Assessing the effectiveness of complex dietary candidate probiotics on growth performance, digestive enzyme activity, antioxidant capability, and intestinal microbiota of koi carp (*Cyprinus carpio* var. *koi*)

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Abstract

The present study aimed to evaluate the effectiveness of three complex dietary candidate probiotics (LDJ, LAC, and LJD) composing different candidate probiotic combinations on the growth performance, digestive enzyme activities, antioxidant parameters, and intestinal microbiota in koi carp (*Cyprinus carpio* var. *koi*). After a 60-days feeding trial, samples from the control and three candidate probiotic groups were obtained and analyzed. The results suggested that all complex dietary candidate probiotic treatments (LDJ, LAC, and LJD) improved the growth performance, nutrition utilization, and intestinal digestive enzyme activities compared with the control group. Furthermore, a dietary supplement with LAC or LJD is more efficient in growth and digestive enzyme than LDJ. The antioxidant enzyme activities, including SOD (superoxide dismutase), CAT (catalase), and GPX (glutathione peroxidase), were intensively improved, the GSH (glutathione) contents increased, and the MDA (malondialdehyde) contents decreased to different extents depending on the tissues and kinds of candidate probiotics. Dietary candidate probiotics also regulated the intestinal microbiota composition: LDJ and LAC increased the proportion of potential candidate probiotics and reduced the level of pollutants reducing bacteria and geosmin producer *Nannocystis.* The LJD group had a similar pattern of the dominant bacteria to the control sample, suggesting a minor disturbance of the gut microbiota of koi carp. Overall, the multi-strain complex candidate probiotics LAC and LJD are more efficient in growth performance and digestive enzymes.

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Introduction

The koi carp (*Cyprinus carpio* var. *koi*) is a common species of ornamental fish for aquaculture (Jha et. al., 2005). However, many types of stress, such as disease, unpredictable environmental conditions, and dietary quality, can cause significant economic losses and limit the sustainable development of koi carp farming (Jha et al., 2007; Lin et al., 2012; Nuwansi et al., 2016). The use of probiotics is currently receiving a lot of attention. Many researchers have confirmed the beneficial effects of dietary inclusion of live microorganisms as probiotics in aquaculture (Hoseinifar et al., 2016; Martínez Cruz et al., 2012). The functions of probiotics involve improving digestive enzymes and antioxidant enzyme activity, regulating intestinal microbiota composition, supplying nutrients, increasing growth efficiency, controlling water quality, and increasing resistance to pathogens of the host (Azari et al., 2011; Gao et al., 2016; Duan et al., 2018; Gobi et al., 2018). Currently, probiotics such as *Bacillus* (Sankar et al., 2017), *Lactiplantibacillus plantarum* (Dawood et al., 2017), *Lactobacillus acidophilus* (Hoseinifar et al., 2015), *Bifidobacterium infantis* (Akbar et al., 2014), *Enterococcus faecalis* (Baños et al., 2019), nitrifying bacteria (Shan et al., 2001), denitrifying bacteria (Jun et al., 2000), photosynthetic bacteria (Delamare-Deboutteville et al., 2019). Live yeast (Tovar-Ramírez et al., 2010) has been widely applied because it can provide multiple benefits to aquatic animals. Although data regarding probiotics is accumulating (Chauhan and Singh 2019; Dawood et al., 2010; Hauville et al., 2016; Kuebutornye, et al., 2020; Van Doan et al., 2020; Wang et al., 2018; Xue et al., 2020), most studies have focused on monostrain probiotics. The effectiveness of multistrain and multispecies probiotics has gained increasing interest. Several studies have demonstrated the potential benefits of complex probiotics in aquatic animals, such as composite *Bacillus* in Nile tilapia (Abarike et al., 2018), Florida pompano, common snook, and red drum larvae (Hauville et al., 2016), whiteleg shrimp (Sadat Hoseini Madani et al., 2018), and common carp (Wang et al., 2017); multi-strains of yeast in sea bass (Tovar et al., 2002); *Bacillus*, lactic acid bacteria combined with yeast in *Labeo rohita* fingerlings (Mohapatra et al., 2012), and *Micrococcus* combined with *Bacillus* in pearl spot and tilapia (Sankar et al., 2017). Wang et al. (2019) have reviewed the better efficiency of multispecies probiotics than in their single-use, including complex *Bacillus* in sea cucumber (Li et al., 2015), *Bacillus*, *lactic acid bacteria* combined with *Arthrobacter* in cobia (*Rachycentron canadum*) (Geng et al., 2012), *Bacillus* and *Pseudomonas* in grass carp (Deng et al., 2014), *Bacillus*, *Shewanella* and *Aeromonas* in shrimp (Hao et al., 2014), *Shewanella* and *Aeromonas* in grass carp (Wu et al., 2015), *Bacillus* and yeast in Atlantic salmon (*Salmo salar*) (Liu et al., 2017), *Bacillus*, lactic acid bacteria combined with *Clostridium* in hybrid grouper (Zhai et al., 2017), *B. subtilis* and *S. cerevisiae* in *Labeo rohita* (Ullah et al., 2020)*, B. licheniformis and B. megaterium* in *Litopenaeus vannamei* (Charan et al., 2014), *B. subtilis*, *B. pumilus* and *B. licheniformis* in *L. vannamei* (Chorong et al., 2019)*. Aeromonas* sp., *Bacillus* sp., and *Citrobacter braakii* in *Oncorhynchus mykiss* (Koca et al., 2015). Researchers believe that the advantage of using multistrain and multispecies preparations may further improve the overall beneficial effects of probiotics (Xue et al., 2020). However, research on probiotic combinations in various aquatic animals is very limited. Moreover, the mechanisms underlying the benefits of multistrain and multispecies are still poorly understood. Therefore, it is important to search for and develop complex probiotics suitable for specific species of aquatic animals.

