

Effect of different Alphamune levels in artificial diet on growth parameters, digestibility and enzyme activity of rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792)

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Abstract

This study was conducted to evaluate the effects of Alphamune, a mixed prebiotic composed of mannan-oligosaccharide and β -glucans, on the growth performance, digestibility and enzyme activity of rainbow trout, *Oncorhynchus mykiss*. A basal diet was formulated using common feed ingredients supplemented with Alphamune at 0, 0.5, 1, 2, and 4 g.kg⁻¹ leading to five experimental diets. Obtained results showed that inclusion of dietary Alphamune significantly increased the final weight and weight gain ($p < 0.01$) of rainbow trout compared to the control group. Feed conversion ratio was also improved after prebiotic administration in comparison with the control group ($p < 0.05$). However, Alphamune supplementation did not change specific growth rate ($p > 0.05$). Also apparent digestibility coefficient (ADC) was not affected by dietary addition of the prebiotic ($p > 0.05$). Our results indicated that amylase and lipase activities were not significantly influenced by administration of different doses of Alphamune. The result also showed that trypsin activity was gradually increased with increasing of the probiotic level ($p > 0.05$). In conclusion, inclusion of the prebiotic Alphamune can improve the nutrient efficiency and growth performance of rainbow trout confirming the positive effect of a mixture of prebiotics on fish.

Keywords: Prebiotic, Alphamune, Growth, Digestibility, Enzyme activity.

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Introduction

As the culture of commercial species intensifies, problems have been reported concerning water quality and disease outbreaks (Austin and Austin, 2007; Shoemaker *et al.*, 2010; Tellez-Banuelos *et al.*, 2010). The water quality management and disease treatment are associated with practices such as appropriate stocking rates and application of chemicals and antibiotics (FAO, 2006; McEwen and Fedorka-Cray, 2002). The increasing economic and social concerns about the use of antibiotics and other chemicals in aquaculture have encouraged more environmentally friendly approaches for increasing growth (Verschuere *et al.*, 2000).

Prebiotics are potentially food supplements that decrease infectious adverse effects and increase feed efficiency (Gibson and Roberfroid 1995). Prebiotics in general include nutrients such as non-digestible carbohydrate, resistant starch, nutrient fiber, sugars, some peptides and proteins as well as some certain lipids that enter the intestine (Fooks and Gibson, 2002). Fructooligosaccharides (FOS), inulin, and other carbohydrates substrate have received considerable attention because of their health benefits to host (Salze *et al.*, 2008).

Several studies have demonstrated that prebiotics can improve growth parameters, disease resistance, villi surface area and microvilli length, and also modulate intestinal micro-biota in various aquatic species (Genc *et al.*,

2007; Li *et al.*, 2007; Staykov *et al.*, 2007; Torrecillas *et al.*, 2007; Zhou *et al.*, 2007; Burr *et al.*, 2008; Salze *et al.*, 2008). Moreover, dietary supplementation of polysaccharides as prebiotics seems to reform bacterial community in fish intestine leading to improved health and feed efficacy in the host (Dimitroglou *et al.*, 2009).

In addition, administration of prebiotics affected feed efficiency and nutrient digestibility (Grisdale-Helland *et al.*, 2008), and enzyme activity (Xu *et al.*, 2009). Prebiotic supplementation improved nutrient and energy digestibility of soybean-meal-based diet in red drum (Burr *et al.*, 2008). On the other hand, it has been suggested that oligosaccharides such as stachyose and raffinose did not interfere with protein or fat digestibility as well as feed utilization in Atlantic salmon, *Salmo salar* (Sorensen *et al.*, 2011).

Alphamune (a mixture of mannan oligosaccharides 15% and β -glucans 24%) as a by-product of *Saccharomyces cerevisiae* can be an alternative to antibiotic growth promoter (Bolu *et al.*, 2009; Olonijolu *et al.*, 2013). Several studies suggested that growth performance and immune parameters of rainbow trout were improved by dietary supplementation of mannanoligosaccharide (Staykov *et al.*, 2007; Yilmaz *et al.*, 2007). Supplementation of β -glucan stimulated immune activity in a wide variety of species, including shrimp, fish, rat, rabbit, guinea pig, sheep, pig, cattle, and human (Ganguly *et al.*, 2013).

