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Effects of dietary compound probiotics and heat-killed compound probiotics on antioxidative capacity, plasma biochemical parameters, intestinal morphology, and microbiota of *Cyprinus carpio haematopterus*

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Abstract

This study investigated the compound probiotics and heat-killed compound probiotics on antioxidants, plasma biochemical parameters, intestinal histopathology, and microbiome of koi fish, *Cyprinus carpio haematopterus*. Koi fish (15.9 ± 0.63 g) were fed with five different diets: C (control, basal diet without probiotics), D3 (3% compound probiotics), D6 (6% compound probiotics), lnaD3 (3% heat-killed compound probiotics), and lnaD6 (6% heat-killed compound probiotics). After 28 days of the feeding trial, results showed that the activities of acid phosphatase (ACP), alkaline phosphatase (AKP), catalase (CAT), and total superoxide dismutase (T-SOD) were significantly improved in koi fish fed with heat-killed compound probiotics compared to the control group ($P < 0.05$). Interestingly, total activities of glutamyl oxaloacetic transaminase (GOT), total protein (TP), albumin (TA), globulin (GLB), and triglyceride (TG) were significantly improved in koi fish fed with compound probiotics compared to the control group ($P < 0.05$). The intestinal muscular and intestinal villi height of lnaD3 was significantly higher than other groups ($P < 0.05$). In addition, compound probiotics and heat-killed compound probiotics increased the diversity of intestinal microbes. Therefore, dietary supplementation with heat-killed compound probiotics can be an effective measure for improving nonspecific immune response, intestinal morphology, and the flora of koi fish.

Keywords Probiotics · Koi fish · Antioxidative capacity · Plasma biochemical parameters · Intestinal morphology · Intestinal microbiota

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Introduction

Koi fish, *Cyprinus carpio haematopterus*, are popular worldwide due to their diverse colors and long-life span (Wang et al. 2021a, b, c). Currently, koi fish are essential for the import and export of ornamental fish and have been widely cultured in China (Putri & Dewi 2019). However, with the intensification of the aquaculture environment, the resistance of their immune system to various diseases has been disrupted. Moreover, these diseases affect aquaculture development and cause significant economic losses (Amenyogbe et al. 2020). Antibiotics are often used to prevent and treat aquatic diseases in traditional aquaculture (Yeganeh et al. 2021). However, the misuse of antibiotics can lead to the development of resistance in pathogens, thus making treatment progressively less effective (Čížek et al. 2010; Santos & Ramos 2016). In addition, the misuse of antibiotics disrupts the intestinal flora and alters the microbiological system, which in turn affects the growth, digestion, and immunity of koi fish (Rekecki et al. 2009; Maynard et al. 2012). For these reasons, alternative methods are required to reduce the use of antibiotics in aquaculture.

According to previous studies, probiotics can enhance immune response (El-Saadony et al. 2021; Islam et al. 2021; Jahan et al. 2021; Haque et al. 2021; Khalafalla et al. 2020) and improve digestion through enzyme activities to improve growth performance of aquatic animals (Giri et al. 2020; Van al. 2019a; Elsabagh et al. 2018; Dowidar et al. 2018; Saputra et al. 2016; Saravanan et al. 2021). Meanwhile, studies have demonstrated that probiotics could decrease mortality and increase immunity in shrimp, *Litopenaeus vannamei*, and sea cucumber, *Apostichopus japonicus* (Chiu et al. 2021; Feng et al. 2020). Therefore, probiotics are recommended to be added to the diet as a functional additive to replace antibiotics to reduce resistance (Yeganeh et al. 2021). However, the application of live bacteria may adversely affect the environment due to the interaction of probiotics with the ecosystem (Dawood et al. 2019). In addition, live probiotics may lose their activity during transportation or in animal intestines. Therefore, replacing live probiotics with dead bacteria is significant because it can benefit the host (Wang et al. 2018).

Probiotics are usually treated by heat (Van et al. 2019b; Giri et al. 2020), so inactivated probiotics can withstand high temperatures while preparing fish feed without affecting their function (Díaz-Rosales et al. 2006; Salinas et al. 2008). It has been demonstrated that dead probiotic cells also have components and metabolites that can exert the same effects as live probiotics (Giri et al. 2020). Although the elevated temperature alters the composition of probiotics, the inactivated probiotics can stimulate immune responses in the host (Yang et al. 2019; Pan et al. 2008). In addition, heat-killed probiotics can affect intestinal bacteria to improve host growth. The heat-killed *Lactobacillus plantarum* is a candidate as one of the functional additives for fish. Studies have shown that heat-killed *Lactobacillus plantarum* is effective in the host in improving growth performance, survival rate, cytokine gene expression, and bacterial resistance (Biswas et al. 2013; Dawood et al. 2015; Van et al. 2019b; Duc et al. 2020). Notably, in another study, it has been reported that heat-killed *Pseudomonas aeruginosa* more effectively than other forms of *Pseudomonas aeruginosa* (Giri et al. 2011). Recently, Wu et al. (2020) reported that after supplementing the diet with two kinds of heat-inactivated probiotics, the compound heat-killed probiotics could increase the 45 expressions of genes related to growth, inflammation, and non-specific immunity. Furthermore, Frouël et al. (2008) found that the mixture of probiotics can improve the endocytosis vesicles of intestinal epithelial cells. Thus, it is important to study the specific effects of heat-killed compound probiotics, especially on the antioxidative status and intestinal health of koi fish.

