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The effect of quorum quenching probiotics on modulated digestive enzymes activity, growth performance, gut microfora and biochemical parameters in Common carp (*Cyprinus carpio*)

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Abstract

In this study, two main bacteria with probiotic ability (*Citrobacter freundii* and *Bacillus foraminis*) with autochthonous quorum quenching (QQ) were isolated from the intestine of *Cyprinus carpio* and their effects on growth performance, gut microbial flora, biochemical indices and digestive enzymes activities (i.e., α-amylase, lipase, trypsin, chymotrypsin, and alkaline phosphatase) of *C. carpio* were determined. Juveniles of *C. carpio* (n=450, weighing 50±10 g) were randomly divided into 6 equal groups (with 3 replications) and fed on diets containing 1×10^9 cfu g^{-1} of *C. freundii* (QQ1, G1), *B. foraminis* (QQ2, G2), *Lactiplantibacillus plantarum* (without characteristics QQ, WQQ, G3), QQ1 + QQ2 (G4), QQ1 + QQ2+WQQ (combine, G5), and a control diet (without probiotic) for 60 continuous days. Results showed that probiotic supplementations had generally significant effects on growth performance. The G5 and G3 had the best effect on specific growth rate (SGR) and feed utilization efficiency in *C. carpio* at days 30 and 60, respectively. The trypsin, protease, and chymotrypsin activities, on day 30 after feeding, significantly increased in G5 when compared with those in the control and the other groups. Significant changes in bacterial intestinal flora were observed in all probiotic groups compared with the control. These results highlighted the potential use of *Bacillus foraminis* (QQ2, G2) alone or in combination with other probiotics (G5) as additive in *C. carpio* diets but are not recommended in the long term. The results indicated that supplementation of isolated bacteria from the intestine of *C. carpio* (i.e., G3) can efficiently improve growth performance, intestinal microbiota and some digestive enzyme activities in juvenile *C. Carpio* in the long term culture. Therefore, it can be used as a growth enhancer like the commercial probiotics.

Key words: *C. carpio*, Intestine bacteria, Growth performance, Digestive enzyme activity, Microbial flora

Introduction

One of the most significant objectives of contemporary aquaculture is the substantial increase in production and the optimisation of profitability (Denev et al, 2009). Over the

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past few decades, the aquaculture industry has developed as a result of the use of a variety of fish species, the implementation of intensive culture systems, and the enhancement of the metabolic assimilation of dietary nutrients. The common carp is one of the most economically significant freshwater fish, typically reared in earthen ponds. The previous investigations have indicated that feed costs account for 84% of the total costs associated with the production of freshwater fish (Saravanan et al, 2021). Carp species can be produced even in lower quality waters, which is an especially valuable characteristic in the Asian and Middle East region. From the aspect of sustainability achieved in carp pond farms, it is essential to perform fish meal-independent and cereal-based fish meat production which provides increasing production of this fish species in the long run (Mohtashamipour et al, 2022). In this context, it has been reported that the highest cost paid for carp production in Iran is related to feed. Therefore, it is important to improve the feed effciency (FE) and decrease the total cost required for carp production per unit of surface (Mohammadian et al, 2024). The concept of functional feeds formulated by probiotics represents a novel approach within the aquaculture industry. The application of probiotics as an alternative to therapeutics in aquaculture is not a novel concept (Panigrahi et al, 2005). However, there has been a notable increase in interest in the safe and highly effective functions of such probiotics (Gatesoupe, 1999). A variety of microorganisms have been assessed as probiotics in aquatic animals (Mohammadian et al, 2024; Xia et al, 2019; Kuebutornye et al, 2019; Mirbakhsh et al, 2013; Faeed et al, 2016). However, quorum quenching bacteria (QQBs) represent the most prevalent probiotic used in aquaculture (Ghanei motlagh et al, 2021). It has been demonstrated that application of functional supplements such as probiotics can effciently improve growth performance, nutritional value and physiological responses of aquatic animals (Mohammadian et al, 2018). Incorporation of probiotics to diet can also improve the activity of digestive enzymes which in turn increase the absorption of nutrients from the gut and provide required energy for fish growth (Mohammadian et al, 2017). Likewise, use of probiotics is an eco-friendly and sustainable approach that reduces the use of harmful chemical compounds particularly antibiotics in aquaculture (Gatesoup,1999; Wang et al, 2008). The *Aeromonas hydrophila* is one of the most important gram-negative pathogenic bacteria in fish that causes hemorrhagic septicemia, ascites and mortality at different ages and in different species of fish (sha et al, 2005). There are several reports available regarding the beneficial influence of bacterial probiotics including live yeast, bifidobacters, *Lactobacillus*, feeding on growth performances and gut microbiota in some aquatic animals such as Shabot (*Tor grypus*) (Mohammadian et al, 2017), Asian Seabass (*Lates calcarifer*) (Mozanzadeh et al, 2023), European sea bass (*Dicentrarchus labrax*) (Tovar et al, 2002; Tovar-Ramırez et al, 2004), sea bream (*Sparus aurata*) (Suzer et al, 2008; Sáenz de Rodrigáñez et al, 2009), beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) (Askarian et al, 2008), Tilapia *(Oreochromis niloticus*) (Standen et al, 2013), Rainbow trout *(Oncorhynchus mykiss*) (Korkea-Aho et al, 2012; Ramos et al, 2013), *Salmo trutta caspius* (Mohammadian et al, 2022). Quorum sensing (QS) is a process by which bacteria monitor their population in a cell-density dependent manner through the synthesis, exchange, and the detection of small intracellular signals (autoinducers) (Xavier and Bassler, 2005). Among the various types of signals that are produced in bacteria (Autoinducer-1, Autoinducerprobably the best studied. Bacteria produces the QS molecules, and when the intensity of these signals reaches a threshold, they diffuse back into the bacterial cell and regulate the expression of QS-related genes such as those involved in biofilm formation and production of virulence determinants (Galloway et al, 2011). Moreover, the isolation of

