

Effect of synbiotic dietary supplementation on survival, growth performance, and digestive enzyme activities of common carp (*Cyprinus carpio*) fingerlings

P. GHASEMPOUR DEHAGHANI¹, M. JAVAHERI BABOLI², A. TAGHAVI MOGHADAM³, S. ZIAEI-NEJAD⁴, M. POURFARHADI⁵

¹Department of Fisheries Science, College of Agriculture, Khuzestan Science and Research Branch, Islamic Azad University, Ahvaz, Iran

²Department of Fisheries Science, College of Agricultural Sciences and Natural Resources, Islamic Azad University, Ahvaz, Iran

³Research Lab of Biological Products, Razi Research Vaccine and Serum Institute, Ahvaz, Iran

⁴Department of Fisheries, Natural Resources Faculty, Khatamalanbia Industrial University of Behbahan, Behbahan, Iran

⁵Department of Fisheries Science, Faculty of Marine Science and Technology, Islamic Azad University, Tehran, Iran

ABSTRACT: Effects of different levels of Biomin[®] IMBO synbiotic, including *Enterococcus faecium* (as probiotic), and fructooligosaccharides (as prebiotic) on survival, growth performance, and digestive enzyme activities of common carp fingerlings (*Cyprinus carpio*) were evaluated. The experiment was carried out in four treatments (each with 3 replicates), including T1 = control with non-synbiotic diet, T2 = 0.5 g/kg synbiotic diet, T3 = 1 g/kg synbiotic diet, and T4 = 1.5 g/kg synbiotic diet. In total 300 fish with an average weight of 10 ± 1 g were distributed in 12 tanks (25 animals per 300 l) and were fed experimental diets over a period of 60 days. The results showed that synbiotic could significantly enhance growth parameters (weight gain, length gain, specific growth rate, percentage weight gain) ($P < 0.05$), but did not exhibit any effect on survival rate ($P > 0.05$) compared with the control. An assay of the digestive enzyme activities demonstrated that the trypsin and chymotrypsin activities of synbiotic groups were considerably increased than those in the control ($P < 0.05$), but there was no significant difference in the levels of α -amylase, lipase, or alkaline phosphatase ($P > 0.05$). This study indicated that different levels of synbiotic have the capability to enhance probiotic substitution, to improve digestive enzyme activity which leads to digestive system efficiency, and finally to increase growth. It seems that the studied synbiotic could serve as a good diet supplement for common carp cultures.

Keywords: additive; prebiotic; probiotic; growth; survival; digestive enzyme

INTRODUCTION

The common carp (*Cyprinus carpio* L.) is one of the most important farmed species in the world's aquaculture especially in Asia where the production was 3 444 203 t in 2010 (FAO 2012). Improving the health conditions and growth performance in commonly farmed fish such as common carp is a topic of extreme interest. Recently, research efforts

have been concentrated on optimizing production with eco-friendly alternatives to the therapeutic use of antibiotics. The use of probiotics in the culture of aquatic organisms is increasing with the demand for good management (Gatesoupe 1999). A probiotic is generally defined as a live microbial food supplement which improves the balance of the host animal's intestinal flora (Fuller 1989). The majority of probiotic studies in fish

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were focused on Gram-positive bacteria, such as lactic acid bacteria (LAB) and *Bacillus* sp., although Gram-negative bacteria (*Aeromonas*, *Alteromonas*, *Photobacterium*, *Pseudomonas*, and *Vibrio* species), microalgae, and yeasts were reported (Wang et al. 2008; Merrifield et al. 2010; Nayak 2010). One potential of LAB is *Enterococcus faecium* that was demonstrated in Nile tilapia (*Oreochromis niloticus*) (Wang et al. 2008), grouper (*Epinephelus coioides*) (Sun et al. 2011), beluga sturgeon (*Huso huso*), and Persian sturgeon (*Acipenser persicus*) (Askarian et al. 2011).

Prebiotics have been defined as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the intestinal tract, and thus associated with health” (Gibson and Roberfroid 1995). The prebiotics used in fish culture, inulin and fructooligosaccharide (FOS), are among the most well-established ones (Van Loo et al. 1999). FOS has been assessed and determined in different species such as Nile tilapia (*Oreochromis niloticus*) (Ibrahim et al. 2010), red drum (*Sciaenops ocellatus*) (Buentello et al. 2010), and beluga sturgeon (*Huso huso*) (Hoseinifar et al. 2011).