This work investigated the effects of three complex probiotics (LDJ-complex *Bacillus*, LAC-complex lactic acid bacteria, and LJD-mixture of *Bacillus*, lactic acid bacteria, nitrifying bacteria, denitrifying bacteria, yeast, and photosynthetic bacteria) on the growth performance, digestive enzyme activities, antioxidant parameters and intestinal microbiota in koi carp (*C. carpio* var. *koi*).

Materials and Methods

Trial Design and Fish Culture

The koi carp (*C. carpio* var. *koi*) were obtained from the Chenhui fish farm in Tianjin, China. The fish were acclimatized to the experimental conditions with a traditional diet for two weeks prior to the feeding experiment. The nutritional composition of the diet is shown in **Table 1**. After acclimation, 240 healthy (normal at eating, disease-free and non-injured) fish of similar size (49.47 ± 3.5 g per fish) were randomly divided into four groups (Control, LDJ, LAC, and LJD). All the fish were cultured in concrete fish tanks (1 m long, 1 m wide, and 0.8 m deep) equipped with continuous aeration. There were 3 tanks with 20 fish each in each group. The control group was fed a conventional diet (Control). The LDJ group was fed a conventional diet supplemented with *Bacillus subtilis* (final content 1×10^6 CFU/g feed). The LAC group was supplemented with *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Bifidobacterium infantis*, and *Enterococcus faecalis* (the final content of each bacterium was 0.25×10^5 CFU/g feed). The LJD group was supplemented with *Bacillus subtilis* (final content 1×10^6 CFU/g feed), lactic acid bacteria (containing *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Bifidobacterium infantis*, and *Enterococcus faecalis*, and the final content of each bacteria was 0.25×10^5 CFU/g feed), nitrifying bacteria (final content 1×10^4 CFU/g feed), denitrifying bacteria (final content 1×10^4 CFU/g feed), yeast (final content 1×10^4 CFU/g feed), and photosynthetic bacteria (final content 1×10^4 CFU/g feed). The complex probiotic powders were all obtained from LIYANG Aquatic Technology Co., Ltd. The probiotic powder was diluted with distilled water and sprayed on pellet diets before use. During the feeding trial, all fish were fed three times at a feeding rate of 1% (body weight) per day and cultured for 60 days. In addition, 1/3 of the water in each pool was replaced with clean, fresh water at the same temperature (28±2 ℃) every two days. All animal experiments in the present study were carried out following EU Directive 2010/63/EU for animal experiments (European Parliament 2020).

Table 1 The nutritional composition of the experimental diet (g/100 g dry diet) for *C. carpio* var. koi

Nutrients	Content (g/100 g dry diet)
Crude protein	≥35
Crude fat	≥4
Crude fiber	≤15
Crude ash	≤15
Calcium	0.5-2.5
Total phosphorus	0.6-1.5
Sodium chloride	0.4-1.0
Lysine	≥1.7
Water content	≤12

Growth Performance
The weight of the fish in each pool was determined at the initial and the end of cultivation. The following formulas were used to measure the weight gain (WG), specific growth rate (SGR, %), feed conversion ratio (FCR, %), and protein efficiency (PER, %):

$$WG = 100 \times (W_t - W_0)/W_0$$
$$SGR = 100 \times (\ln(W_t) - \ln(W_0))/t$$
$$FCR = I_d/(W_t - W_0)$$
$$PER = (W_t - W_0)/(I_d \times P_d)$$

Where, W_0 = initial weight (g); W_t = weight at time t since the beginning (g); t = feeding time (d); I_d = daily intake of feed; P_d = daily protein content intake.

Determination of Antioxidant Status and Digestive Enzyme Activities
At the end of the 60 days feed trial, after fasting for 24 h, all fish (three tanks for each group and 20 fish in each tank) were harvested. The fish's intestines (foregut, midgut, and hindgut), hepatopancreas, spleen, head kidney, middle kidney, gill, and brain were dissected on ice and stored immediately in a -80 °C refrigerator (Thermo Scientific™ Explosion-Proof Refrigerator/Freezers, USA). The tissues were ground in sterile saline (0.85% w/v) under an ice bath with an electric homogenizer (DY89-II, Zhejiang, China), then centrifuged (Microfuge 22R Centrifuge, Beckman, USA) for 10 min at 4 °C for 2400 g. The supernatant was subsequently used to determine the activities of superoxidase dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and contents of malondialdehyde (MDA), glutathione (GSH), and nitric oxide using commercial kits obtained from Nanjing Institute of Bioengineering (Nanjing, China): Superoxide dismutase kit (A001-3-2); Catalase Kit (A007-2-1); Glutathione peroxidase (A005-1-2); Malondialdehyde kit (A003-4-1); Glutathione kit (A006-2-1). The digestive enzyme (protease, amylase, and lipase) activities of the intestine were measured using the Protease kit (A080-1-1), Amylase kit (C016-1-1), and Lipase kit (A054-2-1).