autochthonous bacteria with QQ potential has frequently been reported from freshwater fish. On the other hand, despite the high mortalities caused by freshwater *Aeromonas*, QQ strategy has not been adopted against commonly occurring *Aeromonas* pathogens in fish, particularly, *C. carpio*, an adaptive freshwater fish with high economic importance that has gained much attention from researchers and farmers in the last decade. However, there is no comprehensive investigation of the real probiotic characterization of QQ bacteria prior to their administration to fish farm culture and their application on a commercial scale. There are little reports available regarding the beneficial influence of QQ bacteria including Bacillus, Citrobacter, Bifidobacter, feeding on growth performances and gut microbiota in some aquatic animals such as Rainbow trout *(Oncorhynchus mykiss*) (Torabi et al, 2019), Zebrafish *(Danio rerio*) (Chen et al, 2019), Asian Seabass (*Lates calcarifer*) (Ghanei-Motlagh et al, 2021). In the present study, QQ bacteria with a potential to degrade the dominant range of AHL molecules produced by several significant and prevalent pathogenic *Aeromonas* spp. in fish, were isolated from the intestine of common carp and characterized and their efficacy as autochthonous probiotics was tested for the first time. Due to the lack of information on QQ probiotic application in carp, the goal of this study was to evaluate the effects of two QQ, *Citrobacter freundii* and *Bacillus foraminis*, isolated from the intestine of *C. carpio* and a *Lactobacillus* without characteristics QQ *(Lactobacillus plantarum* WQQ) on growth indices, gut microbiota, and digestive enzymes (α-amylase, lipase, trypsin, chymotrypsin, and alkaline phosphatase) activities in juvenile Common carp (*C. carpio*).

Materials and Methods

Bacteria

Bacterial isolates were recovered using a previously described method (Irianto and Austin, 2002). Briefly, the entire digestive tracts of *C. Carpio* captured from natural water resources of Khuzestan province in Iran were removed and their contents were discarded. The quorum quenching potential of *C. freundii* QQ1 and *B. foraminis* QQ2 was confirmed in our previous study using the agar well diffusion and thin layer chromatography methods. In this study, their QQ activity was also tested against *Yersinia ruckerie* by the degradation assay on Luria-Bertani agar as suggested by Chu et al, (2011). The tested *Y. ruckerie* was able to induce *Chromobacterium violaceum* CV026. This biosensor respondes to exogenous AHLs with *N*-acyl side chains from C_4 to C_8 in length with production of purple pigment violacein. *Pseudomonas fluorescence* P3/pME6863 and *Pseudomonas fluorescence* P3/pME6000 were respectively used as positive and negative controls in AHL degradation assay. The strains CV026, P3/pME6863 and P3/pME6000 were kindly provided by Dr. Torabi Delshad. The *L. plantarum* strains used in this study as none QQ character were primarily identified based on colony and cell morphology, Gram staining, biochemical characteristics, and 16S rRNA gene sequencing (GenBank accession number EU520326 and EU520327) (Mohammadian et al, 2016). These strains were grown for 30 h at 37°C in MRS broth (BD Difco, Sparks, MD, USA).

Diet preparation

The control diet was formulated using the ingredients as subsequently described. The proximate analysis of the basal diet according to the AOAC method was: 37.1% for crude protein, 8.8% for crude lipid, 9.6% ash and 390 Kcal per 100 g for gross energy. Probiotic bacterial suspensions were prepared by centrifuging (15min., 4000 rpm) the 72h TSB cultured bacteria and resuspending them in PBS at the concentration of Macfarland grade 3 (1.2×10^9 cfu mL^{-1}). The probiotic-enriched diets were prepared by gently spraying of the prepared bacterial suspension on the control and mixing that part by part in a drum mixer to obtain a final probiotic concentration of 1×10^9 $cfug^{-1}$. They were packed in sterile propylene containers and stored at 4°C for viability studies for a week.

This dose was chosen based on a previously recommended dose (Takafoyan et al, 2024). Final concentrations of probiotic bacteria in the diet were confirmed by suspending one gram of food in sterile PBS and culturing the serial diluted food suspension in TSA media. Counted bacteria in the food were almost the same as added probiotic bacteria in all batches of probiotic-enriched diets.

Experimental design

Juveniles of *C. carpio* (50±10 g) (Mean±SD) were transferred from a private cyprinid farm in Khuzestan Province, Iran, to the Lab of Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. The fish were acclimated for 2 weeks in indoor 300 L fiberglass tanks and were fed with a standard diet (37.1% crude protein, 8.8% crude lipid, 9.6% ash, and 390 Kcal $100g^{-1}$ gross energy). Then, after verifying the health status of the fish, they were distributed randomly into 12 tanks at an initial density of 25 fish per tank were then divided into 6 treatment groups, including control (n=25), QQ1 (G1, n=25), QQ2 (G2, n=25), *L. plantarum* (G3, n=25 as a whithout characteristics QQ), QQ1 + QQ2 (G4, n=25), QQ1 + QQ2+W QQ (G5, n=25). Final concentration of each probiotic was about 1×10^{9}cfu g^{-1} of the diet (Table 1) (Nikoskelainen *et al*., 2001). The aquaria were supplied with water from external Biofilteres (Athmann, China), at a temperature of 25.9±1.2˚C. The fish were fed with probiotic-contained diets for 60 days (twice a day). During the experimental period, the temperature ranged from 24.5 to 28.5°C, salinity was from 0.6±0.11 % and the dissolved oxygen was 5.9±1.3mgL^{-1}.

Table 1: The experimental design and treatment setting up, applied in this study.

Treatment	G1	G2	G3	G4	G5	Control
Probiotics category	QQ1	QQ2	*L. plantarum* (W QQ)	QQ1 + QQ2	QQ1 + QQ2 + W QQ	Normal saline
Additive quantity (g kg−1)	1×10^{9}	1×10^{9}	1×10^{9}	1×10^{9}	1×10^{9}	0.0

Sampling and analysis of biological parameters

In order to determine growth performance, the weight of all fish in each treatment was measured at the fiberglass tanks. The fish, then, were fed with a standard diet (37.1% crude protein, 8.8% crude lipid, 9.6% ash, and 390 Kcal $100g^{-1}$ gross energy). Growth performance at 30 and 60 days after the feeding was assessed in terms of Body Weight Growth (BWG), Specific Growth Rate (SGR), Food Efficiency Ratio (FER), Food Conversion Ratio (FCR) and Protein Efficiency Ratio (PER). The calculations were performed using the following formulae: BWG %= 100× (FBW–IBW)/ IBW, SGR%= 100× (lnŦFBW–lnIBW)/ days, FCR=feed consumed/ (FBW–IBW), FCE%=(FBW–IBW)/feed consumed×100, PER=IBW/protein intake. IBW is initial body weight, FBW is final body weight and days are days of feeding.

