RESEARCH ARTICLE

The effect of dietary supplementation with *Clostridium butyricum* on the growth performance, immunity, intestinal microbiota and disease resistance of tilapia (*Oreochromis niloticus*)

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Abstract

This study was conducted to assess the effects of dietary *Clostridium butyricum* on the growth, immunity, intestinal microbiota and disease resistance of tilapia (*Oreochromis niloticus*). Three hundreds of tilapia (56.21 ± 0.81 g) were divided into 5 groups and fed a diet supplemented with *C. butyricum* at 0, 1×10^4, 1×10^5, 1×10^6 or 1×10^7 CFU g^{-1} diet (denoted as CG, CB1, CB2, CB3 and CB4, respectively) for 56 days. Then 45 fish from each group were intraperitoneally injected with *Streptococcus agalactiae*, and the mortality was recorded for 14 days. The results showed that dietary *C. butyricum* significantly improved the specific growth rate (SGR) and feed intake in the CB2 group and decreased the cumulative mortality post-challenge with *S. agalactiae* in the CB2, CB3 and CB4 groups. The serum total antioxidant capacity and intestinal interleukin receptor-associated kinase-4 gene expression were significantly increased, and serum malondialdehyde content and diamine oxidase activity were significantly decreased in the CB1, CB2, CB3 and CB4 groups. Serum complement 3 and complement 4 concentrations and intestinal gene expression of tumour necrosis factor α, interleukin 8, and myeloid differentiation factor 88 were significantly higher in the CB2, CB3 and CB4 groups. Intestinal toll-like receptor 2 gene expression was significantly upregulated in the CB3 and CB4 groups. Dietary *C. butyricum* increased the diversity of the intestinal microbiota and the relative abundance of beneficial bacteria (such as *Bacillus*), and decreased the relative abundance of opportunistic pathogenic bacteria (such as *Aeromonas*) in the CB2 group. These results revealed that dietary *C. butyricum* at a suitable dose enhanced growth performance, elevated humoral and intestinal immunity, regulated the intestinal microbial components, and improved disease resistance in tilapia. The optimal dose was 1×10^5 CFU g^{-1} diet.

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Introduction

In recent years, fish has increased in importance as a food source for humans, and aquaculture has rapidly developed [1]. With the expansion of the fish aquaculture industry, fish diseases have increased, resulting in serious economic losses. Antibiotics have been extensively used to prevent and control bacterial diseases [2]. However, the extensive usage of antibiotics may cause some negative effects, such as the emergence of antibiotic-resistant bacteria and antibiotic residues, which may affect the sustainable development of aquaculture and human health [3]. As an alternative, probiotics have received considerable attention in recent years [4]. Studies have shown that probiotics can improve growth [5–10], enhance the immune reponse [11–13], and improve disease resistance in fish [5,6,8–10,13,14]. *Clostridium butyricum*, a typical butyric acid bacterium, is a probiotic that occurs in the intestine of healthy humans [15] and other animals [16] and has been used in a wide range of human and veterinary intestinal diseases [17]. In aquatic animals, studies showed that survival post-challenge with pathogenic bacteria was significantly increased in Chinese drum (*Miichthys miiuy*) [18] and Pacific white shrimp (*Litopenaeus vannamai*) [19,20] fed a diet supplemented with *C. butyricum* for 30 and 42 days, respectively. Short-term supplementation with *C. butyricum* also improved the disease resistance of rainbow trout [21] and gibel carp [22]. Tilapia is the second most farmed fish worldwide [23]. To our knowledge, there are few studies about the effect of dietary *C. butyricum* on tilapia.

Fish disease resistance has been found to be associated with immunity [24,25]. The intestine is an important immune organ [25]. The intestinal immune response is related to inflammation mediated by cytokines [26]. Studies have shown that probiotics upregulate the gene expression of interleukin 8 (*IL-8*) in the intestine of rainbow trout (*Oncorhynchus mykiss*) [27] and grouper *(Epinephelus coioides)* [28] and that of tumour necrosis factor α (*TNF-a*) in the of intestine hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) [29]. However, no studies have examined the effect of *C. butyricum* on cytokines in fish intestine. In HT-29 human colonic epithelial cells, *C. butyricum* improved the gene expression of the pro-inflammatory cytokines *TNF-α* and *IL-8* [17,30] and the anti-inflammatory cytokine *-IL-10* [17,31]. *C. butyricum* improved *IL-6* and *IL-8* gene expression in interstitial cells of Cajal (ICCs) [32]. Toll-like receptor (*TLR*) signalling pathways play an important role in the recognition of probiotics and activation of the intestinal immune system in mammals [28]. *C. butyricum* induced macrophages in inflamed mucosa producing IL-10 to prevent acute experimental colitis via the *TLR2*/Myeloid differentiation factor 88 (*MyD88*) signalling pathway [33]. *TLR2* silencing alleviated *C. butyricum*-induced *IL-6* and *IL-8* expression and significantly inhibited *C. butyricum*-induced cell viability in ICCs [32]. *C. butyricum* upregulated *TLR2* gene expression in HT-29 human colonic epithelial cells [17] and upregulated the gene expression of *TLR2* and *MyD88* in the intestine of weaning rex rabbits [34]. However, the roles of fish TLRs in the C. butyricum-induced intestinal immune response have not been reported. In grouper (*Epinephelus coioides*), the probiotic *Psychrobacter sp*. SE6 upregulated the gene expression of *TLR2* and *MyD88* in the intestine [28], which suggests that *TLR2* signalling may play a key role in the modulation of intestinal immunity by *C. butyricum*.

The intestinal structural integrity is the foundation of intestinal immunity in fish [35]. The integrity of intestinal mucosa cells is reflected in serum diamine oxidase (DAO) activity [36]. Tight junction proteins are critical for maintaining the intercellular structural integrity of the intestine in mice [37,38]. A previous study found that dietary supplementation with *C. butyricum* decreased serum DAO in broiler chickens [39] and piglets [40]. Dietary *C. butyricum* upregulated the protein expression levels of *ZO-1*, *claudin-3*, and *occludin* in the intestines of weaned piglets [40] and the gene expression levels of *ZO-1* and *occludin* in the ileum and colon

of weaning rex rabbits [34]. To date, no reports have addressed the effect of *C. butyricum* on DAO gactivity and tight junction proteins in fish. Intestinal tissue showed close intestinal epithelium connections and healthy morphology in kuruma shrimp (*Marsupenaeus japonicas*) [41] and Pacific white shrimp (*Litopenaeus vannamei*) [42] fed diets supplemented with *C. butyricum*, suggesting that *C. butyricum* might affect intestinal structural integrity in fish, which needs to be further studied.

The intestinal microbiome is part of the host and interacts with the host immune system [43]. Studies revealed some disease association with dysbiosis or abnormal composition of the intestinal microbiome [44]. Studies have shown that *C. butyricum* can modify the intestinal microbiota. In vitro, *C. butyricum* inhibited the growth and adherence of potential pathogens to fish intestinal epithelial cells [45,46]. The potential intestinal pathogens were decreased, and the beneficial bacteria were increased in response to dietary *C. butyricum* in *Litopenaeus vannamei* [47], broiler chickens [48,49] and laying hens [50], and mice [16]. These data suggest that *C. butyricum* might affect the intestinal microbiome in fish, which needs to be further studied.

Therefore, the aim of the present study was to evaluate the effects of dietary supplementation with *C. butyricum* on growth performance and disease resistance in tilapia and the underlying mechanisms involved.

Materials and methods

Bacterial strains

C. butyricum (China Center for Type Culture Collection accession NO. M2014537) used in this experiment was obtained from Sun HY Bio Co., Ltd., Wuhan, China. *Streptococcus agalactiae* was kindly provided by Guangdong Ocean University, Zhanjiang, China.

Diets and feeding management

C. butyricum was cultured with reinforced Clostridium medium (RCM) (tryptone 10 g, beef extract 10 g, yeast extract 3 g, glucose 5 g, soluble starch 1 g, sodium chloride 5 g, sodium acetate 3 g, L-cysteine hydrochloride 0.5 g, distilled water 1000 mL) at 37°C for 24 h under anaerobic conditions. After cultivation, the bacteria were collected by centrifugation and resuspended in sterile saline at a concentration of 1×10^9 CFU g^{-1}. The bacterial cell quantity was examined using the spread RCM plate count method. The formulation of the basal diet is presented in Table 1. The bacterial suspension was gently sprayed on the basal diet, followed by thorough mixing, at five concentrations: 0 (control group), 1×10^4, 1×10^5, 1×10^6 and 1×10^7 CFU g^{-1} diet (denoted as CG, CB1, CB2, CB3 and CB4, respectively). The experimental diets were pelletized and stored at -20°C until use [51,52].

The experimental protocol was approved by the Animal Care Advisory Committee of Huazhong Agricultural University. Juvenile tilapias were obtained from the Far East of Guangxi Agriculture and Animal Husbandry Fishery Development Co., Ltd. (Guangxi, China). Prior to the experiment, tilapia were cultured in experimental conditions (50 L/tank and 30 fish/tank) for 14 d and fed with the basal diet. After adaptation to the farming system, a total of 300 healthy tilapias (initial average weight 56.16 ± 0.82 g) were randomly divided into 15 tanks (50 L) with 20 fish/tank and fed one of five experimental diets to apparent satiety four times daily for 56 days. During the experiment, the water temperature and pH were 25–28°C and 7.0–7.5, respectively, and the dissolved oxygen content remained above 5 mg L^{-1}.

Table 1. Composition and nutrient content of the basal diet.

Ingredients	g kg^{-1} diet	Nutrients content[a]	g kg^{-1} diet
Fish meal	50	Crude protein	323.4
Soy bean meal	300	Crude fat	60.6
Cottonseed meal	130	Crude fibre	48.3
Rapeseed meal	160	Crude ash	47.6
Wheat flour	200		
Rice bran	54		
Cassava starch	40		
Soybean oil	37		
Ca(H$_2$PO$_4$)$_2$	20		
Choline chloride (50%)	3		
Vitamin premix[b]	1		
Mineral premix[b]	5		

[a] The crude protein, crude fat, crude fibre and crude ash content are measured values.

[b] Kindly provided by New Hope Liuhe Co., Ltd, Chengdu, China.

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Sample collection

At the end of the feeding trial, 5 fish per tank were anesthetized using tricaine methane sulfonate (MS222) according to Zhou et al. [53]. Blood samples were collected into non-heparinized tubes from the caudal vein and allowed to clot for 12 h (at 4°C). Then, serum was isolated by centrifugation (5000 g, 5 min) and stored at -20°C until further analysis as described by Hoseinifar et al. [54]. After blood sampling, these fishes were slaughtered and the hindguts were taken out. The luminal contents and hindgut tissues were collected and deposited at −80°C for later analysis. The remaining fishes were used for the challenge test.

Challenge test

The challenge test was approved by the Animal Care Advisory Committee of Huazhong Agricultural University and was conducted after the feeding trial. The remaining 45 fish in each group (triplicate) were anesthetized with MS222, and injected intraperitoneally with 0.3 mL of PBS containing 1.5 x 10^6 CFU bacteria mL^{-1}. Then, these infected fish were returned to the original tank. All water quality parameters were the same as those in the feeding trial. The tanks were monitored every 2 h by a trained aquaculture technician. According to the previous description [55], any dead fish were removed, and any severely morbid fish were immediately anaesthetized and euthanized once noticed. All dead fish were examined for typical symptoms of streptococcosis. The challenge test lasted for 14 days.

The analysis of serum and hindgut tissue samples

According to Li et al. [56], serum total antioxidant capacity (T-AOC) and malondialdehyde (MDA) were analysed via kits provided by Nanjing Jiancheng Biological Engineering Research Institute (Nanjing, China). DAO activity was analysed via kits provided by Hangzhou Nuoyang Biological Technology Co. Ltd. (Hangzhou, China) according to Lei et al. [57]. Kits provided by Zhejiang Elikan Biological Technology Co. Ltd. (Wenzhou, China) were used for the determination of C3 and C4 according to Sun et al. [58].

Thirty hindgut tissue samples, six samples from each group, were used for RNA extraction. Total RNA was extracted using TRIzol according to the manufacturer's instructions (Aidlab

Table 2. The gene specific primers sequences.