Previous studies on prebiotic mostly focused on different types of oligosaccharides alone as a source of prebiotic, but little is known about the efficiency of a mixture of carbohydrate sources.

The main objective of the present study is to determine the effects of alphamune (0, 0.5, 1, 2, and 4 g.kg⁻¹) as a mixed prebiotic on growth performance, nutrient digestibility and enzyme activity of juvenile rainbow trout.

Materials and methods

Experimental system and animal

This study was carried out at the experimental facility of Sari Agricultural and Natural Resource University located in the northern part of Iran, during the winter of 2014. In this study, rainbow trout juveniles, bred at a nearby reproduction facility, were adapted to a commercial diet and the new environment for a week before the start of the experiment. After adaptation, totally 375 fish were divided randomly into the 15 tanks of 500L capacity, with a stocking density of 25 fish (average initial weight of 40.01±0.11 g) per tank. The experiment lasted for 8 weeks. The prebiotic Alphamune used in this study was composed of mannan-oligosaccharide 15%, β-glucans (1-3, 1-6) 24% (Provided by Alpharma Co., Sao Paulo, Brazil).

A basal diet was formulated using locally feed ingredients with estimated gross fat and protein levels of 22.5 and

37.3%, respectively (Table 1). Five incremental levels (0, 0.5, 1, 2, and 4 g.kg⁻¹ prebiotic diet) of Alphamune were added to the basal diet to prepare five experimental diets. All ingredients were ground, mixed and pelletized. A pellet maker with 3 mm diameter die was used for producing the diets. All four diets were air-dried at 50°C at the same time and stored at -20°C until use.

Experimental procedure

During the experiment, fish were fed manually at the rate of 1.17- 2% body weight, three times per day (at 08.00, 13.00 and 18.00 hrs). The treatments were randomly assigned to each of the 15 tanks, having three replicates for each treatment. Water quality parameters were monitored daily to ensure they were in appropriate range for the fish. Water flow which was checked daily for each tank was 8L.min⁻¹.

On day 56, all fish were weighed for calculation of growth parameters. On the same day, three fish were randomly selected from each tank for enzyme activity analyses. No food was added to the rearing tank at night on the day prior to sampling. For enzyme activity measurement, the fish were killed using overdose clove essence solution (Ahmadifar *et al.*, 2014). The whole viscera were removed, intestines were separated from the viscera, and washed with distilled water (Babaei *et al.*, 2011).

Table 1: The percentage of ingredient used in the experimental diet on % dry matter weight basis.

Experimental diet	Amount
Soybean meal	20
Fish meal	43
Wheat gluten	5
Corn	6
Wheat	10
² Oil	13
Binder	1.5
¹ Premix	1.5
Nutrient composition of the experimental diet in g.kg ⁻¹ .	
Dry matter	936.7
Crude protein	377.3
Crude fat	225.0
Carbohydrate	192.3
Crude ash	142.1

¹permix is a half /half mixture of vitamins and minerals
Vitamin premix consisted of (g/kg premix): 1200000 IU
Vitamin A, 400000 IU Vitamin D3, 3000 IU Vitamin E, 1200
mg Vitamin K3, 5400 mg Vitamin C, 200 mg Vitamin B1, 3360
mg Vitamin B2, 7200 mg Vitamin B3, 9000 mg Vitamin B5,
2400 mg Vitamin B6, 600 mg Vitamin B9, 4 mg Vitamin B12,
500mg Antioxidant, up to 1kg carrier.

Mineral premix consisted of (g/kg premix): 2600 mg Mn, 600
mg Cu, 6000mg Fe, 4600mg Zn, 50mg Se, 100mg Iu, 50 mg
Co, 100000 mg choline chloride, up to 1 kg carrier (composed
of wheat bran) .

²an equal mixture of fish oil and sunflower oil.

After removing fresh water with a filter paper, the intestines were frozen in liquid nitrogen and stored at -80°C until further analysis.