Synbiotic is a combination of probiotics and prebiotics. It beneficially affects the host and improves host welfare by improving the survival and colonization of live microbial dietary supplements in the gastrointestinal tract by selective stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria (Gibson and Roberfroid 1995). The use of synbiotic may possibly produce greater benefits rather than the application of individual probiotics (Merrifield et al. 2010). These biotics can be applied through external bathing or dietary supplementation and have been demonstrated to improve growth performance, feed utilization, digestibility of dietary ingredients with enzymes, disease resistance, and stimulation of the immune response of aquatic animals (for reviews see Kesarcodi-Watson et al. 2008; Wang et al. 2008; Merrifield et al. 2010). Such benefits may be due to elevated digestive enzyme activities which have been reported in fish and shellfish fed pro- and prebiotics and the combination of them (synbiotic) (Daniels et al. 2010). Limited data is available regarding the application of synbiotics in aquaculture (Rodriguez-Estrada et al. 2009;

Daniels et al. 2010; Ai et al. 2011; Zhang et al. 2011). Thus, the aim of the present study is to assess the effects of Biomin® IMBO as a synbiotic on the survival, growth performance, and digestive enzyme activities of common carp fingerlings.

MATERIAL AND METHODS

Rearing conditions and experimental design.

Common carp fingerlings with an average body weight of 10 ± 1 g were obtained from a well-known farm in Ahvaz- Khuzestan, Iran, transported to the laboratory of the South Iran Aquaculture Research Center (SIARC), and randomly stocked into 12 tanks (300 l). Fish had been acclimatized to laboratory conditions for 2 weeks before being randomly divided into four equal experimental groups (25 fish for each tank) representing four nutritional groups as three treatments were conducted to evaluate the effect of synbiotic administered to the common carp fingerlings and one group without using synbiotic as control group, in the form of three replicates.

Feeding and synbiotic supplement preparation.

The type of synbiotic applied in this study was Biomin® IMBO (Biomin, Herzogenburg, Austria) composed of probiotic (*Enterococcus faecium* 5×10^{11} CFU/g) and FOS as prebiotic. For a dietary survey, a pellet form size SFC₂ of commercial Common carp feed (BTA Co., Hormozgan, Iran) was used. Nutritional analysis results were as follows: protein: $35 \pm 2\%$, digestible energy 3300 ± 200 kcal/kg, crude fibre $< 2\%$, fat $11 \pm 2\%$.

The proper amounts of synbiotic were mixed with commercial Common carp feed at three levels: T1 = 0 (control), T2 = 0.5, T3 = 1.0, and T4 = 1.5 g synbiotic/1 kg diet. Dietary ingredients of the respective synbiotic and control diets were mixed with required amount of water and then cold pressed, dried for 3 days at room temperature, then stored at 4°C until feeding trial (Sun et al. 2011). The pelleted diets were air-dried, ground and sieved to produce a suitable crumble. The experimental fish were fed three times daily (at 6.00, 12.00, and 18.00 h) for 60 days, and weighed on days 30 and 60 in order to adjust the daily feed rate which was 3–5% of the total biomass (250 ± 25 g).

Water quality management. Water temperature and pH were monitored daily and kept at $30 \pm 0.5^\circ\text{C}$ and 7.5 ± 0.3 , respectively. The fish tanks were daily cleaned by siphoning out the fish faeces and uneaten food debris. Continuous aeration

was provided to each tank through an air stone connected to a central air compressor.

Growth performance and survival. Growth performance parameters were calculated according to the following formulae:

$$\text{Weight gain} = W_2 - W_1$$

$$\text{Percentage weight gain} = 100 \times [(W_2 - W_1)/W_1]$$

$$\text{Length gain} = L_2 - L_1$$

$$\text{Specific growth rate (SGR)} = 100 \times (\ln W_2 - \ln W_1)/T$$

where:

W_1 = initial weight (g)

W_2 = final weight (g)

L_1 = initial length (cm)

L_2 = final length (cm)

T = time (days)

In addition, survival rate was calculated at the end of the experiment:

$$\text{Survival} = (N_f/N_0) \times 100$$

where:

N_0 = initial number of fish

N_f = final number of fish

Digestive enzyme assays. On days 30 and 60 of the experiment, three fish (starved for 24 h) were sampled from each tank for enzymatic analysis. The intestines were isolated and rinsed with cold distilled water (Yanbo et al. 2006) at 4°C. There after, the intestinal enzyme extracts were obtained through homogenization in 100mM Tris-HCl buffer with 0.1mM EDTA and 0.1% Triton X-100 (pH 7.8) was used at a proportion of 1 g tissue per 9 ml buffer. The homogenates were centrifuged at 30 000 g at 4°C for 30 min. After centrifugation, the supernatant was collected and frozen at –80°C (Furne et al. 2008).

The intestinal brush border membrane enzyme (alkaline phosphatase) was determined in accordance with methods applied by Cahu et al. (1999). The samples were homogenized in 30 v/w fractions of Tris (2mM)-mannitol (50mM), pH 7.0, and centrifuged at 19 000 rpm for 30 s. Brush border extracts were prepared as described by Crane et al. (1979). Briefly, tissue homogenates were centrifuged at 9 000 g for 10 min after the addition of 0.1M CaCl₂. The supernatants were transferred to new vials and stored frozen (–80°C) until analysis of enzyme activity or protein content.

Trypsin activity was measured with N- α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrate. BAPNA (1mM in 50mM Tris-HCl, pH 7.5, 20mM CaCl₂) was incubated with the enzyme extract at 37°C. Absorbance was recorded at 410 nm (Erlanger et al. 1961). The molar extinction coefficient of *p*-nitroanilide is 8800 cm²/mg. Trypsin activity units were expressed as a change in absorbance per min/mg protein and trypsin activity units were calculated by the following equation:

Unit/mg protein =

$$\frac{(\text{absorbance } 410/\text{min}) \times 1000 \times \text{ml of reaction mixture}}{8800 \times \text{mg protein in reaction mixture}}$$

Chymotrypsin activity was measured with *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrate. A unit of activity was defined as 1 μ mole of *N*-benzoyl-L-tyrosine ethyl ester released per min at 256 nm (Hummel 1959). The molar extinction coefficient of *p*-nitroanilide is 964 cm²/mg. Chymotrypsin activity units were expressed as a change in absorbance per min/mg protein and trypsin activity units were calculated by the following equation:

$$\text{Unit/mg protein} = \frac{\Delta A/\text{min} \times 1000 \times 3}{964 \times \text{mg protein in assay}} \times \text{DF}$$

where:

A = absorption by spectrophotometry

DF = dilution factor applied

α -Amylase activity was determined by the 3,5-dinitrosalicylic acid (DNS) method (Bernfeld 1951; Worthington 1991). Starch substrate (1% w/v) was diluted in a buffer at pH 6.9, 0.02M Na₂HPO₄, and 0.006M NaCl. The substrate (250 μ l) was incubated with crude extract (50 μ l) and buffer solution (250 μ l) for 3–4 min at 25°C. Then 0.5 ml of 1% DNS solution was added and boiled for 5 min. After boiling, 5 ml of distilled water was added to the mixture and the absorbance of the cooled solution was recorded at 540 nm. Blanks were similarly prepared, but without the crude enzyme extracts. Maltose (0.3–5 μ M/ml) was used for the preparation of the standard curve. The α -amylase specific activity was defined by the μ mol of maltose produced per min/mg protein at the specified condition.

Lipase activity was measured using the titration method specified by Worthington (1991) using olive oil–Arabic gum emulsion. One unit of activity was defined as 1 μ mole of fatty acid released per min.

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Alkaline phosphatase (AP) was quantified at 37°C using 4-nitrophenyl phosphate (PNPP) as substrate in 30mM Na₂CO₃ buffer (pH 9.8). One unit (U) was defined as 1 µg PNPP released per min per ml of brush border homogenate at 407 nm (Bessey et al. 1946).

Total soluble protein was measured by the method of Bradford (1976) using bovine serum albumin as a standard. Enzyme activities were expressed as specific activity (U/mg protein). All the enzymatic assays were run in triplicate.