Gene	Primer	Sequence	NCBI Gene ID
β-actin	Forward	5'– TCCACGAAACCACCTACAACA –3'	100534414
	Reverse	5'– CCAGACGGAGTATTTACGCTCA –3'	
claudin 1	Forward	5'- CTTCACTCTGGTCGCCGTGTC –3'	100705074
	Reverse	5'- GCAGCAAAGCATAGATCCTCCC –3	
occludin	Forward	5'- AATCGGGATAATCTCCTACA –3'	100695261
	Reverse	5'- TTGGTCCTCTTTGCTATTTG –3'	
IL10	Forward	5'- AGATGTCACCCAGTGTAGGAA –3'	100694754
	Reverse	5'- AAGCCAGGTACGTCTCAAAGT –3	
IL-8	Forward	5'- ACCTGTGAAGGCATGGGTGT –3'	100534479
	Reverse	5'- TCGCAGTGGGAGTTGGGAAG –3	
TNF-a	Forward	5'- TCGTCGTCGTGGCTCTTTGT –3'	100534578
	Reverse	5'- GCCTTGGCTTTGCTGCTGAT –3	
TLR2	Forward	5'- CATTCTGCTATCTGTGGTGCTGT –3'	100694547
	Reverse	5'- GCTGCTTTCGCTTGGCTCCTCTA –3	
MyD88	Forward	5'- ATGCCTTCATCTGCTACTGC –3'	100700534
	Reverse	5'- ATCCGTTTACACCTCTTCTCG –3	
IRAK-4	Forward	5'- ATACAAAGGTCTCCTGGATGA –3'	100707440
	Reverse	5'- AGCAAGCCAGTCGGTCTAAT –3	

Biotechnologies, Beijing, China). The quantity and quality of RNA were assessed using an ultraviolet spectrophotometer (Shanghai Sunny Hengping, Shanghai, China); the ratios of absorbance at 260 and 280 nm (A260/A280) were used to assess RNA purity and were between 1.8 and 2.0. Then, cDNA was synthesized using the Prime Script™ RT Reagent Kit according to the manufacturer's protocol (TaKaRa, Tokyo, Japan). Real-time PCR was applied to evaluate gene expression levels using gene-specific primers as shown in Table 2. β-actin was used as the house-keeping gene to standardize the data. Real-time PCR assays were conducted in an ABI 7900 real-time PCR detection system (Applied Biosystems, California, USA) with 20 μL reaction volumes containing: 4 μL of cDNA (10 times dilution), 0.4 μL of forward primer (100 μM), 0.4 μL of reverse primer (100 μM), 10 μL of 2× SYBR Premix Ex Taq™ (TaKaRa, Tokyo, Japan) and 5.2 μL of deionized H_2O. The cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min and then 40 cycles at 95°C for 30 s and 60°C for 30 s. The relative gene expression levels were analysed using the $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen [59].

The analysis of the intestinal microbiota

According to the results of the feeding trial and challenge test, the most effective treatment in the present study was considered to be the CB2 group. Therefore, a comparison of bacterial structure and composition of the intestine between the CG and CB2 groups was performed. Twelve hindgut content samples, six each from the CG and CB2 groups, were used for analysis of the intestinal microbiota. Total microbial DNA was extracted using a TIANamp Stool DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's guidelines, and the quality of the DNA samples was determined by agarose electrophoresis and a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Brisbane, Australia). The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified by PCR (95°C for 2 min, followed by 25 cycles at 95°C for 30s, 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min) using forward primer 338F (5'– ACTCCTACGGGAGGCAGCAG–3') and reverse primer 806R (5'–GGACTACHVGGGTWTCAAT–3'). PCR reactions were conducted in triplicate using a

20 μL reaction mixture consisting of template DNA (10 ng), FastPfu Polymerase (0.4 μL), each primer at 5 μM (0.8 μL), 2.5 mM dNTPs (2 μL) and 5 ×FastPfu Buffer (4 μL). Agarose gels (2%) were used to extract the amplicons, which were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.). The amplicons from each sample were pooled in equimolar amounts and sent to Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China). The Illumina MiSeq platform was used to perform paired-end sequencing (2 × 250/300 bp) of these amplicons according to standard protocols.

In the present study, the Illumina sequencing reads were mainly analysed using QIIME pipeline software (version 1.9.1). The poor/low quality sequences, including those with uncertain nucleotides and three continuous nucleotides with an average quality of less than 20 over a 50bp sliding window, were removed. Operational Taxonomic Units (OTUs) were clustered with 97% similarity cut off using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analysed by RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using a confidence threshold of 0.7. Mothur was also used to calculate the alpha diversity (Shannon index, Chao1, ACE and Sobs) and rarefaction curves. R programming language was used to perform the Venn diagram analysis. Afterwards, all sequence data were deposited to the SRA of the NCBI database under BioProject PRJNA559959.

Calculations and statistical analysis

Specific growth rate (SGR), feed conversion ratio (FCR) and cumulative mortality were calculated for each treatment according to [60,61].

SGR = {[ln (mean final weight) – ln (mean initial weight)] /number of days} x 100

FCR = feed intake (g)/wet weight gain (g)

Cumulative mortality = 100 x the number of dead fish/15

A nonparametric test of two samples was performed to assess the differences in bacterial relative abundance and diversity indices between the CB2 and CG groups. One-way ANOVA and Duncan's multiple comparison test were performed to assess the differences in growth performance, cumulative mortality, serum complement concentration, antioxidant capacity, gene expression, and DAO activity among the five groups. The significance level was set at $P < 0.05$. Statistical analyses were conducted using SPSS Statistics software v. 19.0 (IBM, Armonk, NY, USA). Results are presented as means ± standard deviation (SD).

Results

Growth performance

The effects of dietary *C. butyricum* on SGR, feed intake and FCR of tilapia are presented in Table 3. No fish died during the feeding experiment. SGR and feed intake were significantly

Table 3. Growth Performance of tilapia fed diets supplemented with *C.butyricum*.

	CG	CB1	CB2	CB3	CB4
Initial body weight (g)	55.93±1.25[a]	56.53±0.81[a]	56.00±0.57[a]	56.40±0.52[a]	55.90±1.10[a]
Final body weight (g)	178.77±4.70[a]	187.20±8.55[ab]	197.25±7.42[b]	189.33±9.04[ab]	184.53±7.22[ab]
SGR	2.00±0.01[a]	2.06±0.10[ab]	2.17±0.08[b]	2.09±0.10[ab]	2.06±0.04[ab]
Feed intake (g fish^{-1})	145.68±8.27[a]	156.91±6.65[ab]	163.24±6.39[b]	156.94±7.47[ab]	156.09±3.74[ab]
FCR	1.19±0.04[a]	1.20±0.04[a]	1.16±0.02[a]	1.18±0.03[a]	1.22±0.03[a]

SGR: Specific growth rate. FCR: feed conversion ratio.

Values are means ± standard deviation of three replicate groups. Mean values with the different superscripts in the same row are significantly different (P < 0.05).
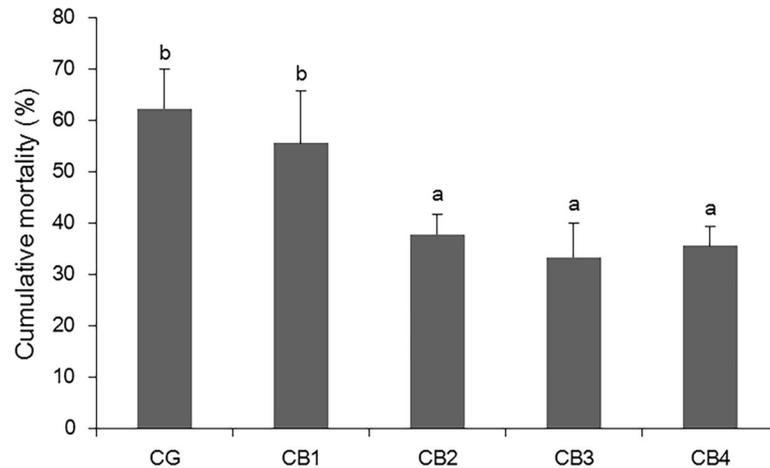
Fig 1. The Effects of dietary *C. butyricum* on the cumulative mortality of tilapia post- challenge with *S. agalactiae*.

improved in tilapia that were fed a diet with *C. butyricum* at a level of 1×10^5 CFU g^{-1} diet ($P < 0.05$), but FCR was not affected by dietary *C. butyricum* supplementation ($P > 0.05$).

Cumulative mortality

The cumulative mortality of fish at 14 days post-challenge with *S. agalactiae* is presented in Fig 1. *C. butyricum* significantly decreased the cumulative mortality of tilapia at levels of 1×10^5, 1×10^6 and 1×10^7 CFU g^{-1} diet ($P < 0.05$). Dead fish showed the typical symptoms of strepto-coccosis such as darkness of the body surface, exophthalmos, and basal fin haemorrhage.

Serum complement concentration

The effects of dietary *C. butyricum* on serum C3 and C4 concentrations of tilapia are presented in Fig 2. Serum C3 and C4 concentrations increased significantly with dietary *C. butyricum* at levels of 1×10^5, 1×10^6 and 1×10^7 CFU g^{-1} diet ($P < 0.05$), with the highest levels observed in the CB3 group receiving the diet supplemented with *C. butyricum* at 1×10^6 CFU g^{-1} diet ($P < 0.05$).
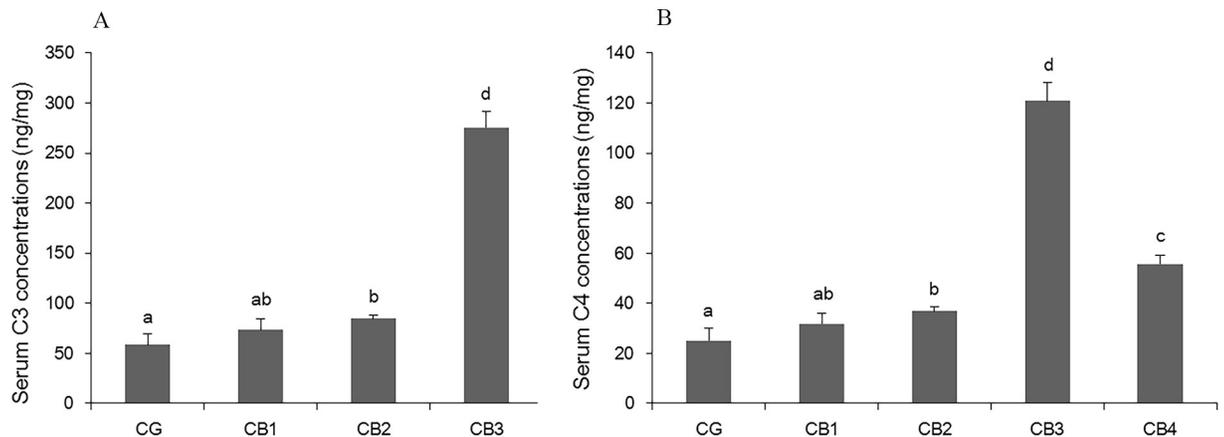
Fig 2. The effects of dietary *C. butyricum* on serum complement 3 (C3) and 4 (C4) concentrations. A: The effects on serum C3; B: The effects on serum C4.
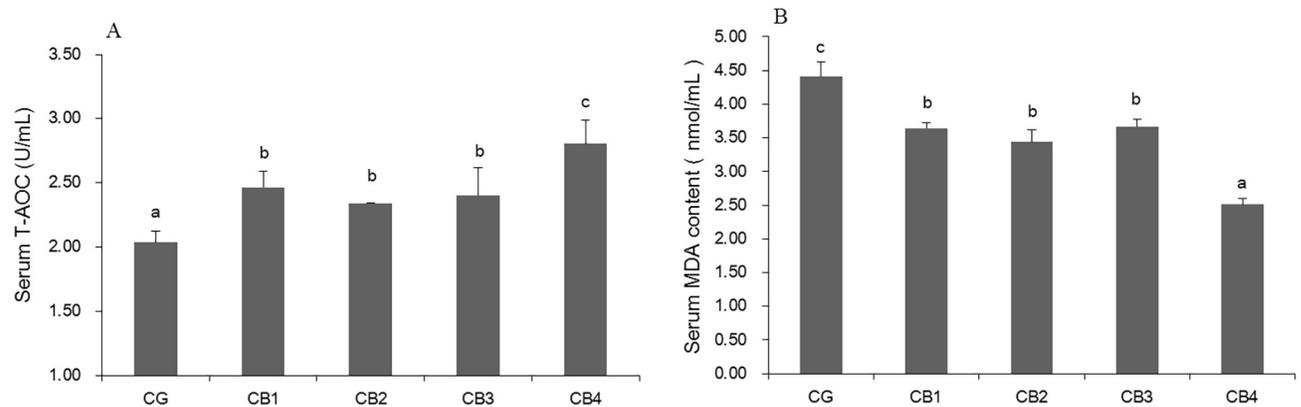
Fig 3. The effects of dietary *C. butyricum* on the serum T-AOC, MDA contents. A: The effects on serum T-AOC; B: The effects on serum MDA.

