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Effects of *Clostridium butyricum* and sodium butyrate on growth performance, immunity, and gut microbiota of mirror carp *Cyprinus carpio* fed with soybean meal based diet

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ABSTRACT

This study investigated the effects of *Clostridium butyricum* (CB) and sodium butyrate (SB) on the growth performance, intestinal immune, and gut microbiota of mirror carp (*Cyprinus carpio*) fed with a high level of soybean meal. Three hundred sixty healthy carp fingerlings (~3.74 g) were divided into eight dietary treatments with four replicates and ten fish per replicate. Which include basal diet (Control group, C0); C0 supplemented with three levels of CB, 3×10^7 cfu/kg (C1), 3×10^8 cfu/kg (C2), 3×10^9 cfu/kg (C3); three levels of SB, 500 mg/kg (S1), 1000 mg/kg (S2), 2 000 mg/kg (S3); and 3×10^8 cfu/kg CB+ 1 000 mg/kg SB (CS), respectively. In 8-week trial, the survival rate of all groups was 100%. Compared with C0, the Weight Gain, Feed Conversion Ratio, Protein Efficiency Ratio were not significantly different in all groups ($P > 0.05$). Crude protein and ether extract contents were increased significantly ($P < 0.05$) when supplemented with different levels of CB and SB, and ash content increased significantly in the CB groups ($P < 0.05$). Serum globulin increased and glucose decreased significantly ($P < 0.05$) in the CB and SB groups. The intestinal T-SOD and glutathione activity in the foregut, midgut, and hindgut were increased ($P < 0.05$), and villus height of foregut and midgut were higher in the CB and SB ($P < 0.05$). Protease activities in the foregut and midgut were significantly increased ($P < 0.05$) in the C2, C3, S2, and S3. The lipase activity of the C2 and C3 in foregut, midgut, and hindgut; S2, S3, and CS in the foregut; S3 in midgut were significantly increased ($P < 0.05$). The gut microbiota was modulated at the phylum and genera level by CB and SB administration. In summary, our results indicated that CB and SB could promote the whole body CP and EE content, intestine immunity, digestive enzyme activity, and intestinal morphology, and modulated gut flora in carp.

1. Introduction

Soybean meal has been used primarily as a protein source in aquafeed production for various fish and shrimp species. However, due to the presence of antinutritional factors, the large use of soybean meal causes a series of adverse effects on the growth and intestinal health of aquaculture animals (Heikkinen et al., 2006). Previous studies in carp *Cyprinus carpio* have shown that when the proportion of replacing fish meal with soy protein isolate (SPI) was more than 60%, the intestinal tract's growth and development were blocked up, and the integrity of epithelium was damaged. Soybean meal influence intestinal mucosa metabolic processes, including lipid, amino acid, sugar, apoptosis, oxidative injury (Zhao and Xu, 2022), and induce enteritis (Luo et al., 2023). The intestinal tract is vital to perform the digestive and absorption function, and intestinal health is of great significance to the healthy culture of fish. Therefore, studies related to enhancing intestinal health when feeding a high soybean-based diet have attracted wide attention from researchers across the globe. In addition, feed additives such as organic acids have shown improvement in gastrointestinal tract function and energy metabolism (Ng and Koh, 2017). Among all organic acids,

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butyrate and its salt have gained more attention due to their beneficial effects on regenerating and repairing the intestine epithelium. Sodium butyrate (SB) is a donor material for butyric acid, which provides energy to colonocytes (Bergman, 1990), along with ameliorating mucosal inflammation, oxidative status, and the epithelial defense barrier (Canani et al., 2011). Previous studies have proved that dietary supplementation with SB could improve the growth performance and enhance immune responses in yellow drum *Nibea albiflora* (Wu et al., 2020), black seabream *Acanthopagrus schlegelii* (Ullah et al., 2020), yellow catfish *Pelteobagrus fulvidraco* (Zhao et al., 2021), and Nile tilapia *Oreochromis niloticus* (Abdel-Tawwab et al., 2021). Besides, butyrate in the gastrointestinal tract is primarily available through anaerobic bacterial glucose fermentation. *Clostridium butyricum* (CB) is a microorganism that can produce butyric acid. Previous studies have demonstrated that CB could improve the growth performance, increased intestine short-chain fatty acid content, intestine digestive capacity, and intestine immune function of Pacific white shrimp *Litopenaeus vannamei* (Duan et al., 2017; Li et al., 2019a), *Miichthys miiuy* (Song et al., 2006), and tilapia (Poolsawat et al., 2020; Li et al., 2019b; Zhang et al., 2020).

Even though there is numerous investigation on the dietary supplementation of CB or SB to terrestrial vertebrates, only a handful of studies have been conducted for fish and shrimp. Furthermore, to the best of our knowledge, no studies compare the effects of CB and SB in soybean-based diets on mirror carp. Therefore, the present study aimed to elucidate the effects of dietary supplementation with CB and SB on growth performance and intestine health of carp fed with a high percentage of soybean meal.

2. Materials and methods

2.1. Diet preparation and experimental design

The study was conducted strictly following the Guidance of the Care and Use of Laboratory Animals in China. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Huzhou University (20180306). The mirror carp (*Cyprinus carpio* Songpu) was provided by the Hulan Experimental Station of Heilongjiang Fisheries Research Institute, Chinese Academy of Fishery Sciences, Harbin, China. The CB with a court of 2.0×10^8 CFU/g was obtained from Lvxue Biotechnology Co. Ltd, China. SB (98% purity) was purchased from Aladdin Shanghai Co., Ltd.

The basal diet (Control group, C0) was formulated principally from dehulled soybean meal, soy protein concentrate, fishmeal, and wheat middlings containing 28.4% crude protein and 6.7% crude fat (Table 1). In addition, seven experimental diets were prepared by adding three levels of CB , 3×10^7 cfu/kg (C1), 3×10^8 cfu/kg (C2), 3×10^9 cfu/kg (C3), and three levels of SB, 500 mg/kg (S1), 1 000 mg/kg (S2), 2 000 mg/kg (S3), respectively in the basal diet. Another dietary treatment were formulated with the combination of CB and SB, 3×10^8 cfu/kg CB+ 1 000 mg/kg SB (CS) in the basal diet. The diet preparation procedures were similar to those previously described in our lab (Ai et al., 2019), dietary ingredients were ground into fines to pass through a 60 mesh sieve, all ingredients in each group were thoroughly mixed in a mixer and prepared particles of 3 mm in diameter. After pelleting, all diets were dried and stored at 4 ℃. The CB count of C0, C1, C2, C3, and CS diets were analyzed and were 0, 4.3×10^7 cfu/kg (C1), 2.5×10^8 cfu/kg (C2), 1.1×10^9 cfu/kg (C3) and 1.2×10^8 cfu/kg(CS), respectively.