Statistical analysis. Data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Bartlett's test) prior to their comparison. All the data were expressed as mean ± SD ($n = 3$). Digestive enzyme activities were compared

by means of one-way ANOVA, and the mean comparison was performed with a Duncan's test at a reliability level of 5%. Data were analyzed using SPSS statistical software (Version 16).

RESULTS

The effects of dietary synbiotic on digestive enzyme activities (α -amylase, trypsin, chymotrypsin, lipase, and alkaline phosphatase) on days 30 and 60 are shown in Figure 1.

α -Amylase activity was increased on days 30 and 60, but there were no significant ($P > 0.05$) differences between the synbiotic groups and control on day 60. α -Amylase activity was significantly greater ($P < 0.05$) in fish fed 1 g/kg dietary syn-

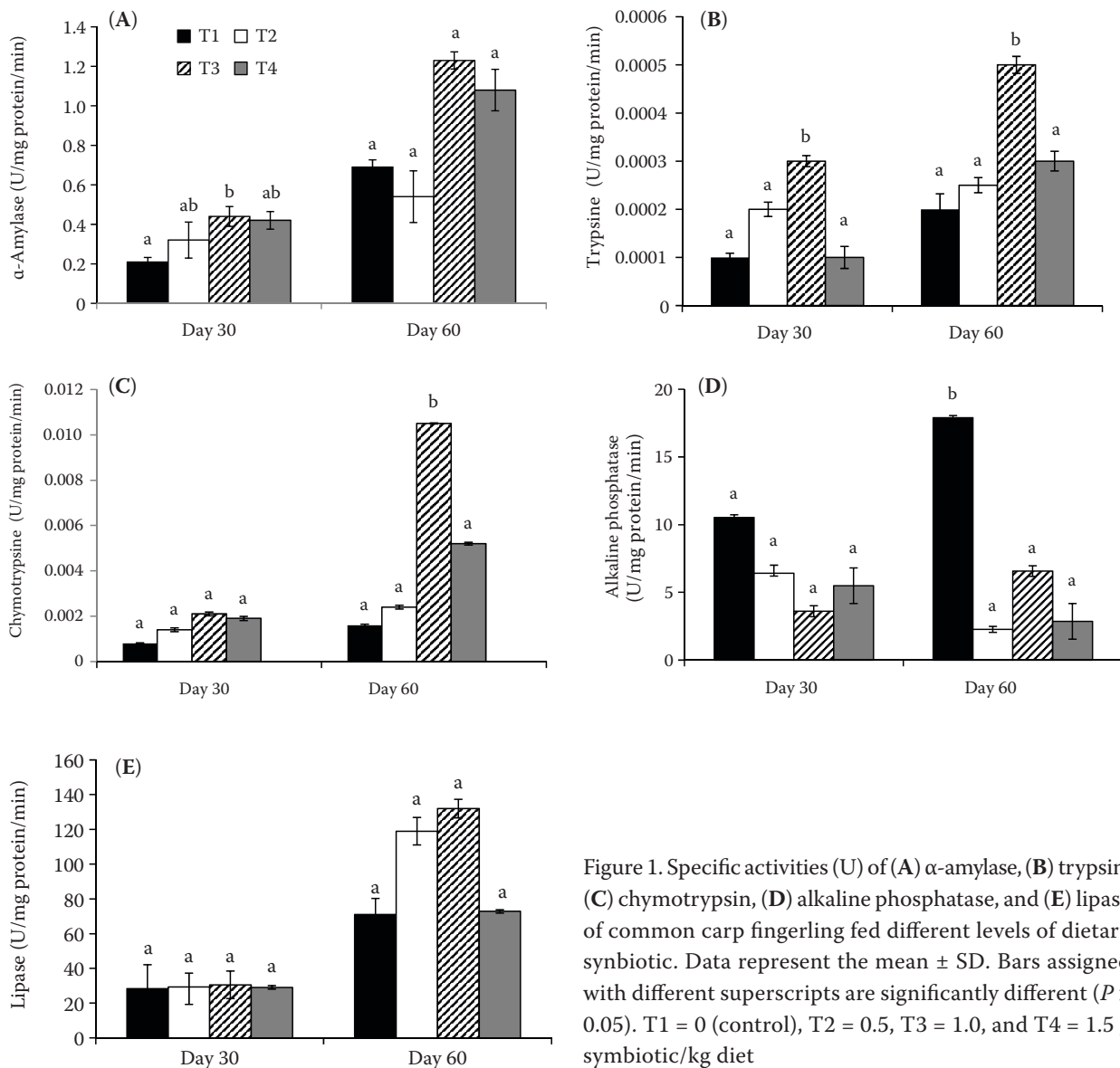


Figure 1. Specific activities (U) of (A) α -amylase, (B) trypsin, (C) chymotrypsin, (D) alkaline phosphatase, and (E) lipase of common carp fingerling fed different levels of dietary synbiotic. Data represent the mean ± SD. Bars assigned with different superscripts are significantly different ($P > 0.05$). T1 = 0 (control), T2 = 0.5, T3 = 1.0, and T4 = 1.5 g synbiotic/kg diet

biotic compared to the control group; however, no difference was seen between treatment 3 and treatments 2 and 4 on day 30 ($P > 0.05$) (Figure 1A).

Trypsin activity was significantly ($P < 0.05$) higher in fish fed 1 g/kg dietary synbiotic compared to the control group on days 30 and 60; however, on day 60, no difference between treatments 2 and 4 and control group was observed ($P > 0.05$). On day 30, the activity was significantly ($P < 0.05$) higher in treatment 3 than in treatment 4 and the control group, but there was no significant ($P > 0.05$) difference between treatments 3 and 2 (Figure 1B).

Chymotrypsin activity was significantly ($P < 0.05$) raised in fish fed 1 g/kg dietary synbiotic compared to the control group on day 60; however on day 60, no difference was observed between the 0.5 g/kg synbiotic, the 1.5 g/kg synbiotic, and the control group ($P > 0.05$). On day 30, no significant ($P > 0.05$) differences in chymotrypsin activity were observed between the synbiotic groups and the control (Figure 1C).