Antioxidant capacity

The effects of dietary *C. butyricum* on the serum T-AOC and MDA contents of tilapia are presented in Fig 3. Serum T-AOC increased significantly with dietary *C. butyricum*, supplementation, with the highest levels observed in the CB4 group receiving the diet supplemented with *C. butyricum* at 1×10^7 CFU g^{-1} diet ($P < 0.05$). Serum MDA content significantly decreased with dietary *C. butyricum* supplementation, with the lowest content observed in the CB4 group receiving *C. butyricum* at 1×10^7 CFU g^{-1} diet ($P < 0.05$).

Gene expression of cytokines, *TLR2*, *MyD88*, and *IRAK-4*

The effects of dietary *C. butyricum* on the gene expression of cytokines, *TLR2*, *MyD88* and *IRAK-4* in the hindgut of tilapia are presented in Fig 4. Compared with the unsupplemented group, gene expression of *TNF-α*, *IL-8* and *MyD88* was significantly upregulated by *C. butyricum* at levels of 1×10^5, 1×10^6 and 1×10^7 CFU g^{-1} diet ($P < 0.05$). *TLR2* gene expression was significantly upregulated by *C. butyricum* supplementation, and significant upregulation was observed at levels of 1×10^6 and 1×10^7 CFU g^{-1} diet relative to the control group ($P < 0.05$). *C. butyricum* significantly upregulated *IRAK-4* gene expression ($P < 0.05$) but did not affect *IL-10* gene expression ($P > 0.05$).

The intestinal physical barrier

The effects of dietary *C. butyricum* on serum DAO activity and the relative gene expression of *claudin-1* and *occludin* in the hindgut of tilapia are presented in Fig 5. Serum DAO activity significantly decreased with dietary *C. butyricum* supplementation and was lowest in the CB4 group receiving *C. butyricum* at 1×10^7 CFU g^{-1} diet ($P < 0.05$). The relative gene expression of *claudin-1* and *occludin* was not significantly affected by *C. butyricum* supplementation ($P > 0.05$).

Intestinal microbiota analyses

A total of 436184 valid sequences were obtained through Illumina sequencing analysis in the present study, with an average of 36348 (SD = 3585) per sample. A total of 2339 OTUs were identified at the 97% similarity level. There were 2036 OTUs shared between CG and CB2, 236 unique OTUS for the CB2 group and 67 unique OTUS for the CG (Fig 6).
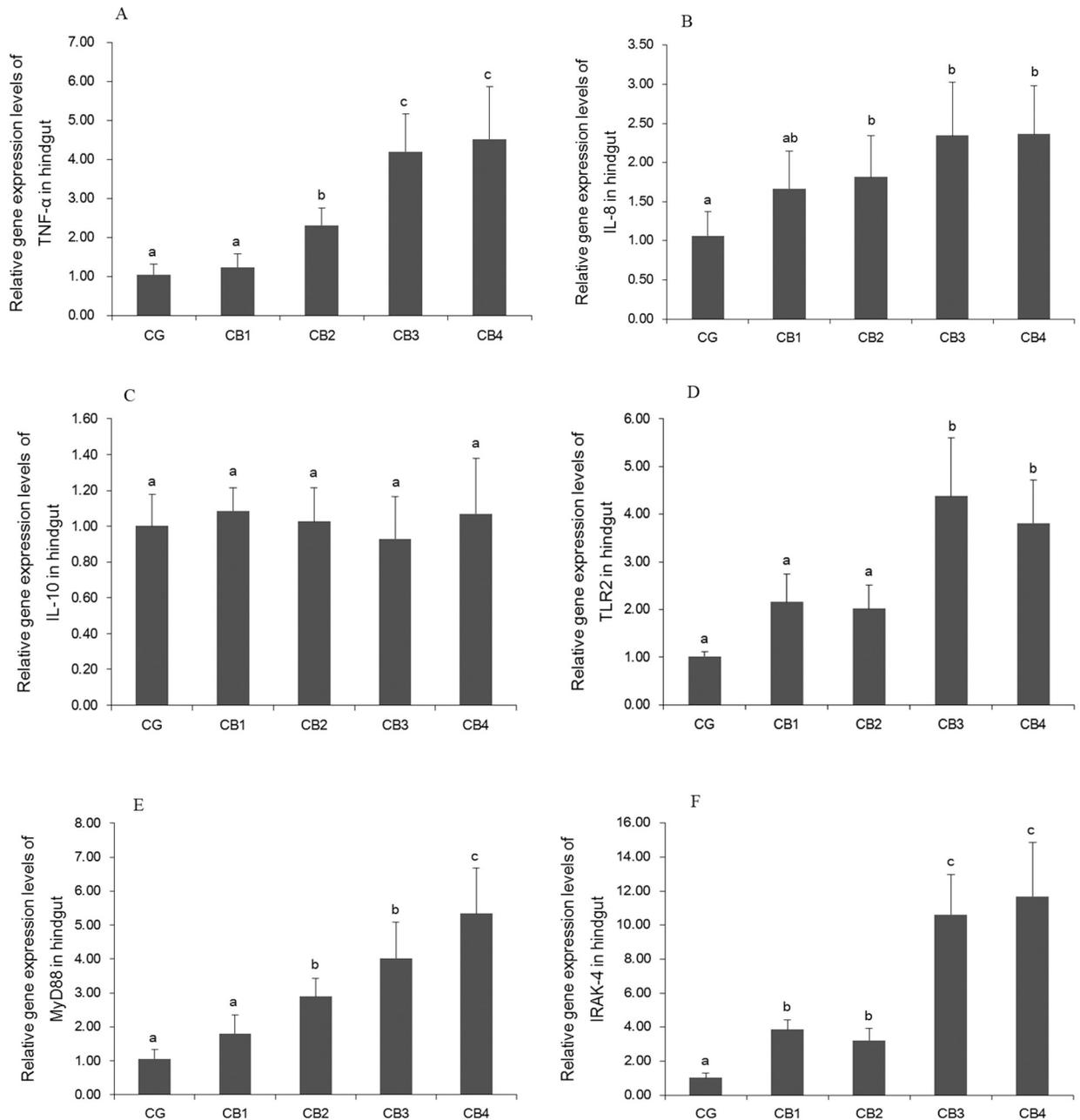
Fig 4. The gene expression of *TNF-α, IL-8, IL-10, TLR2, MyD88, IRAK-4* in the Hindgut of tilapia. A: The effects on the gene expression levels of *TNF-α*; B: The effects on the gene expression levels of *IL-8*; C: The effects on the gene expression levels of *IL-10*; D: The effects on the gene expression levels of *TLR2;* E: The effects on the gene expression levels of *MyD88*; F: The effects on the gene expression levels of *IRAK-4*.

A rarefaction test was performed at the OTU level, and the results are presented in Fig 7. The rarefaction curves tended to approach the saturation plateau, indicating that the majority of microorganisms were revealed in the present study. Alpha diversity indices are presented in Table 4. Compared with the CG group, Sobs, Shannon, Ace and Chao1 indices were significantly increased by dietary supplementation with *C. butyricum* (1 x 10^5 CFU g^{-1} diet) ($P < 0.05$).
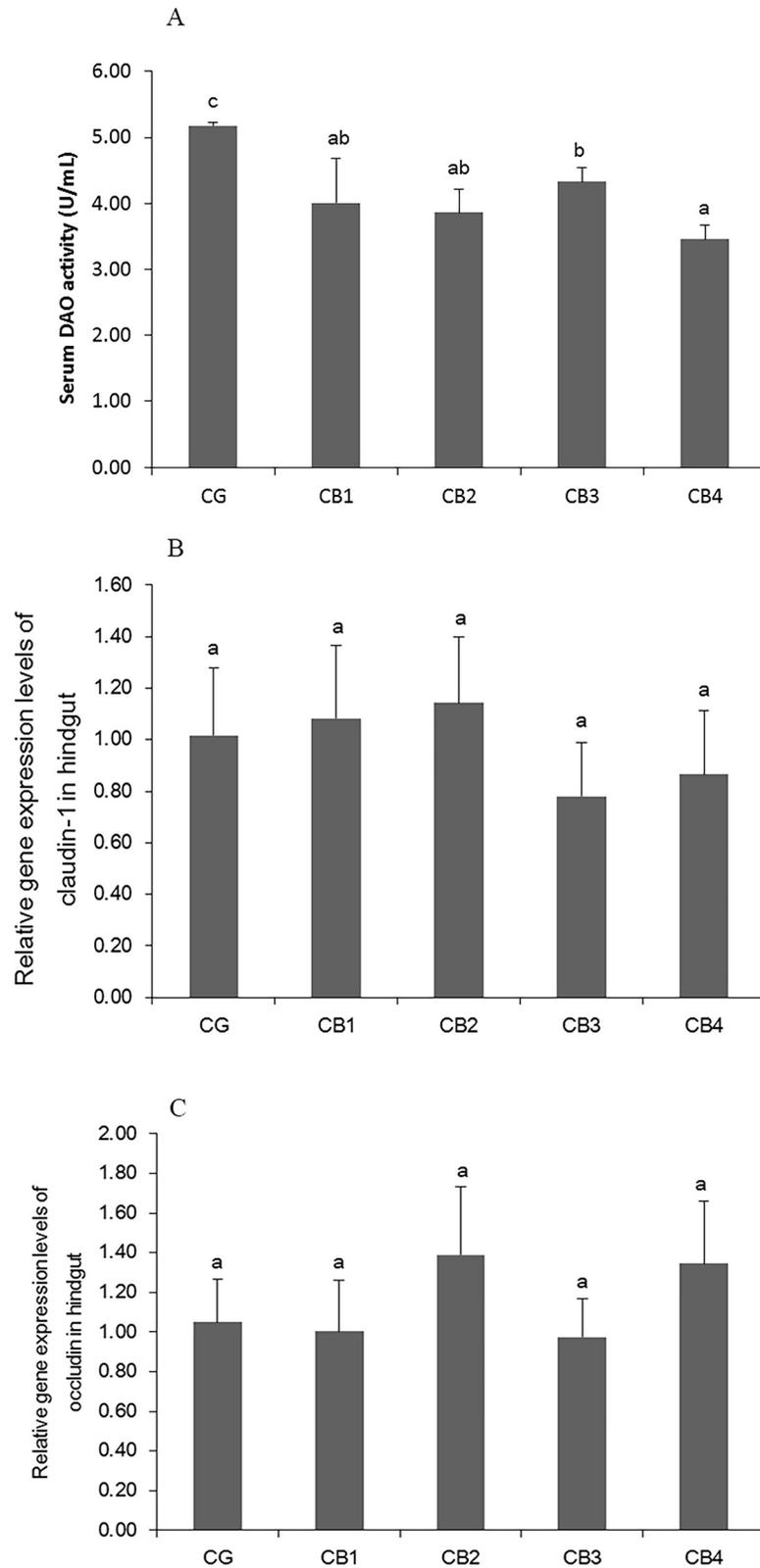
Fig 5. The serum DAO activity and gene expression of *Claudin -1* and *Occludin* in the hindgut. A: The effects on serum DAO activity; B: The effects on the gene expression levels of claudin -1; C: The effects on the gene expression levels of occudin.
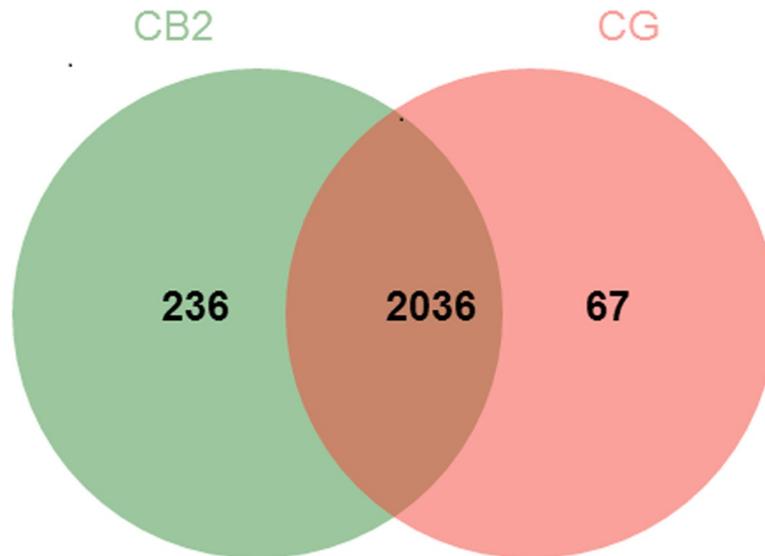
Fig 6. Venn plot showing the shared and unique OTUs.

