PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
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Pakistan Journal of Nutrition 14 (5): 263-268, 2015
ISSN 1680-5194

Growth Performance of Tilapia (*Oreochromis niloticus*) Fed with Probiotic, Prebiotic and Synbiotic in Diet

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Abstract: The aim of this study was to evaluate the growth performance of tilapia which were given probiotic, prebiotic and synbiotic through feed. The probiotic bacteria used was selected from Np 1, Np 3 and Np 5. The prebiotic was extracted from sweet potato var. sukuh, while the synbiotic was a combination between a probiotic and prebiotic. This study was conducted in two phases, the *in vitro* probiotics and prebiotics synergism test and the *in vivo* feeding trial of selected probiotic, prebiotic and synbiotic to tilapia. The *in vivo* assays had four treatments with three replications, i.e., (A) control, (B) probiotic 1% (v/w), (C) prebiotic 2% (v/w) and (D) synbiotic (probiotic 1% (v/w)+prebiotic 2% (v/w)). Results of the *in vitro* assays showed that the three probiotic isolates could grow in media containing the prebiotics and Np 5 isolate demonstrated the best growth. In the *in vivo* assays, the application of the synbiotic resulted in the best growth rate, feed efficiency, digestive enzyme activity, feed digestibility and nutrient retention compared to the control and the other treatments.

Key words: Probiotic, prebiotic, synbiotic, *Oreochromis niloticus*

INTRODUCTION

Tilapia (*Oreochromis niloticus*) is a species of freshwater fish that is widely cultured in the world. This fish has several advantages such as fast growth, thick fleshy, easily cultured and easy to reproduce (Molina *et al*., 2009). Some of these advantages make the development of tilapia culture is now leading to the application of intensive culture systems with high density. On the other hand, the intensive culture of tilapia faces several problems, including the relatively high feed prices which were not followed by the selling price of the product. Therefore, alternative ways to enhance growth through improving digestibility of tilapia and efficiency of feed utilization were needed.

The addition of probiotics, prebiotics and synbiotics in diet are expected to increase the role of the normal flora in the digestive tract of fish to produce exogenous enzymes such as amylase, protease and lipase which can increase the activity of endogenous enzymes to hydrolyze the feed nutrients. According to Merrifield (2010), probiotics applied in fish culture are components of dead or living microbial cells which are administered through feed or the rearing medium and could increase the host resistance to disease, health status, growth performance and feed utilization through the increasing of microbial balance in the host or its environment. In improving nutritional value of feed, probiotics are able to produce some exogenous enzymes for the digestion of feed such as amylase, protease, lipase and cellulase (Wang, 2007). The addition of probiotics to feed has been widely applied to aquaculture practices and has been shown to provide beneficial effects to fish (Kesarcodi-Watson *et al*., 2008).

However, the application of probiotics has weaknesses which are survival, colonization and nutrient competition of probiotics which quite varied. Another approach that can solve these limitations is through the application of prebiotics. Prebiotics are food substances that cannot be digested by the host but can be metabolized by beneficial bacteria and have the ability to improve the host health (Ringo *et al*., 2010). The addition of prebiotics to feed has been applied in aquaculture practices and it plays a role in promoting growth, immune responses, digestive enzyme activity and the composition of beneficial bacteria in the fish digestive tract (Zhou *et al*., 2010; Zhang *et al*., 2012; Soleimani *et al*., 2012; Akrami *et al*., 2013; Hoseinifar *et al*., 2013). As with probiotics application, the effect of prebiotics is also temporary and it strongly depends on the presence of beneficial bacteria in the digestive tract. In this case, a possible approach is by applying synbiotics. Synbiotics are balanced combinations of probiotics and prebiotics in an effort to support the survival and growth of beneficial bacteria in the digestive tract of living organisms (Cerezuela *et al*., 2011). Some studies have shown that the administration of probiotics with

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prebiotics in a host could improve growth, survival and the immune system in prawns (Li *et al*., 2009), lobsters (Daniels *et al*., 2010), sea cucumbers (Zhang *et al*., 2010) and koi (Lin *et al*., 2012). This study aimed to evaluate the growth performance of tilapia fed probiotics, prebiotics and synbiotics in diet.

MATERIALS AND METHODS

Preparation of prebiotic: The extraction process of the prebiotic oligosaccharide referred to Marlis (2008), as much as 10 g of steamed sweet potato flour was suspended in 100 mL of 70% ethanol and stirred for 15 h using a magnetic stirrer at room temperature. The filtrate obtained was concentrated using a vacuum evaporator at 40°C. Then, the oligosaccharide extract was measured for its Total Dissolved Solid (TDS) that was useful in oligosaccharide analysis for the *in vitro* and *in vivo* assays.

