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Effects of Two Probiotics, *Lactobacillus Plantarum* and *Lactobacillus Bulgaricus* on Growth Performance and Intestinal Lactic Acid Bacteria of *Cyprinus Carpio*

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Abstract:

BACKGROUND: The application of probiotics to aquaculture is rather new. Probiotics affect the intestinal microbial flora of fish and subsequently modulate its immune response and growth performance.

OBJECTIVES: This study was conducted to evaluate the effect of food supplementation with *L.plantarum* and *L.bulgaricus* on growth performance and gut microbiota of *Cyprinus carpio*.

METHODS: For this purpose, 480 juveniles of *C. carpio* (40.2 ±6.3 gr Mean ±SD) were randomly divided into three equal groups (each group in three replicates) and fed with diet containing 5×10^7 cfu g^{-1} of *Lactobacillus plantarum* (group A), *Lactobacillus bulgaricus* (group B) and control diet (group C) for 60 days. To evaluate the persistent presence of the bacteria and their effects on the microbiota of the digestive system, remained fish of each group were fed with free probiotic diet from day 60 to 75.

RESULTS: Results showed that most growth indices of probiotic treated groups were increased compared to control group in all sampling points. Although FCR decreased significantly in Groups A (2.9±0.43) and B (2.75±0.37) compared to control (3.88±0.52), SGR, WGP and DWG increased only in Group B compared to control group ($P<0.05$). Two probiotics did not influence fish survival rate compared to control group ($P>0.05$). Intestinal lactobacillus ratio at days 30 and 60 was significantly higher than the control group ($P<0.05$). Group B showed the highest lactobacillus rate among the groups at day 30. Total intestinal bacteria count on day 30 and 60 were significantly higher in probiotic-treated fish compared to the control group ($P<0.05$).

CONCLUSIONS: It can be concluded that *L.bulgaricus* can promote growth indices and intestinal Lactic acid bacterial proportion in common carp. Then it can be a proper candidate for a probiotic in common carp after more trials in farm scale.

Keywords:

Cyprinus carpio, Growth indices, Intestinal bacterial flora, *L. plantarum*, Probiotics

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Introduction

Probiotics are defined as live microbial supplements that benefit the host (Fuller, 1989). Research into the use of probiotics in animal husbandry is increasing with the demand for environmentally friendly agriculture/aquaculture practices and safer food. The beneficial effects of probiotics on fish survival, growth and feed conversion, immune response and disease resistance in aquaculture have been widely recognized (Kaur et al., 2015). In aquaculture, administration of probiotics can be done through a dietary supplement or as a water additive (Taoka et al., 2006). Antibiotic treatment for managing the diseases during aquaculture practices has several negative effects such as the development of drug-resistant bacteria and low efficiency of antibiotic treatment for diseases (Sorroza et al., 2012). Subsequently the use of probiotics has also been suggested to be an alternative method to prevent and control various aquatic diseases by reducing pathogenic organisms in the gastrointestinal tract of fish based on its antagonistic activity at the site of colonization on the host's intestine (Balcazar et al., 2006; Verschuere et al., 2000).

Probiotics can modulate the growth of intestinal microbiota, suppress destructive bacteria and strengthen the body's natural resistance mechanisms. Also, they act on species specific or even strain-specific and immune reactions of the animal, and their interface with intestinal bacterial populations perform an important function (Simon, 2010; Giorgio et al., 2010). Therefore, probiotics have a wide range of valuable effects that include water quality improvement; competitive exclusion of bacterial pathogens through the production of inhibitory compounds; enhancing the nutritional status of the host species by producing supplemental digestive enzymes, etc. (Thompson et al., 1999; Verschuere et al., 2000; Jiang et al., 2013). Lactobacillus has been extensively used as an efficient probiotic (Phian phak et al., 1999) due to their capability to secrete a wide range of exoenzymes and antimicrobial compounds (Moriarty 1998; Soltani et al., 2017).

The present study was designed to evaluate the influence of probiotics *Lactobacillus plantarum* and *Lactobacillus bulgaricus* isolated from native fish: Tor grypus on gut microbiota profile and the subsequent effect on growth performance of *Cyprinus carpio*, a typical species with economic importance worldwide (FAO, 2012).