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A total of 49 phyla, 108 classes, 210 orders, 394 families and 704 genera were identified in the present study. The bacterial compositions at the phylum and genus levels are presented in Fig 8. There were 42 phyla whose relative abundances were significantly different between the CG and CB2 groups, and the top 15 most abundance phyla are presented in Fig 9A. Compared to the control group, the relative abundances of Bacteroidetes, Firmicutes, Candidate-division-SR1, Chloroflexi, Chlorobi, Acidobacteria, Spirochaetae, Nitrospirae, Parcubacteria, Plancto-mycetes and WCHB1-60 were significantly enriched ($P < 0.05$), and those of Fusobacteria and CKC4 were significantly weakened in the CB2 group ($P < 0.05$). Proteobacteria was statistically the same between the two groups ($P > 0.05$). There were 354 genera whose relative abundances were significantly different between the CG and CG2 groups ($P < 0.05$), and the top15 most abundant genera are presented in Fig 9B. Compared with the CG group, the relative abundances of *Cetobacterium*, CKC4, *Aeromonas* and *Gammaproteobacteria* were significantly
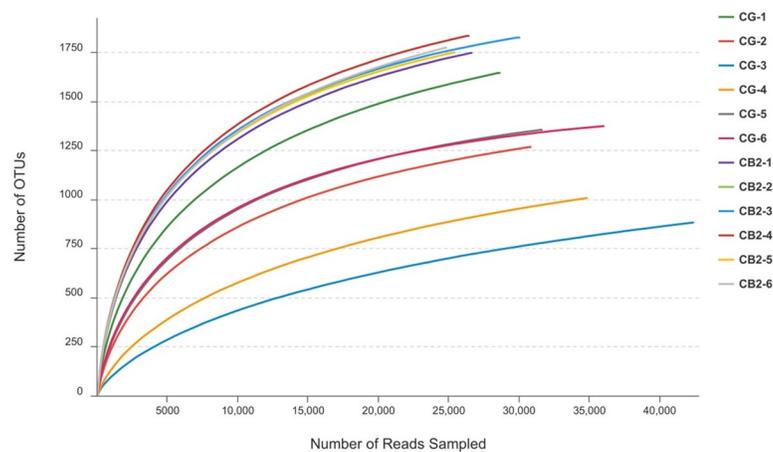
Fig 7. Rarefaction curves of samples.

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Table 4. Comparison of the diversity indices of bacteria communities between the CG and CB2 groups.

Group	Sobs	Ace	Chao1	Shannon
CG	446.67±50.27[a]	489.78±47.28[a]	490.30±44.39[a]	3.32±0.84[a]
CB2	514.00±7.90[b]	547.30±5.30[b]	552.24±5.73[b]	4.99±0.05[b]

Sobs: the observed richness; Ace: abundance-based coverage estimator; Vertical bars represented the means ± standard deviation (N = 6). Data indicated with different letters were significantly different (P < 0.05) between CG and CB2 group.

lower (*P* < 0.05), and those of Candidate_division_SR1_norank, Bacteroidetes_vadinHA17_-norank, *Dechloromonas*, *Zoogloea*, *Oligoflexales_noran*n, *Comamonadaceae*_unclassified, *Draconibacteriaceae*_uncultured, *Saprospiraceae*_uncultured, *Nitrospira*, *Bacillus*, and WCHB1-69_norank were significantly higher in the CB2 group (*P* < 0.05).

Discussion

Probiotics have been extensively researched, and their effects on the growth and health of aquatic animals have been verified in recent years [3]. Compared with other probiotics, such as *Bacillus* and *Lactobacillus*, *C. butyricum* has a stronger tolerance to many antibiotics, higher temperature, and lower pH environment and has a wide range of application prospects [40,62]. Previous studies showed that a suitable dose of dietary *C. butyricum* increased growth performance in Pacific white shrimp (*Litopenaeus vannamei*) [19,20,42], black tiger shrimp (*Penaeus monodon*) [63], *Macrobrachium rosenbergii* [64], Chinese drum (*Miichthys miiuy*) [65] and silver pomfret (*Pampus argenteus*) [66]. The present study also found that dietary *C. butyricum* at a level of 1×10^5 CFU g^{-1} diet significantly improved the growth performance of tilapia. Fish growth performance is related to disease resistance [67]. Mortality post-challenge is an indicator of disease resistance [68,69]. In the present study, cumulative mortality post-challenge with *S. agalactiae* was decreased in tilapia fed a diet supplemented with *C. butyricum* at levels of 1×10^5, 1×10^6 and 1×10^7 CFU g^{-1} diet. Similar results have been obtained in Chinese drum and rainbow trout. Pan et al. [18] reported that survival post-challenge with *V. anguillarum* or *A. hydrophila* was enhanced in Chinese drum fed diets supplemented with *C. butyricum* at the dose of 1×10^8 cells g^{-1} diet. After challenge with *Vibrio parahaemolyticus*, the cumulative mortality of Pacific white shrimp (*Litopenaeus vannamei*) significantly decreased with dietary *C. butyricum* at the dose of 1×10^8 CFU g^{-1} diet [19] and 1×10^5, 1×10^7, 1×10^8, and 1×10^9 CFU g^{-1} diet [20]. These results revealed that *C. butyricum* could improve the disease resistance of aquatic animals.

Disease resistance is often related to humoural immunity, and the complement system is an important part of humoral immunity [70]. In fish, C3 and C4 play important roles in combatting bacterial pathogens [58]. Studies have shown that the probiotics *Pediococcus acidilactici* [54,71], *Lactobacillus rhamnosus* [72], *Enterococcus faecium* [73,74] and *L. plantarum* [5] can improve serum complement component levels in fish. However, no studies have examined the effect of *C. butyricum* on serum complement in fish. In this study, serum C3 and C4 concentrations were improved in tilapias fed a diet supplemented with *C. butyricum* at levels of 1×10^5, 1×10^6, 1×10^7 CFU g^{-1} diet. Similar results were reported in which dietary *C. butyricum* improved serum C3 and C4 concentrations in broiler chickens [48,49]. The improvement of serum complement content induced by *C. butyricum* might be related to lipoteichoic acid (LTA). LTA is a major cell wall component of gram-positive bacteria [75,76]. In human, the classical complement pathway is activated by LTA *in vitro* [77]. In addition, immunity is related to antioxidative status in fish [78]. T-AOC is an anti-oxidative biomarker [79], and MDA is a typical parameter used to reflect oxidative injury [80]. In the present study, serum
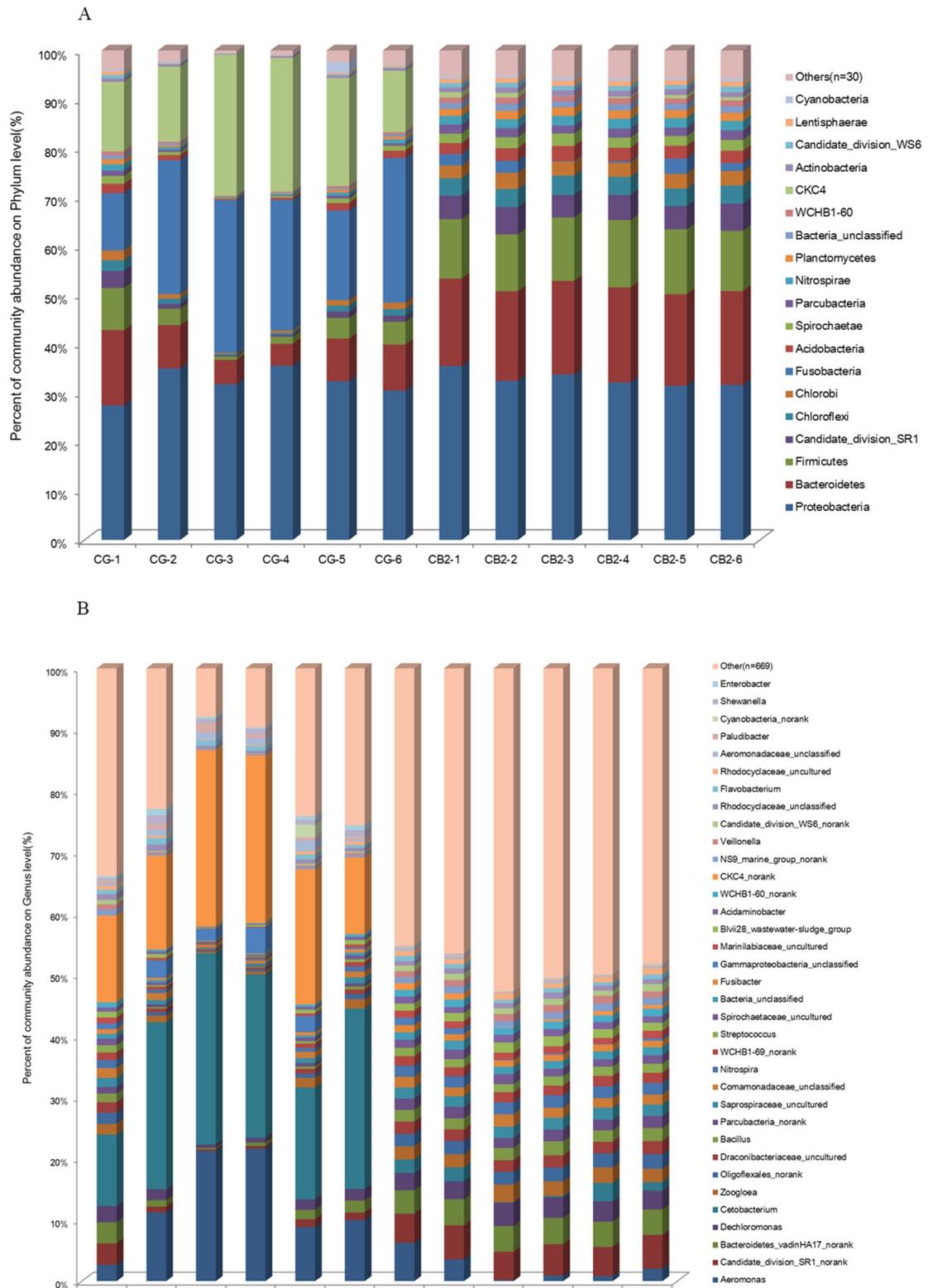
Fig 8. Bacterial composition at the phylum and genus levels of the CB2 and CG groups. A: The bacterial compositions at the phylum level. B: The bacterial compositions at the genus level. Only the phylum and genus whose relative abundance was more than 1% were presented.

