Effect of Bactocell® (*Pediococcus acidilactici*) supplementation on growth performance and antioxidant defense in koi carp (*Cyprinus rubrofuscus*)

Alizadeh Barogh M.[1]; Mahmoodzadeh A.[1]; Kazempoor R.[2*]; Alavinejad Sh.[3]

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Abstract

This study aimed to evaluate the effects of dietary Bactocell® (BA) supplementation on intestinal microbial flora, intestinal morphology, growth performance, and antioxidant defense in koi carp (*Cyprinus rubrofuscus*). A total of 300 fish were randomly divided into four groups with three replicates each. The groups included a control group (basal diet, BD), T1 (0.02 g BA/kg BD), T2 (0.03 g BA/kg BD), and T3 (0.04 g BA/kg BD). Fish were fed the experimental diets for 60 days. Sampling was conducted at the end of the trial to assess growth performance, intestinal microbial flora, and morphology. Plasma antioxidant indices were also evaluated on days 0, 30, and 60. The results showed that the highest increase in Lactobacillus colonies was observed in T1 (p<0.05). Similarly, the greatest increase in intestinal fold length and width was also recorded in T1 (p<0.05). Growth performance was highest in T3, followed by T2 and T1, compared with the control group (p<0.05). In addition, BA supplementation enhanced antioxidant defense and reduced oxidative stress. The highest increase in serum superoxide dismutase (SOD) activity was detected in T1 (p<0.05), while plasma malondialdehyde (MDA) levels did not differ significantly at the end of the trial (p>0.05). Overall, dietary supplementation with BA had positive effects on growth performance and antioxidant defense in ornamental koi carp.

Keywords: *Pediococcus acidilactici,* Koi fish*, Cyprinus rubrofuscus,* SOD, MDA

1-Department of Biology, Roodehen Branch, Islamic Azad University, Rodehen, Iran
2- Department of Veterinary Hygine, SR.C., Islamic Azad University, Tehran, Iran
3- Department of Aquatic Health and Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
*Correspondeing author's Email: r.kazempoor@riau.ac.ir

Introduction

Koi fish are popular ornamental fish that comprise about 30% of China's ornamental fish trade. Compared to goldfish and tropical fish, koi are a compatible, easy-to-breed, beautiful and expensive domestic fish that can be used for personal entertainment or competitive performances around the world, especially in China and Japan (Luo *et al*., 2021). The mortality rate in the ornamental fish trade varies between 2-73% due to the high stress of breeding, transportation, and poor biosecurity (Ploeg, 2007; Larcombe *et al.*, 2025). This statistic indicates the need to use effective strategies to improve safety performance and increase the survival rate of fish to reduce economic losses in the industry. In the past decade, the use of probiotics in the diet has received much attention as a strategy to increase performance and manage aquaculture health (Ringø and Song, 2016; Sayes *et al*., 2018; Alavinejad *et al.*, 2025).

Probiotics are living microbial cells that are used as dietary supplements to improve host health (Mollanourozi *et al*., 2021; Loghmani *et al*., 2022). In recent years, the use of probiotics has expanded significantly in aquaculture due to the colonization of probiotic bacteria in the gastrointestinal tract, inhibition of pathogens, increased survival, increased immune response, and increased growth performance (González-Félix *et al*., 2018; Yang *et al*., 2019).

Despite the growing number of probiotic microorganisms identified, a limited number of probiotic products are legally permissible for use in aquaculture (Caipang and Lazado, 2015). Bactocell® (BA) is a branded functional food additive containing live cells of the *Pediococcus acidilactici* strain of CNCM I-4622, which has been approved by the European Food Safety Authority (EFSA) (EFSA FEEDAP Panel, 2019; Vargas-González *et al.*, 2024). This product is the first probiotic authorized for use in aquaculture in the European Union (Ayyat *et al*., 2018). Stability of intestinal microflora, increase in intestinal villi length, increase in growth, and improved immune function are among the effects of BA in aquaculture (Standen *et al*., 2013; Caipang and Lazado, 2015). Most studies have been limited to edible farmed fish species and few studies have been done on ornamental fish. Due to the recent problems, the present study was performed to evaluate the effects of feeding with different amounts of BA on microbial flora and intestinal morphology, growth performance, and antioxidant response in ornamental Koi fish.