2.2. Carp rearing and management

The experiment was carried out in an indoor aquarium with a recirculating aquaculture system, and the water volume of the tank was 100 L. The fish were acclimated for two weeks before the trial and fed the control diet during the acclimated period. After acclimatization and fasting for 24 h, carp fingerlings with approximately 3.74 g in body weight were randomly assigned to 32 aquariums with four replicates per treatment and ten individuals per aquarium. Fish were hand-fed to apparent satiation three times daily at 8:00, 13:00, and 17:00 for eight weeks. During the experiment, one-third of the water in each tank was changed once daily. The water temperature was controlled at 22 ± 1 °C, pH was maintained at 7.0 ± 0.3, the dissolved oxygen was more than 5.0 mg/L, and the NO_2-N and NH_4^+-N concentrations were not more than 0.02 and 0.5 mg/L.

Table 1
Composition and nutrient levels of the basal diet (Dry matter basis).

Ingredients	%
Fish meal	5.00
Soybean meal	39.80
Concentrated soy protein	14.00
Wheat middling	30.00
Fish oil	1.50
Soybean oil	2.50
Phospholipid	1.00
Vitamin premix[1])	0.30
Mineral Premix[2])	0.20
L-Lysine	0.20
DL-Methionine	0.30
L-Threonine	0.20
Choline chloride	0.50
$Ca(H_2PO_4)_2$	2.00
Glucose	0.50
Sodium carboxymethylcellulose	2.00
Total	100.00
Nutrient levels[3])	
Crude Protein	28.40
Ether extract	6.73
Lysine	2.2
Methionine	0.7
Threonine	1.5

The vitamin premix provided the following per kg of the diet：VA 8 000 IU, VC 500 mg, VD3 3 000 IU, VE 60 mg, VK3 5 mg, VB2 30 mg, VB6 15 mg, VB12 0.5 mg, nicotinic acid 175 mg, D-biotin 2.5 mg, inositol 1 000 mg, folic acid 5 mg, pantothenic acid 50 mg.
[2]) The mineral premix provided the following per kg of the diet：Zn 25 mg, Cu 3 mg, Fe 125 mg, Mn 15 mg, I 0.6 mg, Co 0.1 mg, Se 0.4 mg.
[3]) Crude Protein and Ether extract were measured values, while the others were calculated values.

The growth performance data were calculated using the following formula:

Survival Rate (SR, %) = (Nf /Ni)×100;

Weight Gain (WG, %) = (Wt-W0)×100/W0;

Special Growth Rate (SGR, %/d) = (lnWt-lnW0)×100/t;

Feed Conversion Ratio (FCR) = F/(Wt-W0);

Protein Efficiency Ratio (PER, %) = (Wt-W0)×100/(F×P);

Viscerosomatic Index (VSI, %) = Wh×100/Wt;

Condition Factor (CF, g/cm^3) = (Wt/L^3)×100·

Where Nf and Ni are the final and initial numbers of fish, W_t and W_0 were the final and initial body weight (g) of carp, respectively; F was the feed weight (g); P was the crude protein content of the feed (%), t was the duration of experiment days, and L was the body length of fish (cm).

At the end of the feeding, the fish were anesthetized with tricaine methanesulfonate (MS-222). The fish from each aquarium were individually counted and weighed. Then three fish from each aquarium were randomly selected and stored in a refrigerator at − 20 °C to determine the body composition. In addition, another three fish from each aquarium weight and length were measured, blood samples were obtained from the caudal vein and were dissected, the viscerasomatic weights

were measured. Samples for histology (approximately 5 mm) were taken from the foregut, midgut, and hindgut and immediately put into Bouin's fixative solution for 24 h for intestinal histological observations. The remaining segments of the intestine were frozen in liquid nitrogen and stored at − 80 °C for digestive enzyme analysis.

2.3. Proximate composition analysis

The moisture, ash, crude lipid and crude protein contents in diets and whole body were analysed using the method of AOAC (1995). The moisture content was estimated by drying samples to constant weight in an oven at 105 ℃. The ash content was determined by incinerating samples in a muffle furnace at 550 ℃ for 6 hr. The crude protein content was determined by the Dumas method. The lipid was determined by the Soxhlet extraction method.

2.4. Intestine and serum biochemical analysis

The blood samples were centrifuged at 3000 rpm for 10 min at 4 °C and immediately detected using an automatic biochemical analyzer (Beckman ProCX4, USA). The intestine samples were weighed at 4 °C, 0.98% NaCl solution was added, homogenized at 12000 rpm for 2 min by a homogenizer in an ice bath, and then centrifuged at 3500 rpm for 15 min at 4 °C. The supernatant was used to analyze total superoxide dismutase (T-SOD), glutathione(GSH), malondialdehyde (MDA), protease, lipase, amylase, and protein content by using commercial assay kits (Nanjing Jiancheng Institute of Biological Engineering, China) according to the instruction.

2.5. Morphology of the Intestine

The foregut, midgut, and hindgut intestines (1–2 cm) from 3 fish in each group were sampled individually and immersed in Bouin's solution for histology. The intestine was fixed for 24 h and dehydrated using ascending series ethanols (70%, 80%, 90%, and 100%), then transparent with xylene and embedded in paraffin, and sectioned serially along the vertical axis for 5 μm thickness in a microtome (Leica, RM2016, Germany). After staining with hematoxylin-eosin (HE), we observed and photographed under an inverted phase-contrast microscope (Hitachi, Japan). The villus height, villus width, and intestinal wall thickness were quantified within three different fields, randomly visually selected from triplicates in each group.

2.6. Profiling of intestinal microbiota

Profiling of intestinal microbiota was evaluated according to the method described by Poolsawat et al. (2020). Brifely, two fish per tank were dissected to collect intestinal contents from the middle intestine (pooled as one sample) and then stored at − 80 °C. Samples were examined by 16 S rRNA gene sequencing. One OUT was defined as sequence samples greater than 97% similarity in their 16 S rRNA gene sequences and analyzed using the QIIME platform by Shanghai Meiji Biopharmaceutical Technology Company.

2.7. Statistical analysis

All the experimental data were expressed as mean ± standard deviation (SD) and SPSS25.0 for Windows (SPSS Inc., USA) was used for statistical analysis. Differences between groups were subjected to a one-way analysis of variance (ANOVA). If there is a significant difference, data were compared using Duncan's multiple range test. Results were considered statistically significant at the level of $P < 0.05$.