***In vitro* assays of prebiotics:** This test aimed to test the role of the prebiotics in supporting the growth of probiotics candidate bacteria. The medium used was Trypticase Soy Broth (TSB) which contained 2% prebiotic (TPT 5%) (Marlis, 2008). The control was a TSB medium with and without the addition of several standard sugars: glucose, raffinose, maltotriose, oligofructose (2%). The population of bacteria which grew was calculated based on the optical density with a wave length of 600 nm. The probiotic candidate bacterium with the best growth in the medium which contained the prebiotic was chosen to be used in the *in vivo* assays.

***In vivo* assays of probiotic, prebiotic and synbiotic:** This test aimed to compare the effectiveness of the probiotic, prebiotic and synbiotic on feed digestibility and growth performance of tilapia. The test feed used in this study was dry pellet with a relatively similar protein, fat and carbohydrate content for each treatment (Table 1). This study consisted of four treatments and three replications, including feed without the addition of probiotic or prebiotic (control or A); feed with the addition of 1% probiotic (Wang, 2007) (B); 2% prebiotic, 5% TPT (Marlis, 2008) (C); synbiotic (1% probiotic and 2% prebiotic) (D).

Probiotic and prebiotic added to the feed by spraying using a syringe with adding 2% egg yolk (Wang, 2007). Feeding was done three times a day by at satiation. To maintain water quality, the aquariums were siphoned and 30% of the water volume was replaced daily.

The fish used were monosexual male tilapia weighing 3.53±0.05 g at a density of 15 fish per aquarium. The aquariums used were 50 x 40 x 30 cm^3 sized aquarium. The fish acclimatized for five days before the experiment was carried out. After the acclimatization, the fish were fasted for 24 h before they were fed the treatment feeds. The fish were reared for 60 days for the growth test, while the digestibility test was done separately for 10 days. The feed which had been added Cr_2O_3 as a digestibility indicator was given for ten days and started on the seventh day of experiment, fish feces was collected.

Measurement of the parameters in the growth test: The parameters measured in the growth test were survival rate (SR), daily growth rate (DGR), feed intake (FI) and feed efficiency (FE) that were determined according to Huisman (1987), while protein and fat retention (PR and FR) were calculated according to Takeuchi (1988).

Analysis of enzyme activity in the fish digestive tract: The analysis was conducted at the end of the rearing period. The enzymes activity measured were the activity of amylase and protease referring to the method developed by Bergmeyer and Grassi (1983).

Enumeration of intestinal bacteria population: The enumeration of the intestinal bacteria population was done using the spread plate count technique. The fish intestines (0.1 g) were collected from each aquarium and homogenized in 0.9 mL sterile phosphate buffer saline (PBS; 0.8 g NaCl, 0.2 g KH_2PO_4, 1.5 g Na_2HPO_4, 0.2 g KCl and 1000 mL distilled water). The number of bacterial cells in a sample is calculated by counting the number of colonies growing on the medium multiplied by the dilution factor to obtain the number of colony forming units per gram (CFU/g) (Madigan *et al*., 2003).

Measurement of the parameters in the digestibility test: Digestibility analysis was carried out by drying the feces which collected in a oven at 110°C for 4-6 h. The dried feces was then analyzed for its nutrient and Cr_2O_3 content using a spectrophotometer at a wave length of 350 nm. Nutrient digestibility (protein and carbohydrate digestibility) and total digestibility were calculated according to Watanabe (1988) by the following equations:

$$\text{Nutrient digestibility} = 100-[1-a/a'x\ b'/b]$$

$$\text{Total digestibility} = 100-[1-a/a']$$

where, a (% Cr_2O_3 in the feed); a' (% Cr_2O_3 in the feces); b (% nutrients in the feed); b' (% nutrients in the feces).

Statistical analysis: The data obtained were analyzed using analysis of Variance (ANOVA) with the difference among treatments were determined by the Duncan's Multiple Range test. Statistical analysis was carried out using SPSS 17.

RESULTS

***In vitro* assays of prebiotics:** The results of the effectiveness test for the various prebiotics compounds in supporting the growth of the probiotics bacteria candidate are presented in Fig. 1. In the TSB medium with the addition of the prebiotics, the highest bacterial growth based on the optical density was shown by Np 5 (1.865) followed by Np 3 (1.740) and the lowest was Np 1 (1.680).