Later on, the intestines were homogenized at $0-4^{\circ}\text{C}$ in an electric homogenizer (WIGGEN, D500, Germany). For the homogenization, a 100 mM Tris-HCl buffer with 0.1 mM EDTA and 0.1% Triton X-100, pH 7.8, was used at a proportion of 1 g tissue in 9 ml of buffer (Furne *et al.*, 2008). The homogenates were centrifuged at 30,000 g for 30 min at 4°C (Hermle

Z36HK, Germany). After centrifugation, the supernatant was collected and frozen at -80°C (Babaei *et al.*, 2011).

Trypsin (E.C.3.4.21.4) activity was measured with N- α -benzoyldlarginine-p-nitroanilide (BAPNA) as substrate. BAPNA (1 mM in 50 mM Tris-HCl, pH 7.5, 20 mM CaCl_2) was incubated with the enzyme extract at 37°C . Absorbance was recorded at 410 nm (Erlanger *et al.*, 1961). Trypsin activity unit was calculated by the following

equation presented by (Babaei *et al.*, 2011).

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

Amylase (E.C.3.2.1.1) activity was determined by the 3, 5-dinitrosalicylic acid (DNS) method (Bernfeld, 1951; Worthington, 1991). Lipase (E.C.3.1.1) activity was determined by hydrolysis of n-nitrophenylmyristate. A detailed description related to amylase and lipase measurement is presented by Bolasina *et al.* (2006) and Babaei *et al.* (2011).

Digestibility measurement

During the last week of the experiment, faeces were collected by pipetting from the tank bottom almost four hours after feeding. Daily faeces were pooled until desirable amount of faeces collected. Apparent digestibility coefficients of nutrients in the diet were determined using the indicator method with Cr₂O₃ as a marker (0.6 g kg⁻¹; Amirkolaie *et al.*, 2013). Apparent digestibility (%) of nutrients is expressed as a fractional net absorption of nutrients from the diet calculated according to:

$$\text{ADC} = (1 - [\text{Mar}_{\text{diet}} / \text{Mar}_{\text{faeces}} \times \text{N}_{\text{faeces}} / \text{N}_{\text{diet}}]) \times 100$$

where ADC = apparent digestibility coefficient; Mar_{diet} = dietary chromic oxide concentration; Mar_{faeces} = faecal chromic oxide concentration; N_{diet} = Nutrients of the diet; and N_{faeces} = Nutrients of the faeces.

Chemical analysis

Feed samples were collected and pooled at regular intervals during the experimental period and ground using a 1mm screen before analyses. Feed and faeces were analyzed for dry matter by drying samples for 24 h at 103°C until constant weight was obtained (ISO, 6496 1999). Ash content was determined by incineration in a muffle furnace for 4 h at 550°C (ISO, 5984 1978). Crude protein (N×6.25) was measured by the Kjeldahl method after acid digestion according to ISO, 5983 (1979). Lipid was extracted with petroleum ether in a Soxhlet apparatus. Chromic oxide was measured spectrophotometrically (UV-MS1, Bel Engineering, Italy) following Furukawa and Tsukahara, (1966).

Growth calculation and statistical analysis

Weight gain was determined by the difference between initial and final body weights. Feed conversion ratio (FCR) was calculated per tank from feed intake data and weight gains: FCR = feed consumed (g) / wet body weight gain (g). Specific growth rate (SGR) was calculated as follows and expressed as a percentage per day: $\text{SGR} = 100 (\ln W_{\text{final}} - \ln W_{\text{initial}}) \times \text{days}^{-1}$. Survival rate was calculated as the number of fish at the end of the experiment divided by number of fish at the beginning.

Data are presented as means of each treatment ± standard deviation. All data were checked for normality after

transformation (ArcSIN). Homogeneity of variances was tested using Levene's *F* test. One-way ANOVA was used to determine the effects of Alphamune levels on fish performance and enzyme activity. The means were compared by Tukey's post hoc test.