This study evaluated the effects of compound probiotics and heat-killed compound probiotics on the antioxidative capacity, plasma biochemical parameters, intestinal morphology, and microbiota of koi fish. The result of this study might provide necessary information for applying heat-killed compound probiotics in the sustainable development of koi fish culture.

Materials and methods

Experimental diets and probiotic supplements

Fish meal, soybean meal, and wheat gluten were the primary protein sources to control koi fish diets. Fish oil and soybean oil were the primary lipid sources for the control diets. The main components of probiotics are *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Pseudomonas aeruginosa*, etc., and the effective viable count was 4.0×10^8 CFU/g. The live compound probiotics were inactivated using an automatic temperature control system ventilated oven (DK-s26; Shanghai Samsung Laboratory Instrument Co. Ltd.) at temperatures from 121 to 126 °C for 20 min (Segawa et al. 2008).

In this study, besides the control group C, four experimental diets were prepared with different concentrations of probiotics, two of which contained two levels of compound probiotics (D3 (3% compound probiotics) and D6 (6% compound probiotics)), and the other two contained two levels of heat-killed compound probiotics (lnaD3 (3% heat-killed compound probiotics) and lnaD6 (6% heat-killed compound probiotics)). These components were mixed with water and extruded into 1.5 mm pellets (Pinzheng Equipment Co., Ltd., Changzhou, China). Natural drying to ensure probiotic activity then was put in the − 20 °C immediately. The ingredients and proximate composition of the diets are presented in Table 1.

Experimental daily management and sample collection

All animal experiments complied with the Guidelines of the Care and Use of Laboratory Animals in China, and the study was approved by the ethics committee of Dalian Ocean University. Koi fish were purchased in an ornamental fish commercial hatchery (Fushan Koi Farm, Liaoning, China) and acclimated for 15 days in the tanks. After acclimatization, 300 uniform individuals of Koi fish with similar weights (15.9 ± 0.63 g) were randomly distributed into 15 tanks (capacity: 40 L).

During the experiment, the koi fish were kept in freshwater tanks at 23 °C with 12 h dark and 12 h light, and the pH was 7.8–8.0. Air pumps were used to maintain an adequate oxygen content in the water. The fish were fed at 3% of their body weight at 8:00 and 18:00. The residue and feces were cleaned every day at 17:00 and replaced 1/3 of the water. The feeding trial continued for 28 days.

At the end of the trial, the koi fish were starved for 24 h and weighed body. All koi fish were anesthetized with eugenol after being put on the ice plate. The blood was collected through the caudal vein, then put the blood sample in the tube and liquid nitrogen. In addition, the koi fish was opened with scissors and disinfection with 75% alcohol. Collected gut tracts and liver were quickly frozen in liquid nitrogen. All samples were kept at − 80 °C until further analysis.

Table 1 Ingredients and nutrient composition of koi fish fed with experimental diets (g/100 g)

Ingredients (g/100 g)	C	D3	D6	lnaD3	lnaD6
Steam fish meal[a]	40	40	40	40	40
Soybean meal[b]	25	25	25	25	25
Wheat flour[c]	21	21	19.5	21	19.5
Fish oil[d]	3	3	3	3	3
Soybean oil[e]	2	2	2	2	2
Vitamin mix[f]	1.5	1.5	1.5	1.5	1.5
Mineral mix[g]	1.5	1.5	1.5	1.5	1.5
Calcium dihydrogen phosphate[h]	1	1	1	1	1
Choline chloride	0.5	0.5	0.5	0.5	0.5
Microcrystalline cellulose[i]	4.5	1.5	0	1.5	0
Probiotics (live or heat-killed)[j]	0	3	6	3	6
Total	100	100	100	100	100
Dry matter (% in dry basis)[h]					
Moisture	32.75	33.57	30.55	30.89	37.37
Crude protein	44.27	47.2	45.51	41.74	44.7
Crude lipid	10.18	9.46	9.47	9.98	9.77
Crude ash	12.23	12.97	13.14	13.09	13.05

[a]Steam fish meal: the protein content is 65%. Weihai Xiangrun seafood Co., Ltd., Shandong, China.

[b]Soybean meal: the protein content is 42%

[c]Wheat gluten: the protein content is 78%

[d]Fish oil: Rongcheng Huanyu aquatic fish oil product factory.

[e]Soybean oil: Haijiali Arowana Cereals, Oils and Foods Co., Ltd.

[f]Vitamin mixture (per kg of premix): vitamin A, 1,000,000 IU; vitamin D_3, 300,000 IU; vitamin E, 4000 IU; vitamin K3, 1000 mg; vitamin B_1, 2000 mg; vitamin B_2, 1500 mg; vitamin B_6, 1000 mg; vitamin B_{12}, 5 mg; nicotinic acid, 1000 mg; vitamin C, 5000 mg; Ca pantothenate, 5000 mg; folic acid, 100 mg; inositol, 10,000 mg; carrier glucose; $H_2O \leq 100$ g/kg.

[g]Mineral mixture (0.025 mg/g of premix): NaCl, 107.79; $MgSO_4 \cdot 7\ H_2O$, 380.02; $NaHPO_4 \cdot 2\ H_2O$, 241.91; KH_2PO_4, 665.20; Ca $(H_2PO_4) \cdot 2\ H_2O$, 376.70; Fe citrate, 82.38; Ca lactate,907.10; Al $(OH)_3$, 0.52; $ZnSO_4 \cdot 7\ H_2O$, 9.90; $CuSO_4$, 0.28; $MnSO_4 \cdot 7\ H_2O$, 2.22; Ca $(IO_3)_2$,0.42; $CoSO_4 \cdot H_2O$, 2.77.