Materials and methods

Experimental design and diet preparation

In this experiment, 300 koi fish with a mean weight of 3±1 g and a mean length of 5.5±0.5 cm were adapted for two weeks using Basic diet (BD) (Biomar, France commercial feed) based on 5% body weight. then fish were randomly divided into 4 groups with three replications (25 fish were distributed in each aquarium with a central air pump).

Fish were fed using a BD (Control) and BD+BA (Bactocell®, Lallemand, France) in three concentrations of 0.02, 0.03, and 0.04 g BA / Kg. Groups included control, T1 (0.02 g BA/kg BD), T2 (0.03 g BA/kg BD) and T3 (0.04 g BA / kg BD). The probiotic diet was prepared weekly from a combination of basal diet + desired concentration Bactocell®+corn oil and incubated in ice for 15 minutes for better absorption of bacteria and then kept at 4°C. The fish were fed twice daily based on 5% of body weight (9 am and 4 pm) for 60 days. During the experiment, the hours of darkness: and brightness were set to 12:12. 30% of the tank water was replaced daily with fresh chlorine-free water. Water quality parameters were measured weekly with the JBL PRO SCAN .The mean values for water quality parameters include nitrate (25 mg/L), nitrite (1.0 mg/L), water hardness (>21°d), water carbonate hardness (8°d), pH (7.0), and chlorine content (0 mg/L) were determined.

Colonization of lactobacilli in the intestine

At the end of the experiment, two fish were randomly caught from each aquarium. The fish were anesthetized with clove powder (200 mg/L) and their intestines were removed, weighed separately, homogenized, and then diluted to 9 with saline solution (0.9%, w/v) (Zhang *et al*., 2020). Finally, a volume of 100 µL of different dilutions was spread on an MRS agar medium (Merck, Germany) and incubated for 24 hours at 30°C and the grown colonies were counted (Mahious *et al*., 2006).

Intestinal morphology analysis

At the end of the experiment, the fish were not fed for 24 hours. Then, two fish were randomly taken from each aquarium and after anesthesia with clove powder (200 mg/L) (Zhang *et al*., 2020), the intestines of the fish were removed and placed in 10% formalin. Formalin was replaced after 24 hours to complete the sample stabilization process. Different stages of slide preparation such as dehydrating in a graded series of ethanol, clarification with xylene, embedding in paraffin, block preparation, cross-section using microtome device, hematoxylin-eosin (H&E) staining, and installation were performed, respectively. Measurements of length and width of the intestinal fold were examined using a light microscope (Olympus, DP72, Japan) equipped with a camera (Nikon E600) (Alavinezhad *et al*., 2020). Electronic analysis and dimensioning of intestinal folds (width and height of folds) were measured using CaseViewer software.

Fish growth and feed efficiency

Mean weight, total length (TL), and standard length (SL) of fish in each group were measured using a digital scale (0.01 g) and a measuring board (0.01 cm) at the beginning and end of the experiment.