3. Results

3.1. Growth performance and whole-body composition of carp

At the end of the 8-week trial, the survival rate of all groups was 100%, no significant difference was observed in the WG, SGR, FCR, PE, CF, and VSI between control and all dietary treatments (Table 2). The WG and SGR were the highest, and FCR was the lowest in C1 among all groups and was significantly different with C3 group in SGR ($P < 0.05$) among the CB groups ($P < 0.05$), PER in CS group was significantly higher than that of S1 group ($P < 0.05$).

As shown in Table 3, compared with the C0 group, the crude protein content of C2, C3, S3, CS groups and ether extract content of C3, S3, and CS groups were increased significantly ($P < 0.05$). Ash content in S1 and S3 was increased significantly ($P < 0.05$), moisture content was lower in all experimental groups, and the S3 group was decreased significantly ($P < 0.05$). There were no difference in CP, EE, ash and moisture content among the CB and SB groups.

3.2. Serum biochemical index of carps

Data of the serum biochemical indices were shown in Table 4. Compared with the C0 group, the serum GLB content was significantly higher in the experimental groups ($P < 0.05$). The serum TP content of the C1, C2, and CS groups was significantly increased ($P < 0.05$), GLU content in the experimental groups was decreased significantly ($P < 0.05$ or $P < 0.01$). The serum AST activity of the C1 group was significantly higher than that of other groups and reduced considerably in S2, S3 groups ($P < 0.05$), the serum ALT activity was decreased significantly in C1, C2, C3, S2, and S3 ($P < 0.05$). There were no difference in TP, ALB, GLB, ALT and GLU among the CB and SB groups. AST In C1 was significantly increased than C2 and C3 ($P < 0.01$) and there was no difference among the SB groups.

Table 2
Effects of *Clostridium butyricum* and sodium butyrate on the growth performance of carp.

Items	SR (%)	WG (%)	SGR (%/d)	FCR	PER (%)	CF (g/cm^3)	VSI (%)
C0	100 ± 0.0	744.48 ± 36.46^{ab}	4.10 ± 0.08^{ab}	1.53 ± 0.04^{ab}	229.92 ± 6.43^{ab}	2.88 ± 0.07	4.77 ± 0.62
C1	100 ± 0.0	802.06 ± 39.32^{a}	4.23 ± 0.08^{a}	1.47 ± 0.04^{b}	239.03 ± 6.39^{ab}	3.06 ± 0.18	5.05 ± 0.44
C2	100 ± 0.0	773.46 ± 28.22^{ab}	4.17 ± 0.06^{ab}	1.51 ± 0.03^{ab}	232.96 ± 4.92^{ab}	3.07 ± 0.15	5.10 ± 0.33
C3	100 ± 0.0	744.92 ± 29.50^{ab}	4.08 ± 0.07^{b}	1.52 ± 0.04^{ab}	228.65 ± 6.00^{ab}	3.23 ± 0.36	5.58 ± 0.48
S1	100 ± 0.0	737.63 ± 54.18^{b}	4.08 ± 0.13^{b}	1.57 ± 0.08^{a}	225.22 ± 11.44^{b}	3.05 ± 0.18	4.91 ± 0.94
S2	100 ± 0.0	751.56 ± 36.21^{ab}	4.12 ± 0.08^{b}	1.56 ± 0.05^{ab}	226.37 ± 7.53^{ab}	3.15 ± 0.60	5.65 ± 0.94
S3	100 ± 0.0	794.85 ± 15.80^{ab}	4.21 ± 0.03^{ab}	1.49 ± 0.04^{ab}	236.70 ± 5.83^{ab}	2.84 ± 0.24	5.87 ± 2.15
CS	100 ± 0.0	786.36 ± 44.73^{ab}	4.19 ± 0.10^{ab}	1.48 ± 0.09^{ab}	239.67 ± 14.78^{a}	3.17 ± 0.10	4.76 ± 1.51

In the same column, values with no letter or the same letter superscripts mean no significant difference (P > 0.05), while with different small letter superscripts mean significant difference (P < 0.05).
Abbreviations: SR: Survival Rate; WG: Weight Gain; SGR: Special Growth Rate; FCR: Feed Conversion Ratio; PER: Protein Efficiency Ratio; CF: Condition Factor; VSI: Viscerosomatic Index.

Table 3
Effects of *Clostridium butyricum* and sodium butyrate on the body composition of carp (fresh-weight basis).

Items	CP (%)	EE (%)	ASH (%)	Moisture (%)
C0	17.23 ± 0.86^{b}	2.84 ± 0.57^{b}	2.41 ± 0.17^{c}	75.18 ± 1.85^{a}
C1	19.20 ± 1.05^{ab}	3.77 ± 0.44^{ab}	2.44 ± 0.18^{c}	73.37 ± 1.61^{ab}
C2	20.13 ± 1.16^{a}	3.72 ± 0.19^{ab}	2.54 ± 0.09^{bc}	71.89 ± 1.47^{ab}
C3	19.62 ± 1.00^{a}	3.98 ± 0.24^{a}	2.63 ± 0.17^{abc}	71.78 ± 1.35^{ab}
S1	19.09 ± 1.37^{ab}	3.76 ± 0.50^{ab}	2.97 ± 0.17^{a}	72.17 ± 2.25^{ab}
S2	19.10 ± 1.08^{ab}	3.56 ± 1.05^{ab}	2.77 ± 0.14^{abc}	73.60 ± 2.02^{ab}
S3	20.75 ± 1.78^{a}	3.88 ± 0.46^{a}	2.94 ± 0.55^{ab}	71.09 ± 3.35^{b}
CS	19.81 ± 1.82^{a}	3.87 ± 0.69^{a}	2.78 ± 0.28^{abc}	71.86 ± 1.88^{ab}

In the same column, values with no letter or the same letter superscripts mean no significant difference (P > 0.05), while with different small letter superscripts mean significant difference (P < 0.05).
Abbreviations: CP: crude protein; EE: Ether extract.

Table 4
Effects of *Clostridium butyricum* and sodium butyrate on serum biochemical indices of carp.