[h]Calcium biphosphate: Kemiou Chemical Reagent Co., Ltd., Tianjin, China.

[i]Microcrystalline cellulose: Yinuo Biological Technology Co., Ltd, Zhejiang, China.

[j]Dalian Shengtai Biology Science and Technology Co., Ltd.

[h]Values are analyzed and presented as mean ± SE, $n = 3$.

Non-specific immune parameters analysis

The levels of total acid phosphatase (ACP and spectrophotometric method), alkaline phosphatase (AKP and visible light colorimetry), catalase (CAT and visible light colorimetry), and superoxide dismutase (T-SOD and hydroxylamine method) in the liver of the koi fish were measured using kits purchased by Nanjing Jiancheng Institute of Biological Engineering (Nanjing Jiancheng Bioengineering Institute). All the parameters were performed using visible spectrophotometry (Shanghai Tianmei Scientific instrument Co., Ltd., VIS7200A). ACP and AKP colorimetric measurement wavelengths are 520 nm; CAT colorimetric measurement wavelengths are 405 nm; and T-SOD colorimetric measurement wavelengths are 550 nm. The sample pretreatment, reagent preparation, and determination steps were carried out strictly with the operating instructions.

Blood assays

After the fed trial, the koi fish from each treatment were anesthetized with eugenol (5 mg/L). Then, the blood was collected from the caudal vein using a plastic syringe (syringes were pre-washed with sodium heparin solution (1 g/L) to determine the hemoglobin level). The levels of total activities of glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvic acid transaminase (GPT), total protein (TP), albumin (TA), globulin (GLB), and triglyceride (TG) in the blood of the koi fish were measured by 7600–110 automatic biochemical analyzer (Hitachi, Japan).

Intestinal morphology

The intestines were fixed in Bouin's fixative solution for 24 h and were dehydrated using a series of graded ethanol concentrations in the DIAPATH dehydrator (Donatello). Following that, samples were embedded into melted paraffin wax using an embedding (JB-P5, Wuhan Junjie Electronics Co., Ltd) and sectioned into a 4.0-μm thick slice. Finally, the sections were stained with hematoxylin and eosin. The stained tissue sections were observed using an Olympus CX23 microscope (Olympus Industry Co., Ltd., Guangzhou, China).

Microbial study

Gut samples were taken from the refrigerator at − 80 °C and were subjected to intestinal flora determination. According to the manufacturer's instructions, the total genomic DNA was extracted from intestinal samples using the bacterial DNA kit (E.Z.N.A bacterial Mag-Bind SoilDNA Kit OMEGA). Next, Qubit 3.0 DNA detection kit was used to accurately quantify genomic DNA to determine the amount of DNA used in the PCR reaction. Universal primers 805R (5t-GACTACHVGGGTATCTAA GATCC-3AC and 341F (5A-CCT ACGGGNGGCWGCAG-3CTACGGGNGGCWGCAGA GATCC 3.0 V3-V4 hypervariable region of the 16S rRNA gene, respectively. The following PCR reaction conditions were used: 94 °C heat preservation for 3 min, followed by 94 °C heat preservation for 30 s, 45 °C for 20 s, and 65 °C for 30 s for five cycles; 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s for 20 cycles; finally, an extension for 5 min at 72 °C was performed. Purified PCR products were analyzed by 2% gel electrophoresis to check the library size, and the Qubit3.0 fluorescence quantitative instrument was used to determine the concentration of the library. High-throughput sequencing was performed using the Illumina MiSeq platform. Finally, chimeric sequence detection and OTU selection at 97% sequence similarity were conducted using USEARCH v6.1 64.

Statistical analysis

The statistical analysis was performed by the SPSS20.0 (SPSS, Chicago, IL, USA) software. All data are expressed as mean ± standard error. Two-way ANOVA was employed to test the effects of different probiotics and concentrations and their interactions, excluding the control diet. Data from the test group were compared using Duncan's multiple comparisons (one-way ANOVA). $P < 0.05$ was considered statistically significant.

Results

There was no mortality or abnormally behaved fish in each dietary group during a feeding trial. Furthermore, all dietary groups showed similar specific growth rates (2.18–2.66) and feed intake. Only the relative growth rate of the lnaD6 groups was significantly higher than the other groups ($P < 0.05$).

Hepatopancreatic immune enzyme activity assays

The immune enzymes of the koi fish fed with different diets for 28 days are given in Table 2. In two-way ANOVA, an interactive effect was found in test groups on the ACP and AKP. The ACP activity of the D3 and lnaD3 groups was significantly higher than that of the D6 group ($P < 0.05$). The ACP activity of the lnaD6 group was significantly higher than that of the D3, D6, and lnaD3 groups ($P < 0.05$). The AKP activity of the lnaD6 group was significantly lower than that of the D3, D6, and lnaD3 groups ($P < 0.05$). In the two independent samples' test, the ACP activity of the D3, lnaD3, and lnaD6 groups was significantly higher than the control group ($P < 0.05$). The AKP activity of the D3, D6, lnaD3, and lnaD6 groups was significantly higher than the control group ($P < 0.05$).

In two-way ANOVA, interactive effects were found in test groups on the CAT and T-SOD. The CAT activity of the lnaD6 group was significantly higher than that of the D3, D6, and lnaD3 groups ($P < 0.05$). Similar trends were found in T-SOD. T-SOD was higher in the lnaD6 group than in the D3, D6, and lnaD3 groups ($P < 0.05$). In the two independent samples' test, the CAT activity of the D3, lnaD3, and lnaD6 groups was significantly higher than the control group ($P < 0.05$). The T-SOD activity of D3, D6, lnaD3, and lnaD6 was significantly higher than the control group ($P < 0.05$) (Table 2).