Materials and Methods

Lactobacillus plantarum and *Lactobacillus bulgaricus* were used for supplementation of food. These strains were chosen from over 30 lactic acid bacteria obtained from intestine of healthy wild *T. grypus*, according to their high in-vitro probiotic characteristics (Mohammadian et al., 2014). These strains were primarily identified based on colony and cell morphology, Gram staining, biochemical characteristics, and 16S rRNA gene sequencing. The positive control strain (L. casei PTCC 1608) was obtained from Pastor Institute, Tehran, Iran. They were cultured in the De-Man Regosa and Sharpe (MRS) broth (Pronadisa, Madrid, Spain) at 30 ºC. Bacterial strains preserved in skimmed milk at -80 ºC until used.

Fish: Four hundred and eighty juveniles of *C. carpio* (40.2±6.3 gr Mean±SD) were

obtained from a private cyprinid farm in Hamidieh city, Khuzestan province of Iran. This farm was a specific form without any health problems in their 15 year history. The fish were observed and examined for parasitic and bacterial diseases before the experiment. Fish were reared in the tank (300 L), and fed a basal diet for 2 weeks to acclimate to the experimental diet and conditions.

Water quality: Water quality parameters were recorded during the experiment: temperature, 25±1 ºC; Dissolved oxygen, 7.5±0.8 ppm; pH, 7.8±0.2; NO2 <0.01ppm and NH3 <0.1ppm, NO3< 0.1 ppm, Electrical Conductivity (EC) =575 µSiemens/cm. Total Dissolved Solid (TDS) =625 ppm. Water exchange rate was 20% of water volume daily.

Experimental procedure: The experiment was performed in aquarium room of Veterinary Faculty, Shahid Chamran University of Ahvaz, Iran.

Fish were weighed after anesthetization with 2-phenoxy ethanol (400 ppm), afterward fish were divided into three equal experimental groups with three replicates each following a randomized block design. It was run in 12 aquaria of 100 L using 40 fish per each aquarium. Each diet was randomly assigned to triplicate aquaria and control group was fed with free bacteria food. Fish were hand fed according to manufacturer's guide to apparent satiation twice daily 3% of biomass/day. The feeding trial lasted for 75 days. During the experimental period, the temperature range was 25±1 ºC, salinity from 600 ±75 ppm and the dissolved oxygen was approximately 7 $mg.1^{-1}$.

Experimental diet preparation (The experimental groups include): Group A: fed basal diet + *L. plantarum*; Group B: fed basal diet + *L. bulgaricus* and Group C: fed basal diet (control); A commercial pellet diet, Faradaneh Co., Shahrekord, Iran, FFC code: (38% protein, 7% lipid, 7%Fiber, 8.3% Ash, 9% moisture) was grinded and the defined quantities of the lyophilized probiotic (5×10^7 CFU g^{-1}) of dry powder were added to the basal diet. After homogenization the mixture was pelleted with10% water incorporation, dried in a ventilation oven (48 h, at 40 ºC) and maintained at 4 ºC in vacuum bags. During the trial, diets were hand fed to apparent satiety during the study. The basal commercial diet was used as a control, and passed through the same processing, excluding probiotic addition (Giri et al., 2013).

Growth performance indices: In order to evaluate growth performance, biometrical index of fish was measured at day zero at the beginning of trial, day 30, day 60 and day 75. Specific growth rate (SGR), feed conversion ratio (FCR) and Protein efficiency ratio (PER), daily weight growth (DWG), condition factor (CF) and Weight gain percentage (WGP) were calculated according to the following equations:

SGR (%/day) = [ln final body weight (g) - ln initial body weight (g) / experimental period (80 days)] × 100

FCR = Food intake (g) / weight gain (g)

PER = Wet weight gain (g) / total protein intake (g)

DWG = Average final weight (g) – Average initial weight (g) / experimental period (80 days)

CF = Weight (g) / [standard length (cm)] 3

WGP= [Final weight (g) - Initial Weight (g)/ Final weight (g)] × 100

Survival rate = (Number of live fishes at the end of experiment / total number of fishes in each group) × 100

Intestinal microbiota: Intestinal bac-

Table 1. Growth performance indices of the experimental groups in different sampling points (the results are given as mean ± SD). Latin lower case letters on standard error show a significant difference in level 0.05 in each column, and Latin capital letters on standard error show a significant difference in level 0.05 in each row.

