB* signaling also disrupts immune homeostasis and triggers inflammation and disease [22]. In the present study, *TNF-α*, *IL-1β*, *IL-8*, and *NF-κB* gene expression levels were all significantly decreased at the level of 25% FCSM replacement. In contrast, excessive substitution levels significantly increased *TNF-α*, *IL-1β*, *IL-8*, and *NF-κB* gene expression levels, indicating that higher substitution levels of FCSM can induce inflammatory responses in the intestine. *IL-10* is an anti-inflammatory factor which plays a role in down-regulating inflammatory response and antagonizing inflammatory mediators [25]. Gene expression of the pro-inflammatory factor *IL-10* was significantly higher than that of the control group at 12.5%, 25% and 50% substitution levels, suggesting that intestinal inflammation improves at appropriate substitution ranges.

The intestinal tract in fish is widely recognized as a significant physical barrier, and maintaining its structural integrity is essential for effectively resisting foreign antigens [26]. The presence of compromised or diminished physical barrier function within the intestinal tract of fish is associated with an elevated susceptibility to pathogen infection and a hindered ability to suppress pathogen growth [12]. *ZO-1* is involved, in its function as a scaffolding protein, by determining the tightness of the epithelial barrier and contributing to the polarity of the epithelial cells [5]. The extracellular loop of *Occludin* goes straight into the TJ complex and interacts with the transmembrane domain to alter the permeability of extracellular selectivity [27]. In the present study, the mRNA transcript levels of *ZO-1* were up-regulated, but the relative expression levels of the *Occludin* gene in the CSM25 and FM groups were not significantly different. *Claudin-3* is a tightening TJ protein that aids in sealing the paracellular intestinal barrier [28]. In this study, the *Claudin-3* gene had

the highest relative expression in the CSM25 group, indicating that the intestinal barrier closure was strongest at the 25% substitution level. *Claudin-15* is widely distributed in the tight junctions of the villi and crypt cells of the small and large intestines. In this study, the relative expression of *Claudin-15* in the CSM12.5, CSM25, and CSM100 groups was significantly higher than that in the FM group, suggesting that the structural integrity of intestinal intercellular structures was better in the CSM12.5, CSM25, and CSM100 groups. The results of the above studies suggest that appropriate substitution ratios can ameliorate golden pompano intestinal physical barrier damage induced by FCSM. This is similar to the results of a study on the genes related to the improvement of intestinal tight junction in yellow catfish by fermented rapeseed meal [29]. Therefore, FCSM may alleviate the damage, associated with cottonseed meal, to the intestinal structure of golden pompano by degrading anti-nutritional factors, thereby improving the intestinal health of fish.

The microbial ecosystem of animals consists of microbial communities and their host microenvironment. The intestinal microenvironment consists of intestinal microorganisms and is the most critical microenvironment of the animal body [30]. The intestinal microbiota play a crucial role in various physiological processes, including metabolism, nutrient absorption, growth and development, and immune function. Therefore, it is very important for body health to investigate changes in fish intestinal flora. In the present study, the OTU Wayne diagram found that 71 OTU were common in all groups, indicating that there are still inherent core flora at different levels of FCSM addition in the same culture environment. These results suggested that the tested variations in diet did not exert large, long-term alterations on the intestinal microbiota of golden pompano.