A

Student's t-test bar plot on Phylum level

B

Student's t-test bar plot on Genus level
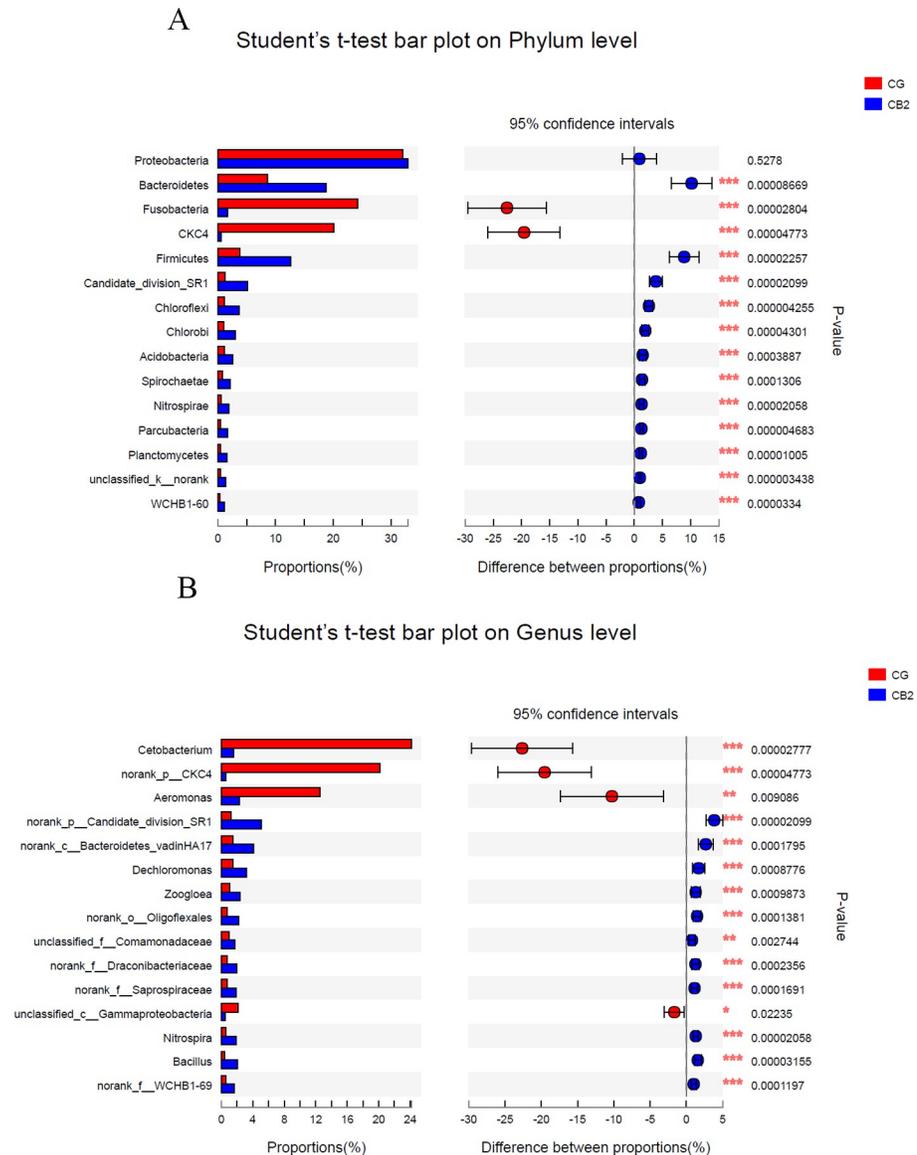
Fig 9. The differences at the phylum and genus level between the CB2 and CG groups. A: at the phylum level. B: at the genus level. The vertical axis represented the species name at the phylum or genus level. Each column corresponding to the species represented the average relative abundance of the species in the CG and CB2 groups. Different colors represented different groups. Only the top15 most abundant genera were presented. 0.01< P < = 0.05 *, 0.001< P < = 0.01 **, P < = 0.001 ***.

T-AOC was significantly increased and MDA content was significantly decreased in all four *C. butyricum* supplementary groups. Similar results were reported showing that dietary *C. butyricum* improved the antioxidant capacity in shrimp [20,41,42] and fish [66,81]. The effect of *C. butyricum* on antioxidant capacity of aquatic animals might be attributed to butyrate and H_2. *C. butyricum* can produce butyric acid [82,83] and H_2 [84]. Butyrate has been shown to reduce H_2O_2-induced DNA damage in human colon tumor cell [85] and to decrease MDA concentrations in the Caco-2 human colon carcinoma cell line [86]. Zhou et al. [87] reported that H_2 reduced oxidative stress in acute pancreatitis both *in vitro* and *in vivo*.

The intestine is an important immune organ in fish [35]. Disease resistance in fish is related to intestinal immunity [25]. Cytokines play important roles in intestinal immunity [88]. Studies have shown that the gene expression of *TNF-α* and *IL-8* in the fish intestine is upregulated by the dietary probiotics *P. acidilactici* [12] and *L. plantarum* [27]. Our results showed that the gene expression of *TNF-α* and *IL-8* in the intestine was upregulated in tilapias fed a diet supplemented with *C.butyricum* at doses of 1×10^5, 1×10^6 and 1×10^7 CFU g^{-1} diet. Similar trends were reported in HT-29 human colonic epithelial cells [17,30] and the jejunal mucosa of broiler chickens [39]. The effect of *C. butyricum* on cytokines might be related to butyric acid. Sodium butyrate was found to upregulate the gene expression levels of *TNF-α* in the intestine of juvenile common carp [89] and in bovine mammary epithelial cells [90]. IL-10 is an anti-inflammatory cytokine that suppresses excessive immune responses [33]. In the present study, the gene expression of *IL-10* was not influenced by dietary *C. butyricum*. This result differs from previous reports that *C. butyricum* upregulated the gene expression of *IL-10* in HT-29 human colonic epithelial cells [17,31]. This difference might be related to immune status. In mouse intestinal macrophages, *C. butyricum* failed to increase the production of IL-10 in the steady state, whereas it induced IL-10 production under inflamed conditions [33]. The TLR2/ MyD88/IRAK-4 signalling pathway plays an important role in intestinal immune responses, inducing the production of cytokines such as TNF-α and IL-8, in mammals [91]. Study has shown that the probiotic *Psychrobacter sp.* SE6 upregulated gene expression of *TLR2* and *MyD88* in the intestine of grouper (*Epinephelus coioides*) [28]. There has been no investigation of the effect of TLR signalling pathways in immune responses induced by *C. butyricum* in aquatic animals. In the present study, *TLR2* gene expression was upregulated by dietary *C. butyricum* at levels of 1×10^6 and 1×10^7 CFU g^{-1} diet. *MyD88* gene expression was significantly upregulated by dietary *C. butyricum* at levels of 1×10^5, 1×10^6 and 1×10^7 CFU g^{-1} diet. The gene expression of *IRAK-4* was significantly upregulated in all four *C. butyricum* supplementary groups. Similar results were obtained showing that *C. butyricum* upregulate *TLR2* gene expression in HT-29 human colonic epithelial cells [17], and upregulated the gene expression of *TLR2* and *MyD88* in the intestines of weaning rex rabbits [34]. The correlation analysis showed that *TNF-α* gene expression was significantly and positively related to gene expression of *TLR2* (r = +0.932, P < 0.05), *MyD88* (r = +0.969, P < 0.05) and *IRAK-4* (r = +0.960, P < 0.05) and that *IL-8* gene expression was significantly and positively related to gene expression of *TLR2* (r = +0.957, P < 0.05), *MyD88* (r = +0.936, P < 0.05) and *IRAK-4* (r = +0.936, P < 0.05). These results indicated that the upregulation of *TNF-α* and *IL-8* in response to dietary *C. butyricum* supplementation might be related to the upregulation of *TLR2/ MyD88/IRAK-4* signalling molecules in tilapia. To the best of our knowledge, this is the first study of the effects of *C. butyricum* on *TLR2/MyD88/IRAK-4* signalling molecules in fish. The regulation of *TLR2/MyD88/IRAK-4* signalling molecules by *C. butyricum* might be related to LTA. Rashidi et al. [92] reported that the gene expression of *TLR2* and *MyD88* was upregulated by LTA in human whole endometrial cells.

On the other hand, the effect of *C. butyricum* on intestinal immunity may be related to the modulation of intestinal microbial components. Studies have shown that the diversity and composition of the intestinal microbiota affect immune responses, including the regulation of immune cells, cytokines and TLR signalling molecule [93–95]. It has been reported that probiotics can regulate intestinal communities in tilapia [29,96–98]. In the present study, we investigated intestinal microbial composition in the CB2 and CG groups. Our results showed that a total of 49 phyla were identified, the relative abundances of 42 phyla were significantly different between the CG and CB2 groups, and the phylum with the greatest change was Fusobacteria, followed by CKC4, Bacteroidetes and Firmicutes. *Cetobacterium* is present in high numbers in many fish species [99–102] and produces large quantities of vitamin B12 [103]. In

the present study, *Cetobacterium* was the dominant bacteria of Fusobacteria in the two groups, and its relative abundance was significantly decreased in the CB2 group. Similar to our results, a previous study showed that *Cetobacterium* abundance was significantly decreased by a multi-species probiotic (from 13.8% to 0.02%) in tilapia [96]. The reason that *Cetobacterium* was inhibited by probiotics was unclear and needs further research. CKC4, a phylum in the SILVA database, was detected in the intestines of zebrafish [104], white shrimp (*Penaeus vannamei*) [105] and Chinese mitten crab (*Eriocheir sinensis*) [106]. In the present study, dietary *C. butyricum* significantly decreased the relative abundance of CKC4. However, there is a lack of detailed information about the function of CKC4 in the fish intestine. The specific mechanism needs further research. Firmicutes is an abundant phylum in the tilapia intestine [96] and provides a good index of intestinal health [47]. In the present study, Firmicutes was enriched in the CB2 group relative to the CG group. Meanwhile, the relative abundance of *Bacillus* was significantly improved in the CB2 group. These results suggested that dietary *C. butyricum* could stimulate the growth of beneficial Firmicutes bacteria, such as *Bacillus*, in the tilapia intestine. A similar result was reported showing that Firmicutes, such as *Bacillus* and *Clostridium*, were abundant in Pacific white shrimp (*Litopenaeus vannamei*) fed a diet supplemented with *C. butyricum* [47]. Various opportunistic pathogens cause disease in stressed fish and fish affected by concurrent infection [107]. *Aeromonas hydrophila* is a conditional pathogen [108]. In the present study, the relative abundance of *Aeromonas* was significantly decreased in the CB2 group. A similar result was reported in Pacific white shrimp (*Litopenaeus vannamei*), showing that dietary *C. butyricum* inhibited some intestinal opportunistic pathogen genera [47]. In vitro, *C. butyricum* exhibited significant inhibitory activity on the growth and adherence of pathogenic bacteria to fish intestinal epithelial cells [45,46]. These results revealed that *C. butyricum* might decrease the risk of opportunistic pathogens to invade the host. Compared with disease states, there was a higher microbial diversity index in healthy humans [109], mice [110], pigs [111] and fish [110]. Stress caused a reduction in intestinal microbial diversity in fish [97]. In this study, alpha-diversity indices, including Sobs, Shannon, ACE and Chao1, were significantly increased by dietary *C. butyricum*. Similarly, *C. butyricum* increased the alpha-diversity indices, including ACE and Chao1, in Pacific white shrimp (*Litopenaeus vannamei*) [47]. These results indicated that *C. butyricum* improved the homeostasis of the intestinal microbial community.

In fish, the intestinal immunity is correlated with intestinal structural integrity [35]. DAO is an index of intestinal mucosal cell integrity [39]. In this study, serum DAO activity was decreased in tilapia that were fed diets supplemented with *C. butyricum* at all four levels. Similar results were reported in broiler chickens [39] and weaned piglets [40]. These results indicate that *C. butyricum* can improve intestinal mucosal cell integrity in fish. The observed decrease of DAO activity in the serum of fish fed *C. butyricum* might be associated with butyric acid. Butyric acid is a major respiratory fuel, is trophic to the colon and exerts proliferative effects on colonocytes [112]. Fang et al. [113] reported that serum DAO was significantly decreased by sodium butyrate in weanling piglets. In previous work, the intestinal intercellular structural integrity was found to be related to tight junction proteins, such as occludin and claudin, in the Caco-2 human colon carcinoma cell line [114]. Dietary *C. Butyricum* upregulated the protein expression levels of *ZO-1*, *claudin-3*, *occludin* in intestine of weaned piglets [40] and the gene expression levels of *ZO-1* and *occludin* in ileum and colon of weaning rex rabbits [34]. In the present study, *C. butyricum* had no effect on the gene expression of *claudin-1* and *occludin* in tilapia intestine. Nébot-Vivinus et al. [115] reported that in the T84 human colon epithelial cell line, multispecies probiotic LT did not affect the expression of *occludin* under basal conditions, but the expression of *occludin* was increased upon

lipopolysaccharide stimulation. The effects of probiotics on tight junction protein might be related to health.