Results

The water temperature and pH ranged between 12-14°C and 7.0-7.6, respectively, during the experiment. Oxygen concentration always remained above 6.5 mg/l in the outlet of the fish tanks. The inclusion of different levels of the prebiotic influenced final weight, weight gain and FCR during the 8-week experiment ($p < 0.05$; Table 2). Rainbow trout significantly gained more weight with administration of 0.5 g.kg⁻¹ of Alphamune ($p < 0.05$). However, further inclusion of the prebiotic did not change weight gain and final weight of the fish. Similar to the weight gain, FCR increased in rainbow trout fed diet contained 0.5 g.kg⁻¹ prebiotic diet ($p < 0.05$), but further inclusion levels did not change FCR compared to

control diet. SGR and survival rate of the fish were not influenced by the administration of Alphamune ($p > 0.05$). The digestibility results demonstrated that inclusion of dietary prebiotic did not affect digestibility of nutrient in rainbow trout, although there were trends toward higher digestibility of nutrients in rainbow trout fed 0.5 g.kg⁻¹ prebiotic diet (Table 3).

Enzyme activity analysis revealed that amylase and lipase activities were not significantly influenced by administration of different doses of Alphamune ($p > 0.05$; Table 4). However, trypsin activity was influenced by the prebiotic ($p < 0.05$). Trypsin activity was gradually increased with increasing of the probiotic level with maximum activity that observed in rainbow trout fed 4 g.kg⁻¹ prebiotic diet ($p < 0.05$).

Table 2: Growth performance in rainbow trout fed on different levels of Alphamune (ALP g.kg⁻¹) over 56 days experimental period. All values are means \pm standard deviation of triplicate tanks/treatment.

Growth parameters	Diets				
	Control	ALP 0.5	ALP 1	ALP 2	ALP 4
Initial weight (g)	40.07 \pm 0.05	40.08 \pm 0.11	39.97 \pm 0.05	40.02 \pm 0.08	39.92 \pm 0.09
Final weight (g)	97.47 \pm 2.34 ^b	101.44 \pm 1.31 ^a	98.65 \pm 1.23 ^{ab}	99.23 \pm 1.66 ^{ab}	97.45 \pm 0.17 ^b
Weight gain (g)	57.41 \pm 2.37 ^b	61.35 \pm 1.41 ^a	58.68 \pm 1.18 ^{ab}	59.02 \pm 1.73 ^{ab}	57.53 \pm 0.32 ^b
SGR (%/day)	1.59 \pm 0.45	1.65 \pm 0.03	1.61 \pm 0.03	1.62 \pm 0.04	1.59 \pm 0.02
FCR	1.08 \pm 0.03 ^a	1.03 \pm 0.02 ^b	1.07 \pm 0.02 ^{ab}	1.07 \pm 0.04 ^{ab}	1.08 \pm 0.02 ^a
Survival rate (%)	92.66 \pm 2.3	96.22 \pm 4.0	94.24 \pm 4.2	94.32 \pm 3.6	92.71 \pm 2.3

Different superscript letters in the same row show significant differences.

Table 3: Nutrient digestibility in rainbow trout fed on different levels of Alphamun (ALP g.kg⁻¹) over 56 days experimental period. All values are means±standard deviation of triplicate (tanks)/treatment.

Digestibility	Diets				
	Control	ALP 0.5	ALP 1	ALP 2	ALP 4
Dry matter (%)	73.93 ±4.72	78.81 ± 0.81	76.55 ± 2.57	76.68 ± 4.75	74.93 ± 0.50
Crude protein (%)	90.57 ± 1.56	91.32 ± 0.34	91.96 ± 1.18	91.54 ± 1.88	91.76 ± 0.47
Crude fat (%)	87.48 ± 2.05	88.21 ±1.93	88.07 ± 1.37	88.66 ± 3.99	88.73 ± 0.85
Crude ash (%)	43.19± 23.43	54.15± 3.15	49.85± 7.71	55.66± 7.25	54.88± 1.84