Items	TP /(g/L)	ALB /(g/L)	GLB /(g/L)	ALT /(U/L)	AST /(U/L)	GLU /(mmol/L)
C0	25.58 ± 7.31^{b}	15.32 ± 2.91	10.27 ± 4.77^{b}	8.75 ± 1.50^{ab}	175.50 ± 25.04^{bc}	3.80 ± 1.13^{a}
C1	30.47 ± 1.48^{a}	15.32 ± 1.91	15.15 ± 0.62^{a}	8.00 ± 1.15^{abc}	230.50 ± 44.40^{a}	1.40 ± 0.22^{bc}
C2	30.41 ± 0.91^{a}	16.37 ± 1.00	14.05 ± 0.79^{a}	9.00 ± 0.82^{a}	155.75 ± 29.20 ^{cd}	1.78 ± 0.79^{bc}
C3	29.12 ± 3.12^{ab}	15.15 ± 0.91	13.97 ± 2.58^{a}	7.50 ± 3.32^{abc}	142.50 ± 19.81 ^{cd}	0.98 ± 0.87^{c}
S1	29.33 ± 3.95^{ab}	15.15 ± 2.80	14.18 ± 2.05^{a}	6.00 ± 0.82^{bc}	153.25 ± 18.17 ^{cd}	2.05 ± 0.37^{b}
S2	28.58 ± 2.08^{ab}	14.57 ± 1.90	14.02 ± 1.41^{a}	5.75 ± 1.50^{c}	139.50 ± 21.24 ^{cd}	2.05 ± 0.60^{b}
S3	29.33 ± 1.88^{ab}	15.35 ± 2.01	13.98 ± 1.29^{a}	5.50 ± 2.38^{c}	132.75 ± 12.61^{d}	2.35 ± 0.44^{b}
CS	31.07 ± 3.49^{a}	15.57 ± 0.79	15.33 ± 0.88^{a}	8.75 ± 0.50^{ab}	202.75 ± 17.04^{ab}	0.98 ± 0.15^{c}

In the same column, values with no letter or the same letter superscripts mean no significant difference (P > 0.05), while with different small letter superscripts mean significant difference (P < 0.05).
Abbreviations: TP: Total Protein; ALB: Albumin; GLB: Globulin; ALT: Alanine Transaminase; AST: Aspartate Transaminase; GLU: Glucose.

3.3. Antioxidant index of carps

Data on the T-SOD, GSH activity, and MDA content of the intestine were shown in Table 5. The intestinal T-SOD and GSH activity in the foregut, midgut, and hindgut of carp were increased when supplemented with different levels of CB, SB, and both ($P < 0.05$ or $P < 0.01$) except GSH of Midgut, Hindgut in C1, Midgut in S1 group and T-SOD of foregut, hindgut in C1, hindgut in S1. The intestinal MDA content was decreased when supplemented with different levels of CB and SB in C2, C3 group and S2, S3 group ($P < 0.05$ or $P < 0.01$) except S3 group of the hindgut, MDA content of C1 and CS group in foregut were decreased too ($P < 0.05$). Compared with C1, T-SOD and GSH of the foregut, midgut, and hindgut were significantly increased and MDA decreased ($P < 0.05$) in C2 and C3. Compared with S1, T-SOD and GSH of the foregut, midgut, and hindgut were significantly increased and MDA decreased ($P < 0.05$) in S2 and S3 except T-SOD of foregut in S3, MDA of Midgut in S2,S3 and hindgut in S3, GSH of hindgut in S2.

3.4. Intestinal digestive enzyme activity of carps

The results of the digestive enzyme activity of the intestine were shown in Fig. 1. Compared with the C0, protease activity in C2, C3, and S2, S3 groups of foregut and midgut were significantly increased ($P < 0.05$ or $P < 0.01$), the protease activities of all groups in hindgut were not different ($P > 0.05$). The lipase activity of C2, C3 groups in foregut, midgut, and hindgut, S2, S3, CS group in the foregut, S3 group in midgut were significantly increased ($P < 0.05$). Amylase activity in the midgut of S2 group was increased significantly ($P < 0.05$). In contrast, the amylase activity of other groups in the foregut, midgut, and hindgut was not different ($P > 0.05$). Compared with C1, protease of foregut and midgut in C2 and C3, lipase of midgut in C2 and C3, hindgut in C2 were significantly increased ($P < 0.05$). Compare with S1, protease of foregut and midgut in S2 and S3, lipase of foregut in S3, amylase of midgut in S2 were significantly increased ($P < 0.05$).

3.5. Intestinal morphology

Compared with the C0 group, the villus height of foregut and midgut was higher in all experimental groups, and the foregut of S2, S3, and midgut of C1, C3 groups, hindgut of S3 was significantly increased ($P < 0.05$) (Table 6). Villus width of foregut, hindgut in group S2, midgut in group S3 was significantly increased ($P < 0.05$). The intestinal wall thickness of the S2 group in the foregut and hindgut and CS group in

Table 5
Effects of *Clostridium butyricum* and sodium butyrate on intestinal antioxidant indices of carp (U/mg prot) .

Items	T-SOD			MDA			GSH		
	Foregut	Midgut	Hindgut	Foregut	Midgut	Hindgut	Foregut	Midgut	Hindgut
C0	82.89 ± 5.57^{c}	74.73 ± 2.54^{c}	89.55 ± 4.10^{c}	1.75 ± 0.09^{a}	1.30 ± 0.05^{a}	1.59 ± 0.06^{a}	33.68 ± 3.16^{c}	28.42 ± 2.36^{c}	34.91 ± 2.54^{c}
C1	88.68 ± 7.80^{c}	81.04 ± 1.68^{b}	93.52 ± 1.55^{c}	1.60 ± 0.07^{b}	1.24 ± 0.06^{a}	1.59 ± 0.13^{a}	38.00 ± 4.37^{b}	30.08 ± 4.81^{bc}	36.77 ± 3.04^{c}
C2	103.63 ± 5.16^{b}	96.23 ± 4.71^{a}	113.17 ± 2.64^{ab}	1.35 ± 0.06^{c}	1.07 ± 0.06^{c}	1.29 ± 0.07^{b}	44.33 ± 6.91^{a}	39.59 ± 6.54^{a}	46.38 ± 4.26^{ab}
C3	105.98 ± 6.68^{b}	102.48 ± 3.54^{a}	111.88 ± 3.83^{ab}	1.44 ± 0.09^{c}	1.03 ± 0.12^{c}	1.24 ± 0.05^{b}	45.56 ± 3.03^{a}	40.20 ± 4.48^{a}	46.63 ± 2.82^{a}
S1	101.93 ± 3.11^{b}	84.98 ± 4.92^{b}	94.65 ± 4.11^{c}	1.66 ± 0.06^{ab}	1.21 ± 0.05^{ab}	1.51 ± 0.02^{a}	39.02 ± 1.18^{b}	31.83 ± 2.61^{bc}	41.45 ± 1.91^{b}
S2	116.00 ± 1.97^{a}	99.88 ± 5.07^{a}	114.82 ± 3.26^{a}	1.40 ± 0.06^{c}	1.10 ± 0.14^{bc}	1.28 ± 0.08^{b}	45.72 ± 2.59^{a}	39.41 ± 5.84^{a}	42.88 ± 3.97^{ab}
S3	108.58 ± 4.00^{b}	99.99 ± 5.85^{a}	111.63 ± 2.95^{ab}	1.40 ± 0.06^{c}	1.11 ± 0.05^{bc}	1.49 ± 0.06^{a}	42.91 ± 2.49^{a}	38.49 ± 4.27^{a}	46.64 ± 3.90^{a}
CS	106.70 ± 2.54^{b}	86.23 ± 4.61^{b}	108.62 ± 3.65^{b}	1.57 ± 0.05^{b}	1.25 ± 0.08^{a}	1.44 ± 0.15^{a}	41.57 ± 4.59^{a}	34.94 ± 3.69^{ab}	42.02 ± 3.15^{ab}