Digestive enzyme activity

To analyze the activity of digestive enzymes, on days 30, and 60 following probiotic feeding, the fish were starved for 24 h. Then, 3 fish were taken randomly. The intestine was dissected out under sterile conditions and at low temperature (around 4ºC, near icepack). After the samples were homogenized in a cold homogenizing buffer containing 50 mMTris–HCl, pH 8.0 (1:9v/w) followed by centrifugation (13.500 ×g; 30 min at 4°C). The supernatant was collected and kept at −80°C in small portions for later determinations (Rungruangsak-Torrissen et al, 2002; Rungruagsak-Torrissen and Fosseidengen, 2007). Total protein content of the supernatant was assayed according to a

Ŧ Natural logarithm having based 10

(Bradford, 1976) method using bovine serum albumin as standard. Trypsin activity was measured using N α -Benzoyl- L - arginine ethyl ester N α -Benzoyl- L - arginine ethyl ester (BAEE) as the substrate (Erlanger et al, 1961). Banzoyl-L-Tyrosine ethyl ester Ester (BTEE) was used as a substrate to determine enzyme activity of chymotrypsin (Hummel, 1959). ALP activity was kinetically measured using 4-nitrophenyl phosphate (PNPP) as substrate by a commercial kit (Pars Azmoon Co., Tehran, Iran). α-amylase activity was measured according to the modified Bernfeld method as described previously (Areekijseree et al, 2004) using starch solution as substrate. Amylase specific activity was expressed as μmol maltose produced h^{-1} mg $protein^{-1}$. Lipase activity was determined based on the measurement of fatty acids release due to enzymatic hydrolysis of triglycerides in stabilized emulsion of olive oil (Borlongan, 1990). Enzyme activities were measured as the change in absorbance using a spectrophotometer (UV-2802S; Unico, Shanghai, China) and expressed as specific activity (U mg^{1}protein) (Sun et al, 2012).

One of the objectives of this study was to select a suitable modified method for improving nutrient utilization. Feeding habit and metabolic flexibility of carbohydrate–protein utilizations to reach this objective was measured in probiotics fed on *C. carpio* according to Hofer and Schiemer (1981) and Thongprajukaew et al, (2011). To do this, α-mylase/Trypsin ratio was calculated for each treatment.

Intestinal bacterial community

The specimens of posterior intestine were aseptically removed and homogenized with sterilized PBS (1:10 w/v). The homogenates were serially diluted and 100 μL of tenfold diluted suspensions were spread on different agar media including tryptone soy agar (TSA), MRSA (de Man, Rogosa and Sharpe) and mannitol egg yolk polymyxin (MYP) in triplicates to determine the populations of total aerobic heterotrophic bacteria, LAB spp. and *Bacillus* spp. respectively. All plates were incubated for 48 h at 29 °C and the counted colonies were expressed as denary logarithm of colony forming units (CFU) per ml homogenized suspension (Ghanei-Motlagh et al, 2021b).

Statistical analysis

All statistical tests were performed using SPSS software (SPSS, Release 16.0, SPSS, Chicago, IL, USA). Two-way analysis of variance (ANOVA) and general linear model were used to evaluate the effect of time and treatments on each variable. One-way analysis of ANOVA was done to determine the differences between different variables. Differences were considered statistically significant when $P<0.05$ and the results are expressed as mean ± SD.

Result

Growth performance

The final weight, weight gain, daily weight gain, relative growth rate and specific growth rate of *C. carpio* significantly increased when they were fed with diets containing *B. foraminis* (Q2) *and* Combine group (Q1+Q2+WQ) at 30 days and *L. Planturum* (WQ) for 60 days (Table 2). The minimal FCR and higher protein efficiency ratio (PER) was observed in fish fed with dietary Combine group (Q1+Q2+WQ) and control group while the highest FCR was observed in the Q1 and Q1+Q2 fed groups at 30 days and the minimal FCR and higher PERwas observed in fish fed with dietary WQ group for 60 days (Table 2). The lowest protein efficiency ratio was found in the fish fed Q1+Q2 containing food at 30 days and Combine group (Q1+Q2+WQ) for 60 days.

Table 2: Growth performance of *C. carpio* fed either regular feed or feed supplemented with probiotics for 60 days