	Treatment	Day 30	Day 60	Day 75
CF	*L. Bulgaricus*	1.32±0.12 ^{aA}	1.31±0.07 ^{aA}	1.29±0.08 ^{aA}
	L. Plantarum	1.33±0.09 ^{aA}	1.30±0.09 ^{aA}	1.33±0.1 ^{aA}
	Control	1.37±0.1 ^{aA}	1.35±0.11 ^{aA}	1.30±0.07 ^{aA}
SGR	*L. Bulgaricus*	0.14 ± 0.05 ^{aA}	0.13 ± 0.02 ^{aA}	0.12 ± 0.01 ^{aA}
	L. Plantarum	0.12 ± 0.03 ^{aA}	0.11± 0.02 ^{abA}	0.11± 0.03 ^{abA}
	Control	0.1± 0.01 ^{aA}	0.09± 0.02 ^{bA}	0.09± 0.01 ^{bA}
PER	*L. Bulgaricus*	1.31± 0.18 ^{aA}	1.27± 0.13 ^{aA}	1.31± 0.18 ^{aA}
	L. Plantarum	1.37± 0.22 ^{aA}	1.28± 0.24 ^{aA}	1.27± 0.16 ^{aA}
	Control	1.24± 0.18 ^{aA}	1.17± 0.19 ^{aA}	1.16± 0.05 ^{aA}
WGP	*L. Bulgaricus*	10.34±2.86 ^{aA}	8.38±0.55 ^{aAB}	4.46±0.41 ^{aB}
	L. Plantarum	8.89±1.65 ^{aA}	6.27±1.26 ^{bAB}	3.90±0.61 ^{abB}
	Control	7.48±1.97 ^{bA}	6.88±1.14 ^{bA}	3.13±0.72 ^{bB}
SR	*L. Bulgaricus*	100 ^{aA}	100 ^{aA}	100 ^{aA}
	L. Plantarum	100 ^{aA}	98.3± 3.3 ^{aA}	97.5±2.8 ^{aA}
	Control	100 ^{aA}	100 ^{aA}	98.3± 3.3 ^{aA}

terial micro floral analyses were done as described by Merrifield et al. (2010). Intestinal content samples were taken on days 30, 60 and 75 of the experiment after 24 h starvation. One gram of isolated intestinal contents was homogenized in tissue grinders (Kontes, Vineland, NJ, USA), and vortexed vigorously in 9.0 ml of sterile saline (0.85% W/V). Serial dilutions of intestinal content were prepared from 10-2 to 10-7 in sterile saline, 0.1 ml of 10-7 were spread onto duplicate tryptone soya agar plates to determine total aerobic heterotrophic populations and the same amount of 10-2 dilution was pure on MRS media for the LAB using the spread plate method. MRS and TSA plates were incubated at 30 ○C for 48 h and colony forming units (CFU g^{-1}) were calculated for each sample. For MRS plates anaerobic jar was used.

Statistical analysis: Two-way analysis of variance (ANOVA) was used to analyze the data. Multiple comparisons were performed with Tukey's test to analyze the differences between treatments. All statistical tests were performed using SPSS software (SPSS, Release 16.0, SPSS, Chicago, IL, USA). Differences were considered statistically significant when $P<0.05$ and the results are expressed as mean ± SD.