Weight changes, body weight gain (WG), specific growth rate (SGR), and length changes were calculated at the end of the 8-week feeding experiment. The calculations were performed as follows (Opiyo *et al*., 2019):

Weight gain (WG) = Final weight (g) – Initial weight (g)
Specific Growth Rate (SGR %) = 100 (lnWt – lnW0)/t
Total Length Changes = Final TL (cm) – Initial TL (cm)
Standard Length Changes = Final SL (cm) – Initial SL (cm)

Serum sample preparation and antioxidation status determination

Fish were randomly caught from each aquarium for blood sampling (n=3 fish per aquarium) on days 0, 30, and 60, and antioxidant factors were assessed according to the method of Zhang *et al*. (2020). According to this method, the fish were anesthetized with clove powder (200 mg/L) and blood was collected from the caudal vein. The collected blood was clotted overnight at 4°C, centrifuged at 4000× g for 30 minutes, and the supernatant was kept at -80°C until use. Serum superoxide dismutase activity (SOD) and malondialdehyde (MDA) levels in serum were measured with commercial assay kits (ZellBio, Germany) according to the manufacturer's instructions.

Data analysis

The normality of the data was determined by the Kolmogorov-Smirnov test and the paired t-test at 5 % was then employed to compare means and the relation between the measured factors. Significant differences between treatments were considered by one-way analysis of variance (One-way ANOVA). Duncan's test was used at a significant level of 0.05 to compare means. Statistical analyzes were performed by SPSS 21 and Excel 2013 software.

Results

Colonization of lactobacilli in the intestine

The results of the counted number of colonies belonging to intestinal microbial flora in the MRS medium on the 60th day showed that the highest number of colonies was in the T1 (1.3×10^9 cfu/mL). Also, there was a significant difference between T2 and the control group with T1 and T3 ($p<0.05$) (Table 1).

Table 1. Number of lactobacilli colonies in koi (*Cyrpinus rubrofuscus*) intestinal microbial flora cultured with MRS medium (10^9 cfu / ml) in the experimental treatments on day 60.

Group	Number of colonies (10^9 cfu / mL)
Control	0.01 ^{c}
T1	1.3 ^{a}
T2	0.03 ^{c}
T3	0.09 ^{b}

Different lowercase letters in each column indicate a significant difference between different treatments.

Intestinal morphology

The histomorphometric comparison showed that there was a significant difference between the diameter and length of the intestinal fold in the control

group and other treatments with different concentrations of probiotics and the highest increase in diameter and length of the fold was observed in the T1 group (p<0.05) (Fig. 1).