In the same column, values with no letter or the same letter superscripts mean no significant difference (P > 0.05), while with different small letter superscripts mean significant difference (P < 0.05).
Abbreviations:T-SOD:Total Superoxide Dismutase; MDA: malondialdehyde; GSH::Glutathione.

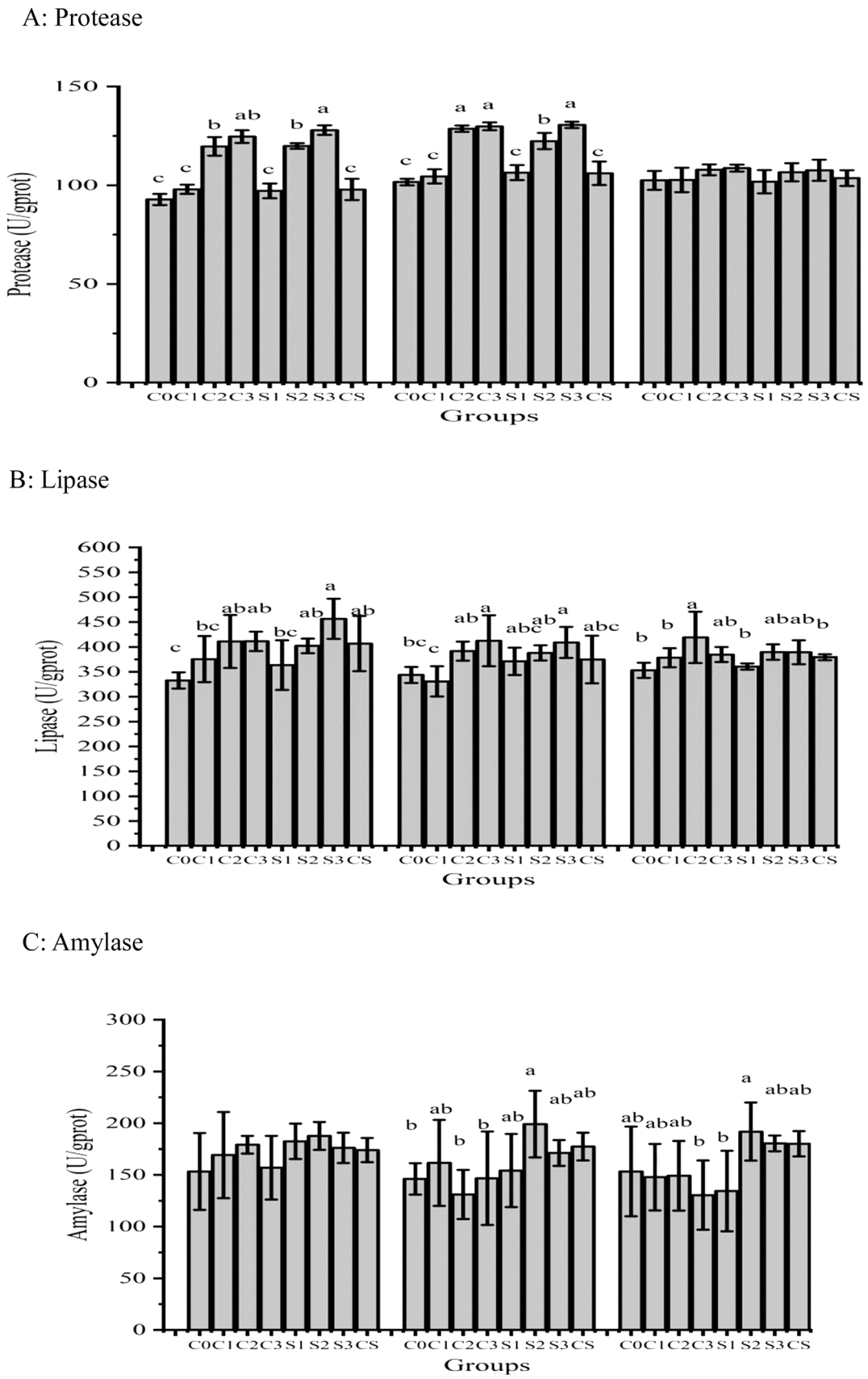

Fig. 1. Effects of *Clostridium butyricum* and sodium butyrate on intestinal digestive enzyme of carp U/g prot In the same row, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$).

Table 6
Effects of *Clostridium butyricum* and sodium butyrate on intestinal morphology of carp (μm) .