Parameters	Groups	Day 30	Day 60
IW	Q1	59.2 ± 1.4^{a}	70.1 ± 2.7^{b}
	Q2	65.96 ± 1.11^{a}	80.36 ± 3.52^{a}
	WQ	65.1 ± 2.52^{a}	78.3 ± 2.5^{ab}
	Q1+Q2	64.7 ± 2.2^{a}	75.3 ± 1.5^{ab}
	Q1+Q2+WQ	66.54 ± 1.32^{a}	81.73 ± 4.15^{a}
	Control	61.8 ± 2.22^{a}	69.36 ± 1.5^{b}
FW	Q1	70.1 ± 2.7^{b}	102.5 ± 8.7^{b}
	Q2	80.36 ± 3.52^{a}	104.07 ± 2.56^{b}
	WQ	78.3 ± 2.5^{ab}	119.43 ± 3.55^{a}
	Q1+Q2	75.3 ± 1.5^{ab}	105.5 ± 4.9^{b}
	Q1+Q2+WQ	81.73 ± 4.15^{a}	105.16 ± 5.55^{b}
	Control	69.36 ± 1.5^{b}	103.03 ± 3.9^{b}
FCR	Q1	$3.3\pm0.26^{b,A}$	$2.07\pm0.12^{a,B}$
	Q2	$2.9\pm0.11^{ab,B}$	$4.4\pm0.6^{b,A}$
	WQ	$2.7\pm0.32^{ab,A}$	$1.62\pm0.01^{a,B}$
	Q1+Q2	$3.6\pm0.2^{b,A}$	$2.21\pm0.25^{a,B}$
	Q1+Q2+WQ	$2.41\pm0.22^{a,B}$	$4.04\pm0.1^{b,A}$
	Control	$2.44\pm0.12^{a,A}$	$2.098\pm0.05^{a,A}$
SGR	Q1	$0.56\pm0.04^{ab,B}$	$1.26\pm0.1^{a,A}$
	Q2	$0.65\pm0.09^{a,A}$	$0.87\pm0.05^{b,A}$
	WQ	$0.61\pm0.02^{ab,B}$	$1.4\pm0.01^{a,A}$
	Q1+Q2	$0.51\pm0.06^{ab,B}$	$1.12\pm0.1^{a,A}$
	Q1+Q2+WQ	$0.68\pm0.02^{a,A}$	$0.85\pm0.01^{b,A}$
	Control	$0.43\pm0.06^{b,B}$	$1.01\pm0.01^{ab,A}$
PER	Q1	$0.93\pm0.16^{ab,B}$	$1.51\pm0.2^{a,A}$
	Q2	$1.07\pm0.11^{ab,A}$	$0.8\pm0.08^{b,A}$
	WQ	$1.14\pm0.22^{a,B}$	$1.93\pm0.12^{a,A}$
	Q1+Q2	$0.88\pm0.02^{b,B}$	$1.4\pm0.334^{a,A}$
	Q1+Q2+WQ	$1.33\pm0.15^{a,A}$	$0.97\pm0.02^{b,B}$
	Control	$1.28\pm0.12^{a,A}$	$1.13\pm0.134^{ab,A}$
DWG	Q1	$0.36\pm0.08^{b,B}$	$1.071\pm0.02^{ab,A}$
	Q2	$0.47\pm0.017^{ab,B}$	$0.87\pm0.01^{b,A}$
	WQ	$0.44\pm0.02^{ab,B}$	$1.37\pm0.02^{a,A}$
	Q1+Q2	$0.35\pm0.03^{b,B}$	$1.076\pm0.044^{ab,A}$
	Q1+Q2+WQ	$0.5\pm0.02^{a,A}$	$0.95\pm0.02^{b,A}$
	Control	$0.28\pm0.03^{b,A}$	$1.017\pm0.044^{ab,B}$
RGR	Q1	$18.6\pm0.25^{ab,B}$	$46.5\pm0.75^{a,A}$
	Q2	$21.15\pm0.61^{a,A}$	$29.16\pm0.26^{b,A}$
	WQ	$20.3\pm1.21^{a,B}$	$52.6\pm0.55^{a,A}$
	Q1+Q2	$16.1\pm0.37^{ab,B}$	$40.27\pm0.76^{ab,A}$
	Q1+Q2+WQ	$22.3\pm1.21^{a,A}$	$33.6\pm0.55^{b,A}$
	Control	$13.1\pm0.37^{b,B}$	$42.27\pm0.76^{ab,A}$
FER	Q1	$29.3\pm2.7^{b,B}$	$48.6\pm1.2^{ab,A}$
	Q2	$34.33\pm0.47^{b,A}$	$27.2\pm0.44^{b,A}$
	WQ	$36.7\pm0.8^{aa,B}$	$61.76\pm1.18^{a,A}$
	Q1+Q2	$28.12\pm0.09^{b,B}$	$47.08\pm3.02^{ab,A}$
	Q1+Q2+WQ	$42.7\pm0.8^{a,A}$	$31.76\pm1.18^{b,A}$
	Control	$41.12\pm0.09^{a,A}$	$49.08\pm3.02^{ab,A}$

* For each parameter, values (Mean ± SD) bearing different uppercase letters or different lowercase letters represent significant differences within each column or each row, respectively ($P<0.05$).

* Abbreviations: IW, Initial Weight; FW, Final Weight; %WG, FCR, Feed Conversion Ratio; SGR: Specific Growth Rate; DWG, Daily Weight Gain; PER, Protein Efficiency Ratio, RGR, Relative Growth Rate; FER, Feed Efficiency Ratio.