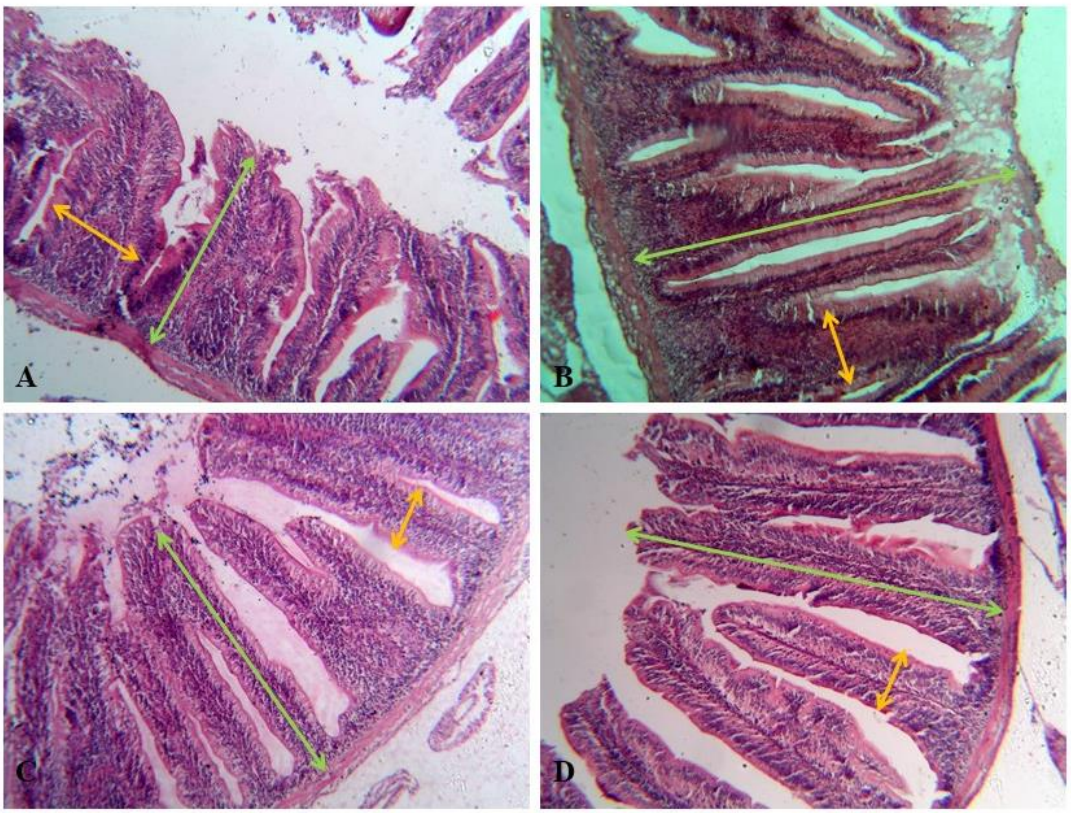

Figure 1: Transverse section photomicrographs of koi (*Cyprinus rubrofuscus*) mid-intestine. A: Control group, B: T1 group, C: T2 group, D: T3 group. Orange Arrow: Width of the fold, Green Arrow: fold height.

Fish growth and feed efficiency

Comparison of initial weight showed that there was no significant difference between treatments with control group (p>0.05). Comparison final weight showed that there was a significant difference between T1 and T2 with control and T3 (p<0.05). There was also a significant difference between the initial and final weights on zero and 60th days (p<0.05) (Table 2).

Table 2: Comparison of initial (day 0) and final (day 60) koi (*Cyprinus rubrofuscus*) body weights during the study period (Mean ± Standard deviation).

Group	Control	T1	T2	T3
Initial Weight	3.97±0.3 ^{Ab}	3.34±0.36 ^{Ab}	3.43±0.39 ^{Ab}	3.39±0.46 ^{Ab}
Final Weight	5.85±0.66 ^{Ca}	11.53±1.01 ^{Ba}	11.79±0.93 ^{Ba}	13.21±0.99 ^{Aa}
Weight gain (WG)	1.88±0.47 ^{B}	8.18±0.9 ^{A}	8.96±3.79 ^{A}	9.82±0.83 ^{A}

Different uppercase letters in each row indicate significant differences between treatments; Different lowercase letters in each column indicate a significant difference between different days in treatment.

The results of SGR showed that there is a significant difference between T2 and T3 with T1 and control (p<0.05). Also, the highest amount of SGR was observed in T2 (p<0.05) (Fig. 2).

A comparison of the initial TL showed that there was no significant difference between treatments (p>0.05).