Blood hematology and biochemistry

The blood index of the koi fish fed with different diets for 28 days is given in Table 3. In addition to GLB, interactive effects were found in two-way ANOVA of other indicators. GOT, GPT, TP, and TA were significantly lower in the lnaD6 group than in D3, D6, and lnaD3 groups ($P < 0.05$). TG in D6 was significantly lower than in D3, lnaD3, and lnaD6 groups ($P < 0.05$). In the two independent samples' test, the GOT, TP, and GLB of the D3 and D6 groups were significantly higher than the control group ($P < 0.05$), on the contrary, the GOT, TP, and GLB of lnaD3 and lnaD6 groups were significantly lower than the control group ($P < 0.05$). The GPT of the D3, D6, and lnaD3 groups was significantly higher than the control group ($P < 0.05$), and the GPT of the lnaD6 group was significantly lower than the control group ($P < 0.05$). The TA of the D3 and D6 groups was significantly higher than the control group ($P < 0.05$), and the TA of the lnaD6 group was significantly lower than the control group ($P < 0.05$). All test groups in TG were significantly lower than the control group ($P < 0.05$).

Table 2 The hepatopancreatic immune enzyme of koi fish fed with experimental diets

Parameters	Treatment					Probability (P value)3		
	Control	D3 (3%)	D6 (6%)	lnaD3 (3%)	lnaD6 (6%)	D or lnaD	concentration	D or lnaD × concentration
ACP (U/gprot)	277.28 ± 14.38	356.93 ± 8.33^{b*}	249.66 ± 2.80^{a*}	416.41 ± 24.23^{b*}	551.20 ± 30.89^{c*}	0.000	0.104	0.000
AKP (U/gprot)	0.02 ± 0.01	0.05 ± 0.00^{b*}	0.08 ± 0.00^{d*}	0.07 ± 0.00^{c*}	0.03 ± 0.00^{a*}	0.000	0.513	0.000
CAT (U/mgprot)	31.31 ± 0.49	40.73 ± 0.65^{b*}	26.26 ± 0.99^{a*}	43.02 ± 1.83^{b*}	50.90 ± 1.10^{c*}	0.000	0.009	0.000
T-SOD (U/mgprot)	526.53 ± 4.24	653.80 ± 20.80^{b*}	553.48 ± 2.98^{a*}	663.89 ± 9.40^{b*}	770.35 ± 34.88^{c*}	0.001	0.886	0.001

Data were expressed as mean ± S.E.M. from triplicate groups. Data with different superscript letters in one row represent a significant difference from the test group ($P < 0.05$).

Significant effects were determined by two-way ANOVA. Control is not included in two-way ANOVA. D or lnaD × concentration, interaction.

Significant effects were determined by a two independent samples' test in comparison of the control group and other experimental groups.

Data with * in one row represent a significant difference from the control group ($P < 0.05$).

Table 3 The plasma biochemical parameters of koi fish fed with different probiotics

Parameters	Treatments					Probability (*P* value)3		
	Control	D3	D6	lnaD3	lnaD6	D or lnaD	concentration	D or lnaD × concentration
GOT (IU/L)	217 ± 3.79	$239 \pm 4.04^{c*}$	$309 \pm 4.63^{d*}$	$148 \pm 3.51^{b*}$	$97 \pm 19.71^{a*}$	0.000	0.034	0.000
GPT (U/L)	11.32 ± 0.88	$15.34 \pm 0.33^{b*}$	$23.31 \pm 0.66^{c*}$	$15.68 \pm 14.32^{b*}$	$6.39 \pm 0.88^{a*}$	0.000	0.299	0.000
TP (g/dL)	24.64 ± 3.78	$31.61 \pm 4.04^{c*}$	$34.33 \pm 4.63^{d*}$	$15.63 \pm 3.51^{b*}$	$11.68 \pm 2.64^{a*}$	0.000	0.169	0.000
TA (g/L)	14 ± 0.01	$17.23 \pm 15.99^{d*}$	$23 \pm 18.03^{c*}$	11.66 ± 8.79^{b}	$9.37 \pm 7.90^{a*}$	0.000	0.046	0.000
GLB (g/L)	10.3 ± 1.76	13 ± 1.15	12.67 ± 0.33	$4 \pm 0.00^{*}$	$2 \pm 0.00^{*}$	0.000	0.088	0.203
TG (mg/dL)	0.88 ± 0.80	$0.63 \pm 0.56^{b*}$	$0.53 \pm 0.46^{a*}$	$0.58 \pm 0.54^{b*}$	$0.62 \pm 0.55^{b*}$	0.106	0.074	0.001

Intestinal morphology

In two-way ANOVA, interactive effects were found on the intestinal muscular in test groups. The intestinal muscular thickness of the lnaD3 group was significantly higher than that of the D3, D6, and lnaD6 groups ($P<0.05$). The intestinal muscular thickness was significantly lower in the lnaD6 group than in D3, D6, and lnaD3 groups ($P<0.05$).

In the two independent samples' test, the intestinal villi height of the lnaD3 group was significantly higher than the control group ($P<0.05$). The intestinal villus width of the lnaD6 group was significantly higher than in the control group ($P<0.05$). All the test groups were significantly higher than the control group ($P<0.05$) (Fig. 1) (Table 4).