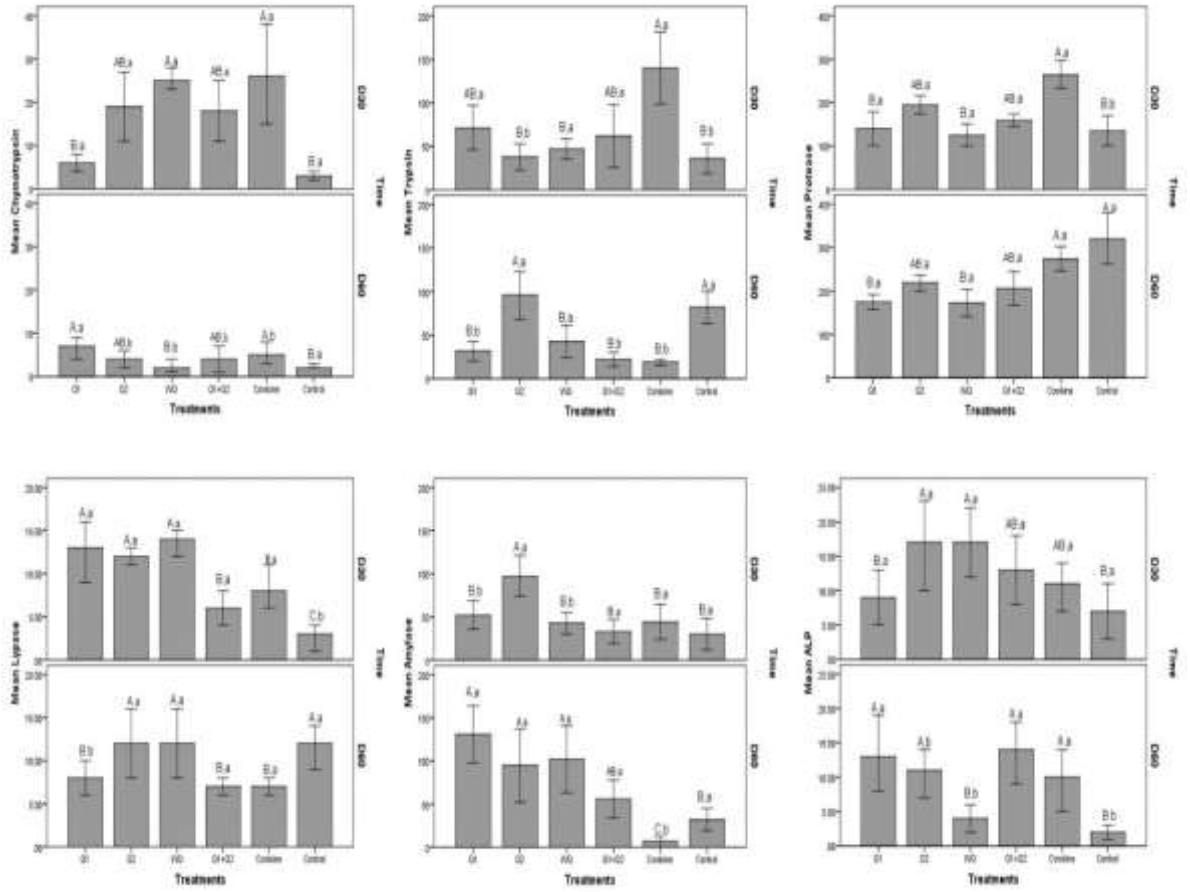

Figure 1: Digestive enzyme activity in *Cyprinus caroio* **treated with QQ1 (*C. freundii* ~1×10^9 cfu g^{-1}), QQ2 (*B. foraminis* ~1×10^9 cfu g^{-1}), WQQ (*Lactobacillus planturum* ~1×10^9 cfu g^{-1}), QQ1+QQ2 (*C. freundii* and *B. foraminis* ~1×10^9 cfu g^{-1}), QQ2 (*B. foraminis* ~1×10^9 cfu g^{-1}), QQ1+QQ2 +WQQ (*C. freundii* and *B. foraminis and Lactobacillus planturum* ~1×10^9 cfu g^{-1}) and without probiotic (control). Values is shown as means±standard error (n = 9). Legends as mentioned in Fig. 1.**
***For each parameter, values (Mean ± SD) bearing different lowercase letters or different uppercase letters represent significant differences within each column or each row, respectively (P<0.05).**

Digestive enzyme activity

The specific activity of trypsin was increased during the 30 days of treatment in Combine group (QQ1+QQ2+WQQ) group, while its activity declined at the end of the experiment. The highest specific activity of trypsin was determined in QQ2 group on the 60th day of the experiment (Figure 1). No significant differences were found among the other groups on days 30 or 60 of feeding (P>0.05).

The specific activity of α-amylase increased in all experimental groups during the 30 days of probiotic feeding and thereafter slightly increased (except combine group) until the end of the experiment. Its highest activity was found in QQ2 group on day 30 of the experiment (Figure 1). The highest was found in QQ1, QQ2 and WQQ groups on day 60 of the experiment (Figure 1).

The specific activity of ALP was significantly elevated on the 30th day of the feeding in G1 and G2 groups (P<0.05). The highest activity of ALP was observed on day 30 past the feeding in QQ2 and WQQ groups (Figure 1). We found changes in ALP activity of QQ1 group, 60 days after the probiotic feeding. The highest activity of ALP was observed on day 60 past the feeding in QQ1 and QQ1+QQ2 groups.

Chymotrypsin activity was influenced by probiotic administration, so that on the 30th day of the test, Combine group (Q1+Q2+WQ) and WQ groups had the highest chymotrypsin activity and a significant difference with the control group (P<0.05). The results on the 60th day of the feeding showed that all experimental groups had declined chymotrypsin activity.

Lipase activity had no significant (P>0.05) differences among the probiotic-fed groups during the feeding period (Figure 1). The highest activity of Lipase was observed on day 30 past the feeding in QQ1, QQ2 and WQQ groups (Figure 1). We found changes in Lipase activity of QQ2 and WQ group, 60 days after the probiotic feeding. Protease activity was influenced by probiotic administration, so that on the 30th day of the test, combine group had the highest Protease activity and a significant difference with the control group (P<0.05). The results on the 60th day of the feeding showed that all experimental groups had declined Protease activity except the control group.