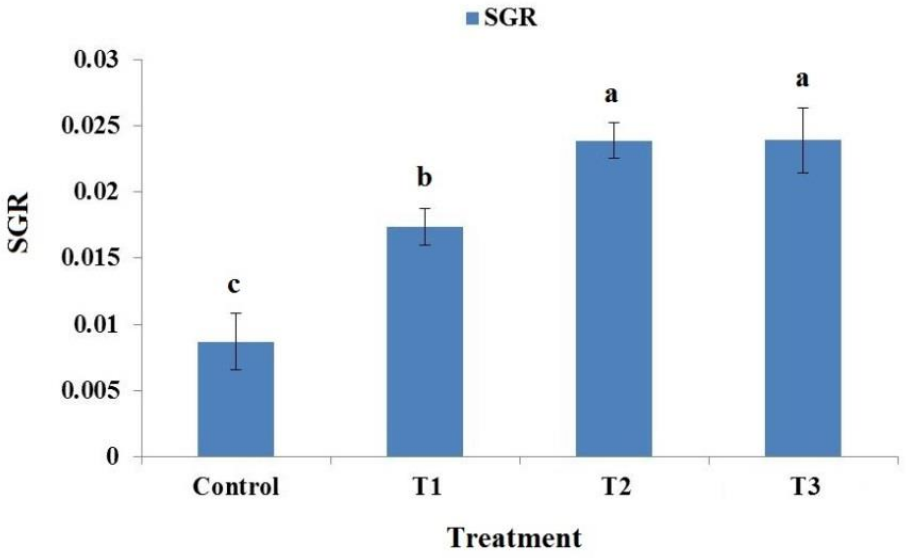

Figure 2: Comparison of calculated specific growth rates (SGR) (Mean ± Standard deviation) between different koi (*Cyprinus rubrofuscus*) treatment groups during the study period. Different lowercase letters indicate a significant difference between different treatments.

A comparison of the initial TL showed that there was no significant difference between treatments (p>0.05). A comparison of the final TL showed that there was a significant difference between T1 and T3 with the control and T2 groups (p<0.05). There was also a significant difference between the initial and final TL on zero and 60th days (p<0.05). A comparison of the initial SL showed that there was no significant difference between treatments (p>0.05). A comparison of the final SL showed that there was a significant difference between T1 and T3 with the control and T2 groups (p<0.05). There was also a significant difference between the initial and final SL on zero and 60th days (p<0.05) (Table 3).

Table 3: Comparison of initial (day 0) and final length (day 60) length measurements of koi (*Cyrpinus rubrofuscus*) during the study period (Mean ± Standard deviation).

Parameter	Group	Control	T1	T2	T3
Total Length (cm)	Initial TL	5.72±0.57 ^{Ab}	5.3±0.37 ^{Ab}	5.09±0.38 ^{Ab}	5.65±0.69 ^{Ab}
	Final TL	7.03±0.47 ^{Ca}	10.06±1.31 ^{Aa}	8.97±1.29 ^{Ba}	10.27±1.33 ^{Aa}
	TL Changes	1.31±0.66 ^{C}	4.76±1.47 ^{A}	3.88±1.47 ^{B}	4.61±1.42 ^{A}
Standard Length (cm)	Initial SL	4.65±0.45 ^{Ab}	4.38±0.39 ^{Ab}	4.32±0.38 ^{Ab}	4.59±0.00 ^{Ab}
	Final SL	5.91±0.76 ^{Ca}	8.47±1.06 ^{Aa}	7.27±1.26 ^{Ba}	8.16±0.87 ^{Aa}
	SL Changes	1.27±0.87 ^{D}	4.09±1.21 ^{A}	2.95±1.33 ^{C}	3.57±0.94 ^{B}

Different uppercase letters in each row indicate significant differences between treatments; Different lowercase letters in each column indicate a significant difference between different days in treatment.

Antioxidant status

SOD

The results of SOD activity in different groups and days are shown in Table 4. No significant differences were observed in the control group on different days (p>0.05). SOD did not show a significant change in groups T1, T2, and T3 on day 30 compared to the previous sampling (p>0.05), but an increase in SOD was observed in these groups on day 60 (p<0.05). Also, the results of SOD activity on different days showed no significant difference between the groups on days 0 and 30 (p>0.05), while a significant difference was observed between the groups on day 60 and the highest value was recorded in group T1 (p<0.05).

Table 4: Comparison of serum superoxide dismutase (SOD) in experimental treatments during the study period (Mean ± Standard error).

Day	Control	T1	T2	T3
0	0.06±0.00 ^{Aa}	0.064±0.002 ^{Ba}	0.058±0.003 ^{Ba}	0.062±0.002 ^{Ba}
30	0.06±0.00 ^{Aa}	0.067±0.001 ^{Ba}	0.06±0.003 ^{Ba}	0.066±0.002 ^{Ba}
60	0.06±0.00 ^{Ad}	0.154±0.001 ^{Aa}	0.084±0.002 ^{Ab}	0.072±0.002 ^{Ac}

Different uppercase letters in each column indicate a significant difference between the experiment days; Different lowercase letters in each row indicate a significant difference between different treatments.