Conclusions

Dietary *C. butyricum* at a suitable dose significantly improved the growth performance, humoural and intestinal immunity, and integrity of the intestinal structure, elevated the diversity of the intestinal microbiota and the relative abundance of beneficial bacteria, decreased the relative abundance of opportunistic pathogenic bacteria, and enhanced the disease resistance in tilapia. According to the growth performance, immunity and disease resistance, the optimal dose of dietary *C. butyricum* was 1 x 10^5 CFU g^{-1} diet.

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Writing – review & editing: Hongqin Li, Desheng Qi, Lin Feng.

References

1. Hai NV. The use of probiotics in aquaculture. J Appl Microbiol. 2015; 119: 917–935. https://doi.org/10.1111/jam.12886 PMID: 26119489

2. Wang A, Ran C, Wang Y, Zhang Z, Ding Q, Yang Y, et al. Use of probiotics in aquaculture of China-a review of the past decade. Fish Shellfish Immunol. 2019; 86: 734–755. https://doi.org/10.1016/j.fsi.2018.12.026 PMID: 30553887

3. Chauhan A, Singh R. Probiotics in aquaculture: a promising emerging alternative approach. Symbiosis. 2019; 77: 99–113.

4. Lazado CC, Caipang CM, Estante EG. Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions. Fish Shellfish Immunol. 2015; 45: 2–12. https://doi.org/10.1016/j.fsi.2015.02.023 PMID: 25703713

5. Son VM, Chang CC, Wu MC, Guu YK, Chiu CH, Cheng W. Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses, and disease resistance of the grouper *Epinephelus coioides*. Fish Shellfish Immunol. 2009; 26: 691–698. https://doi.org/10.1016/j.fsi.2009.02.018 PMID: 19264134

6. Gupta A, Gupta P, Dhawan A. Dietary supplementation of probiotics affects growth, immune response and disease resistance of *Cyprinus carpio* fry. Fish Shellfish Immunol. 2014; 41: 113–119. https://doi.org/10.1016/j.fsi.2014.08.023 PMID: 25160796

7. Ramos MA, Goncalves JF, Batista S, Costas B, Pires MA, Rema P, et al. Growth, immune responses and intestinal morphology of rainbow trout (*Oncorhynchus mykiss*) supplemented with commercial probiotics. Fish Shellfish Immunol. 2015; 45: 19–26. https://doi.org/10.1016/j.fsi.2015.04.001 PMID: 25865055

8. Aly SM, Ahmed YA-G, Ghareeb AA-A, Mohamed MF. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish Shellfish Immunol. 2008; 25: 128–136. https://doi.org/10.1016/j.fsi.2008.03.013 PMID: 18450477

9. Aly SM, Mohamed MF, John G. Effect of probiotics on the survival, growth and challenge infection in tilapia nilotica (*Oreochromis niloticus*). Aquac Res. 2008; 39: 647–656.

10. Geng X, Dong XH, Tan BP, Yang QH, Chi SY, Liu HY, et al. Effects of dietary chitosan and *Bacillus subtilis* on the growth performance, non-specific immunity and disease resistance of cobia, *Rachycentron canadum*. Fish Shellfish Immunol. 2011; 31: 400–406. https://doi.org/10.1016/j.fsi.2011.06.006 PMID: 21693191

11. Panigrahi A, Kiron V, Satoh S, Hirono I, Kobayashi T, Sugita H, et al. Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. Dev Comp Immunol. 2007; 31: 372–382. https://doi.org/10.1016/j.dci.2006.07.004 PMID: 17045337

12. Standen BT, Rawling MD, Davies SJ, Castex M, Foey A, Gioacchini G, et al. Probiotic Pediococcus acidilactici modulates both localised intestinal- and peripheral-immunity in tilapia (Oreochromis niloticus). Fish Shellfish Immunol. 2013; 35: 1097–1104. https://doi.org/10.1016/j.fsi.2013.07.018 PMID: 23871840

13. Selim KM, Reda RM. Improvement of immunity and disease resistance in the nile tilapia, *Oreochromis niloticus*, by dietary supplementation with *Bacillus amyloliquefaciens*. Fish Shellfish Immunol. 2015; 44: 496–503. https://doi.org/10.1016/j.fsi.2015.03.004 PMID: 25783002

14. Iwashita MK, Nakandakare IB, Terhune JS, Wood T, Ranzani-Paiva MJ. Dietary supplementation with *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus oryzae* enhance immunity and disease resistance against *Aeromonas hydrophila* and *Streptococcus iniae* infection in juvenile tilapia *Oreochromis niloticus*. Fish Shellfish Immunol. 2015; 43: 60–66. https://doi.org/10.1016/j.fsi.2014.12.008 PMID: 25530581

15. Zhao X, Guo Y, Liu H, Gao J, Nie W. *Clostridium butyricum* reduce lipogenesis through bacterial wall components and butyrate. Appl Microbiol Biotechnol. 2014; 98: 7549–7557. https://doi.org/10.1007/s00253-014-5829-x PMID: 24878750

16. Kong Q, He GQ, Jia JL, Zhu QL, Ruan H. Oral administration of *Clostridium butyricum* for modulating gastrointestinal microflora in mice. Curr Microbiol. 2011; 62: 512–517. https://doi.org/10.1007/s00284-010-9737-8 PMID: 20711781

17. Gao Q, Qi L, Wu T, Xia T, Wang J. Immunomodulatory effects of *Clostridium butyricum* on human enterocyte-like HT-29 cells. Anim Cells Syst. 2013; 17: 121–126.

18. Pan X, Wu T, Song Z, Tang H, Zhao Z. Immune responses and enhanced disease resistance in Chinese drum, *Miichthys miiuy* (Basilewsky), after oral administration of live or dead cells of *Clostridium butyrium* CB2. J Fish Dis. 2008; 31: 679–686. https://doi.org/10.1111/j.1365-2761.2008.00955.x PMID: 18786030

19. Li H, Tian X, Zhao K, Jiang W, Dong S. Effect of *Clostridium butyricum* in different forms on growth performance, disease resistance, expression of genes involved in immune responses and mTOR signaling pathway of *Litopenaeus vannamai*. Fish Shellfish Immunol. 2019; 87: 13–21. https://doi.org/10.1016/j.fsi.2018.12.069 PMID: 30599253

20. Li H-D, Tian X-L, Dong S-L. Growth performance, non-specific immunity, intestinal histology and disease resistance of *Litopenaeus vannamei* fed on a diet supplemented with live cells of *Clostridium butyricum*. Aquaculture. 2019; 498: 470–481.

21. Sakai M, Yoshida T, Atsuta S, Kobayashi M. Enhancement of resistance to vibriosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum), by oral administration of *Clostridium butyricum* bacterin. J Fish Dis. 1995; 18: 187–190.

22. Li T, Ke F, Gui J-F, Zhou L, Zhang X-J, Zhang Q-Y. Protective effect of *Clostridium butyricum* against *Carassius auratus* herpesvirus in gibel carp. Aquac Int. 2019; 27: 905–914.

23. Hai NV. Research findings from the use of probiotics in tilapia aquaculture: a review. Fish Shellfish Immunol. 2015; 45: 592–597. https://doi.org/10.1016/j.fsi.2015.05.026 PMID: 26003738

24. Wu P, Jiang J, Liu Y, Hu K, Jiang WD, Li SH, et al. Dietary choline modulates immune responses, and gene expressions of TOR and eIF4E-binding protein2 in immune organs of juvenile Jian carp

(*Cyprinus carpio* var. Jian). Fish Shellfish Immunol. 2013; 35: 697–706. https://doi.org/10.1016/j.fsi.2013.05.030 PMID: 23774323

25. Wen LM, Jiang WD, Liu Y, Wu P, Zhao J, Jiang J, et al. Evaluation the effect of thiamin deficiency on intestinal immunity of young grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol. 2015; 46: 501–515. https://doi.org/10.1016/j.fsi.2015.07.001 PMID: 26159094

26. Wang B, Feng L, Chen GF, Jiang WD, Liu Y, Kuang SY, et al. Jian carp (*Cyprinus carpio* var. Jian) intestinal immune responses, antioxidant status and tight junction protein mRNA expression are modulated via Nrf2 and PKC in response to dietary arginine deficiency. Fish Shellfish Immunol. 2016; 51: 116–124. https://doi.org/10.1016/j.fsi.2015.10.032 PMID: 26518504

27. Perez-Sanchez T, Balcazar JL, Merrifield DL, Carnevali O, Gioacchini G, de Blas I, et al. Expression of immune-related genes in rainbow trout (Oncorhynchus mykiss) induced by probiotic bacteria during Lactococcus garvieae infection. Fish Shellfish Immunol. 2011; 31: 196–201. https://doi.org/10.1016/j.fsi.2011.05.005 PMID: 21620974

28. Sun Y-Z, Xia H-Q, Yang H-L, Wang Y-L, Zou W-C. TLR2 signaling may play a key role in the probiotic modulation of intestinal microbiota in grouper *Epinephelus coioides*. Aquaculture. 2014; 430: 50–56.

29. He S, Zhang Y, Xu L, Yang Y, Marubashi T, Zhou Z, et al. Effects of dietary *Bacillus subtilis* C-3102 on the production, intestinal cytokine expression and autochthonous bacteria of hybrid tilapia *Oreochromis niloticus* ♀× *Oreochromis aureus* ♂. Aquaculture. 2013; 412–413: 125–130.

30. Gao Q, Qi L, Wu T, Wang J. *Clostridium butyricum* activates TLR2-mediated MyD88-independent signaling pathway in HT-29 cells. Mol Cell Biochem. 2012; 361: 31–37. https://doi.org/10.1007/s11010-011-1084-y PMID: 21956671

31. Gao Q, Qi L, Wu T, Wang J. An important role of interleukin-10 in counteracting excessive immune response in HT-29 cells exposed to *Clostridium butyricum*. BMC Microbiol. 2012; 12: 100. https://doi.org/10.1186/1471-2180-12-100 PMID: 22681958

32. Sui SJ, Tian ZB, Wang QC, Chen R, Nie J, Li JS, et al. *Clostridium butyricum* promotes intestinal motility by regulation of TLR2 in interstitial cells of Cajal. Eur Rev Med Pharmacol Sci. 2018; 22: 4730–4738. https://doi.org/10.26355/eurrev_201807_15533 PMID: 30058712

33. Kanai T, Mikami Y, Hayashi A. A breakthrough in probiotics: *Clostridium butyricum* regulates gut homeostasis and anti-inflammatory response in inflammatory bowel disease. J Gastroenterol. 2015; 50: 928–939. https://doi.org/10.1007/s00535-015-1084-x PMID: 25940150

34. Liu L, Zeng D, Yang M, Wen B, Lai J, Zhou Y, et al. Probiotic *Clostridium butyricum* improves the growth performance, immune function, and gut microbiota of weaning rex rabbits. Probiotics Antimicrob Proteins. 2018. https://doi.org/10.1007/s12602-018-9476-x PMID: 30324399

35. Li L, Feng L, Jiang WD, Jiang J, Wu P, Kuang SY, et al. Dietary pantothenic acid deficiency and excess depress the growth, intestinal mucosal immune and physical functions by regulating NF-kappaB, TOR, Nrf2 and MLCK signaling pathways in grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol. 2015; 45: 399–413. https://doi.org/10.1016/j.fsi.2015.04.030 PMID: 25957886

36. Ruan Z, Liu S, Zhou Y, Mi S, Liu G, Wu X, et al. Chlorogenic acid decreases intestinal permeability and increases expression of intestinal tight junction proteins in weaned rats challenged with LPS. PLoS One. 2014; 9: e97815. https://doi.org/10.1371/journal.pone.0097815 PMID: 24887396

37. Gong Y, Li H, Li Y. Effects of *Bacillus subtilis* on epithelial tight junctions of mice with inflammatory bowel disease. J Interferon Cytokine Res. 2016; 36: 75–85. https://doi.org/10.1089/jir.2015.0030 PMID: 26720180

38. Anderson RC, Cookson AL, McNabb WC, Park Z, McCann MJ, Kelly WJ, et al. *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiol. 2010; 10: 316. https://doi.org/10.1186/1471-2180-10-316 PMID: 21143932