Digestibility and growth performance: The results of the effectiveness test of probiotic, prebiotic and synbiotic on feed digestibility and growth performance of tilapia are presented in Table 2. The highest feed intake was found in the addition of the synbiotic (1067.20±21.08 g) which was significantly different ($p<0.05$) from the other treatments. This was followed by the prebiotic treatment (1020.33±5.10 g), the probiotic treatment (999.00±24.44 g), the lowest amount was in the control at 961.93±9.21 g.

Based on Table 2, it can be seen that the highest population of bacteria was found in D (7.44±0.02 logCFU/g), followed by C (7.26±0.06 logCFU/g), B (6.44±0.04 logCFU/g) and the lowest was A or control (6.02±0.03 logCFU/g). Furthermore, the highest amylase activity also was found in D (0.601±0.01 U/min/mL) that was significantly different with the other treatments ($p<0.05$). This was followed by B (0.466±0.002 U/min/mL), C (0.385±0.03 U/min/mL) and the lowest was in the control (0.316±0.02 U/min/mL). In addition, the highest protease activity also was found in D (0.47±0.01 U/min/mL) that was significantly different ($p<0.05$) with the other treatments, followed by C (0.33±0.02 U/min/mL), B (0.14±0.02 U/min/mL) and the lowest was in the control (0.12±0.02 U/min/mL).

The values of protein digestibility, carbohydrate digestibility and total digestibility of each treatment (Table 2) showed that the highest protein digestibility was found in D (82.41±0.84%) followed by C (71.91±0.26%), B (61.88±0.50%) and the lowest protein digestibility value was found in the control (39.24±0.97%). Carbohydrate digestibility from highest to lowest were D (89.37±0.88%), followed by B (84.40±3.72%), C (82.72±0.37%) and the lowest digestibility was found in the control (63.45±0.35%). In addition, the highest total digestibility value also was shown by D (71.05±2.49%), followed by C (57.3±0.45%); B (51.40±0.87%) and the lowest was in the control (43.40±1.94%).

The highest DGR was found in D (4.18±0.02%), followed by C (3.95±0.05%), B (3.72±0.03%) and the lowest was in the control (3.56±0.05%). Furthermore, the highest feed efficiency was found in D (55.46±0.65%) that was significantly different with the other treatments. This was followed by C (50.61±1.15%), B (43.66±1.80%) and A (41.40±1.23%). These growth performance values above were supported by the values of protein retention and fat retention in Table 2. which showed that the highest protein retention was found in D (30.47±0.54%), followed by C (27.26±1.12%), B (23.08±0.78%) and the control (21.13±0.84%). Similar results were shown in fat retention, in which the highest value was found in D (36.32±5.20%), followed by C (33.04±4.05%), B (30.34±0.37%) and the control (26.92±0.65%).

Table 1: Composition of artificial feed in the study

	Treatment (%)			
Feed ingredients	A	B	C	D
Fish meal	23.00	23.00	23.00	23.00
Soy meal	18.00	18.00	18.00	18.00
Tapioca flour	16.00	16.00	16.00	16.00
Wheat pollard	15.00	15.00	15.00	15.00
Wheat flour	18.00	18.00	18.00	18.00
Vitamin C	1.00	1.00	1.00	1.00
Fish oil	3.00	3.00	3.00	3.00
Palm oil	2.00	2.00	2.00	2.00
Vitamin and mineral mix	1.00	1.00	1.00	1.00
Filler	3.00	2.00	1.00	0.00
Probiotics	0.00	1.00	0.00	1.00
Prebiotics	0.00	0.00	2.00	2.00
Protein	23.24	23.56	23.66	23.57
Fat	8.17	8.57	8.55	8.04
BETN[1]	43.68	43.20	43.23	43.21
Total Energy[2])	256.72	259.88	260.14	255.64
C/P (Kcal/kg)	11.05	11.03	10.99	10.85

Nitrogen-Free Extracts, DE: Digestible Energy = carbohydrate: 2.5 Kcal DE; protein: 3.5 Kcal DE, fat: 8.1 Kcal DE

DISCUSSION

***In vitro* assays of prebiotics:** The test results for 24 h showed that each bacterial isolate which were tested on media containing the prebiotic and glucose resulted in a higher growth rate than the bacteria isolate on media containing raffinose, oligofructose and maltotriose. In the study by Hernandez-Hernandez *et al.* (2012), it was shown that several strains of the bacteria *Lactobacillus* spp. which were tested *in vitro* for 24 h on media containing galactooligosaccharide from lactose (GOS-La), galactooligosaccharide from lactulose (GOS-Lu), lactulose and glucose resulted in similar growth rates. However, from 24 h until the last observation at the 120 h, the growth rate of all the *Lactobacillus* spp. strains decreased rapidly except in the prebiotic GOS-Lu and GOS-La media, which were relatively stable. This was because the oligomers with high molecular weights were more capable of supporting bacterial growth than other substrates with lower molecular weight (Vernazza *et al.*, 2006). In this study, Np 5 showed the highest growth rate in all media compared to the other bacteria isolate. This suggested that Np 5 was able to utilize the prebiotic as an energy source for growth. Therefore, Np 5 was selected as the probiotic bacteria for the next stage of the experiment.