Analysis of Microbiota
At the end of the experiment, the intestinal contents of three fish from each pool were collected and mixed, and the three samples in the same group were combined into a single sample and then frozen in liquid nitrogen. The samples were transported to Allwegene Technology Inc. (Beijing, China) immediately under a cold solid carbon dioxide (dry ice) environment. Total gut microbiota DNA was isolated and purified, and the 16S rDNA gene region V3-V4 was targeted for amplification using PCR primers (5'-GTACTCCTACGGGAGGCAGCA-3' /5'-GTGGACTACHVGGGTWTCTAAT-3') using an Illumina MiSeq instrument (HiSeq 2000, Illumina/Miseq, USA). After denoising and chimera removal, sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97%. Each OUT was taxonomically assigned using BLASTn against a curated database derived from RDPII and NCBI. The OTUs identified as the Chloroplast were artificially removed, and the statistics were analyzed based on the composition of the remaining OTUs.

Statistical Analysis
After routine processing of test data by Excel, the difference between treatments was conducted using a one-way analysis of variance (ANOVA) with SPSS version 16.0 software using multiple comparison methods (Least Significant Difference, LSD) at a significance level of 0.05 (except for the analysis of intestinal microbiota). All data were presented as mean values with standard deviations (mean ± SD).

Results

Growth Performance and Feed Utilization

   Table 2 presents the effects of dietary complex candidate probiotics on the growth performance of koi carp. Compared with the control group, the probiotic groups (LDJ, LAC, and LJD) showed similar significant improvements in WG (*P* < 0.05) and decreased FCR to comparable levels (*P* < 0.05). The LAC group had a higher (*P* < 0.05) SGR level than the control and other probiotic groups. The LJD and LAC groups showed significantly higher (*P* < 0.05) PER than the control and LDJ groups.

Table 2 Effects of complex dietary probiotics on the growth performance and feed utilization of *C. carpio* var. koi (Mean ±SD, n = 3)

	Control	LDJ	LAC	LJD
WG (g)	90.46±5.26[b]	123.61±11.15[a]	128.96±9.03[a]	120.04±7.38[a]
SGR (%)	0.72±0.14[b]	0.87±0.08[ab]	0.94±0.07[a]	0.82±0.12[ab]
FCR (%)	2.36±0.28[a]	1.53±0.16[b]	1.42±0.08[b]	1.51±0.15[b]
PER (%)	5.74±0.44[b]	6.21±0.2[b]	8.17±0.32[a]	7.46±0.6[a]

Note: The control group was fed a conventional diet adding an equivalent amount of sterile saline to the probiotic groups. The LDJ, LAC, and LJD group was fed the diet supplemented with complex *Bacillus*, combined lactic acid bacteria, and a mixture of *Bacillus*, lactic acid bacteria, nitrifying bacteria, denitrifying bacteria, yeast, and photosynthetic bacteria, respectively. WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency. The results are presented as means ± standard deviation, n = 3. Different superscripted letters (a and b) indicate a significant difference (*P* < 0.05) between the data in the same row.

Intestinal Digestive Enzyme Activities

   Table 3 shows the digestive enzyme activities in the gut (foregut, midgut, hindgut) of each group of carp, including proteases, amylases, and lipases. There was no significant difference (*P* > 0.05) in protease activities between the control, LDJ, LAC, and LJD groups in the whole intestine (foregut, midgut, and hindgut). Compared with the control group, fish in the LDJ group had significantly (*P < 0.05*) improved midgut and hindgut amylase activities and foregut and midgut lipase activities. Fish in the LAC group had increased (*P* < 0.05) lipase activity in all parts of the gut and enhanced amylase activity in the midgut and hindgut. The LJD group showed remarkably (*P* < 0.05) enhanced amylase and lipase activities in all parts of the intestine.

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Table 3 Effects of complex dietary probiotics on the intestinal digestive enzyme activities of *C. carpio* var. koi (Mean ±SD, n = 3)

Tissue	Enzyme activity (U/mg·prot)	Treatment			
		Control	LDJ	LAC	LJD
Foregut	protease	336.6±28.02	326.39±24.92	347.15±32.77	340.68±26.54
	amylase	0.83±0.1[b]	0.91±0.1[ab]	0.93±0.06[ab]	1.03±0.09[a]
	lipase	3.53±0.17[d]	5.62±0.2[c]	8.71±0.2[a]	6.43±0.17[b]
Midgut	protease	314.99±9.59	304.39±6.32	328.81±18.9	322.82±12.21
	amylase	1.61±0.17[c]	4.8±0.1[a]	4.31±0.2[b]	4.38±0.14[b]
	lipase	4.27±0.11[b]	6.57±0.43[a]	7.05±0.17[a]	6.61±0.35[a]
Hindgut	protease	331.9±19.82	349.34±16.52	358.36±13.07	360.64±10.71
	amylase	2.26±0.11[b]	3.55±0.25[a]	3.59±0.16[a]	3.15±0.46[a]
	lipase	37.01±4.12[c]	44.78±3.95[bc]	57.29±6.15[a]	48.52±4.48[ab]

Note: The control group was fed a conventional diet adding an equivalent amount of sterile saline to the probiotic groups. The LDJ, LAC, and LJD group was fed the diet supplemented with complex *Bacillus*, combined lactic acid bacteria, and a mixture of *Bacillus*, lactic acid bacteria, nitrifying bacteria, denitrifying bacteria, yeast, and photosynthetic bacteria, respectively. Different superscripted letters (a, b, c, and d) indicate a significant difference ($P < 0.05$) between the data in the same row.

