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Effects of prebiotic Immunogen on growth performance, intestinal bacteria colonization, and survival rate in *Rutilus frisii* fry

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ABSTRACT

This study was carried out to determine the effects of Immunogen as a prebiotic on growth-related parameters and gut micro-flora in *R. frisii* fry. A basal diet was formulated using common feed ingredients supplemented with Immunogen at 0, 1, 1.5, and 2 g.kg^{-1} leading to four experimental diets. Fish were randomly distributed in 500 L fiberglass tanks (1×1×0.5 m). The experiment lasted for eight weeks and the water temperature ranged between 21-24 °C. *R. frisii* fry with an initial weight of 300 ± 1.7 mg and were randomly distributed in the experimental tanks. At the end of the experiment, growth performance, feed efficiency, and gut micro-flora were assessed. Results showed that inclusion of 1g.kg^{-1} Immunogen improved final weight, feed conversion efficiency (FCR), and specific growth rate (SGR) ($p<0.01$) in the fry. A larger survival rate of *R. frisii* fry was observed at 1 g kg^{-1} prebiotic inclusion level ($p<0.01$). However, body protein, fat, and ash were not influenced by prebiotic inclusion. Supplementation of 1 g.kg^{-1} prebiotic increased the total count of bacteria ($p<0.05$), but bacterial count did not change at 1.5 and 2 g.kg^{-1} prebiotic inclusion compared to control diet. In conclusion, Immunogen administration of 1 g. kg^{-1} is capable of improving the nutrients efficiency and performance of *R. frisii* fry through growth stimulation of beneficial intestinal bacteria.

Keywords: Bacterial count, Feed conversion, Kutum, Prebiotic, Survival rate, Immunogen

INTRODUCTION

The shortage of natural resources such as freshwater and land has led to the intensification of aquaculture system (Piedrahita, 2003). However, increasing fish density may also increase the incidences of diseases in fish farms due to deteriorated water quality (Losordo *et al*., 1999). Intensification can also increase stress level in farmed fish, which, in turn, may threat their immune system and exacerbate the incidences of bacterial infections (Liltved & Cripps, 1999). Antibiotics have long been introduced as a solution to treat those infections (Burr *et al*., 2005), but long-term administration of antibiotics to treat bacterial diseases of farmed fish has widely been criticized because of bacterial resistance to antibiotics, elimination of gut microbial flora, high cost of these drugs, and potential side effects (Boyd and Massaaut, 1999; Esiobu *et al*., 2002). The public's concern about the negative impacts of antibiotics have sharply reduced their uses for aquaculture industry in the United States and Europe (Burr *et al*., 2005). Therefore, alternative techniques have been introduced to replace chemical drugs, among which the contribution of prebiotics may be significant. Prebiotics are potentially food

supplements that decrease infectious adverse effects and increase feed efficiency by stimulating the growth of beneficial bacteria (Gibson & Roberfroid, 1995). Prebiotics generally 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 & Gibson, 2002). 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 animals (Genc *et al.*, 2007; Li *et al*., 2007; Staykov *et al*., 2007; Zhou *et al*. 2007; Torrecillas *et al*., 2007; Burr *et al.*, 2008; Salze *et al*., 2008). Moreover, dietary supplementation of polysaccharide prebiotic seems to reform bacterial community in fish intestine, which was found to improve health and feed efficacy in the host (Dimitroglou *et al*., 2009). Yet, there is still little information on beneficial bacteria and the by-products produced by dietary non-digestible carbohydrate. Immunogen as a commercial prebiotic contains various stimulating components such as mannan oligosaccharide and β-glucans, which have been used as feed additives in various animals. Supplementation of Immunogen improved performance and disease resistance of Huso huso (Mohajer Esterabadi *et al.*, 2010), common carp, *Cyprinus carpio*, (Ebrahimi *et al*. 2012), and reproductive performance of platy, *Xiphophorus maculatus* (Hajibeglou & Sudagar 2011). *R. frisii* is one of the most commercially valuable fish species in the southern coast of the Caspian Sea (Abedi *et al.*, 2012; Khara *et al*., 2012), which migrates from the sea to freshwater inlets (Heidari *et al*., 2009). Due to a sharp decline observed in the natural population, establishment of R. kutum restocking centers has been recognized as a solution to recover the natural stocks (Heyrati *et al.*, 2006). At restocking centers, dietary prebiotics supplementation may improve fish growth, immune response, and survival rate. While some literature is available on the effects of prebiotic on fish performance and micro-flora characteristics, there is little information related to Immunogen on *R. frisii*. Hence, the main objective of the current study was to identify the effects of Immunogen on growth-related parameters and gut micro-flora in *R. frisii* fry.