Gut microflora

Richness and diversity

Figure 2 indicates that the sample dilution curve tended to be flat. The coverage of all the sequenced samples ranged up to 0.999 in all the treatments, demonstrating that the sequencing result had a sufficient amount of sequencing data (Table 5). The Shannon index, Chao1, Ace estimators, and Simpson index represented the richness and alpha diversity of the intestinal microbiota of the koi fish gut microbial community. The order of Shannon, Chao1, and Ace estimators of all treatment groups was higher than that of the control group. The Simpson order index of all treatment groups was lower than that of the control group.

Composition of intestinal microbiota

The relative abundance of the intestinal flora of each group at the phylum level is shown in Fig. 3. Results showed that the most dominant bacteria were *Fusobacteria* in the intestine of fish at the phylum level. The relative abundance of *Bacteroidetes* in the D3, lnaD3, and lnaD6 groups was higher than that in the control group. Notably, the relative abundance of *Proteobacteria* increased in the lnaD6 group.

At the genus level in Fig. 4, the *Cetobacterium* was dominant in the intestines of fish. The relative abundance of *Bacteroides* increased in the D3, lnaD3, and lnaD6 groups. Moreover, the relative abundance of *Aeromonas* was increased in lanD6.

Discussion

This study explores the interactive effects of activity and concentration of probiotics in aquaculture. Probiotics can increase teleost fish immunity in vivo (Aly et al. 2008; Balcázar et al. 2007; Daz-Rosales et al. 2009).

ACP and AKP are probiotic indicators that have an impact on aquatic animal health (Yi et al. 2018). ACP is an enzyme found in lysosomes. It is a macrophage activation marker that can eliminate exogenous microorganisms (Li et al. 2019). In this experiment, koi fish fed on diets containing heat-killed compound probiotics had higher ACP activity than the other groups. This was consistent with the results for carp *Labeo rohita* (Giri et al. 2013) and hybrid grouper *Epinephelus lanceolatus anceolafuscoguttatus us*(Li et al. 2019). However, koi fish fed on diets containing 6% compound probiotics

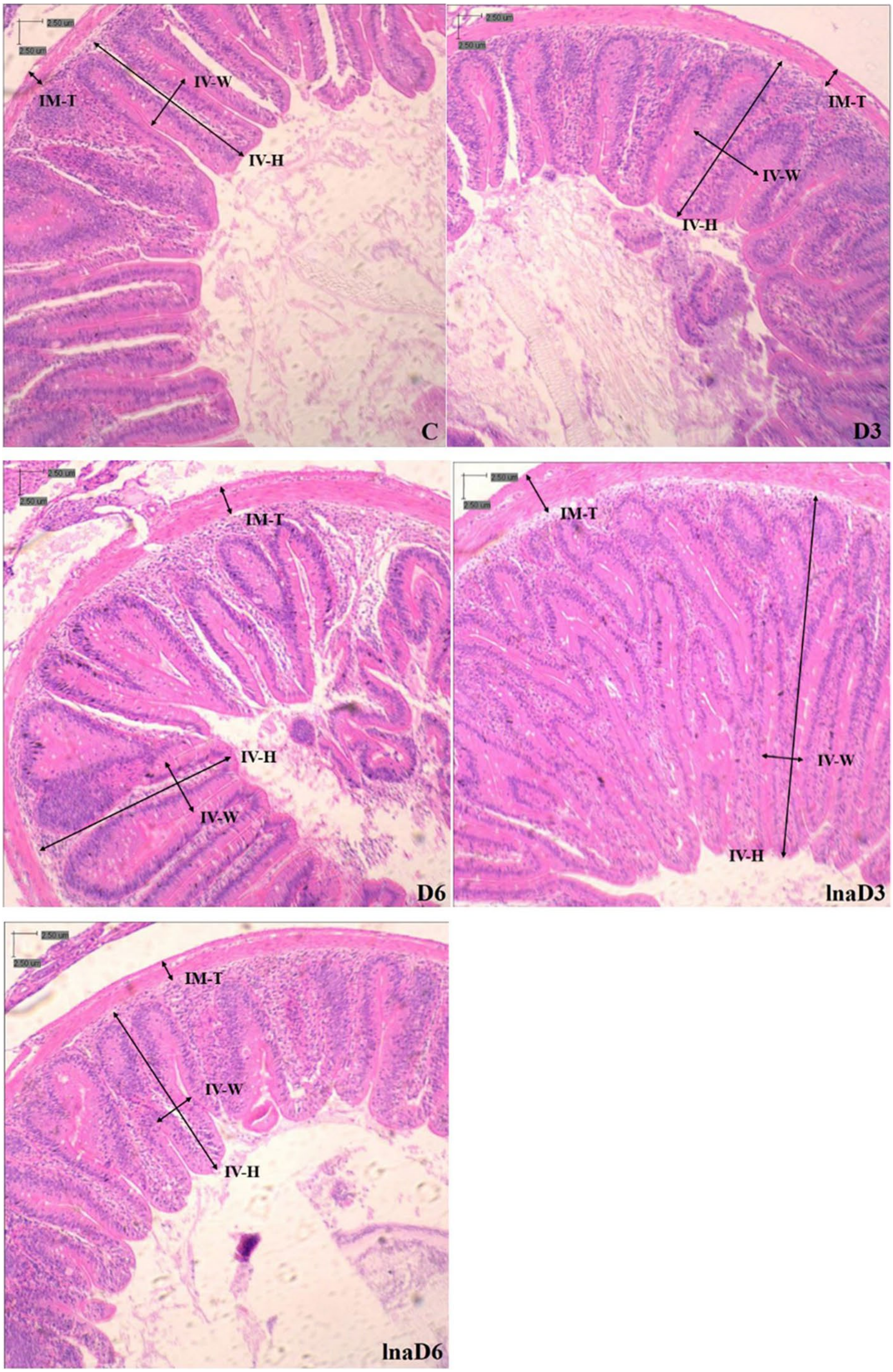

Fig. 1 HE staining of midgut villus structure of koi fed with compound probiotics or heat-killed compound probiotics (100×). Scale bar = 2.5 μm. Abbreviations: IV-H, intestinal villus height; IV-W, intestinal villus width; IM-T, intestinal muscular thickness