Antioxidant Status

The effects of dietary probiotics on antioxidant enzyme activity (SOD, CAT, and GPX), GSH, and lipid peroxidation (MDA) levels in hepatopancreas, spleen, head kidney, middle kidney, gill, and koi carp brain were determined after a 60-day feeding trial, and the results were shown in **Figure 1 (A-E).** Compared to the control group, all three complex candidate probiotics all significantly improved ($P < 0.05$) the activity of SOD in all detected tissues observed in koi carp (**Figure 1A**). The probiotic groups (LDJ, LAC, and LJD) had higher CAT activity in the middle kidney, gill, and brain (**Figure 1B**) than the control sample. However, candidate probiotics had no significant influence ($P > 0.05$) on CAT activities in the spleen and head kidney. In the promotion of GPX activity (**Figure 1C**), LJD was more efficient than in promoting GPX activities than other probiotics. LDJ also increased the GPX activity in the hepatopancreas, spleen, and gill. LAC group had an increased GPX activity in the gill compared to the control sample ($P < 0.05$). However, there was no significant difference ($P > 0.05$) in GPX activity in the head kidney of fish fed or not given candidate probiotics. The GSH levels varied in different tissues and groups (Fig. 1D). Compared with the control group, the LDJ group had higher GSH levels in the spleen, middle kidney, and gill but lower levels in the hepatopancreas, head kidney, and similar level in the brain. The LAC group showed elevated GSH levels only in the gill, with lower or similar levels in other tissues. LJD increased the GSH levels in the head kidney, middle kidney, and brain. The MDA levels in the hepatopancreas, middle kidney, and gill were reduced by applying candidate probiotics (**Figure 1E**). Among the three probiotic groups, LDJ and LAC had the lowest MDA levels in the middle kidney and grill, respectively, suggesting greater efficiency of LDJ and LAC in protecting the cellular membrane from lipid oxidation.
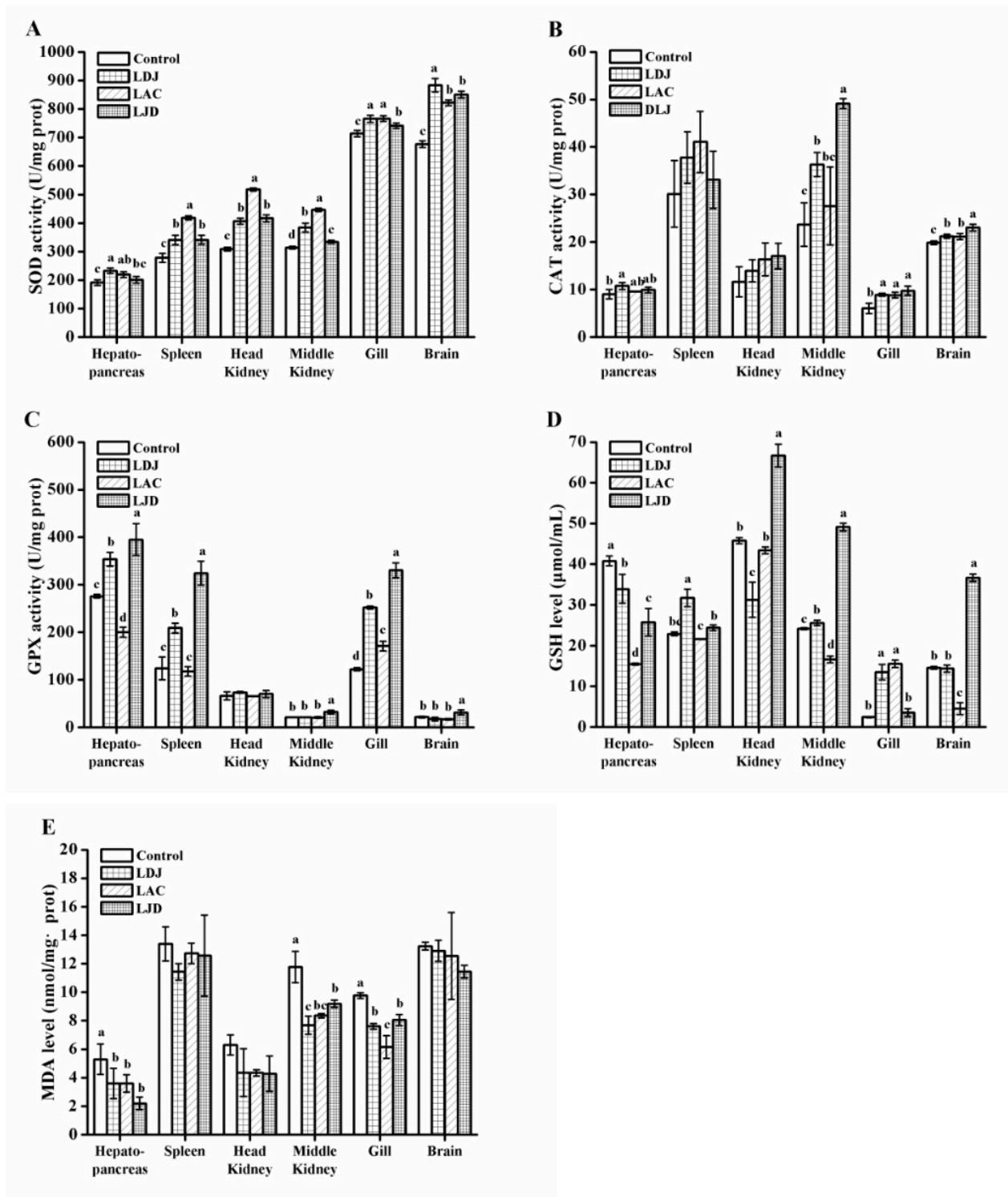
Figure 1 The effects of complex dietary probiotics on the antioxidant status, GSH, and NO level in the tissues of *C. carpio* var. *koi*. (A) SOD: Superoxidase dismutase; (B) CAT: Catalase; (C) GPX: Glutathione peroxidase; (D) GSH: Glutathione; (E) MDA: Malondialdehyde; U: unit; prot: protein; NT: not detected. Different superscript letters indicate a significant difference between data in different groups ($p < 0.05$).