Digestibility and growth performance: The feed intake of the probiotic, prebiotic and synbiotic treatment were

Table 2: Feed intake (FI), bacteria population (BP), amylase activity (AA), protease activity (PA), protein digestibility (PD), carbohydrate digestibility (CD), total digestibility (TD), protein retention (PR), fat retention (FR), daily growth rate (DGR), survival rate (SR) and feed efficiency (FE) in tilapia (*Oreochromis niloticus*)

	Treatment			
Parameter	A	B	C	D
FI (g)	961.93±9.21^{a}	999.00±24.44^{b}	1020.33±5.10^{b}	1067.20±21.08^{c}
BP (log CFU/g)	6.02±0.03^{a}	6.44±0.04^{b}	7.26±0.06^{c}	7.44±0.02^{d}
AA (U/min/mL)	0.316±0.02^{a}	0.466±0.002^{b}	0.385±0.03^{ab}	0.601±0.01^{c}
PA (U/min/mL)	0.12±0.02^{a}	0.14±0.02^{a}	0.33±0.02^{b}	0.47±0.01^{c}
PD (%)	39.24±0.97^{a}	61.88±0.50^{b}	71.91±0.26^{c}	82.41±0.84^{d}
CD (%)	63.45±0.35^{a}	84.40±3.72^{b}	82.72±0.37^{c}	89.37±0.88^{d}
TD (%)	43.40±1.94^{a}	51.40±0.87^{b}	57.3±0.45^{b}	71.05±2.49^{c}
PR (%)	21.13±0.84^{a}	23.08±0.78^{b}	27.26±1.12^{c}	30.47±0.54^{d}
FR (%)	26.92±0.65^{a}	30.34±0.37^{ab}	33.04±4.05^{ab}	36.32±5.20^{b}
DGR (%)	3.56±0.05^{a}	3.72±0.03^{b}	3.95±0.05^{c}	4.18±0.02^{d}
SR (%)	100±0.00	100±0.00	100±0.00	100±0.00
FE (%)	41.40±1.23^{a}	43.66±1.80^{a}	50.61±1.15^{b}	55.46±0.65^{c}

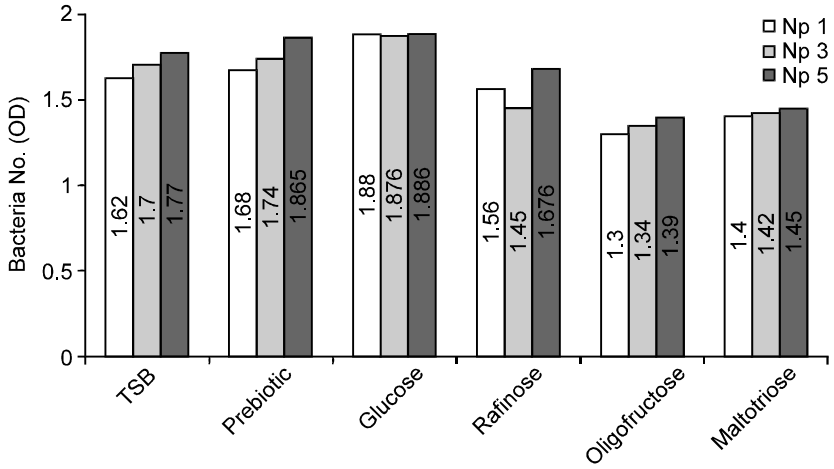

Fig. 1: Growth of probiotics candidate bacteria on TSB media with the addition of prebiotic and several sugar sources

higher than the control. The higher digestibility, the more feed is digested, making the gastric-emptying rate faster so that feed intake will increase. According to Zokaeifar *et al*. (2012), a better appetite in the host that fed with feed supplemented with *Bacillus subtilis* compared to the control group during the treatment period was because there was no remains of undigested feed.