Intestinal bacterial microflora level is closely related to digestive function and overall gastrointestinal health [31]. In terms of community composition, we found that the dominant intestinal bacteria of the golden pompano belonged to three phyla: Proteobacteria, Firmicutes, and Tenercutes [32]; this has also been reported for the turbot [33]. *Proteobacteria* can catabolize feed ingredients [34]. The increased abundance may allow *T. ovatus* to absorb nutrients from the feed. Proteobacteria, a class of bacteria characterized by their Gram-negative cell wall structure, serves a significant function in the breakdown and fermentation of polysaccharides, proteins, and various organic substances. Moreover, they constitute the prevailing microbial population within the intestinal ecosystem of numerous fish species [35]. It is well known that many kinds of Vibrio, Photobacterium, and Mycoplasma are pathogenic bacteria [19,36]. These microorganisms are found within the gastrointestinal tract. The degradation of the aquatic environment leads to an escalation in nutrient deficits and physiological dysfunctions, hence heightening the vulnerability of fish to these infections [37]. Research shows that the balance of the intestinal microflora was altered primarily by probiotic supplementation and, to a lesser extent, by the energy content of the diet [33]. The majority of Fusobacteriales strains identified in fish intestinal samples are attributed to Cetobacterium, a prevalent and extensively distributed species found within the gastrointestinal tracts of fish [38]. In this experiment, the dominant intestinal flora of the golden pompano was composed of *Firmicutes* and *proteobacteria*. However, with the change in the proportion of FCSM instead of fishmeal in feed, the absolute dominant flora between the groups also changed. At the phylum level, the dominant phylum of the control group for the CSM12.5 group, CSM50 group, and CSM100 group was firmicutes, while the dominant phylum of the CSM25 group was proteobacteria. The results of the above studies showed that the substitution of FM by FCSM did not lead to changes in the dominant strains of the intestinal flora of golden pompano.

At the genus level, the dominant genera in the intestinal flora of the *T. ovatus* are *Photobacterium* and *Paraclostridium*. *Photobacterium*, a bacterium classified as Gram-negative, has a combination of anaerobic and aerobic characteristics, displaying both respiratory and fermentative metabolic pathways. The inclusion of Bacillus probiotics in the diet has been found to promote the proliferation of lactic acid bacteria within the gastrointestinal system, leading to a decrease in pH levels. Consequently, this creates an unfavorable environment for the growth and survival of pathogenic bacteria, such as *E. coli* and *Salmonella*

spp. [39]. Photobacterium, a Gram-negative bacterium, is partly anaerobic and has respiratory and fermentative metabolism types. Photobacterium can infect the shrimp Litopenaeus vannamei to cause its mortality, and may be a common pathogen of shrimps [19]. The Photobacterium levels in the CSM25 group and CSM50 group were significantly lower than that in the control group, indicating that the abundance of Photobacterium decreased when the amount of FCSM added to the feed was 25–50% [40].

Beta diversity analysis is mainly used to compare differences in the overall structure of microbial communities in each sample. Non-metric multidimensional scaling (NMDS) is a data analysis method that simplifies the research objects of multidimensional space to low-dimensional space for locating, analysis, and classification while retaining the original relationship between objects [41]. In this experiment, we found that the gut microbial composition of the FM group was significantly different from that of the CSM12.5, CSM25, CSM50, and CSM100 groups, and that there were significant differences in the composition of the CSM12.5 and CSM50 groups and the CSM25 and CSM100 groups, indicating that varying levels of fermented cotton meal substitution in the feeds resulted in significant differences in gut microbial composition. Complex microbiota interactions may explain the fact that fermented fishmeal increased the diversity of the gut microbiota of the vanabin carp, a finding which is similar to the results of our study [42].

5. Conclusions

In conclusion, by analyzing the intestinal enzyme activity, inflammation, physical barrier-related gene expression, and intestinal microecology of juvenile golden pompano (*T. ovatus*), this investigation demonstrated in its results that FCSM replacement of FM affected the intestinal health status of the fish. Replacing 25% of FM with FCSM had no significant effect on LPS and chymotrypsin in juvenile golden pompano, but AMY activity was significantly increased. However, when 50–100% was replaced, LPS and chymotrypsin were significantly reduced, indicating that the substitution of 25% FM by FCSM did not affect intestinal digestion. It was demonstrated that an appropriate substitution ratio could improve nutrient absorption, reduce intestinal inflammation, and improve intestinal physical barrier damage, while not affecting intestinal microecology. However, substitution of a high proportion of FM with FCSM negatively affects the intestinal microflora and nutrient absorption capacity of fish.

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Conflicts of Interest: The authors declare no conflict of interest.

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