Lipase activity was increased in fish fed 1 g/kg dietary synbiotic compared to the other groups on days 30 and 60, but there were no significant ($P > 0.05$) differences between the synbiotic groups and the control on days 30 and 60 (Figure 1D).

Alkaline phosphatase activity in this study was reversed with activity of the other enzymes. Alkaline phosphatase activity in the control group was increased on days 30 and 60 and was significantly ($P < 0.05$) greater in the control compared with the synbiotic group on day 60 (Figure 1E).

Growth performance of common carp fed diets supplemented with varying levels of dietary synbiotic is presented in Table 1. Compared with the control treatment, common carp fingerlings fed 1.5 g/kg dietary synbiotic displayed improved ($P < 0.05$) growth performance, including weight gain, length gain, percentage weight gain, and specific growth rate (SGR) on days 30 and 60, but no significant difference was observed between treatments 2, 3, and 4 that were fed synbiotic. At the end of the trial, survival rate was high in all treatments with no significant ($P > 0.05$) differences observed. Mortality was similar in all groups.

DISCUSSION

To our knowledge, this study was the first to investigate the effects of Biomin[®] IMBO as a synbiotic on the survival, growth performance, and digestive enzyme activities of common carp (*Cyprinus carpio*) fingerlings. Recently, probiotics and prebiotics have become integral parts of aquaculture practices for improving growth performance (Nayak 2010; Ringo et al. 2010; Mehrabi et al. 2011). Synbiotics, the combined application of probiotics and prebiotics, is based on the principle of providing a probiont with a competitive advantage over competing endogenous populations; thus, it effectively improves the survival and implantation of the live microbial dietary supplement in the gastrointestinal tract of the host (Gibson and Robefroid 1995). It could be concluded that the addition of probionts in basal diets improved growth performance, feed

Table 1. Growth parameters of common carp fingerlings on different treatments on days 30 and 60 of experiment