Results

Results showed that oral administration of two native probiotic bacteria, *Lactobacillus plantarum* and *Lactobacillus bulgaricus*, significantly influenced most growth indices compared to control group in all sampling points (Table 1). Although FCR decreased significantly in Groups A (2.9±0.43) and B (2.75±0.37) compared to control (3.88±0.52), SGR, WGP and DWG increased only in Group B compared to control group ($P<0.05$). Two probiotics did not influence fish survival rate in 60 days compared to control group ($P>0.05$). It is notable that growth indices were promoted in probiotic-treated groups even 15 days after ceasing the probiotic administration (day 75). No significant difference was seen

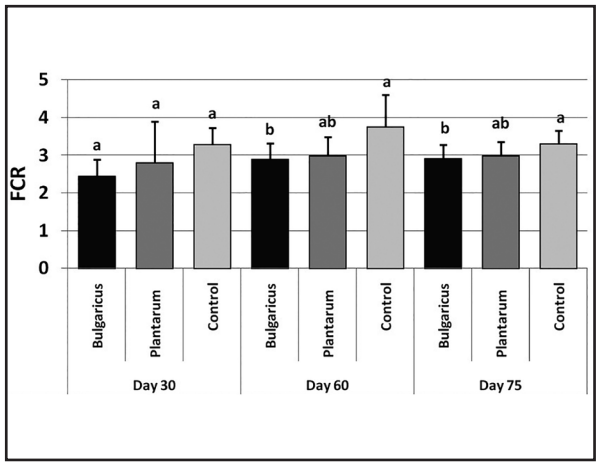

Figure 1. Food Conversion Rate of the experimental groups in different sampling steps (the results are given as mean ± SD). Means with different letters are significantly different (p< 0.05).

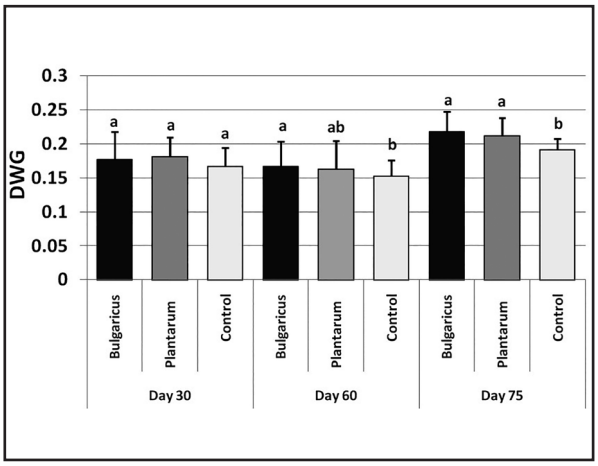

Figure 2. Daily Weight Gain of the experimental groups in different sampling steps (the results are given as mean ± SD). Means with different letters are significantly different (p< 0.05).

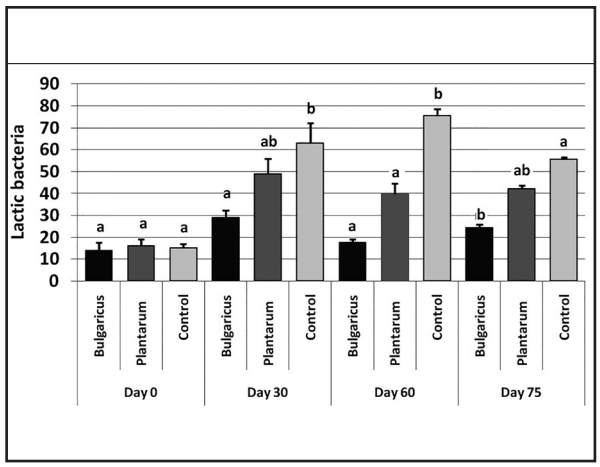

Figure 3. Lactic bacteria levels of the experimental groups in different sampling steps (the results are given as mean ± SD). Means with different letters are significantly different (P< 0.05).

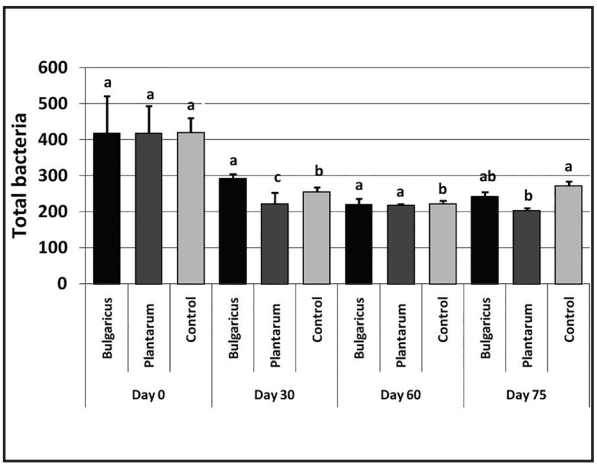

Figure 4. Lactic acid bacteria levels of the experimental groups in different sampling steps (the results are given as mean ± SD). Means with different letters are significantly different (P< 0.05).

among the groups in condition factor (CF) (P>0.05).