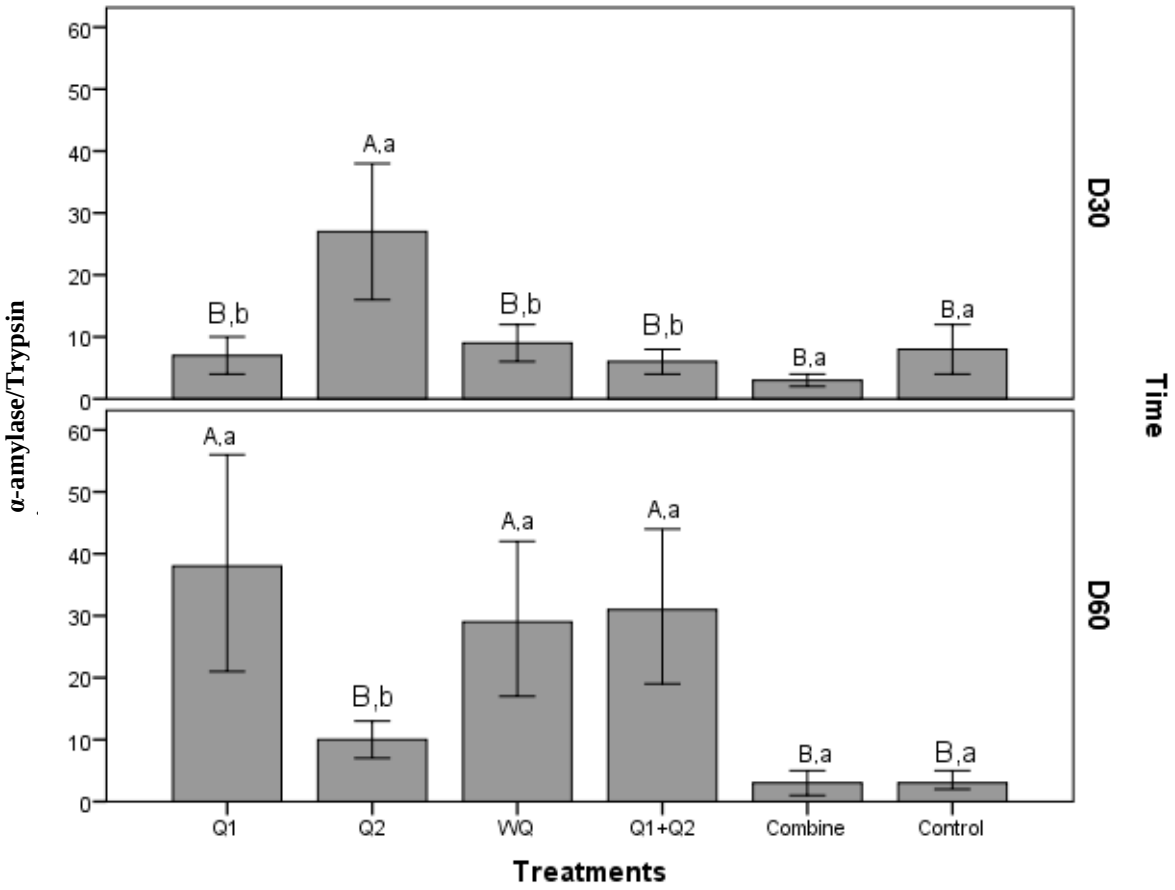

Figure 2: α-amylase/Trypsin ratio in *Cyprinus caroio* **treated with QQ1 (*C. freundii* ~1×10^9 cfu g^{-1}), QQ2 (*B. foraminis* ~1×10^9 cfu g^{-1}), WQQ (***Lactobacillus planturum*** ~1×10^9 cfu g^{-1}), QQ1+QQ2 (*C. freundii* and *B. foraminis* ~1×10^9 cfu g^{-1}), QQ2 (*B. foraminis* ~1×10^9 cfu g^{-1}), QQ1+QQ2 +WQQ (*C. freundii* and *B. foraminis* and *Lactobacillus planturum* ~1×10^9 cfu g^{-1}) and without probiotic (control). Values is shown as means±standard error (n = 9). Legends as mentioned in Fig. 1.**

***For each parameter, values (Mean ± SD) bearing different lowercase letters or different uppercase letters represent significant differences within each column or each row, respectively (P<0.05).**

The highest amylase to trypsin ratio (A/T ratio, P<0.05) was found in fish-fed QQ2 diet on the 30th day of the treatment. The highest activity of A/T ratio was observed

on day 60 past the feeding in QQ1, QQ1+QQ2 and WQQ groups (Figure 3) (Figure 2).

Microbiological assay

Before the probiotic feeding, the fish had low detectable lactobacilli level in the entire intestines. The viable count of LABs significantly increased in a time-dependent manner in the intestine of fish-fed WQQ and combine (QQ1+QQ2+WQQ) group probiotics-contained diets. Although fish-fed QQ2 supplemented diets increased viable counts 60 days after the probiotic feeding, the number of viable LABs in QQ1 was significantly reduced on the 60th day of the experiment (Table 3). On the 30th day of the experiment, the total count of bacteria was significantly increased in the control group compared to the other group ($P<0.05$). After 60 days of feeding with diets containing probiotic, no significant alteration in the total count of bacteria was observed between the groups ($P>0.05$). Moreover, *Bacillus* cultivable bacterial counts were found at significantly higher numbers in the groups fed with diets containing QQ2 and combine (Q1+Q2+WQ) group than in the control group at days 30 and 60 ($P<0.05$).

Table 3: Total viable counts (means ± SEM (n = 9)), total lactic acid bacteria (LAB) and *Bacillus* from the digestive tract of *C. carpio*.

Parameters	Groups	Day 30	Day 60
Total Count Bacteria ($\times10^5$ CFU/g)	Q1	176.6±53.5 ^{Aa}	156.3±47.5 ^{Aa}
	Q2	196.3±19.5 ^{Bb}	238.2±35.3 ^{Aa}
	WQ	169.0±27.16 ^{Aa}	206.0±66.1 ^{Aa}
	Q1+Q2	136.6±19.6 ^{Aa}	179.0±33.5 ^{Aa}
	Q1+Q2+WQ	180.0±22.64 ^{Aa}	194.0±25.5 ^{Aa}
	Control	246.0±19.6 ^{Aa}	253.0±33.5 ^{Aa}
Lactic Acid Bacteria ($\times10^2$ CFU/g)	Q1	54.0±7.63 ^{Ba}	46.0±33.5 ^{Ca}
	Q2	65.0±7.63 ^{Bb}	199.45±26.2 ^{Ba}
	WQ	233.0±1.15 ^{Aa}	268.66±3.2 ^{Aa}
	Q1+Q2	72.0±14.3 ^{Ba}	102.3±10.4 ^{BCa}
	Q1+Q2+WQ	237.0±10.3 ^{Aa}	276.33±10.4 ^{Aa}
	Control	18.0±10.3 ^{Ca}	14.33±10.4 ^{Ca}
Bacillus spp. ($\times10^4$ CFU/g)	Q1	ND	ND
	Q2	4.8 ± 0.11^{Ab}	13.31 ± 0.08^{Aa}
	WQ	ND	ND
	Q1+Q2	4.74 ± 0.1^{Aa}	3.77 ± 0.06^{Ba}
	Q1+Q2+WQ	4.8 ± 0.11^{,Ab}	8.81 ± 0.08^{Aa}
	Control	3.34 ± 0.1^{Aa}	5.87 ± 0.06^{Ba}

* ND: No detected. Different lowercase letters indicate statistically significant differences between each of the experimental groups at various sampling time points (row) ($P<0.05$). Different capital letters denote significant differences between the experimental groups at a specified time point (column) ($P<0.05$).

Discussion

Probiotics are microbes that have positive effects on fsh welfare when they are administered in adequate numbers in diet or water. Probiotics should be compatible with their target species to colonize the host's intestine and establish their positive effects (Mohammadian et al., 2019c). In this study, two autochthonous QQ bacteria (*C. ferundi* and *B. foraminis*) with probiotic action were isolated from the intestine of *C. carpio* and their effects on growth performance, gut microbial flora, biochemical indices. Digestive enzyme activity was determined during a 60-day feeding. We also compared their properties with a WQ LAB strain *(L. plantarum*). Our results showed that *B. foraminis* and Combine group improved the growth performance of *C. carpio* more effectively when compared with other tested group on