39. Zhang L, Zhang L, Zhan X, Zeng X, Zhou L, Cao G, et al. Effects of dietary supplementation of probiotic, *Clostridium butyricum*, on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with *Escherichia coli* K88. J Anim Sci Biotechnol. 2016; 7: 3. https://doi.org/10.1186/s40104-016-0061-4 PMID: 26819705

40. Li HH, Li YP, Zhu Q, Qiao JY, Wang WJ. Dietary supplementation with *Clostridium butyricum* helps to improve the intestinal barrier function of weaned piglets challenged with enterotoxigenic *Escherichia coli* K88. J Appl Microbiol. 2018; 125: 964–975. https://doi.org/10.1111/jam.13936 PMID: 29851202

41. Duan Y, Zhang Y, Dong H, Wang Y, Zhang J. Effect of the dietary probiotic *Clostridium butyricum* on growth, intestine antioxidant capacity and resistance to high temperature stress in kuruma shrimp *Marsupenaeus japonicus*. J Therm Biol. 2017; 66: 93–100. https://doi.org/10.1016/j.jtherbio.2017.04.004 PMID: 28477915

42. Duan Y, Zhang Y, Dong H, Wang Y, Zheng X, Zhang J. Effect of dietary *Clostridium butyricum* on growth, intestine health status and resistance to ammonia stress in Pacific white shrimp *Litopenaeus*

vannamei. Fish Shellfish Immunol. 2017; 65: 25–33. https://doi.org/10.1016/j.fsi.2017.03.048 PMID: 28359948

43. Li H, Gong Y, Xie Y, Sun Q, Li Y. *Clostridium butyricum* protects the epithelial barrier by maintaining tight junction protein expression and regulating microflora in a murine model of dextran sodium sulfate-induced colitis. Scand J Gastroenterol. 2018; 53: 1031–1042. https://doi.org/10.1080/00365521.2016.1192678 PMID: 30141701

44. Hagihara M, Yamashita R, Matsumoto A, Mori T, Inagaki T, Nonogaki T, et al. The impact of probiotic *Clostridium butyricum* MIYAIRI 588 on murine gut metabolic alterations. J Infect Chemother. 2019; 25: 571–577. https://doi.org/10.1016/j.jiac.2019.02.008 PMID: 31101528

45. Gao Q, Xiao Y, Sun P, Peng S, Yin F, Ma X, et al. *In vitro* protective efficacy of *Clostridium butyricum* against fish pathogen infections. Indian J Microbiol. 2013; 53: 453–459. https://doi.org/10.1007/s12088-013-0394-z PMID: 24426150

46. Pan X, Wu T, Zhang L, Song Z, Tang H, Zhao Z. *In vitro* evaluation on adherence and antimicrobial properties of a candidate probiotic *Clostridium butyricum* CB2 for farmed fish. J Appl Microbiol. 2008; 105: 1623–1629. https://doi.org/10.1111/j.1365-2672.2008.03885.x PMID: 18795975

47. Duan Y, Wang Y, Dong H, Ding X, Liu Q, Li H, et al. Changes in the intestine microbial, digestive, and immune-related genes of litopenaeus vannamei in response to dietary probiotic *Clostridium butyricum* supplementation. Front Microbiol. 2018; 9: 2191. https://doi.org/10.3389/fmicb.2018.02191 PMID: 30283419

48. Zhang L, Cao GT, Zeng XF, Zhou L, Ferket PR, Xiao YP, et al. Effects of *Clostridium butyricum* on growth performance, immune function, and cecal microflora in broiler chickens challenged with *Escherichia coli* K88. Poult Sci. 2014; 93: 46–53. https://doi.org/10.3382/ps.2013-03412 PMID: 24570422

49. Yang CM, Cao GT, Ferket PR, Liu TT, Zhou L, Zhang L, et al. Effects of probiotic, *Clostridium butyricum*, on growth performance, immune function, and cecal microflora in broiler chickens. Poult Sci. 2012; 91: 2121–2129. https://doi.org/10.3382/ps.2011-02131 PMID: 22912445

50. Zhan HQ, Dong XY, Li LL, Zheng YX, Gong YJ, Zou XT. Effects of dietary supplementation with *Clostridium butyricum* on laying performance, egg quality, serum parameters, and cecal microflora of laying hens in the late phase of production. Poult Sci. 2019; 98: 896–903. https://doi.org/10.3382/ps/pey436 PMID: 30285187

51. Lin Y-H, Ku C-Y, Shiau S-Y. Estimation of dietary magnesium requirements of juvenile tilapia, *Oreochromis niloticus×Oreochromis aureus*, reared in freshwater and seawater. Aquaculture. 2013; 380–383: 47–51.

52. Shiau SY, Lo PS. Dietary choline requirements of juvenile hybrid tilapia, *Oreochromis niloticus* x *O. aureus*. J Nutr. 2000; 130: 100–103. https://doi.org/10.1093/jn/130.1.100 PMID: 10613774

53. Zhou P, Wang M, Xie F, Deng D-F, Zhou Q. Effects of dietary carbohydrate to lipid ratios on growth performance, digestive enzyme and hepatic carbohydrate metabolic enzyme activities of large yellow croaker (*Larmichthys crocea*). Aquaculture. 2016; 452: 45–51.

54. Hoseinifar SH, Mirvaghefi A, Amoozegar MA, Sharifian M, Esteban MA. Modulation of innate immune response, mucosal parameters and disease resistance in rainbow trout (*Oncorhynchus mykiss*) upon synbiotic feeding. Fish Shellfish Immunol. 2015; 45: 27–32. https://doi.org/10.1016/j.fsi.2015.03.029 PMID: 25827628

55. Bayha KM, Ortell N, Ryan CN, Griffitt KJ, Krasnec M, Sena J, et al. Crude oil impairs immune function and increases susceptibility to pathogenic bacteria in southern flounder. PLoS One. 2017; 12: e0176559. https://doi.org/10.1371/journal.pone.0176559 PMID: 28464028

56. Li W, Zhang X, Song W, Deng B, Liang Q, Fu L, et al. Effects of *Bacillus* preparations on immunity and antioxidant activities in grass carp (*Ctenopharyngodon idellus*). Fish Physiol Biochem. 2012; 38: 1585–1592. https://doi.org/10.1007/s10695-012-9652-y PMID: 22585415

57. Lei K, Li YL, Yu DY, Rajput IR, Li WF. Influence of dietary inclusion of *Bacillus licheniformis* on laying performance, egg quality, antioxidant enzyme activities, and intestinal barrier function of laying hens. Poult Sci. 2013; 92: 2389–2395. https://doi.org/10.3382/ps.2012-02686 PMID: 23960122

58. Sun YZ, Yang HL, Ma RL, Lin WY. Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*. Fish Shellfish Immunol. 2010; 29: 803–809. https://doi.org/10.1016/j.fsi.2010.07.018 PMID: 20637875

59. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001; 25: 402–408. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

60. Zhao J, Liu Y, Jiang J, Wu P, Chen G, Jiang W, et al. Effects of dietary isoleucine on growth, the digestion and absorption capacity and gene expression in hepatopancreas and intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquaculture. 2012; 368–369: 117–128.

61. Wang J, Xu H, Zuo R, Mai K, Xu W, Ai Q. Effects of oxidised dietary fish oil and high-dose vitamin E supplementation on growth performance, feed utilisation and antioxidant defence enzyme activities of juvenile large yellow croaker (*Larmichthys crocea*). Br J Nutr. 2016; 115: 1531–1538. https://doi.org/10.1017/S0007114516000398 PMID: 26948923

62. Liao XD, Ma G, Cai J, Fu Y, Yan XY, Wei XB, et al. Effects of *Clostridium butyricum* on growth performance, antioxidation, and immune function of broilers. Poult Sci. 2015; 94: 662–667. https://doi.org/10.3382/ps/pev038 PMID: 25717087

63. Duan Y, Zhang J, Huang J, Jiang S. Effects of dietary *Clostridium butyricum* on the growth, digestive enzyme activity, antioxidant capacity, and resistance to nitrite stress of Penaeus monodon. Probiotics Antimicrob Proteins. 2019; 11: 938–945. https://doi.org/10.1007/s12602-018-9421-z PMID: 29858778

64. Sumon MS, Ahmmed F, Khushi SS, Ahmmed MK, Rouf MA, Chisty MAH, et al. Growth performance, digestive enzyme activity and immune response of *Macrobrachium rosenbergii* fed with probiotic *Clostridium butyricum* incorporated diets. J King Saud Univ Sci. 2018; 30: 21–28.

65. Song ZF, Wu TX, Cai LS, Zhang LJ, Zheng XD. Effects of dietary supplementation with *Clostridium butyricum* on the growth performance and humoral immune response in *Miichthys miiuy*. J Zhejiang Univ Sci B. 2006; 7: 596–602. https://doi.org/10.1631/jzus.2006.B0596 PMID: 16773736

66. Gao Q, Xiao C, Min M, Zhang C, Peng S, Shi Z. Effects of probiotics dietary supplementation on growth performance, innate immunity and digestive enzymes of silver pomfret, *Pampus argenteus*. Indian J Anim Res. 2016; 50: 936–941.

67. Xu HJ, Jiang WD, Feng L, Liu Y, Wu P, Jiang J, et al. Dietary vitamin C deficiency depresses the growth, head kidney and spleen immunity and structural integrity by regulating NF-kappaB, TOR, Nrf2, apoptosis and MLCK signaling in young grass carp (Ctenopharyngodon idella). Fish Shellfish Immunol. 2016; 52: 111–138. https://doi.org/10.1016/j.fsi.2016.02.033 PMID: 26944716

68. Wei Z, Yi L, Xu W, Zhou H, Zhang Y, Zhang W, et al. Effects of dietary nucleotides on growth, non-specific immune response and disease resistance of sea cucumber *Apostichopus japonicas*. Fish Shellfish Immunol. 2015; 47: 1–6. https://doi.org/10.1016/j.fsi.2015.08.017 PMID: 26299704

69. Zhou Q, Wang L, Wang H, Xie F, Wang T. Effect of dietary vitamin C on the growth performance and innate immunity of juvenile cobia (*Rachycentron canadum*). Fish Shellfish Immunol. 2012; 32: 969–975. https://doi.org/10.1016/j.fsi.2012.01.024 PMID: 22366311

70. Zhao J, Liu Y, Jiang J, Wu P, Jiang W, Li S, et al. Effects of dietary isoleucine on the immune response, antioxidant status and gene expression in the head kidney of juvenile Jian carp (*Cyprinus carpio* var. Jian). Fish Shellfish Immunol. 2013; 35: 572–580. https://doi.org/10.1016/j.fsi.2013.05.027 PMID: 23742869

71. Neissi A, Rafiee G, Nematollahi M, Razavi SH, Maniei F. Influence of supplemented diet with *Pediococcus acidilactici* on non-specific immunity and stress indicators in green terror (*Aequidens rivulatus*) during hypoxia. Fish Shellfish Immunol. 2015; 45: 13–18. https://doi.org/10.1016/j.fsi.2015.04.008 PMID: 25891275

72. Pirarat N, Pinpimai K, Endo M, Katagiri T, Ponpornpisit A, Chansue N, et al. Modulation of intestinal morphology and immunity in nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG. Res Vet Sci. 2011; 91: e92–e97. https://doi.org/10.1016/j.rvsc.2011.02.014 PMID: 21536310

73. Wang Y-B, Tian Z-Q, Yao J-T, Li W-F. Effect of probiotics, *Enteroccus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response. Aquaculture. 2008; 277: 203–207.

74. Sun YZ, Yang HL, Ma RL, Song K, Li JS. Effect of *Lactococcus lactis* and *Enterococcus faecium* on growth performance, digestive enzymes and immune response of grouper *Epinephelus coioides*. Aquac Nutr. 2012; 18: 281–289.