Items	Villus height			Villus width			Intestinal wall thickness		
	Foregut	Midgut	Hindgut	Foregut	Midgut	Hindgut	Foregut	Midgut	Hindgut
C0	356.14 ± 104.25^{b}	267.81 ± 14.56^{b}	255.96 ± 35.40^{b}	56.42 ± 2.36^{b}	50.64 ± 3.62^{b}	61.66 ± 5.66^{b}	31.43 ± 10.36^{b}	28.79 ± 6.13^{ab}	25.45 ± 4.81^{c}
C1	416.36 ± 78.77^{ab}	339.23 ± 26.70^{a}	317.94 ± 5.55^{b}	70.99 ± 17.75^{ab}	58.45 ± 7.39^{ab}	57.80 ± 5.21^{b}	36.48 ± 11.35^{ab}	25.17 ± 6.58^{b}	23.07 ± 10.74^{c}
C2	418.50 ± 110.10^{ab}	318.07 ± 25.22^{ab}	276.17 ± 59.64^{b}	60.62 ± 10.28^{ab}	55.94 ± 2.78^{ab}	65.57 ± 3.15^{b}	40.25 ± 11.38^{ab}	29.00 ± 3.30^{ab}	32.62 ± 10.41^{bc}
C3	420.04 ± 31.17^{ab}	346.79 ± 9.72^{a}	256.73 ± 55.00^{b}	63.61 ± 10.91^{ab}	55.92 ± 2.05^{ab}	57.98 ± 5.91^{b}	38.02 ± 3.36^{ab}	28.85 ± 1.9^{ab}	31.82 ± 8.87^{bc}
S1	416.89 ± 38.66^{ab}	254.61 ± 54.90^{b}	309.23 ± 16.56^{b}	65.22 ± 0.73^{ab}	51.94 ± 8.52^{ab}	57.95 ± 3.86^{b}	38.46 ± 4.21^{ab}	26.18 ± 4.72^{b}	27.39 ± 3.23^{c}
S2	507.51 ± 25.80^{a}	266.56 ± 14.60^{b}	559.38 ± 57.54^{a}	70.97 ± 11.03^{a}	55.92 ± 8.12^{ab}	78.51 ± 12.69^{a}	48.04 ± 5.14^{a}	27.58 ± 7.12^{b}	54.32 ± 4.65^{a}
S3	488.59 ± 64.67^{a}	311.09 ± 74.45^{ab}	295.55 ± 44.80^{b}	61.58 ± 9.00^{ab}	61.95 ± 9.76^{a}	59.22 ± 10.26^{b}	39.07 ± 12.59^{ab}	36.55 ± 6.42^{a}	34.88 ± 7.56^{bc}
CS	463.53 ± 13.49^{ab}	294.34 ± 53.30^{ab}	280.67 ± 11.24^{b}	65.38 ± 9.38^{ab}	52.97 ± 5.13^{ab}	56.87 ± 0.39^{b}	45.28 ± 8.39^{ab}	31.37 ± 2.71^{ab}	43.06 ± 5.15^{b}

In the same column, values with no letter or the same letter superscripts mean no significant difference (P > 0.05), while with different small letter superscripts mean significant difference (P < 0.05).

the hindgut were significantly increased ($P < 0.05$). There were no difference in villus height, villus width and intestinal wall thickness among CB group. Compared with S1, villus height, villus width and intestinal wall thickness of hindgut in S2, intestinal wall thickness of midgut in S3 were significantly increased ($P < 0.05$).

3.6. The intestinal microbiota of carp

The coverage of each group has reached more than 99.9%, which indicates that the sequencing quantity of the sample can fully reflect the microbial situation in the intestinal contents of carp (Table 7). Analysis of Chao1 index showed no significant difference between different groups, and analysis of Simpson showed that the community diversity was not changed by CB and SB ($P > 0.05$). In other words, CB and SB administration did not significantly alter the abundance and α-diversity of the gut microbiota.

As showed in Fig. 2.A., at the phylum level, the gut microbiota mainly contained four phyla: *Fusobacteria*, *Bacteroidetes*, *Firmicutes,* and *Proteobacteria*. The dominant bacteria in each group were *Fusobacteria*. The relative abundance of *Fusobacteria* decreased in the group that supplemented with SB. The relative abundance of *Proteobacteria* was increased in all experimental groups, and the relative abundance of *Bacteroides* increased in the C2, C3, and S1, S3 groups. The gut microbiota's structure in the CS group has changed; *Actinobacteria*, *Verrucomicrobia*, *Saccharibacteria*, *Chloroflexi,* and *Cyanobacteria* were found. At the genus level (Fig. 2 B), *Cetobacterium* was the most prevalent in all groups. The proportion of *Cetobacterium* decreased, *Bacteroides* increased in groups S1 and S3 compared with C0, *Erysipelotrichacese* decreased in C2, C3, and CS groups. Many genera, which included *Legionella, Candidatus, Saccharibacteria, Cyanobacteria, Citrobacter,* were found in the CS group.

Table 7
The α diversity of bacterial 16 S rRNA gene in intestinal contents of carp.

Items	Chao1	shannon	coverage
C0	94.80 ± 14.46	0.32 ± 0.13	99.96 ± 0.02
C1	103.05 ± 19.22	0.51 ± 0.04	99.96 ± 0.01
C2	99.98 ± 11.88	0.44 ± 0.28	99.94 ± 0.03
C3	96.79 ± 18.59	0.37 ± 0.19	99.94 ± 0.01
S1	84.34 ± 18.40	0.81 ± 0.23	99.95 ± 0.02
S2	108.44 ± 16.07	0.70 ± 0.14	99.95 ± 0.03
S3	95.80 ± 17.30	0.91 ± 0.21	99.98 ± 0.01
CS	96.05 ± 16.94	0.57 ± 0.13	99.95 ± 0.03

4. Discussion

Intestine is important for the digestive and immune organs, and its health is essential for its growth performance. It was found that diets with more than 20% soybean meal (SBM) inclusion level fed to mirror carp demonstrated inflammatory response in the distal gut segment of this omnivorous species (Urań et al., 2008). Similarly, replacing fish meal with a high proportion of soybean meal can affect the inflammatory status of the intestinal mucosa of rainbow trout (Sealey et al., 2009). Therefore, it is important to protect intestinal health when feeding fish with high level soybean meal in the diet. Butyrate can promote intestinal mucosal development and plays an important role in intestinal health. Previous experiments have illustrated that dietary supplementation with CB could improve growth performance and enhance intestine health in *Macrobrachium rosenbergii* (Sumon et al., 2018), *Litopenaeus vannamai* (Li et al., 2019a, 2019b), Pacific white shrimp *Penaeus vannamei* (Luo et al., 2021), *Miichthys miiuy* (Song et al., 2006), Large Yellow Croaker *Larimichthys crocea* (Yin et al., 2021) and tilapia (Ahmed and Sadek, 2014; Poolsawat et al., 2020; Zhang et al., 2020). Similarly, researchers have reported that dietary SB showed significant improvement of gut health and growth performance of Nile tilapia (Ahmed and Sadek, 2014; Abdel-Tawwab et al., 2021), European sea bass *Dicentrarchus labrax* (Rimoldi et al., 2016), yellow drum (Wu et al., 2020), black seabream (Ullah et al., 2020), yellow catfish (Zhao et al., 2021). Nevertheless, the WGR, FCR, PE, CF, and VSI were not different significantly by supplementation of CB and SB in this study. This finding was consistent with previous studies of CB on carp (Meng et al., 2021) and SB on rainbow trout *Oncorhynchus mykiss* (Gao et al., 2011), common carp (Liu et al., 2014). similar results were also found in previous studies. Supplementation 0.2% SB did not affect growth performance of European sea bass (Rimoldi etal, 2016) and largemouth bass (*Micropterus salmoides*) (Chen et al., 2021) fed with a high soybean meal diet.