The addition of probiotic, prebiotic and synbiotic to feed aimed to increase the population of probiotic in tilapia digestive tract so that the action mechanism of the probiotic in producing exogenous enzymes for digestion will increase (Merrifield, 2010; Cerezuela *et al*., 2011). The addition of prebiotic to feed is believed to stimulate the growth of normal micro flora in tilapia digestive tract, making the population of the bacteria in fish which were given the prebiotic and synbiotic treatments higher than the probiotic treatment and control. Similar results were obtained by Mahious *et al*. (2006) where the addition of raffinose to feed had increased the composition of probiotics bacteria in the digestive tract of turbot. According to Delgado *et al*. (2011), prebiotics in synbiotics application can potentially improve the survival rate and increase the role of probiotics and the beneficial microorganisms.

Microorganisms are able to adapt to its environment which is rich in complex molecules by secreting exogenous enzymes. Exogenous enzymes catalyze the hydrolyzation of macromolecules into simpler molecules. NP5 probiotic bacteria are bacteria which have an amylolytic activity (Putra *et al*., 2010), presumably this causes amylase enzyme activity in the probiotic treatment to be higher than in the prebiotic treatment and control. The increasing of amylase activity due to the addition of probiotic bacteria are also found in tilapia fed *Bacillus subtilis* (Taoka *et al*., 2007) and white shrimp fed *Bacillus* spp (Wang, 2007).

Prebiotics are non-digestible food ingredients which have beneficial effects on the host and are related to the macro biota modulation (Ringo *et al*., 2010). It is suggested that the high protease activity in the prebiotic and synbiotic treatments probably derived from other bacteria whose growth is increased due to the addition of prebiotic to the feed. The prebiotic stimulated the growth of other beneficial bacteria or the normal micro

flora found in tilapia digestive tract beside from the probiotic bacteria supplemented. Li *et al.* (2007) also found that FOS supports the growth of certain bacteria species in the digestive tract of white shrimp. Similar results were obtained by Helland *et al.* (2008), the addition of mannan oligosaccharide (MOS) to feed increased the composition of normal micro flora and the activity of digestive enzymes in Atlantic salmon. According to Delgado *et al.* (2010), prebiotics may produce short chain fatty acid (SCFA) which causes intestinal pH decrease in order to inhibit the growth of pathogenic bacteria and stimulate the population of beneficial bacteria for the host.

The increased activity of digestive enzymes in tilapia digestive tract has a positive correlation to feed digestibility. The higher carbohydrate digestibility in the probiotic treatment compared to the prebiotic treatment is strongly related to the activity of amylase enzyme which was higher in the probiotic treatment due to the role of the amylolytic probiotic added to the feed. The total digestibility represented the amount of nutrients in the feed which the fish were able to digest. The study results showed that the highest total digestibility was found in the synbiotic treatment. A combination between the probiotic and prebiotic in the feed increased the activity of tilapia digestive enzyme, so more of the nutrients in the feed were digested. The increased activity of the digestive enzymes could help the host in degrading nutrients, improving digestibility and increasing feed efficiency (Cerezuela *et al.*, 2011).

After the digestion process, nutrients are absorbed by the fish body. The amount of nutrients which could be absorbed from the feed and stored in the fish body is represented by the retention rate. The results of the study showed that the addition of probiotic, prebiotic and synbiotic to feed increased protein retention and fat retention compared to the control. This was because of the high enzymatic activity of the fish in the experiment. Protease will break down protein into simpler compounds so that they are easier to be absorbed and the amount of protein stored in the body will be higher. In the prebiotic and synbiotic treatments, it is suggested that the addition of prebiotic to the feed stimulated the growth of other normal micro flora which had lipolytic activities, so the fat retention in the prebiotic and synbiotic treatment were higher than the other treatments. In the study by Soleimani *et al.* (2012), it was reported that the supplementation of the prebiotic fructooligosaccharide (FOS) in feed increased the activity of endogenous enzymes by bacteria in the Caspian roach (*Rutilus rutilus*) fry.

The addition of synbiotics to feed resulted in the highest enzyme activity, digestibility and nutrient retention values compared to the other treatments. This has a positive correlation to the daily growth rate and feed efficiency, in which the addition of synbiotic to feed resulted in a better growth performance than the control. Similar results also occurred in the study on the addition of synbiotics to feed which could improve body weight gaining, specific growth rate and feed conversion ratio in European lobster (*Homarus gammarus* L.) (Daniels *et al.*, 2010), yellow croaker (*Larimichthys crocea*) (Ai *et al.*, 2011) and Siberian sturgeon (*Acipenser baerii*) (Geraylou *et al.*, 2013). Based on this study, it can be concluded that the addition of synbiotic to feed resulted in the highest growth rate, feed efficiency, enzyme activity, feed digestibility and nutrient retention compared to the control and other treatments.

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