MDA

The results of MDA activity in different groups and days are shown in Table 5. There were no significant differences in the control groups on different days (p>0.05). MDA showed a significant gradual decrease in groups T1, T2, and T3 on days 30 and 60 (p>0.05). Also, the results of MDA activity on different days showed no significant difference between the groups on day zero (p>0.05), while, there was a significant difference between the treatments with the control group on days 30 and 60 and the highest value was recorded in the control group (p<0.05).

Table 5: Comparison of malondialdehyde (MDA) in experimental treatments during the study period (Mean ± Standard error).

Day	Control	T1	T2	T3
0	0.415±0.014 ^{Aa}	0.392±0.005 ^{Aa}	0.386±0.011 ^{Aa}	0.405±0.006 ^{Aa}
30	0.435±0.016 ^{Aa}	0.258±0.008 ^{Bb}	0.219±0.004 ^{Bb}	0.191±0.009 ^{Bb}
60	0.444±0.00 ^{Aa}	0.163±0.005 ^{Cb}	0.15±0.004 ^{Cb}	0.135±0.005 ^{Cb}

Different uppercase letters in each column indicate a significant difference between the experiment days; Different lowercase letters in each row indicate a significant difference between different treatments.

Discussion

The use of probiotics has been considered by many researchers due to their numerous positive effects on the growth and immune response of aquatic animals (Cao *et al*., 2019). The ability to survive, adhere and colonize the gastrointestinal tract is one of the characteristics of probiotic bacteria (Li *et al*. 2019). The most important and first mechanism of using probiotics is the regulation of intestinal microbiota (Huo *et al*., 2021). The results of intestinal microbial flora in this study showed an

increase in the colonization of lactic acid bacteria in fish-fed BA. In this regard, Caipang and Lazado (2015) reported the effect of BA on the stability of intestinal microflora in aquatic animals. The results of other studies also show an increase in the population of probiotic-induced lactic acid bacteria in fish intestines (Merrifield *et al*., 2010; Ferguson *et al*., 2010), which is in line with our observations..

Another effect of probiotic nutrition in aquatic intestines is the effect on intestinal morphology. In this study investigation of the length and width of the intestinal folds showed their increase in the groups fed with BA. Similar studies have reported an increase in the length of the fish intestine folds due to BA feeding (Merrifield *et al*., 2010; Cerezuela *et al*., 2012; Standen *et al*., 2013; Elshafy *et al.,* 2024). An increase in the intestinal villi of fish fed with other types of probiotics has also been reported (Cao *et al*., 2019; Elahi *et al*., 2020; Alavinezhad *et al*., 2020). A possible mechanism for increasing the length of intestinal fold is due to the production of short-chain fatty acids from sugars by probiotic bacteria grown in the gastrointestinal tract, because short-chain fatty acids (especially butyric acid) are the main source of energy in intestinal epithelial cells. The results of microbial flora and the length of intestinal folds in the present study confirm the results of Pelicano *et al*. (2005).

Based on the results, feeding with different amounts of BA led to an increase in growth indices in fish. Numerous studies have reported increased growth in fish fed a variety of probiotics (Eleraky *and Reda*, 2014; Kanwal and Tayyeb, 2019; Alavinezhad *et al*., 2020). Eleraky *and Reda* (2014) reported that probiotic nutrition increases food intake, fat, and total protein content, and ultimately increases the growth of *Cyprinus carpio*. Probiotics can directly produce enzymes such as lipase, amylase, cellulase, protease, and chitinase. They can also indirectly stimulate the activity of enzymes by increasing the beneficial bacteria in the gastrointestinal tract and increasing the digestive capacity of the host (Alavinejad *et al.*, 2025). Therefore, they increase food productivity and improve growth performance (Lazado *et al*., 2012; Caipang and Lazado, 2015). Probiotics also increase the level of food absorption and facilitate the digestive process by increasing the length and number of intestinal folds (Yang *et al*., 2019). In this study, the greatest effect on the microbial flora and length of intestinal folds was in the group fed with the lowest concentration of BA, while the highest growth rate was reported in the group fed with the highest concentration of BA. In this study, only the total growth rate of lactic acid bacteria was measured. Therefore, the need for additional and more specific studies is essential. In addition, the function of probiotics is not necessarily dependent on colonization in the gastrointestinal tract and may be due to metabolites produced by bacteria (Qin *et al*., 2018). An increase in the length and width of intestinal folds increases the