It was hypothesized that the growth performance was associated with digestive enzyme activity and small intestinal mucosa morphology. It is also important to note that the villus height and digestive enzyme in the intestine in this study were increased with CB and SB dietary treatment compared to the control. Previous study have indicated that CB treatment could improve intestinal barrier function, boost digestion and absorption, and gut microflora (Liao et al., 2015; Song et al., 2006; Poolsawat et al., 2020; Yin et al., 2021). Similar findings were reported by Dawood et al. (2019), who found that dietary supplementation of Nile tilapia with SB improved villus height and width in a different section of the intestinal tract. Chen et al. (2021) indicated that

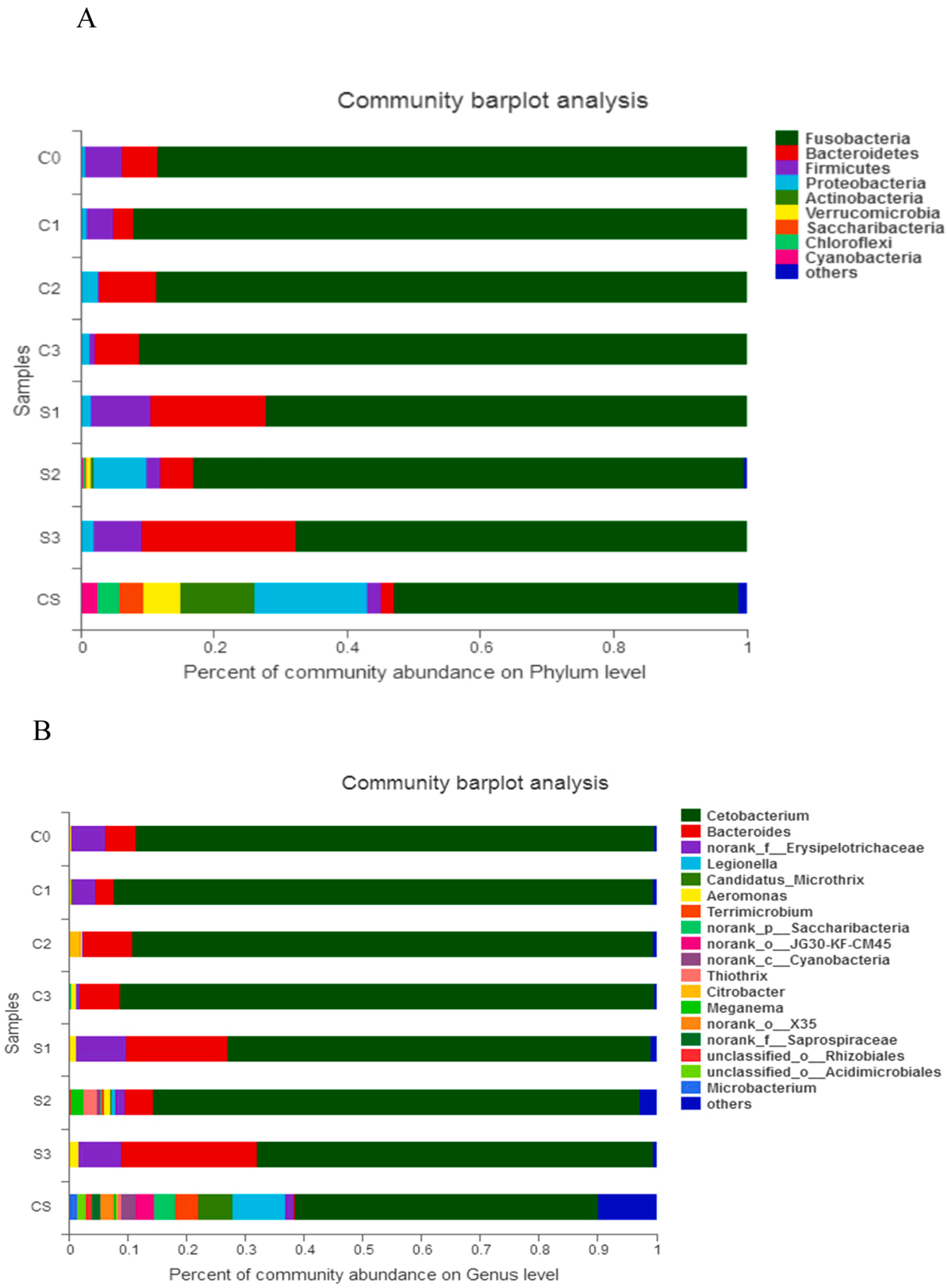

Fig. 2. Bacterial composition at the phylum and genus levels of different groups. A: The bacterial compositions at the phylum level. B: The bacterial compositions at the genus level.

supplementing the soybean meal diet with 0.2% SB improved the intestinal morphology and reduced gut oxidative stress and the expression levels of pro-inflammatory cytokines (IL-1β and TNF-1α) of largemouth bass. Nile tilapia fed SB and *Spirulina platensis* enriched diets showed an increase in villi length, villi width, crypt depth, villi surface in the anterior and middle part of the fish intestine (Shalata et al., 2021). Butyric acid is a major respiratory fuel (Topping et al., 2001), trophic to the intestine, exerts proliferative effects on intestinal cells, enhancing epithelial cell proliferation and differentiation and subsequently improving intestinal absorptive function (Canani et al., 2011). The mechanism by which CB improves immunity and intestine healthy is considered through butyric acid, a kind of short-chain fatty acids, which can provide energy and promote the proliferation, and maturation of intestinal epithelium (Duan et al., 2017; Meng et al., 2021), maintain epithelial cell in gut (Junghare et al., 2012).

The CP and EE composition of carp were increased significantly by supplementation of CB and SB, which contradicts the previous study by Poolsawat et al. (2019) in tilapia *Oreochromis niloticus*×*O. aureus* fed with CB and by Ullah et al. (2020) in black sea bream *Acanthopagrus schlegelii* provided with SB. The ash composition in the SB group increased in the present study showing an improvement in the bioavailability of minerals such as calcium, phosphorous, and other elements by SB supplementation. Moreover, dietary supplementation of CB and SB in the diets demonstrated improvement in the bioavailability of nutrient substances in this study. Therefore, CB and SB have similar effects on the host's digestion and absorption, thus creating positive effects on growth performance and feed utilization.