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Intestinal Microbiota Composition

    A total of 164 OTUs (operational taxonomic units) were obtained for all groups of fish. A Venn diagram was generated showing the number of shared (91) and unique OTUs among the four groups (**Figure 2A**). Probiotic supplement samples (LDJ, LAC, and LJD) showed a slight decrease in OUT numbers compared with control. The alpha diversity of gut microbiota differed between groups (**Figure 2B-E**). Compared with the control group, the alpha diversity of the probiotic groups all decreased according to Chao 1 index (**Figure 2B**), observed species (**Figure 2C**), PD (phylogenetic diversity) (**Figure 2D**), and Shannon index (**Figure 2E**). The LAC had the lowest alpha diversity among the four groups. Principal component analysis (**Figure 2F**) showed that fish fed diet containing probiotics (LDJ and LJD) had different levels of principal components compared with the control group. The LAC had a similar principal component 2 with the control group but differed at the level of principal component 1.
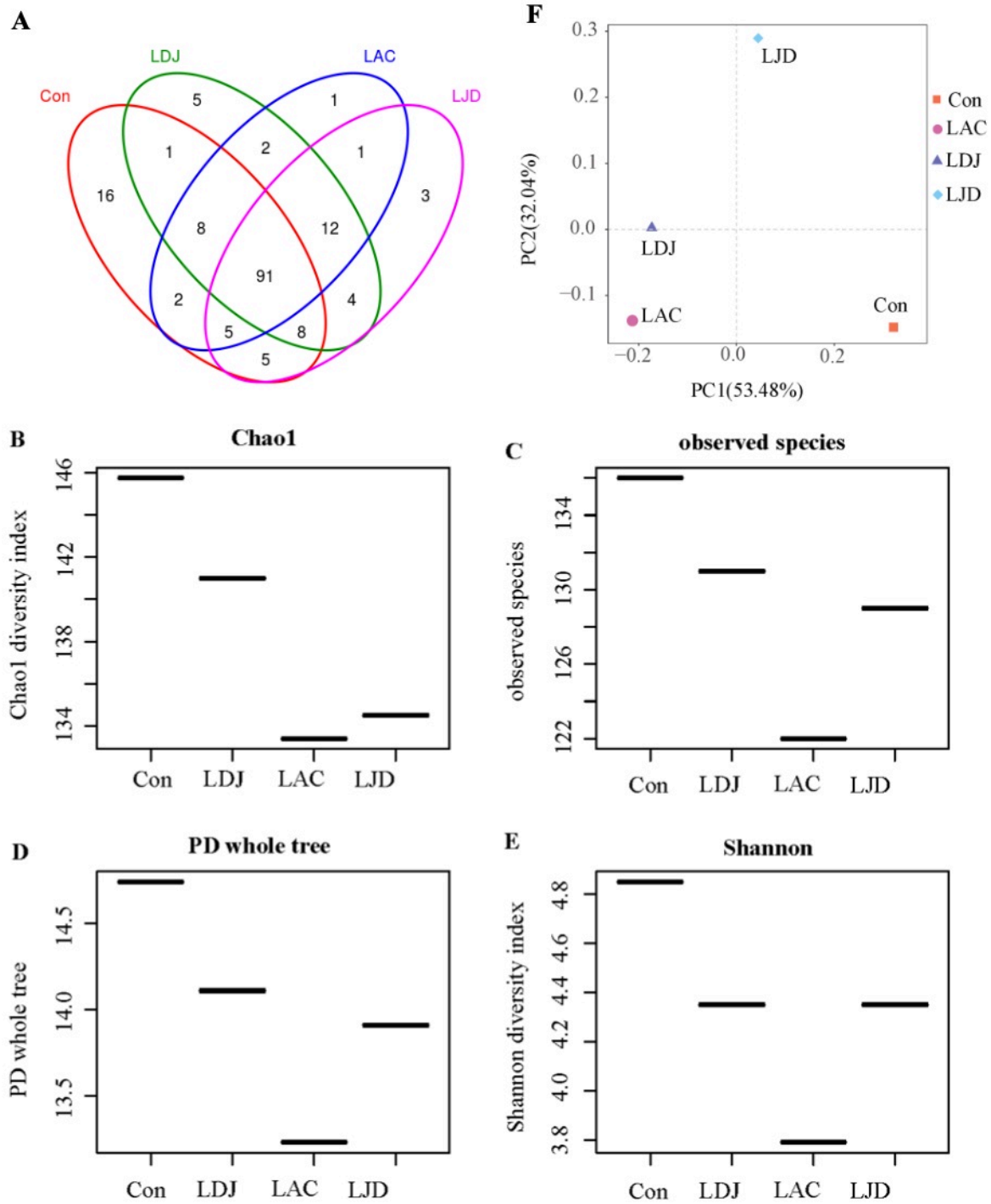
Figure 2 Influence of complex dietary probiotics on the diversity of gut microbiota in *C. carpio* var. *koi*. (A) Venn diagrams showing core microbiota OTU distributions. Alpha diversity of intestinal bacteria based on Chao 1(B), observed species (C), PD (phylogenetic diversity) (D), and Shannon index (E). Beta diversity revealed by principal component analysis (F). (Con, fish fed control diet; DLJ, LAC, and LJD fish fed diets supplemented with different complex probiotic products).