level of uptake and increases growth in the host. However, the main reason for the lack of coordination between the group with the highest increase in folds and the group with the highest increase in growth could be due to better performance of digestive enzymes in the group fed with higher concentrations of BA, which requires further studies.

The results of antioxidant factors showed an increase in SOD in the serum of fish fed with the probiotic. Other researchers have reported similar results (Weifen *et al*., 2012; Liu *et al*., 2017; Gobi *et al*., 2018). Generally, probiotics can affect host antioxidant defense through various mechanisms such as the ability to chelate metal ions, Modulation of gut microbiota, Producing Antioxidant metabolites, and Prevention of reactive oxygen species (ROS) production (Hoseinifar *et al*., 2020). Weifen *et al*. (2012) stated that the use of *B. licheniformis* and *B. subtilis* in diet and pond water of grass carp *(Ctenopharyngodon idella*) induces the secretion of antioxidant enzymes which can eliminate excess free radicals produced by high metabolism and adverse environmental stress. Therefore, it regulates the balance of free radicals in the body and increases SOD (Weifen *et al*., 2012). Also, probiotics are involved in reducing oxidative stress by reducing the activity of NADPH (NOX) as the main source of production of ROS (Gomez-Guzman *et al*. 2015), reducing the expression of cyclooxygenase (COX) as an effective enzyme in the production of ROS (Hussain *et al*. 2003; Brzozowski *et al*. 2006), and reducing expression Cytochrome P450 (CYP) enzymes as effective enzymes in oxidative metabolism (Trinder *et al*. 2015; Salem *et al*. 2018). In addition, the use of probiotics produces metabolites such as lactic acid and acetic acid, which decrease the intestinal pH, regulate the composition of intestinal microbiota, prevent the spread of harmful bacteria, and ultimately reduce oxidative stress (Merrifield *et al*. 2014; Ringø *et al*. 2018). The results of this study showed the positive effects of BA on the bacterial flora of fish intestines. Based on the observed antioxidant effects, it is suggested that mechanisms of action of the BA on antioxidant defence be investigated in future studies. SOD and MDA are final peroxidation products of unsaturated fatty acids and are an indicator of oxidative stress and antioxidant status (Gaweł *et al*., 2004). High concentrations of MDA increase free radicals and exacerbate cell membrane damage (Su *et al*., 2019). The results of this study showed a decrease in MDA levels in fish fed BA, which is consistent with the results of Zhai *et al*. (2014) and Chen *et al*. (2020). Generally, the increase in SOD along with the decrease in MDA confirms the antioxidant effect in the present study.

Conclusion

According to the obtained results, it can be concluded that consumption of BA commercial dietary supplement significantly improved the microbial flora and intestinal morphology, increased growth performance, and improved the antioxidant capacity of Koi fish serum. Therefore, this

commercial supplement can be used as a practical supplement in the cultivation of ornamental and edible fish.

Authors and contributors

Mehdi AlizadehBarogh: Conceptualization, Methodology, Writing – original draft, Afshin Mahmoodzadeh: Conceptualization, Data curation, Formal analysis, Supervision, Reza Kazempoor: Conceptualization, Supervision, Writing – review & editing.

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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