The highest SGR and PER were seen in *L.bulgaricus* group at day 30 (0.14 ± 0.05 and 1.34 ±0.31) followed by *L.plantarum* (0.12 ± 0.03 and 1.37±0.22) which were significantly higher than the control group (P<0.05)

Intestinal lactobacillus ratio of two probiotic treated groups at days 30 and 60 was significantly higher than the control group (P<0.05). Group B showed the highest lactobacillus rate among the groups. Total intestinal bacteria count on day 30 and 60 was significantly higher in probiotic-treated fish compared to the control group (P<0.05). A significant increase in LAB/Total intestinal bacteria ratio of intestinal flora was seen in probiotic-treated groups of the experiment (P<0.05).

Discussion

In recent years, investigators have been trying to introduce better probiotics to promote the efficiency of aquaculture (Mo-

hammadian et al., 2016). Lactobacilli can be appropriate probiotics in fish. There are various reports on the improvement of aquatic animal growth after administration of lactobacilli (Nikoskelainen et al., 2003). In this study, two LABs (*L. bulgaricus* and *L. planturum*) with probiotic potency were isolated from the intestine of *T. grypus* and their effects on growth performance and gut microbial flora were determined during a 60-day feeding. The present study revealed that supplementation of common carp fed with *L.plantarum* and *L.bulgaricus* promoted the growth performance indices and changed the intestinal bacterial microflora. In similar work Lactic acid bacteria strains as feed additive have been shown to promote fish growth in aquaculture (Ibrahim et al., 2004). A mixture of Lactobacillus sp. isolated from chicken gastrointestinal tract improved the growth indices and survival rate of juvenile Penaeus monodon. (Phian phak et al., 1999). An enhancement of the growth rate of the tilapia (*O. niloticus*), following the use of probiotics in water body was reported. Similarly, Bogut et al. (1999) and Noh et al. (1994) found that food supplementation with *Streptococcus faecium* induce growth enhancement and change in intestinal bacterial flora in common carp. Regarding investigations on other aquatic species (shrimp), our findings were in agreement with the study on Indian white shrimp, *F. indicus*, (Ziae Nejad et al., 2006), and white shrimp *P. vannamei* (Wang 2007).

Our results showed that in vivo probiotic potency of *L.bulgaricus* is significantly higher than *L.plantarum* in common carp. This bacteria was not only successful to promote the growth indices, but also to change positively the intestinal bacterial flora of fish in common carp. This characteristic is very imperative for commercial aquaculture (Giri et al., 2013). Phian phak et al. (1999) found that *Bacillus* sp. supplemented with shrimp diet increased the weight gain and SGR. The main objective of probiotic administration is the improvement of fish growth indices, health status and resistance to opportunistic diseases, through immune stimulation by modification of intestinal microbiota. The growth enhancement can be a result of the improvement of dietary nutrient assimilation, for instance, an increase in the availability of metabolic substrates like vitamins (LeBlanc et al., 2011) or digestive enzymes (Bairagi et al., 2002) produced by microbiota. Given the obvious importance of growth in aquaculture activity, the potential as a growth promoter is a significant attribute that can be considered in the use of probiotics as feed additives. Bureau et al. (2000) report that probiotic supplementation into the diet (live food and/or extruded pellet food) induce a significant increase in both growth performance and husbandry parameters compared to basal diets or no supplemented groups. Similar findings were recorded in fresh water species such as Nile tilapia *O. niloticus* and common carp, C.carpio, (Wang and Xu, 2006) and marine fish species such as red drum, Sciaenops ocellatus (Li et al., 2005). Several authors pointed out that one of the main modes of action and beneficial effects of probiotics in aquaculture organisms is enhancement of nutrition of host species through the production of supplemental digestive enzymes and higher growth and feed efficacy, inhibition of intestinal disorders and predigestion of antinutritional elements present in the ingredients (Thompson et al., 1999; Verschuere et al., 2000). In detail, after transition through the stomach, they germinate