Treatment	Sampling days	Weight gain (g)	Percentage weight gain (g)	Length gain (cm)	SGR (%)
T1	30	6.91 ± 3.13 ^a	65.27 ± 12.55 ^a	1.26 ± 0.28 ^a	0.82 ± 1.29 ^a
	60	7.69 ± 0.94 ^a	72.58 ± 8.88 ^a	1.52 ± 0.25 ^a	0.90 ± 0.08 ^a
T2	30	7.80 ± 0.57 ^a	73.61 ± 5.42 ^a	1.48 ± 0.15 ^a	0.91 ± 0.05 ^a
	60	11.49 ± 1.39 ^b	108.47 ± 13.17 ^b	2.27 ± 0.24 ^{ab}	1.21 ± 0.100 ^b
T3	30	9.67 ± 0.84 ^{ab}	91.3 ± 7.92 ^{ab}	1.77 ± 0.02 ^a	1.07 ± 0.06 ^{ab}
	60	11.44 ± 0.12 ^b	108.04 ± 1.20 ^b	2.48 ± 0.29 ^b	1.22 ± 0.009 ^b
T4	30	11.04 ± 0.36 ^b	104.22 ± 4.47 ^b	2.10 ± 0.47 ^a	1.18 ± 0.02 ^b
	60	13.93 ± 1.29 ^b	131.52 ± 13.21 ^b	2.91 ± 0.11 ^b	1.39 ± 0.09 ^b

T1 = 0 (control), T2 = 0.5, T3 = 1.0, and T4 = 1.5 g symbiotic/kg diet

SGR = specific growth rate

On day 30 values in rows with different superscripts denote a significant difference ($P < 0.05$), and on day 60 values in columns with different superscripts denote a significant difference ($P < 0.05$)

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utilization, and digestive enzyme activities (Wang and Xu 2006) and the improved enzyme activities obtained with the supplemented diets suggest that the addition of probiotics improves diet digestibility, including protein, starch, and fats (Wang and Xu 2006). The use of synbiotics may provide greater benefits rather than the application of individual probiotics (Merrifield et al. 2010).

The present and previous studies have demonstrated that LAB and other probiotic strains can improve the growth rate and/or feed utilization of fish (Lara-Flores et al. 2003; Suzer et al. 2008; Sun et al. 2011). Such benefits may be due to the elevated digestive enzyme activities which have been reported in fish and shellfish fed oligosaccharides prebiotics (Merrifield et al. 2010; Ringo et al. 2010; Soleimani et al. 2012). So, in this study, trypsin and chymotrypsin activity was significantly enhanced in fish fed a synbiotic diet. Fingerlings fed 1 g per each kg of dietary synbiotic showed better digestive enzyme activities and significantly higher trypsin and chymotrypsin activities compared with the control treatment. These two enzymes belong to alkaline proteases and play a considerable role in food digestion (Jobling 1995). These enhanced protease activities might be beneficial to the digestion of dietary protein, which might in turn contribute to the better feed utilization in common carp fingerlings. Different levels of synbiotic, however, have no effect on α -amylase and lipase activity. The lack of significant differences in terms of α -amylase and lipase activity may be explained by the low fat and carbohydrate content of the assimilated food items. Whether these elevated activities are elevated by endogenous (host) and/or exogenous microbial activities has not been fully elucidated (Soleimani et al. 2012).

Results similar to our findings (the increase in digestive enzyme activities and therefore improved feed utilization through the use of probiotics) have also been demonstrated in common carp (*Cyprinus carpio*) (Wang and Xu 2006), in Indian white shrimp (*Fenneropenaeus indicus*) by *Bacillus* sp. (Ziaei-Nejad et al. 2006), in gilthead sea bream (*Sparus aurata* L.) by *Lactobacillus* sp. (Suzer et al. 2008), in grouper (*Epinephelus coioides*) by *Psychrobacter* sp. (Sun et al. 2011), in beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) by lactic acid bacteria (LAB) (Askarian et al. 2011), in grouper (*E. coioides*) by *Lactococcus lactis* and *E. faecium* (Sun et al. 2011), and

in artemia (*Artemia urmiana*) by *B. subtilis* and *B. licheniformis* (Ahmadnia Motlagh et al. 2012). Similar results have also been reported in previous studies on prebiotics in fresh water crayfish (*Cherax destructor*) fed mannan oligosaccharides (MOS) (Sang et al. 2011) and Caspian roach (*Rutilus rutilus*) fingerlings fed fructooligosaccharides (FOS) (Soleimani et al. 2012).