The 34 dominant bacteria at the OTU level were artificially divided into seven classes, as shown in **Figure 3**. The proportion of potential candidate probiotics Fusobacteriales (order, OTU2) increased in the LDJ group. However, Bacillales (order, OTU47) and Firmicutes (phylum, OTU27) did not increase in any probiotic group compared to the control sample (**Figure 3A**). The proportion of Erythrobacteraceae (family, OTU11) and *Luteimonas* (genus, OTU25) with potential pigment-producing ability increased in the LAC group (**Figure 3B**). This should favor the coloration of the fish. Bacteria capable of metabolizing ammonia/nitrite or other organic pollutants accounted for 14-17% of the gut microbiota in various samples (**Figure 3C**). Compared to the control group, the total proportion of the pollutant-reducing bacteria did not increase in any probiotic groups. But the LDJ group had a higher proportion of *Prochlorococcus* (genus, OTU17). The LAC group has a higher level of *Hyphomicrobium* (genus, OTU24) (**Figure 3C**). The eight photosynthetic bacteria compromise most (over 35%) of the intestinal microbiota (**Figure 3D**). The total proportion of photosynthetic bacteria in the LAC group was over 60%. The level of *Tabrizicola* (genus, OTU4) in the LAC group was much higher than in other groups (**Figure 3D**). Four OUTs (OUT50, 48, 23 and 12) of bacteria with unknown functions comprise 1.9-4.6% of the total microbiota (**Figure 3E**). The two OTUs (*Nnnocystis* and *Leptolyngbya*) with contradictory-function were similar in the control and LJD groups but lower in LDJ and LAC groups (**Figure 3F**). As shown in **Figure 3G**, the proportion of potential pathogens *Legionella* (genus, OUT14) and Methylobacteriaceae (family, OTU79) was lowest (0.04%) in the LAC sample and 0.03% in the LDJ sample and was highest (7.1%) in LDJ group and 0.6% in LAC sample, respectively.
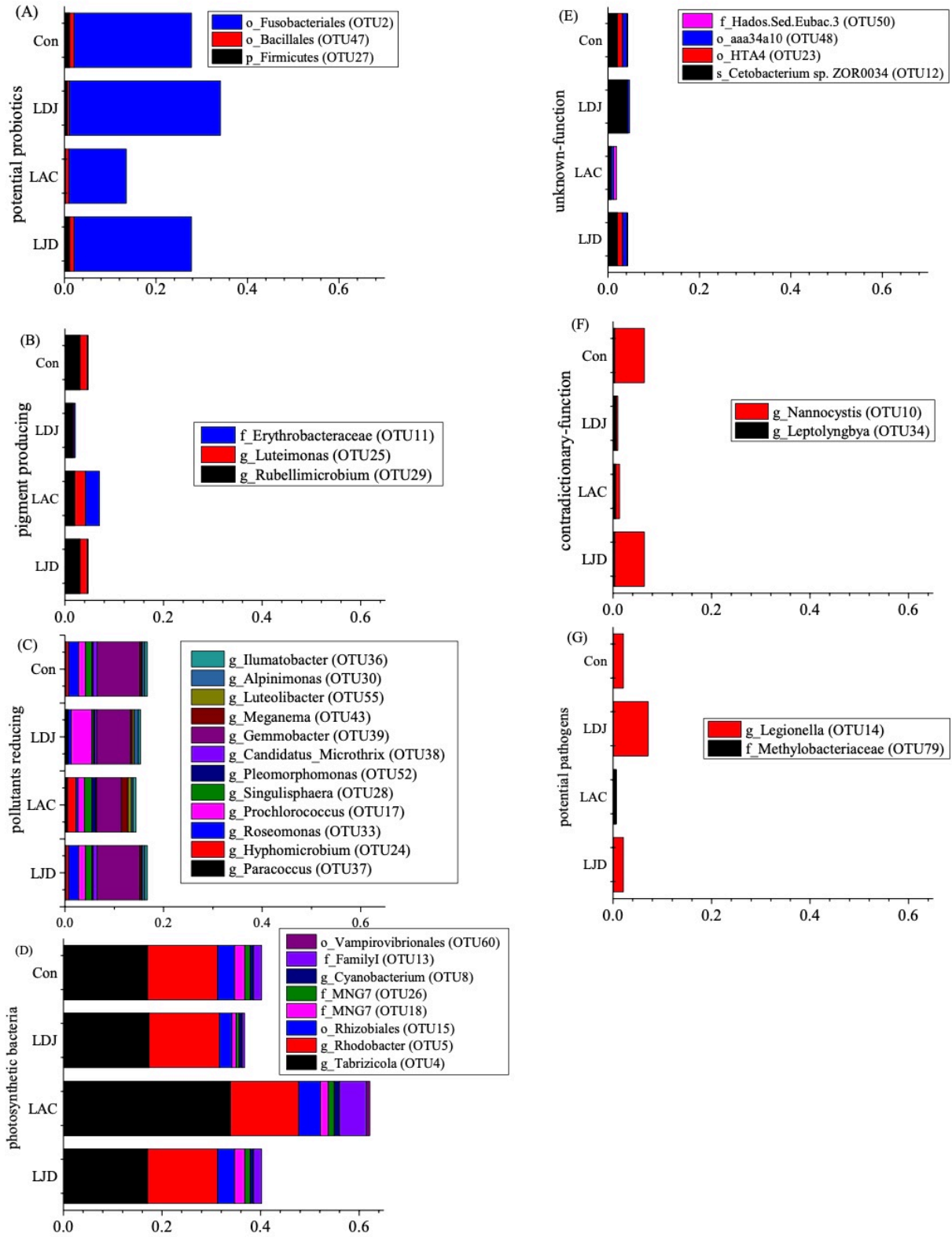
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Figure 3 Influence of complex dietary probiotics on the proportion of dominant (over 0.5%) OTUs of the gut microbiota in *C. carpio* var. *koi*. (A) potential probiotics, (B) pigment-producing bacteria, (C) bacteria that could metabolize ammonia/nitrite or other organic pollutants, (D) photosynthetic bacteria, (E) bacteria with unknown functions, (F) bacteria that have contradictory-function, (G) potential pathogens. (Con, fish-fed control diet; DLJ, LAC, and LJD, fish-fed diets supplemented with different complex probiotic products).