Table 4 The intestinal morphology of koi fish fed with different probiotics

Parameters	Treatments (μm)					Probability (*P* value)3		
	Control	D3	D6	lnaD3	lnaD6	D or lnaD	concentration	D or lnaD × concentration
Intestinal villi height	91.50 ± 3.26	$65.63 \pm 8.32^{a*}$	$85.79 \pm 3.59^{ab*}$	$122.68 \pm 18.01^{b*}$	$76.88 \pm 1.05^{a*}$	0.044	0.240	0.011
Intestinal villus width	27.02 ± 1.33	$21.37 \pm 1.54^{*}$	$21.13 \pm 0.99^{*}$	$16.95 \pm 0.59^{*}$	$27.22 \pm 2.62^{*}$	0.900	0.515	0.002
Intestinal muscular thickness	8.18 ± 0.59	$12.58 \pm 0.50^{b*}$	$11.57 \pm 0.65^{b*}$	$18.02 \pm 0.82^{c*}$	$9.42 \pm 0.42^{a*}$	0.740	0.426	0.000

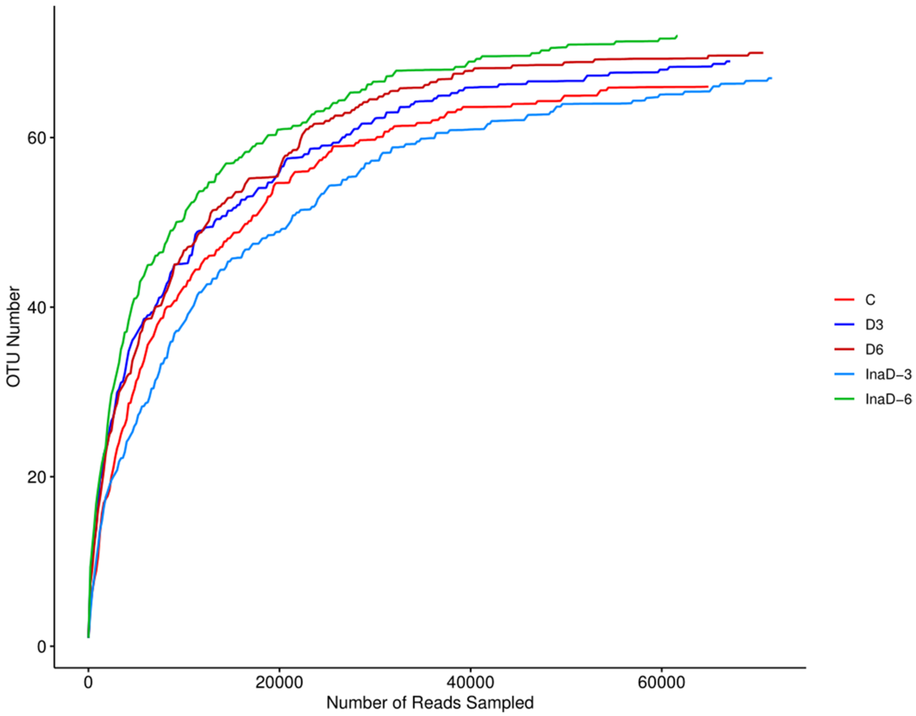

Fig. 2 Rarefaction curve of OTU

Table 5 Alpha diversity analysis of intestinal microflora of koi fish fed with probiotics and heat-killed probiotics

Sample	Shannon	Chao1	Ace	Simpson	Coverage
C	0.270847	67.11	67.90	0.910816	0.999923
D3	0.798838	81.00	72.65	0.632145	0.999866
D6	0.332240	71.50	71.31	0.903019	0.999943
lnaD3	0.420317	72.00	72.87	0.824049	0.999860
lnaD6	1.252213	73.50	74.60	0.412163	0.999903

had lower ACP activity than the control group. Thus, an overdose of probiotics is likely to harm the immune system of koi fish.

AKP is an extracellular enzyme that is activated by macrophages (Kuebutornye et al. 2020). AKP activity in koi fish fed compound probiotics in the D6 group was significantly higher than AKP activity in koi fish fed the control diet in this experiment. According to Wang et al. (2021a), live *Lactococcus lactis* Z-2 dramatically boosted the AKP activity in carp *Cyprinus carpio*. Similar findings were made by Yi et al. (2018) who discovered that feeding fish *velezensis* JW can enhance AKP activity. Additionally, like live probiotics, heat-killed probiotics can enhance the stimulation of immunological responses (Pan et al. 2008). The AKP activity of the lnaD3 group fed with heat-killed compound probiotics was significantly higher than the control group. However, the AKP activity of the lnaD6 group fed with heat-killed compound probiotics was significantly lower than other test groups. Even though live and heat-killed probiotics elicit the same