75. Wang J, Qi L, Mei L, Wu Z, Wang H. *C. butyricum* lipoteichoic acid inhibits the inflammatory response and apoptosis in HT-29 cells induced by *S. aureus* lipoteichoic acid. Int J Biol Macromol. 2016; 88: 81–87. https://doi.org/10.1016/j.ijbiomac.2016.03.054 PMID: 27020942

76. Villeger R, Saad N, Grenier K, Falourd X, Foucat L, Urdaci MC, et al. Characterization of lipoteichoic acid structures from three probiotic *Bacillus* strains: involvement of D-alanine in their biological activity. Antonie Van Leeuwenhoek. 2014; 106: 693–706. https://doi.org/10.1007/s10482-014-0239-8 PMID: 25090957

77. Monefeldt K, Helgeland K, Tollefsen T. *In vitro* activation of the classical pathway of complement by a streptococcal lipoteichoic acid. Oral Microbiol Immunol. 1994; 9: 70–76. PMID: 8008432

78. Biller-Takahashi JD, Takahashi LS, Mingatto FE, Urbinati EC. The immune system is limited by oxidative stress: dietary selenium promotes optimal antioxidative status and greatest immune defense in

pacu *Piaractus mesopotamicus*. Fish Shellfish Immunol. 2015; 47: 360–367. https://doi.org/10.1016/j.fsi.2015.09.022 PMID: 26370542

79. Duan GL, Wang CN, Liu YJ, Yu Q, Tang XL, Ni X, et al. Resveratrol alleviates endotoxemia-associated adrenal insufficiency by suppressing oxidative/nitrative stress. Endocr J. 2016; 63: 569–580. https://doi.org/10.1507/endocrj.EJ15-0610 PMID: 27052214

80. Wang FY, Liu JM, Luo HH, Liu AH, Jiang Y. Potential protective effects of *Clostridium butyricum* on experimental gastric ulcers in mice. World J Gastroenterol. 2015; 21: 8340–8351. https://doi.org/10.3748/wjg.v21.i27.8340 PMID: 26217085

81. He R-P, Feng J, Tian X-L, Dong S-L, Wen B. Effects of dietary supplementation of probiotics on the growth, activities of digestive and non-specific immune enzymes in hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀). Aquac Res. 2017; 48: 5782–5790.

82. Zhang B, Yang X, Guo Y, Long F. Effects of dietary lipids and *Clostridium butyricum* on the performance and the digestive tract of broiler chickens. Arch Anim Nutr. 2011; 65: 329–339. PMID: 21888038

83. Nakanishi S, Kataoka K, Kuwahara T, Ohnishi Y. Effects of high amylose maize starch and *Clostridium butyricum* on metabolism in colonic microbiota and formation of azoxymethane-induced aberrant crypt foci in the rat colon. Microbiol Immunol. 2003; 47: 951–958. https://doi.org/10.1111/j.1348-0421.2003.tb03469.x PMID: 14695445

84. Dan J, Fang Z, Chin SX, Tian XF, Su TC. Biohydrogen production from hydrolysates of selected tropical biomass wastes with *Clostridium butyricum*. Sci Rep. 2016; 6: 27205. https://doi.org/10.1038/srep27205 PMID: 27251222

85. Rosignoli P, Fabiani R, De Bartolomeo A, Spinozzi F, Agea E, Pelli MA, et al. Protective activity of butyrate on hydrogen peroxide-induced DNA damage in isolated human colonocytes and HT29 tumour cells. Carcinogenesis. 2001; 22: 1675–1680. https://doi.org/10.1093/carcin/22.10.1675 PMID: 11577008

86. Courtois F, Seidman EG, Delvin E, Asselin C, Bernotti S, Ledoux M, et al. Membrane peroxidation by lipopolysaccharide and iron-ascorbate adversely affects Caco-2 cell function: beneficial role of butyric acid. Am J Clin Nutr. 2003; 77: 744–750. https://doi.org/10.1093/ajcn/77.3.744 PMID: 12600871

87. Zhou HX, Han B, Hou LM, An TT, Jia G, Cheng ZX, et al. Protective effects of hydrogen gas on experimental acute pancreatitis. PLoS One. 2016; 11: e0154483. https://doi.org/10.1371/journal.pone.0154483 PMID: 27115738

88. Zhao J, Feng L, Liu Y, Jiang W, Wu P, Jiang J, et al. Effect of dietary isoleucine on the immunity, antioxidant status, tight junctions and microflora in the intestine of juvenile Jian carp (Cyprinus carpio var. Jian). Fish Shellfish Immunol. 2014; 41: 663–673. https://doi.org/10.1016/j.fsi.2014.10.002 PMID: 25451003

89. Liu W, Yang Y, Zhang J, Gatlin DM, Ringo E, Zhou Z. Effects of dietary microencapsulated sodium butyrate on growth, intestinal mucosal morphology, immune response and adhesive bacteria in juvenile common carp (*Cyprinus carpio*) pre-fed with or without oxidised oil. Br J Nutr. 2014; 112: 15–29. https://doi.org/10.1017/S0007114514000610 PMID: 24774835

90. Alva-Murillo N, Medina-Estrada I, Baez-Magana M, Ochoa-Zarzosa A, Lopez-Meza JE. The activation of the TLR2/p38 pathway by sodium butyrate in bovine mammary epithelial cells is involved in the reduction of *Staphylococcus aureus* internalization. Mol Immunol. 2015; 68: 445–455. https://doi.org/10.1016/j.molimm.2015.09.025 PMID: 26471700

91. Kim TW, Staschke K, Bulek K, Yao J, Peters K, Oh KH, et al. A critical role for IRAK4 kinase activity in toll-like receptor-mediated innate immunity. J Exp Med. 2007; 204: 1025–1036. https://doi.org/10.1084/jem.20061825 PMID: 17470642

92. Rashidi N, Mirahmadian M, Jeddi-Tehrani M, Rezania S, Ghasemi J, Kazemnejad S, et al. Lipopolysaccharide- and lipoteichoic acid-mediated pro-inflammatory cytokine production and modulation of TLR2, TLR4 and MyD88 expression in human endometrial cells. J Reprod Infertil. 2015; 16: 72–81. PMID: 25927023

93. Kamada N, Seo SU, Chen GY, Nunez G. Role of the gut microbiota in immunity and inflammatory disease. Nat Rev Immunol. 2013; 13: 321–335. https://doi.org/10.1038/nri3430 PMID: 23618829

94. Molloy MJ, Bouladoux N, Belkaid Y. Intestinal microbiota: shaping local and systemic immune responses. Semin Immunol. 2012; 24: 58–66. https://doi.org/10.1016/j.smim.2011.11.008 PMID: 22178452

95. Nowak P, Troseid M, Avershina E, Barqasho B, Neogi U, Holm K, et al. Gut microbiota diversity predicts immune status in HIV-1 infection. AIDS. 2015; 29: 2409–2418. https://doi.org/10.1097/QAD.0000000000000869 PMID: 26355675

96. Standen BT, Rodiles A, Peggs DL, Davies SJ, Santos GA, Merrifield DL. Modulation of the intestinal microbiota and morphology of tilapia, *Oreochromis niloticus*, following the application of a multi-

species probiotic. Appl Microbiol Biotechnol. 2015; 99: 8403–8417. https://doi.org/10.1007/s00253-015-6702-2 PMID: 26115752

97. Zhai Q, Yu L, Li T, Zhu J, Zhang C, Zhao J, et al. Effect of dietary probiotic supplementation on intestinal microbiota and physiological conditions of nile tilapia (*Oreochromis niloticus*) under waterborne cadmium exposure. Antonie Van Leeuwenhoek. 2017; 110: 501–513. https://doi.org/10.1007/s10482-016-0819-x PMID: 28028640

98. Ferguson RM, Merrifield DL, Harper GM, Rawling MD, Mustafa S, Picchietti S, et al. The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). J Appl Microbiol. 2010; 109: 851–862. https://doi.org/10.1111/j.1365-2672.2010.04713.x PMID: 20353430

99. Zhang Z, Li D, Refaey MM, Xu W. High spatial and temporal variations of microbial community along the southern Catfish gastrointestinal tract: insights into dynamic food digestion. Front Microbiol. 2017; 8: 1531. https://doi.org/10.3389/fmicb.2017.01531 PMID: 28848535

100. She R, Li TT, Luo D, Li JB, Yin LY, Li H, et al. Changes in the intestinal microbiota of gibel carp (*Carassius gibelio*) associated with *Cyprinid herpesvirus* 2 (CyHV-2) infection. Curr Microbiol. 2017; 74: 1130–1136. https://doi.org/10.1007/s00284-017-1294-y PMID: 28748273

101. Larsen AM, Mohammed HH, Arias CR. Characterization of the gut microbiota of three commercially valuable warmwater fish species. J Appl Microbiol. 2014; 116: 1396–1404. https://doi.org/10.1111/jam.12475 PMID: 24529218

102. Li T, Long M, Gatesoupe FJ, Zhang Q, Li A, Gong X. Comparative analysis of the intestinal bacterial communities in different species of carp by pyrosequencing. Microb Ecol. 2015; 69: 25–36. https://doi.org/10.1007/s00248-014-0480-8 PMID: 25145494

103. Tsuchiya C, Sakata T, Sugita H. Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. Lett Appl Microbiol. 2008; 46: 43–48. https://doi.org/10.1111/j.1472-765X.2007.02258.x PMID: 17944860

104. Liu Y, Yao Y, Li H, Qiao F, Wu J, Du ZY, et al. Influence of endogenous and exogenous estrogenic endocrine on intestinal microbiota in Zebrafish. PLoS One. 2016; 11: e0163895. https://doi.org/10.1371/journal.pone.0163895 PMID: 27701432

105. Gainza O, Ramirez C, Ramos AS, Romero J. Intestinal microbiota of white shrimp *Penaeus vannamei* under intensive cultivation conditions in ecuador. Microb Ecol. 2018; 75: 562–568. https://doi.org/10.1007/s00248-017-1066-z PMID: 28929202

106. Zhang M, Sun Y, Chen L, Cai C, Qiao F, Du Z, et al. Symbiotic bacteria in gills and guts of Chinese mitten crab (*Eriocheir sinensis*) differ from the free-living bacteria in water. PLoS One. 2016; 11: e0148135. https://doi.org/10.1371/journal.pone.0148135 PMID: 26820139

107. van der Marel M, Schroers V, Neuhaus H, Steinhagen D. Chemotaxis towards, adhesion to, and growth in carp gut mucus of two *Aeromonas hydrophila* strains with different pathogenicity for common carp, *Cyprinus carpio* L. J Fish Dis. 2008; 31: 321–330. https://doi.org/10.1111/j.1365-2761.2008.00902.x PMID: 18355183

108. Harikrishnan R, Balasundaram C. Modern trends in *Aeromonas hydrophila* disease management with fish. Reviews in Fisheries Science. 2005; 13: 281–320.

109. Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Folsch UR, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut. 2004; 53: 685–693. https://doi.org/10.1136/gut.2003.025403 PMID: 15082587

110. Sun J, Wang F, Ling Z, Yu X, Chen W, Li H, et al. *Clostridium butyricum* attenuates cerebral ischemia/reperfusion injury in diabetic mice via modulation of gut microbiota. Brain Res. 2016; 1642: 180–188. https://doi.org/10.1016/j.brainres.2016.03.042 PMID: 27037183

111. Konstantinov SR, Favier CF, Zhu WY, Williams BA, Klüß J, Souffrant W, et al. Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. Anim Res. 2004; 53: 317–324.

112. Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev. 2001; 81: 1031–1064. https://doi.org/10.1152/physrev.2001.81.3.1031 PMID: 11427691

113. Fang CL, Sun H, Wu J, Niu HH, Feng J. Effects of sodium butyrate on growth performance, haematological and immunological characteristics of weanling piglets. J Anim Physiol Anim Nutr (Berl). 2014; 98: 680–685.

114. Jiang WD, Tang RJ, Liu Y, Kuang SY, Jiang J, Wu P, et al. Manganese deficiency or excess caused the depression of intestinal immunity, induction of inflammation and dysfunction of the intestinal physical barrier, as regulated by NF-kappaB, TOR and Nrf2 signalling, in grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol. 2015; 46: 406–416. https://doi.org/10.1016/j.fsi.2015.06.007 PMID: 26072140

115. Nebot-Vivinus M, Harkat C, Bzioueche H, Cartier C, Plichon-Dainese R, Moussa L, et al. Multispecies probiotic protects gut barrier function in experimental models. World J Gastroenterol. 2014; 20: 6832–6843. https://doi.org/10.3748/wjg.v20.i22.6832 PMID: 24944474