day 3o. The promotion of the growth rate, FER and PER in *B. foraminis* fed group occurred concomitantly with increasing protein turnover. *C. carpio* fed WQQ supplemented diets showed higher growth performance, SGR, RGR, PER, FCR and FER in relation to other experimental groups on day 60. This result was in agreement with those observed by Liu et al, (2013) who observed the highest growth performance in hybrid tilapia fed two different *Lactobacillus* strains and Reda et al. (2018) who observed the highest growth performance in African catfsh (*Clarias gariepinus*) fed with host-associated *B.cereus* 39HN compared to fish fed with a commercial probiotic (*B. amyloliquefaciens*). Different actions of probiotics on growth performance of treated fish, found in our experiment support the suggestion that each probiotic strain may interact with the host in a different way (Bomba et al, 2002; Sun et al, 2012; Mohammadian et al, 2017). It may also be explained by the greater adaptive capacity of *B. foraminis* in aquatic environments in comparison to *C. freundii* and *L. Plantarum* at short time culture (30 days). At this time of culture, it was also found that supplementation of food with *B. foraminis* could improve the feed utilization of *C. carpio* in higher rates than other probiotics. In the previous investigations, the positive effects of probiotics on growth performance have been attributed to a variety of factors., intestinal bacteria shared in the decomposition of nutrients, such as enzymes, minerals and vitamins, and thus, facilitate feed utilization, digestion and absorption. Growth indices such as PER, FCR and FER increased among *C. carpio* fed on a diet containing WQ (*L. Plantarum*) for 60 days. These findings are similar to that obtained by (Lin et al, 2017; Adorian et al, 2019; Mohammadian et al, 2022). It has been indicated that probiotics in feeds with a certain concentration display a growth promoting effect and can be beneficial for commercial fish production. In practical terms, this means that probiotic can decrease the amount of feed necessary for animal growth resulting in production cost reduction. Another probable reason of this difference can refer to autochthonous characteristic of *B. foraminis* which may provide higher digestible nutrients for the host and digests higher dietary protein/amino acids when compared to the allochthonous bacteria. Considering these findings, we concluded that different QQ bacteria especially *B. foraminis* isolated from intestine of *C. carpio* can improve the growth performance of cultured juvenile *C. carpio* when administrated as a food additive. Such probiotics are recommended to be used as a commercial growth promoter to facilitate extensive culture of *C. carpio* in future.

Probiotics by increasing digestive enzymes activity can enhance growth previously confirmed in different fish species, such as gilthead sea bream (*Sparus aurata*) (Suzer et al., 2008), silver pomfret (*Pampus argenteus*) (Gao et al, 2016), common carp (*C. carpio*) (Mohammadian et al, 2022), Rohu (*Labeo rohita*) (Saravanan et al, 2021) and as well as in olive flounder (*Paralichthys olivaceus*) (Ye et al., 2011). Administration of probiotics isolated from the gut of *C. carpio* had an effective action on the activities of different digestive enzymes. On day 30 of the probiotic feeding, the specific activities of lipase were higher in all probiotics-fed fishes in relation to the control group. In the current study, QQ2 and combine group had higher proteases and chymotrypsin and ALP activities than other groups, suggesting that using only bacillus with blend of all probiotic bacteria strains in the combine group had more stimulatory effect on digestive enzymes compare to those fed with QQ1. It seems that the presence of *B. foraminis* may be provided with wide range of stimulatory bioactive compounds or enzymes that enhanced enzyme activities in this group. In agreement with results from the previous studies (Francis et al, 2001;

Lara-Flores et al, 2010), we found higher ALP activity in *C. carpio* treated with QQ2 and WQQ containing diets for 30 days. The increase in the activity of AP reflects a possible development of brush border membranes of enterocytes that can be stimulated by the probiotics (Cuvier-Péres and Kestemont, 2001). Activities of this brush border enzyme have been reported to be indicators of the intensity of nutrient absorption in the enterocytes of fish (Gawlicka et al, 2000). High ALP activity also has been reported to be an indicator of carbohydrate and lipid absorption (Calhau et al, 2000; German et al, 2004; Lalles, 2019). Taken these findings, it has been concluded that higher growth performance rate in *C. carpio* fed probiotics may be due to the improvement of enterocytes function as well as better conversion and utilization of feed in brush border.

The trypsin, α-amylase, and chymotrypsin in the *C. carpio* digestive system may not only be secreted from ingested bacteria but may also be derived from indigenous origin, as in grouper (*Epinephelus coioides)* (Sun et al, 2012), barramundi (*Lates calcarifer*) (Siddik et al, 2022) and in beluga (*Huso huso*) (Askarian et al, 2008). Because gram-positive bacteria like members of the genus *Bacillus, Citribacter* and *Lactobacillus*, secrete a wide range of exoenzymes, the origin of enzymatic activities in fish-fed probiotics could not be distinguished (Moriarty, 1996; Moriarty, 1998; Suzer et al, 2008). It has been confirmed that relatively higher activities of digestive enzymes result in growth performance improvement. It is believed that probiotics influence digestive processes by enhancing the population of beneficial microorganisms, microbial enzyme activity, improving the intestinal microbial balance, consequently improving the digestibility and absorption of food and feed utilization (Mohapatra *et al*., 2012; Askarian *et al*., 2008). Our results were in agreement with the study in sea bass (*Dicentrarchus labrax*) larvae (Tovar-Ramírez *et al*., 2004), common carp (*Cyprinus carpio*) (Yanbo and Zirong, 2006), Indian white shrimp (*Fenneropenaeus indicus*) (Ziaei-Nejad et al, 2006), shrimp (*Litopenaeus vannamei*) (Wang, 2007), Skrodenyte-Arbaciauskiene (2007); gilthead sea bream (*Sparus aurata*, L.) (Suzer et al, 2008), grouper (*E. coioides*) (Sun et al, 2011), rainbow trout (*Oncorhynchus mykiss*) (Andani et al, 2012) Kuebutornye and Abarike (2019) demonstrating the improvement of survival rate, growth parameters, and digestive enzyme activities.

Regarding the above results, we encountered contradictory findings because α-amylase, lipase and ALP activities were generally lower in *C. carpio* in combine group than those in fish of QQ1, QQ2 and QQ1+QQ2 group at days 30, while combine group had the highest growth performance. On the other hand, Proteolytic activities (The trypsin, protease, and chymotrypsin), were consistent with the growth results. One possibility is that different probiotics in the diet may affect the gut microbiological and biochemical parameters independently (Balcázar and Rojas-Luna, 2007). According to these findings, it has been concluded that higher growth performance rate in *C. carpio* fed probiotics may be due to the improvement of protein turnover function as well as better conversion and utilization of feed in gut of omnivorous fish. Additional studies are required in order to clarify this hypothesis in detail. A/T ratio had no association with fish growth because it was at the highest level in QQ2 group, while the highest fish growth was observed in combine group at days 30 but in days 60, it was consistent with the growth results. The significantly higher growth performance and A/T ratio in WQQ groups respectively, may indicate higher energy requirement for protein utilization and growth in WQQ group than the other groups at long time culture (Thongprajukaew et al, 2011; Ghanei motlagh et al, 2022).