in the intestine and use a large number of sugars (carbohydrates) for their growth and produce a range of relevant digestive enzymes (amylase, protease and lipase) (El-Haroun et al., 2006). Further, probiotics can change the gut epithelium construction and improve nutrient absorption by providing a more absorptive surface area (Pirarat et al., 2011 Gupta et al., 2014). In addition to growth efficiency, it is essential to know the safety level of candidate probiotics. In this investigational period we did not observe any sign of stress or mortality among treatments, all fish were healthy with the usual level of activity and appetite. The survival rate was not affected by oral administration of probiotics. In this study the FCR of two lactobacillus species was significantly lower than the control group ($P \leq 0.05$). Similarly, improved FCR was observed in rainbow trout fed with *E. faecium* enriched diet (Merrifield et al., 2010). However, there are significant data showing positive effect of probiotics on growth performance of different fish species, while some reports show no significant benefits regarding the use of probiotics as growth promoters. Administration of *Bacillus licheniformis* and *Bacillus subtilis* had no significant positive impact on growth rate of juvenile rainbow trout (Merrifield et al., 2010), catfish (Shelby et al., 2007), and tilapia (Shelby et al., 2006). In the study by Reyes-Becerril et al. overall growth of probiotic + marine silage treated group was higher than control but other factors such as FCR did not changed significantly (Reyes-Becerril et al., 2012). It appears that different features like probiotic and fish species, age, dosage and length of probiotic intake, nurture conditions like temperature, etc. can make significant differences in the result of using probiotics in aquaculture (Ramos et al., 2013). To our best knowledge, this is the first report of the dietary administration of *L.bulgaricus* as a probiotic which improved the growth indices of fish. Interestingly, slightly enhanced WG and SGR were observed in *lactobacillus plantarum* group, but not to asignificant extent. Similarly, Merrifield et al. (2010), found slight improvement of WG and SGR in rainbow trout after 10 weeks of feeding with *E. faecium* containing diet. This difference can be referred to the other factors, like differences in their survival rates in the gut and interaction with host microbiota. This supports the suggestion that each probiotic strain may interact with the host in a different way and different period of administration (Bomba et al., 2002), so further studies would be necessary to optimize the dose and frequency of probiotic administration to maximize their benefits. Mechanism of the LAB on the growth rate and feed utilization of fish, is not totally clear (Lara Flores et al., 2003). The enhanced growth rate and/or feed utilization of fish might be due to the increase in digestive enzyme activities induced by probiotics (Wang and Xu 2006; Suzer et al., 2008). On the other hand, extracellular digestive enzymes secreted by probiotics themselves may also have contributed to the growth performance of host (Bairagi et al., 2002).

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 common carp with a diet containing *L. bulgaricus* and *L. plantarum* could increase the viable LABs counts in the intestine. These results were in agreement with 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), in grouper (*E. coioides*) by *Psychrobacter* sp. (Sun et al., 2011), and Siberian sturgeon (Geraylou et al., 2013a; Geraylou et al., 2013b). Fifteen days after cessation of feeding with *L.bulgaricus* enriched diet (from day 60 to day 75), the number of gut Lactobacilli dramatically decreased. This result was in agreement with the observations by other researchers (Suzer et al., 2008; Dash et al., 2014) shown in full washing out of probiotic bacteria seven days after the probiotic withdrawal. 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. The exact mechanism behind this variation was unclear, but probably *L. plantarum* is not harbored in the intestine beyond 4 weeks after administration of the diet. It may be due to structural differences in the cell wall compositions of different LAB strains (Geraylou et al., 2013a), or antagonism action of various gut bacteria. In the present study, the highest numbers of LABs concomitant of the highest growth rate were found in the intestine of common carp fed *L. bulgaricus*. The effect of probiotics on the intestinal LAB rates and their relationship with growth and feed utilization in aquatic animals needs further study. In conclusion, *L.bulgaricus*, can be a proper candidate as probiotic in carp. Considering these findings, we concluded that lactic acid bacteria, *L. bulgaricus*, isolated from the intestine of *T. grypus* increased the rate of beneficial bacterial in bacterial microflora in common carp intestine and consequently induce growth promotion in treated fish. Such probiotics are recommended to be used as a commercial growth promoter to facilitate an extensive culture of common carp in future.