MATERIALS AND METHODS

Experimental system and animal

This study was carried out using the experimental facility of Shahid Rajaee aquaculture complex located in the northern part of Iran. Fry *R. frisii* were bred using the reproduction facility of the complex and adapted to the experimental conditions a few days before the start of the experiment. Afterward, the fry with an average weight of 300 ± 1.7 mg were divided randomly into twelve 0.5 m^3 tanks through individual counting with an initial stocking density of 500 fish per tank. The experiment lasted for 8 weeks. The prebiotic Immunogen used in this study is composed of mannan oligosaccharide (18%) and β-glucans (1-3, 1-6)(30%) (Provided by Soroush Radian Co., Tehran, Iran). A basal diet was formulated using locally grown feed ingredients (Table 1). The inclusion of Immunogen into the basal diet at the rates of 0, 1, 1.5, and 2 g kg^{-1} was led to four experimental diets.

Experimental procedure

Fish were weighed on the 1st and the last days of the experiment. The experimental diets were given to the fish at a rate of 10% of biomass (Takeuchi *et al.*, 2002) three times per day (8.00, 13.00, and 18.00 hrs).

The diets were randomly assigned to one of the 12 tanks, with three replications per diet. Randomization was done by numbering the diets and selecting three tanks randomly for each diet. Water quality parameters were monitored daily to ensure they were in appropriate range for the fish. The water temperature and pH ranged between 20-23 °C and 7.3-7.9, respectively, during the experiment. Oxygen concentration was measured in a randomly selected tank by a digital oxygen detector, which always

remained above 6.1 mg.L^{-1}. On Day 56, all fish were weighed. Afterward, 30 fish were randomly selected from each tank and sacrificed using overdosed (400 mg.L^{-1}) clove essence solution for measurement of body composition.

Table 1. The percentage of ingredients used in the basal diet on % dry matter weight basis.

Experimental diet	Amount
Soybean meal	21
Fish meal	34
Wheat meal	10
Corn meal	5
Wheat gluten	2.5
Meat meal	12
Cotton seed meal	4
Barley meal	4
Alfa alfa meal	1
Molasses	2
Salt (NaCl)	0.5
Toxin binder	1
Dicalcium phosphate	1
Vit & Min. Premix[1]	2
Nutrient composition of the basal diet in g.kg^{-1}	
Dry matter	915.2
Crude protein	375.3
Crude fat	107.4
Crude ash	98.2

Premix consisted of equal proportions of vitamins and minerals
Vitamin premix consisted of (g.kg^{-1} 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, 500 mg Antioxidant, up to 1kg carrier.
Mineral premix consisted of (g.kg^{-1} premix): 2600 mg Mn, 600 mg Cu, 6000 mg Fe, 4600 mg Zn, 50 mg Se, 100 mg IU, 50 mg Co, 100000 mg choline chloride, up to 1 kg carrier (composed of wheat bran).

Chemical analysis and bacteria count

Feed and fish body were analysed for dry matter through drying samples for 24 h at 103°C until constant weight (ISO 6496, 1983). 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 applying the Kjeldahl method after acid digestion according to ISO 5983 (1979). Lipid was extracted by petroleum ether extraction in a Soxhlet apparatus (ISO 6492, 1999). At the end of the experiment, 10 fries were collected from each treatment and their intestine samples were tested for bacterial counts. Prior to dissection and homogenization, the fry were rinsed with sterilized distilled water, cleaned with ethanol (70.0%) and then washed again with sterilized distilled water to eliminate all exterior bacteria. The intestine samples were dissected out in sterile conditions. For microbiological analysis, three samples from the middle part of the intestine were taken.

All samples were diluted using sterilized normal saline solution (0.85% NaCl w/v) and then placed into nutrient agar plates for bacterial counts (Ebrahimi *et al*. 2012).

Fish performance

Weight gain was determined by the difference between initial and final body weights (Amirkolaie *et al.*, 2005). Feed conversion ratio (FCR) was calculated per tank from feed intake data and weight gains: FCR = feed consumed (g) / wet body weight gain (g) (Amirkolaie *et al.* 2005). Specific growth rate (SGR) was calculated as follows and expressed as a percentage:

SGR = 100 (Ln W_{final}- Ln $W_{initial}$) × Days $^{-1}$ (Amirkolaie *et al.,* 2005). The calculations were based on the dry weight of the diets.

Statistical analysis

Data are presented as means of each treatment with standard deviations. All data were verified for normality (Kolmogorov-Smirnov test) after ArcSine transformation. One-way ANOVA was used to determine the effects of Immunogen on fish performance and bacteria community. Tukey's test was used to compare differences between the means. For all statistical analyses, each tank was considered as the experimental unit.

RESULTS

Data on the growth performance of *R. kutum* are presented in Table 2. *R. frisii* fry significantly gained higher weight with the administration of 1 g.kg^{-1} of Immunogen ($p<0.05$). Along with the growth, FCR and SGR were also significantly improved in R. kutum fed the prebiotic diets in comparison to control diet ($p<0.05$).

Nevertheless, growth-related parameters were similar to control diet in fish fed prebiotic levels of 1.5 and 2 g.kg-^{1}.