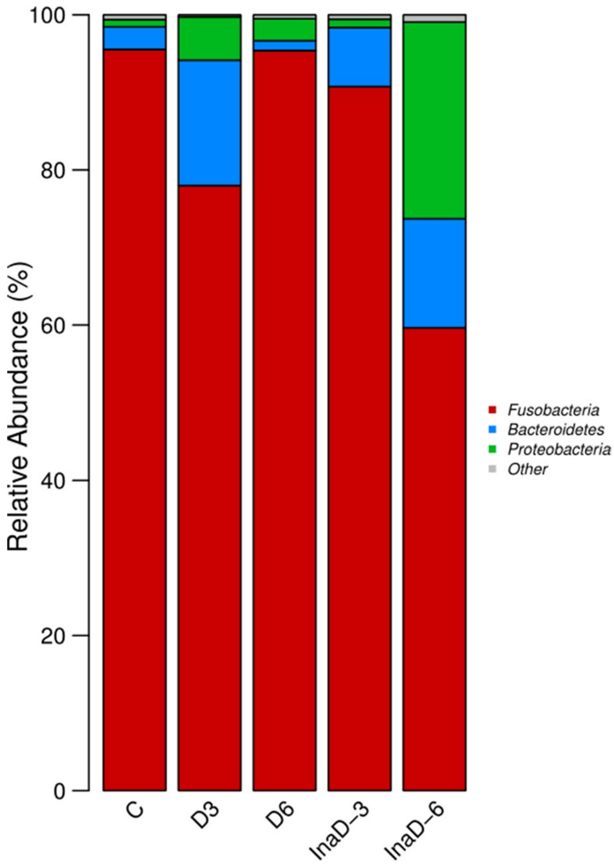

Fig. 3 Composition and relative abundance of dominant microbes at the phylum level for samples

immune response, the underlying mechanisms of action may differ. (Taverniti & Guglielmetti 2011).

CAT is an antioxidant enzyme found in the fish's first line of oxidant defense (Kuebutornye et al. 2020). In the meantime, CAT can mitigate the damage by decomposing excess hydrogen peroxide (Wang et al. 2021b). Yang et al. (2020) discovered that feeding living and heat-killed *B. cereus* can improve CAT activity, with live probiotics outperforming heat-killed probiotics. On the contrary, our study found that heat-killed compound probiotics-supplemented groups had significantly higher CAT activity than other groups. This inconsistency could be attributed to different methods of killing probiotics and treatment duration (Munoz-Atienza et al. 2015). SOD is a type of living organism with enzymatic defenses (Wang et al. 2017). According to Gayed et al. (2021), feeding probiotics can significantly increase SOD activity. Similarly, our findings indicated that feeding probiotics or heat-killed probiotics could improve SOD activity significantly more than the control group. It is worth noting that the lnaD6 group fed with heat-killed probiotics was significantly higher than in other groups. This finding demonstrated that heat-killed compound probiotics can improve SOD activity more than live probiotics. The evidence presented above suggests that a diet supplemented with heat-killed compound probiotics can improve the health of koi fish. Overall, feeding 3% heat-killed probiotics can improve the immunity of koi fish.

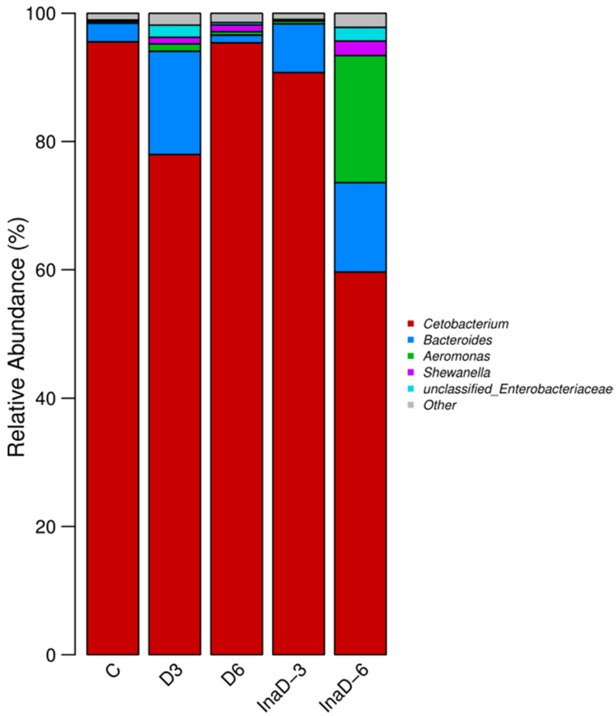

Fig. 4 Composition and relative abundance of dominant microbes at the genus level for samples