It is hypothesized that CB and SB supplementation could improve the antioxidant ability and increase the nonspecific immune function of mirror carp. Plasma GLB is the most important immune substance in the plasma (Jha et al., 2007). In this study, the serum GLB content was significantly higher in CB and SB treatments. This observation is similar to the previous findings that IgM of Black sea bream has significantly increased in the SB supplemented diet (Ullah et al., 2020). In addition, the intestinal T-SOD and GSH activity in the foregut, midgut, and hindgut of carp were increased, and the intestinal MDA content was decreased when supplemented with different CB and SB levels. This was consistent with previous findings that demonstrated CB-supplemented diets could improve the antioxidant ability in Pacific white shrimp (Duan et al., 2017), *Macrobrachium rosenbergii* (Wangari et al., 2020), and tilapia (Li et al., 2019b; Zhang et al., 2020). Similarly, previous studies reported that SB significantly increases the antioxidant enzyme activity of black sea bream (Ullah et al., 2020) and rice field eel *Monopterus albus* (Zhang et al., 2020). AST and ALT could be utilized as a marker to detect any toxic effects which may cause hepatic dysfunction (Abdel-Tawwab et al., 2021). No significant differences in AST and ALT activities when fed with diets supplemented with CB on *Macrobrachium rosenbergii* (Wangari et al., 2020) and SB nanoparticles on tilapia (Abdel-Tawwab et al., 2021). In this study, the serum AST activity decreased in the S3 group, and ALT activity decreased in S2 and S3. These results indicated an improvement in fish health when fed SB enriched diets. Similar result was recorded in Nile tilapia fed with SB and *Spirulina platensis* by Shalata et al. (2021).

The gut microbiota play a central role in the host's digestive function, gastric development, mucosal tolerance, immunity, and disease resistance (Chen et al., 2021). Supplementation with soybean meal often reduces intestinal microbial diversity (Liu et al., 2019). The administration of probiotics and prebiotics can regulate the gut microbiota. Supplementation of SB in a high soybean meal diet had beneficial effects on the gut microbiota of largemouth bass (Chen et al., 2021). The research on tilapia showed that dietary CB significantly increased the alpha diversity and composition of intestinal microbiota (Li et al., 2019b). In this study, the administration of CB and SB could modulate the gut microbiota at the phylum level. The relative abundance of *Fusobacteria* decreased in the group supplemented with SB, and the relative abundance of *Proteobacteria* was increased in all experimental groups. The gut microbiota at the genera level was modulated in this study, the proportion of *Cetobacterium* decreased, *Bacteroides* increased in groups S1 and S3 groups, and *Erysipelotrichacese* decreased in C2, C3, and CS groups. Thus, they are consistent with previous experimental data on carp (Meng et al., 2021). Poolsawat et al. (2020) also found a significant enhancement in the relative abundance of the phyla *Planctomycetes*, *Proteobacteria,* and *Chloroflexi* in CB supplementation on tilapia. Dietary supplementation with SB increased the relative abundance of *Bacteroides*, *Lachnospiraceae_unclassified*, *Lachnospiraceae_uncultured, Lactobacillus*, and *Peptococcus* of largemouth bass, which could partially account for the amelioration of gut inflammation of fish fed high soybean meal (Chen et al., 2021).

The amount of CB and SB will have a great impact on the growth performance, immunity and intestinal health. The growth performance were not different but the antioxidant capacity and digestive enzyme activity increased with increasing addition in our experiment. This finding was consistent with previous studies. The supplementation of CB at (1.7–3.1 $\times 10^8$ CFU/kg feed) in the diet promoted the growth performance, feed utilization, gut health and microbiota community of tilapia (Poolsawat et al., 2020). But some studies suggest that higher additions are better effective. 10^{11} and 10^{12} cfu/kg CB could significantly improve the growth performance, immunity capacity and resistance against *V. parahaemolyticus* of *L. vannamei*, and have a positive effect on the intestinal morphological structure (Li et al., 2019a). Dietary CB could improve growth performance of large yellow croaker larvae probably through promoting intestinal development, improving immune enzyme activities, and modulating gut microbiota, the optimal CB supplementation dosage is 5–10 $\times$ 10^9 CFU g^{-1} (Yin et al., 2021). For SB, the growth performance were not different but the gut structures, digestive enzyme activity, and antioxidant activity in the 1 000 mg/kg SB were better among all dietary treatments. Similar results were demonstrated by Zhao et al. (2020) that addition of SB in diets of yellow catfish in the range of 500–1 000 mg/kg is recommended and significantly increased weigh gain, feed efficiency, digestive tract health. But Rimoldi et al. (2016) who fed European sea bass with a high percentage of soybean meal supplemented with 2 000 mg/kg SB. Consider the cost and the application effect, the recommended addition amount of SB and CB is 3 $\times$ 10^8 cfu/kg and 1000 mg/kg.

CB could produce butyric acid that can maintain gastrointestinal health, and could produce a wide range of relevant digestive enzymes (amylase, lipase and protease) which improve the digestibility, produce a lot of essential vitamin B (Ahmed and Sadek, 2014). If the combined use of CB and SB could produce even stronger effects need to study. The highest (weight gain, specific growth rate, and FCR) of Nile tilapia were obtained with feeding diet containing SB plus *enteroccus faecum* (Ahmed and Sadek, 2014). But no additive effect on growth performance, gut structures, digestive enzyme activity, and antioxidant activity of using both CB and SB was found in our study, therefore, simultaneous use is not recommended. Furthermore, the results showed that dietary supplementation of CB and SB combined changed gut microbiota structure clearly; in phylum level, *Actinobacteria*, *Verrucomicrobia*, *Saccharibacteria*, *Chloroflexi, Cyanobacteria,* and genus level, *Legionella, Candidatus, Saccharibacteria* were found. However, the mechanism of the synergistic effect of CB and SB on gut microbiota was limited in aquatic animals due to its complications, and further studies is warranted.

5. Conclusion

In summary, CB and SB could promote the body protein and EE content, intestine digestive enzyme activity, and intestinal morphology of carp when fed a high soybean meal diet. Moreover, CB or SB administration modulated gut flora in carp, especially when administrated them both together.

CRediT authorship contribution statement

Yi Du: Feeding, samples analysis, statistical analysis, data interpretation, manuscript drafting; Long Cheng: samples analysis, statistical analysis, manuscript drafting; Jianhua Zhao: samples analysis; Clement R. de Cruz: manuscript revision; Hong Xu: samples analysis; Liansheng Wang: conception, and manuscript revision; Qiyou Xu: conception, manuscript drafting, and manuscript revision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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