Discussion

Candidate Probiotics Promote the Growth Performance and Feed Utilization

The results of our study showed that supplementation with LDJ, LAC and LJD, which are candidate compound probiotics, significantly increased the WG, SGR, and PER and decreased the FCR of koi carp (*C. carpio* var. *koi*). In practice, the reduced FCR and increased PER obtained from candidate probiotic supplements represent a reduction in feed requirements for fish growth, leading to reduced feed costs (Hardy et al., 2002). Furthermore, the growth promotion effects of complex *Bacillus*, lactic acid bacteria, yeast, or other candidate probiotics have been demonstrated in several species of aquatic animals, such as *Labeo rohita* fingerlings (Mohapatra et al., 2012), *Oreochromis niloticus* (Essa et al., 2010), sea cucumber (Li at al., 2015), Atlantic salmon (*Salmo salar*) (Liu at al., 2017), cobia (*Rachycentron canadum*) (Geng et al., 2012), and shrimp (Hao et al., 2014).

The intestine plays a crucial role in the digestion of agastric fish, such as koi carp (Marković et al., 2016; Curry, 1939). Therefore, better activity of intestinal digestive enzyme can be beneficial for nutrient digestion and subsequently improve the growth of aquatic animals (Gobi et al., 2018; Kuebutornye et al., 2020). In our work, dietary supplementation of the three complex candidate probiotics significantly enhanced intestinal amylase and lipase activities in koi carp. Among them, the LAC group exhibited higher activities of amylase and lipase, which contributed to the highest WG, SGR, and PER and the lowest FCR among all samples detected. Besides directly stimulating intestinal enzymes, probiotics such as *Bacillus subtilis* (Afrilasari et al., 2016; Simair et al., 2017; Saengsanga et al., 2016), lactic acid bacteria (García-Cano et al., 2019; Padmavathi et al., 2018; Nespolo et al., 2020), and yeast (Charoenchai et al., 1997) can produce various hydrolytic enzymes during metabolic processes. Those hydrolytic enzymes have been suggested to be another reason for promoting host growth (NavinChandran et al., 2014; Huddy and Coyne, 2014).

Candidate Probiotics Enhance Antioxidant Status

Antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), constitute the first line of antioxidant enzymatic defense (Ighodaro and Akinloye, 2018) and the innate immune system for fish (Whyte, 2008; Tort et al., 2003; Smith at al., 2019). The antioxidant enzymes or substances are important for the balance of reactive oxygen species (ROS) (Tang et al., 2019) and the reduction of malondialdehyde (MDA) caused by excess ROS (Fidan et al., 2008), thus can maintain fish health (Chauhan and Singh, 2019; Johnson, 2002). In our work, we found that dietary supplementation of the three complex candidate probiotics all increased the activities of SOD, CAT, and GPX, and reduced the levels of MDA in several tissues of koi carp. It is generally accepted that probiotics can stimulate the organism's antioxidant enzyme secretion system and enhance the secretion of antioxidants (Weifen et al., 2012). In addition, probiotics can produce antioxidants such as SOD and GSH (Moriarty, 1998; Gopi et al., 2014; Moktan et al., 2008; Juan and Chou, 2010; Kullisaar et al., 2002; Chang and Hassan, 1997), which are beneficial in enhancing antioxidant activity.