On the contrary, dietary administration of live yeast *Debaryomyces hansenii* HF1 or *Saccharomyces cerevisiae* X2180 improved the intestinal amylase and trypsin activities, but decreased the growth rate of sea bass (*Dicentrarchus labrax*) larvae (Tovar et al. 2002). Therefore, the effect of probiotics on the digestive enzymes and their relationship with growth and feed utilization in aquatic animals need further study. In the present study, different concentrations of synbiotic had different effects on enzyme activity. Alkaline phosphatase activity was significantly greater in the control compared to the synbiotic groups. The enzymatic activity in the treatments of 0.5 g into each kg of dietary synbiotic and 1.5 g into each kg of dietary synbiotic was reduced on day 60 compared to day 30. Contrarily, *Lactobacillus* spp. bacteria increased alkaline phosphatase activity in gilthead sea bream (*Sparus aurata* L.) larvae (Suzer et al. 2008).

Subsequently, better growth performance was observed in common carp fed synbiotic with a trend towards the best results being achieved at a level of 1.5 g into each kg of dietary synbiotic. Growth enhancement as a result of probiotic and prebiotic administration has been reported in several previous studies on a variety of fish and shellfish species fed dietary prebiotics (Mahious et al. 2005; Daniels et al. 2010; Hoseinifar et al. 2010; Mehrabi et al. 2011; Sun et al. 2011; Soleimani et al. 2012). The results of the present study revealed that the supplementation of LAB to the food of common carp significantly improved SGR. To our knowledge, improved growth performance from the use of probiotic and prebiotic has been reported in *Homarus gammarus* L. (Daniels et al. 2010), *Oreochromis niloticus* (Wang et al. 2008), *Epinephelus coioides* (Sun et al. 2011), and *Rutilus rutilus* (Soleimani et al. 2012). The enhanced growth performance might be because of the increased digestive enzyme activity induced by the probiotics, as it has been reported that Gram-positive bacteria, particularly members of the genus *Lactobacillus*, have the ability to secrete a wide range of exo-enzymes

(Moriarty 1998; Suzer et al. 2008). Considering the results, it can be concluded that there were no significant differences in survival between the synbiotic group and the control, and the survival rate in all treatments was 100%. Nevertheless, a study on the effect of Biomin® IMBO synbiotic on rainbow trout fingerlings reported an increase in growth parameters and survival relative to the control group (Mehrabi et al. 2011). These different findings may refer to differences in quality (amount), manner of administration of *E. faecium* probiotic and FOS prebiotic, and the target species. Various mechanisms have been proposed to explain the beneficial effects of probiotics, such as: (i) antagonism towards pathogens, (ii) competition for adhesion sites, (iii) competition for nutrients, (iv) improvement of water quality, (v) stimulation of host immune responses, and (vi) enzymatic contribution to digestion (Askarian et al. 2011).

We could not distinguish between the activity due to enzymes synthesized by the common carp fingerlings and due to enzymes synthesized by the probiotic strains colonized in the fish fingerlings' digestive tracts. However, the exogenous enzymes produced by the probiotic would represent, at most, only a small contribution to the total enzyme activity of the gut (Ding et al. 2004; Ziaei-Nejad et al. 2006; Xu et al. 2009), and the presence of the probiotic might stimulate the production of endogenous enzymes by the common carp fingerling. The observed increases in specific activities of digestive enzymes in probiotic treatments might have led to enhanced digestion and increased absorption of food (Xu et al. 2009). Studies show that digestive enzyme activities are affected by life stage, amount, and the chemical composition of feed, and the nutritional requirements of the fish (Ahmadnia Motlagh et al. 2012). Moreover, the activity of these enzymes may fluctuate depending on the age and type of feed. However, to realize just what the digestive enzymes activities are, more research is needed.

CONCLUSION

It seems that *E. faecium* bacteria with fructooligosaccharide, which are available in synbiotic Biomin® IMBO, have increased the digestibility and absorption of feed and consequently the SGR by enhancing the activities of digestive enzymes and their secretion.

As it is clear from the results of this experiment, *E. faecium* bacteria are capable of creating dominant bacterial flora in guts of experimental carp and consequently LAB are also increased and experimental treatments which are influenced by this bacteria have obviously demonstrated the increasing activity of trypsin and chymotrypsin enzymes. This issue should be further investigated.

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Corresponding Author

Pegah Ghasempour Dehaghani, MSc., Islamic Azad University, Science and Research Branch, Department of Fisheries, Khuzestan, P.O. Box 163, Iran
Phone: +98 611 445 7612, mobile: +98 916 310 6940, e-mail: Paqua6940@gmail.com