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تأثیر دو پروبیوتیک لاکتوباسیلوس پلانتاروم و لاکتوباسیلوس بولگاریکوس بر روی عملکرد رشد و فلور میکروبی روده کپور معمولی (*Cyprinus carpio*)

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چکیده

زمینه مطالعه: استفاده از پروبیوتیک‌ها در آبزی پروری تکنیکی نسبتاً جدید است. پروبیوتیک‌ها با تأثیر فلور میکروبی روده ماهی، پاسخ ایمنی و عملکرد رشد را بهبود می‌بخشند.

هدف: این پژوهش جهت ارزیابی تأثیر مکمل‌های غذایی شامل لاکتوباسیلوس پلانتاروم و لاکتوباسیلوس بولگاریکوس بر روی عملکرد رشد و فلور میکروبی روده کپور معمولی صورت گرفت.

روش کار: به این منظور ۴۸۰ قطعه بچه ماهی کپور معمولی (با میانگین وزن $3/6 \pm 40$ g) به‌طور تصادفی در ۴ گروه مساوی (با ۳ تکرار) تقسیم شدند و تغذیه با جیره‌ی محتوی (5×10^{7} cfu g^{-1}) لاکتوباسیلوس پلانتاروم (گروه A)، لاکتوباسیلوس بولگاریکوس (گروه B) و جیره‌ی فاقد مکمل پروبیوتیکی (گروه C) برای ۶۰ روز صورت گرفت. جهت ارزیابی میزان حضور باکتری و تأثیر روی فلور میکروبی سیستم گوارش، تغذیه از روز ۶۰ تا ۷۵ با جیره فاقد پروبیوتیک صورت گرفت.

نتایج: نتایج نشان داد که مقدار اکثر شاخص‌های رشد در دو تیمار پروبیوتیکی افزایش معنی‌داری نسبت به گروه کنترل داشت ($P<0/05$). هرچند ضریب تبدیل غذایی (FCR) در گروه A ($2/9\pm0/43$) و B ($2/75\pm0/37$) نسبت به گروه کنترل ($3/88\pm0/52$) کاهش معنی‌داری نشان داد، ولی ضریب رشد ویژه، درصد افزایش وزن و افزایش وزن روزانه در گروه B نسبت به سایر گروه‌ها به‌طور معنی‌داری افزایش یافت ($P<0/05$). درصد بقای تیمارها تحت تأثیر تیمارهای پروبیوتیکی قرار نگرفت ($P>0/05$). لاکتوباسیلوس‌های روده در روز ۳۰ و ۶۰ آزمایش به‌طور معنی‌داری در مقایسه با گروه شاهد افزایش پیدا کردند ($P<0/05$). در روز ۳۰ آزمایش گروه B بیشترین مقدار لاکتوباسیلوس در مقایسه با گروه شاهد را نشان داد ($P<0/05$). کل باکتری‌های روده در روز ۳۰ آزمایش به‌طور معنی‌داری در گروه‌های تغذیه‌شده با پروبیوتیک در مقایسه با گروه شاهد تغییر کرد ($P<0/05$).

نتیجه‌گیری نهایی: به‌طورکلی می‌توان نتیجه گرفت که لاکتوباسیلوس‌های، استعداد پروبیوتیکی بالایی در بهبود رشد و فلور باکتریایی روده داشته و بعد از آزمایش‌های فارمی گزینه خوبی به‌عنوان پروبیوتیک ماهی کپور است.

واژه‌های کلیدی: کپور معمولی (*Cyprinus carpio*)، شاخص‌های رشد، فلور میکروبی روده، لاکتوباسیلوس پلانتاروم، پروبیوتیک

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