Discussion

The results of this experiment show that the addition of 0.5g/kg Alphamune as a prebiotic improves growth performance of the fish. This is similar to results of Li and Gatlin (2005), Staykov *et al.* (2007), and Ebrahimi *et al.* (2012) who observed higher feed efficiencies in hybrid striped bass, rainbow trout and common carp fed GroBiotic® prebiotic, mannan-oligosaccharide and Immunogen, respectively. Although the main cause of prebiotic effect on the fish in the current study is not fully understood, it has been stated that colonization of beneficial bacteria induced by dietary prebiotic might increase the synthesis of essential nutrients such as fatty acids, protein, and vitamins (Irianto and Austin, 2002; Hajibeglou and Sudagar, 2011). Lower numbers of dead and deformed fry in four species of ornamental fish, for instance, were the results of vitamins B₁ and B₁₂ syntheses by a prebiotic bacterial strain, *Bacillus subtilis* (Ghosh *et al.*, 2007). A positive impact of Alphamune on fish growth found in this study may be attributed to an up-regulation of the digestive enzymes

activities as it has been demonstrated in poultry and fish (Xu *et al.*, 2003, 2009).

Alphamune supplementation did not change nutrient digestibility in rainbow trout, although a number of observations suggested positive impacts of prebiotics on digestibility (Burr *et al.*, 2008). This condition may support the idea that the beneficial effect of Alphamun on the growth is not too much related to the nutrient digestibility and, probably, an improve in the immune system is more significant. This finding is similar to that of Raggi and Gatlin (2012) who observed that inclusion of 1% of four prebiotics (GroBiotic-A, mannanoligosaccharide, galactooligosaccharide, fructooligosaccharide) did not improve nutrient digestibility in goldfish. In addition, lipid digestibility was lower for diet containing the above four perbiotics (Raggi and Gatlin, 2012). Significant reductions in lipid digestibility were also observed in red drum fed diets supplemented with inulin, mannanoligosaccharide, and galactooligosaccharide (Burr *et al.*, 2008). However, these results are in contrast to those obtained in red drum in which prebiotics supplementation

increased nutrient digestibility (Burr *et al.*, 2008). It appears that the inclusion levels of prebiotic in the current experiment (0.5-4 g.kg⁻¹) were not too high to up-regulate the activity of digestive enzymes.

In this study, ash digestibility was not significantly affected by the prebiotic supplementation, but the estimates were always higher compared to control diet. Oligosaccharides fermentation may decline intestinal pH rendering increased minerals availability (Olsen *et al.*, 2001; Valancony *et al.*, 2001; Mussatto and Mancilha, 2007) resulting in a higher ash digestibility.

Addition of prebiotic was initially supposed to increase digestibility of nutrients by increasing enzyme activity. However, only trypsin activity was improved by a high dose of the prebiotic while other treatments did not change enzyme activities in rainbow trout. This finding is not similar to that of Xu *et al.* (2009) who observed larger digestive enzyme activities in red drum fed prebiotic diets. The limited effect of prebiotic on enzyme activity may be related to the composition of this supplement. Alphamune is composed of 15% mannan-oligosaccharide and 24% β -glucans (1-3, 1-6). The interaction effect between these two ingredients may have had a negative impact on enzyme activity in the fish. Pure oligosaccharide such as xylooligosaccharide, on the other hand, led to a higher enzyme activity in allogynogenetic crucian carp (Xu *et al.*,

2009). Limited enzyme activities of rainbow trout treated with Alphamune might also be associated with the activity of beneficial microbial community. It can be speculated that Alphamune supplementation may not induce enzyme producer bacterial community. Further works are necessary to focus on bacterial community using different types and levels of carbohydrates at different environmental conditions in order to fully assess possible factors affecting enzyme activity.

In conclusion, the results of the current study demonstrate that the administration of Alphamune as a dietary supplement can improve growth performance in rainbow trout, but limited effect on ADC and digestive enzyme activity. Limited impact of the prebiotic on ADC may suggest that the beneficial effect of Alphamun on the growth is not too much related to the nutrient digestibility and, probably, an improve in immune system is more significant. Further investigations are needed to verify the impacts of different types of carbohydrates and their interactions on ADC and enzyme activities.

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