Hematologic parameters can reflect fish liver health (Lemaire et al. 1991; Megarani et al. 2020). Meanwhile, hematologic parameters are an important guide for determining fish health (Kesbiç et al. 2020). Dawood et al. (2015, 2019) reported that fish fed with heat-killed probiotics can decrease the GOT and GPT levels. This is consistent with the results of the present study. Serum GOT and GPT of the koi fish fed with 6% heat-killed probiotics were lower than other groups. The GOT and GPT will be higher when the liver is destroyed (Lemaire et al. 1991). As a result, the fish-fed heat-killed compound probiotics appeared to be in good health. Blood protein profiles (TP, ALB, and GLO) are produced in the liver and are considered markers of fish humoral immunity (Patriche et al. 2009). Tan et al. (2017) found that blood protein profiles can reflect the body's protein metabolism and the liver's functional status and that elevated levels of TP and ALB in the liver are a sign that the liver's capacity for protein synthesis has risen. Dawood et al. (2019) discovered that TP levels were significantly higher in fish-fed heat-killed probiotics. Conversely, some reports claimed that probiotics had little effect on TP (Shelby et al. 2006; Abd El-Rhman et al. 2009). In contrast to previous studies, we discovered that TP was significantly higher in koi fish-fed probiotics and reduced in koi fish-fed heat-killed compound probiotics. It is thought to be because different probiotics have different durations of action and effects. Dawood et al. (2015) discovered that heat-killed probiotics can reduce TG levels. We came to the same conclusion in our report. Probiotics can significantly reduce TG levels. In fish, TG could be involved in the regulation of blood lipid derivatives (Falcinelli et al. 2015). This result indicated that the fish had low plasma triglyceride levels, and compound probiotics in the feed could improve koi fish fat metabolism (Dawood et al. 2015).

The effects of different probiotics on intestinal morphology have been investigated in many fish species (Lee et al. 2017; Ramos et al. 2015, 2017; Zhu et al. 2021). The intestine is a vital digestive and defense organ in fish (Mohammadian et al. 2022). Studies have revealed that *B. cereus* and *B. subtilis* can protect the intestinal barrier (Xue et al. 2020). When Nile tilapia *Oreochromis niloticus* were fed *cerevisiae*, the fish's intestinal mucosal fold, lamina propria, and enterocyte width all increased (Islam et al. 2021). However, Ramos et al. (2017) observed no significant effect on the mid-intestine villi length using probiotic formula. Unlike other results, our findings showed that intestinal villi height and intestinal villi width were lower in D3- and D6-fed probiotics. Differences in results could be attributed to differences in digestive system location and probiotic species measured (Demirci et al. 2021). In our study, the intestinal villi height and intestinal muscular thickness were higher in the lnaD3 group fed with heat-killed compound probiotics. Dawood et al. (2019) fed the tilapia *Oreochromis niloticus* with the heat-killed *Lactobacillus plantarum* and found villus length significantly increased. Hien et al. observed that in snakehead *Channa striata* fed with heat-killed *Lactobacillus plantarum*, the height and length of the intestinal villi were significantly higher than those fed the control diet (Hien et al. 2021). Nofouzi et al. found that the intestinal villus length of the rainbow trout *Oncorhynchus mykiss* was mainly enhanced in the heat-killed *Tsukamurella inchonensis* group (Nofouzi et al. 2019). Increasing the length of the intestinal villi may increase the absorption surface area, resulting in improved nutrient utilization and growth performance (Khojasteh, 2012). The increase in muscle thickness can enhance the digestion and absorption capacity of the intestinal tract (Peng et al. 2022). The findings showed that heat-killed probiotics can improve digestion by increasing the height of the fish intestinal villi and the thickness of the intestinal muscular layer.

Probiotics can influence changes in the intestinal microbial community (Nayak 2010). Moreover, dominant intestinal bacteria are required to maintain intestinal homeostasis (Guangxin et al. 2022; Zhu et al. 2021). In the current study, probiotic supplements or heat-killed probiotics increased the richness of gut microbiota in fish. Carnevali et al. (2017) investigated that the higher richness indicated implies that probiotics could improve organisms' ability to adapt to bacterial diversity. *Proteobacteria*, *Firmicutes*, and *Actinobacteria* are the primary bacteria found in the fish gut (Wu et al. 2012). *Fusobacteria* and *Bacteroidetes* were the dominant phyla in all groups in this study. At the phylum level, the addition of heat-killed compound probiotics increased the relative abundance of *Bacteroidetes* and *Proteobacteria*. *Bacteroidetes* have digestive enzymes, according to previous research (Karlsson et al. 2011). Furthermore, *Proteobacteria* can promote nutrient cycling and are linked to the immune system (Gomez et al. 2013; Cardona et al. 2016). This demonstrates that heat-killed compound probiotics benefit the digestive system and immunity.

Interestingly, *Aeromonas* was discovered in the intestines of the lnaD6 group at the genus level. Many extracellular proteins can be produced by *Aeromonas*, including amylase, chitinase, elastase, aerolysin, nuclease, gelatinase, lecithinase, lipase, and protease (Aberoum et al. 2010). As a result, heat-killed compound probiotics may affect the digestive ability of koi fish. In addition, the *Shewanella* was found in D3, D6, and lnaD6. Several studies have explored the antimicrobial activity of *Shewanella species* against various fish pathogens (Zadeh et al. 2010; Díaz-Rosales et al. 2009; Makridis et al. 2008). From this point of view, the application of compound probiotics and heat-killed compound probiotics can improve koi fish resistance to pathogenic bacteria.

In conclusion, the effects of compound probiotics and heat-killed probiotics on the antioxidative capacity, plasma biochemical parameters, intestinal morphology, and microbiota of koi fish were demonstrated in this study. The appropriate amount of heat-killed compound probiotics are better than compound probiotics based on the species, feed composition, type of probiotics, and experimental environment of koi fish. However, the number of probiotics used should be noted. As a result, heat-killed compound probiotics could be used in the healthy management of koi fish aquaculture. However, more research is needed to determine the mechanism of action of heat-killed compound probiotics.

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Data availability All data, models, and code generated or used during the study appear in the submitted article.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval The experimental processes in this research confirmed to the Guidelines of the Care and Use of Laboratory Animals in China, and the study was approved by the ethics committee of Dalian Ocean University.

Conflict of interest The authors declare no competing interests.

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