The gut microbiota can play an important role in the health and growth of the aquatic animals (Vine et al, 2004). Our results showed that feeding of *C. carpio* with diet containing *L. plantarum* could increase counts of viable LABs. These results were in agreement with the previous reports that probiotics have been used as growth promoters in Atlantic salmon and rainbow trout (Robertson et al, 2000), Tilapia (Ferguson et al, 2010), Rainbow trout (Merrifield et al, 2010) , Shrimp (Castex et al, 2008), zebrafish (Alavinejad et al, 2022; Zang et al, 2019) and Siberian sturgeon (Geraylou et al, 2013a; Geraylou et al, 2013b). Findings of this study clearly demonstrate that the probiotic-contained feed must be given to fish continuously to retain the probiotic-bacteria level in the gut. In our study when fish were fed on *L. plantarum* containing diets, changes were less apparent in the diversity of the microbiota in 60 days, compared to those in fish of QQ1 and QQ2 groups. In the present study, the highest numbers of LABs concomitant of the highest growth rate were found in the intestine of *C. carpio* fed *L. Plantarum* (WQQ). It suggests that the numbers of viable LABs are more efficient than enzyme activity in enhancing the growth parameters of *C. carpio*. To confirm this hypothesis we found that combine group could increase counts of the microbiota in the intestine compared to the control and other probiotic groups at days 30, but these changes were not translated into increased enzyme activity in the gut. Taking these findings into consideration, we concluded that feeding of *C. carpio* with combination of QQ1, QQ2 and *L. plantarum* may balance intestinal microbial flora resulting in improvement of food absorption and enhancement of growth performance at short time culture (Irianto and Austin, 2002).

In this study, isolation of *Bacillus* spp. from the intestine of common carp was possible after feeding with QQ2 probiotics for 4 weeks compared to the other groups in which no isolation occurred. Moreover, total cultivable bacterial counts were significantly lower in the probiotic groups than the control group ($P<0.05$). This could be associated with high survival of these strains within the gastrointestinal tract from which they isolated and their appropriate abilities to adhere on mucus as described in our previous study (Ghanei-Motlagh et al, 2020). After 60 days feeding with QQ2 probionts, significant alteration in populations of *Bucillus* spp. was observed between the treatments ($P>0.05$).

In conclusion, the ability of *B. foraminis* and some deal of *C. ferundi* isolated strains to augment growth performance and enzyme activity, colonize and modify the intestinal microbiota as a potential probiotic strains on day 30 were confirmed. But application of WQQ (*L. plantarum*) probiotics is recommended as supplementation for other cultural fish because the diet with these probiotic bacteria increases digestion, absorption of protein, and other nutrients in the gastrointestinal tract due to the increase of intestinal proteolytic enzyme activity. Finally we recommend a similar study be done on cultured shrimp species, because this industry needs to pay attention to health management.

The study suggests that incorporating QQ1, QQ2 and *Lactobacillus plantarum*, as dietary supplements in common carp feed can significantly enhance growth indicators and the food conversion ratio, making them viable candidates for use in aquaculture practices at short time culture.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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اثر پروبیوتیک‌های ضد درک حد نصاب بر تعدیل فعالیت آنزیم‌های گوارشی، فلور میکروبی، عملکرد رشد و پارامترهای بیوشیمیایی در ماهی کپور معمولی (*Cyprinus carpio*)

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چکیده

در مطالعه حاضر، دو پروبیوتیک اصلی (*Citrobacter freundii* و *Bacillus foraminis*) با ویژگی ضد درک حد نصاب از روده ماهی کپور معمولی جداسازی شدند و اثرات آن‌ها بر عملکرد رشد، فلور میکروبی روده، شاخص‌های بیوشیمیایی و فعالیت آنزیم‌های گوارشی، مانند الفا آمیلاز، لیپاز، تریپسین، کیموتریپسین، و آلکالین فسفاتاز در ماهی کپور معمولی مورد بررسی قرار گرفت. بچه ماهیان (تعداد ۴۵۰ قطعه با وزن ۱۰±۵۰ گرم) به طور تصادفی به ۶ گروه مساوی (با ۳ تکرار) تقسیم شدند و با جیره‌های حاوی QQ1 (*C. freundii* به میزان ۱×۱۰۹ CFU/g)، QQ2 (*B. foraminis* به میزان ۱×۱۰۹ CFU/g)، WQQ (*L. plantarum* باکتری فاقد ویژگی ضد درک حد نصاب) به میزان ۱×۱۰۹ CFU/g)، QQ1 +QQ2 (*B. foraminis* و *C. freundii* به میزان مساوی ۱×۱۰۹ CFU/g)، گروه ترکیبی (*B. foraminis* و *C. freundii* و *L. plantarum* به میزان مساوی ۱×۱۰۹ CFU/g) و یک رژیم غذایی کنترل (بدون پروبیوتیک) برای ۶۰ روز به طور مداوم تغذیه شدند. نتایج نشان داد که مکمل‌های پروبیوتیک به طور کلی اثرات معنی‌داری بر عملکرد رشد داشتند. گروه‌های ترکیبی و پلانتاروم به ترتیب در روزهای ۳۰ و ۶۰ بهترین اثر را بر نرخ رشد ویژه (SGR) و راندمان مصرف خوراک در کپور معمولی داشتند. فعالیت‌های تریپسین، پروتئاز و کیموتریپسین در روز ۳۰ پس از تغذیه، در تیمار ترکیبی در مقایسه با گروه شاهد و سایر گروه‌ها به طور معنی‌داری افزایش یافت. تغییرات معنی‌داری در فلور باکتریایی روده در تمامی گروه‌های پروبیوتیک نسبت به گروه شاهد مشاهده شد. این نتایج استفاده بالقوه از QQ2 (*B. foraminis*) را به تنهایی یا در ترکیب سایر پروبیوتیک‌ها (تیمار ترکیبی) به عنوان افزودنی در رژیم غذایی کپور معمولی نشان داد اما در طولانی مدت توصیه نمی‌شود. نتایج نشان داد که مکمل‌سازی باکتری‌های جدا شده از روده کپور معمولی (مانند *لاکتوپلانتی باسیلوس پلانتاروم*) می‌تواند عملکرد رشد، میکروبیوتای روده و برخی فعالیت‌های آنزیم گوارشی را در بچه ماهیان کپور معمولی در دوره‌های پرورش طولانی‌مدت بهبود بخشد. بنابراین، می‌توان آن را مانند پروبیوتیک‌های تجاری به عنوان تقویت کننده رشد استفاده نمود.

کلمات کلیدی: کپور معمولی، باکتری‌های روده، عملکرد رشد، فعالیت آنزیم‌های گوارشی، فلور میکروبی

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