Regarding the complex application of candidate probiotics, multiple researchers have reported greater efficiency of *Bacillus*, lactic acid bacteria, and yeast in combination than a single probiotic. A mixture of *Bacillus* strains can enhance common carp's antioxidant ability

(Wang et al., 2017). Combining *Bacillus*, lactic acid bacteria, and *Arthrobacter* increases the innate immunity in cobia (*Rachycentron canadum*) (Geng et al., 2012). Combining *Shewanella xiamenensis* and *Aeromonas veronii* enhanced innate immunity (Wu et al., 2015). A dietary probiotic mix of *Bacillus* stimulated non-specific immune responses in the sea cucumber (Li et al., 2015). A dietary combination of *Bacillus* and lactic acid enhanced the activity of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) of hybrid grouper (Zhai et al., 2017). Dietary *Bacillus* and yeast supplementation promoted Atlantic salmon's non-specific immunity (*Salmo salar*) (Liu et al., 2017). In the present study, combined candidate probiotics containing complex *Bacillus* (LDJ) and lactic acid bacteria (LAC) showed different advantages in improving the antioxidant status of koi carp, depending on the detected parameters and tissues. The complex of *Bacillus*, lactic acid bacteria, yeast, nitrifying bacteria, denitrifying bacteria, and photosynthetic bacteria (LJD) was more efficient in improving the CAT, GPX activity, increasing GSH levels, and reducing MDA levels in several tissues.

Candidate Probiotics Remodel the Intestinal Bacterial Community
      The intestinal microbiota comprises a diverse and vast population of microbes, which plays a crucial role in fish's health and adaptation to multiple stress (Ding et al., 2020; Wang et al., 2018). Probiotic supplementation in the fish diet is well recognized for regulating the physiological functions of digestive tract microbiota, reducing the incidence of opportunistic pathogens (Balcázar et al., 2006; Dimitroglou et al., 2011), improving nutritional performance (Gaggìa et al., 2010), and maintaining the health and growth performance of the host (Allameh et al., 2017; Wang et al., 2006).
      In the present study, dietary complex probiotics decreased the alpha diversity of the microbial community. The LDJ group increased the abundance of potential candidate probiotics (Fuscobacteriales order, OTU2) compared to the control sample. Fuscobacteriales order is commonly found in the gut microbiota of freshwater fish (Borrelli et al., 2016; Roeseler et al., 2011). *Cetobacterium somerae* is a bacterium within the order Fusobacteriales, a species widely distributed in the guts of freshwater fish (Borsodi et al., 2017). Some strains of *Cetobacterium somerae* can produce vitamin B_{12} (Tsuchiya et al., 2008). Vitamin B_{12} (cobalamin) is important for fish health and can affect the host-microbe symbioses (Degnan et al., 2014). Therefore, increased Fuscobacteriales level caused by compound dietary probiotics may benefit the fish. Pigment-producing bacteria are also potential probiotic candidates for ornamental fish. The LAC group has a higher proportion of Erythrobacteraceae than the control group. A prominent feature of many members of Erythrobactaeriaceae is pigmentation (pink/red/orange/yellow) due to the production of carotenoids (Borsodi et al., 2017).
      Bacteria that can metabolize ammonia/nitrite or other organic pollutants are essential for maintaining water quality (Borsodi et al., 2017; Wang et al., 2019). The LDJ and LAC had lower total levels of pollutants-reducing bacteria and a reduced level of *Gemmobacter* genus compared to the control sample. *Gemmobacter* was one of the predominant genera in the biofilm samples of the simultaneous partial nitrification, anammox, and denitrification (SNAD) bioreactor (Lu et al., 2020). The reduction in carbon or nitrogen-reducing bacteria could be attributed to improved digestibility and improved feed efficiency, which is demonstrated in the present study.
      Photosynthetic bacteria are also important for good water quality. The LAC group had a higher proportion of the genus *Tabrizicola*, an important aerobic anoxygenic phototrophic bacteria (Borsodi et al., 2017), suggesting an enhancing effect of *Tabrizicola* by LAC. Several *Nannocystis* species were reported to be geosmin producers (Dickschat et al., 2007). The levels of *Nannocystis* in the LAC and LJD were low, but LDJ had a higher level of when compared to the control group. Since carp are used as ornamental fish, the level of geosmin

producers is negligible. Sensory properties should be considered when feeding LDJ, a complex probiotic product, to food fish. *Legionella* is a genus of pathogenic Gram-negative bacteria for humans (Borsodi et al., 2017). However, the LDJ group had a higher level of *Legionella* genus, which could threaten aquaculture farmers.

The overall intestinal microbiota patterns of the LJD group (which contains complex *Bacillus*, lactic acid bacteria, yeast, nitrifying bacteria, denitrifying bacteria, and photosynthetic bacteria) are like the control group. The result suggested a minor disturbance of the gut microbiota by LJD. This may be more beneficial for maintaining gut microbiota homeostasis and the cultured fish's health.

Conclusion

Our data highlight that dietary supplementation of two complex probiotics (LAC, and LJD) was more efficient in promoting growth performance, feed utilization, digestive enzyme activities, and antioxidant status in koi carp (*C. carpio* var. *koi*). Overall, LJD containing multi-strain complex candidate probiotics showed greater efficiency in growth, feed utilization, and antioxidant promotion and a minor disturbance in the gut microbiota in koi carp (*Cyprinus carpio* var. *koi*). Further research on the underlying mechanisms is crucial for applying multi-complex probiotics in aquaculture.

Acknowledgments

Conflict of Interest
The authors have no conflict of interest to declare.

Authors' Contributions
Dong-Qing Bai and Guang Yang designed the experiments. Jing-Ru Wang and Yang Wang analyzed the data and wrote the manuscript. Qing-Hao Meng performed the experiments. Guo-Xia Zhu and Yun-Long Luo contributed to the editing of the manuscript.

Ethical Approval
The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All animal experiments were approved by the Animal Experiment Ethics Committee at Tianjin Agricultural University.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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