Similar to growth parameters, fish displayed higher survival rates with 1 g.kg^{-1} prebiotic in comparison to the other treatments ($p<0.05$). The body composition results demonstrated that the inclusion of dietary prebiotic did not affect the carcass composition of *R. kutum* (Table 3; $p>0.05$). Bacterial counts were also affected by the prebiotic inclusion (Table 4). Total count of bacteria together with Gram positive bacteria count were increased by the addition of Immunogen (1 g.kg^{-1}); but, bacterial counts in fish fed prebiotic levels of 1.5 and 2 g.kg^{-1} were similar to those in control diet ($p<0.05$).

Table 2. Growth performance in *R. frisii* fry fed different levels of Immunogen (IMU) over 56 days of experimental period. All values are means ± standard deviation of triplicate tanks/treatment (n=3).

Parameters	Diets			
	Control	IMU1 g.kg^{-1}	IMU 1.5 g.kg^{-1}	IMU 2 g.kg^{-1}
Initial weight (mg)	301.1 ± 1.3	299.2 ± 2.1	300.3 ± 1.8	302.4 ± 1.5
Final weight (mg)	706.0 ± 3.5^{b}	718.6 ± 2.6^{a}	705.8 ± 3.3^{b}	708.9 ± 4.2^{b}
SGR (%/day)	2.09 ± 0.04^{b}	2.29 ± 0.03^{a}	2.15 ± 0.04^{ab}	2.12 ± 0.07^{ab}
FCR	2.20 ± 0.04^{b}	2.07 ± 0.09^{a}	2.09 ± 0.02^{a}	2.23 ± 0.02^{b}
Survival rate (%)	87.4 ± 2.3^{b}	92.3 ± 1.2^{a}	87.1 ± 2.5^{b}	87.1 ± 2.6^{b}

Different superscript letters in the same row show significant differences ($P<0.05$).

Table 3. Body composition in *R. frisii* fed different levels of Immunogen (IMU) over 56 days of experimental period. All values are means ± standard deviation of triplicate tanks/treatment (n=3).

Proximate composition	Diets			
	Control	IMU1 g/kg	IMU 1.5 g/kg	IMU 2 g/kg
Dry mater	24.95 ± 0.32	24.56 ± 1.01	23.25 ± 2.77	24.26 ± 1.24
Protein	15.28 ± 1.22	15.18 ± 1.46	15.07 ± 0.91	14.92 ± 0.09
Fat	8.15 ± 0.18	8.03 ± 0.28	7.79 ± 0.96	7.55 ± 0.26
Ash	1.90 ± 0.23	1.91 ± 0.16	1.94 ± 0.13	1.97 ± 0.21

Table 4. Total counts of bacteria in the intestine of *R. frisii* fed different levels of Immunogen over 56 days of experimental period. All values are means ± standard deviation of triplicate tanks/treatment (n=3).

	Diet			
Bacterial community	**Control**	**IMU 1 g.kg^{-1}**	**IMU 1.5 g.kg^{-1}**	**IMU 2 g.kg^{-1}**
Total count (cfu.g^{-1})	$6.57 \times 10^4 \pm 910^b$	$6.52 \times 10^5 \pm 3100^b$	$7.09 \times 10^4 \pm 953^b$	$7.12 \times 10^4 \pm 821^b$
Gram positive*	$1.66 \times 10^4 \pm 152^b$	$1.41 \times 10^5 \pm 1527^a$	$1.87 \times 10^4 \pm 1616^b$	$1.79 \times 10^4 \pm 655^b$
Gram negative**	$4.91 \times 10^4 \pm 1892^b$	$5.11 \times 10^5 \pm 4509^a$	$5.22 \times 10^4 \pm 360^b$	$5.33 \times 10^4 \pm 1178^b$
Gram negative**	$4.91 \times 10^4 \pm 1892^b$	$5.11 \times 10^5 \pm 4509^a$	$5.22 \times 10^4 \pm 360^b$	$5.33 \times 10^4 \pm 1178^b$

Different superscript letters in the same row show significant differences (P<0.05).

DISCUSSION

The results of this experiment shows that the addition of 1 g kg^{-1} Immunogen as a prebiotic improves growth performance and survival rate of *R. frisii*. These are similar to results of Li and Gatlin (2005), Staykov *et al*., (2007), and Mohajer Esterabadi *et al.* (2010), who observed higher feed efficiencies in hybrid striped bass, rainbow trout, and *H. huso* fed Grobiotic® prebiotic, mannan oligosacchraide, and Immunogen, respectively. Although the main cause of prebiotic effect on *R. frisii* is not fully understood, colonization of beneficial Gram positive bacteria induced by dietary Immunogen might have increased the synthesis of essential nutrients such as fatty acids, protein, and vitamins (Hajibeglou and Sudagar 2011). A balanced production of essential nutrients especially fatty acids by micro-organisms was also mentioned as a reason for a better growth performance in fish (Irianto and Austin 2002). The beneficial bacteria may involve in production of essential vitamins leading to health improvement and reduced mortality. Lower numbers of dead and deformed fry in four species of ornamental fish, for instance, were reported to be the results of vitamins B1 and B12 synthesis by a prebiotic bacterial strain, *Bacillus subtilis* (Ghosh *et al.*, 2007). An improved survival rate along with the growth promotion of Gram positive bacteria at 1g kg^{-1} administration level in the current study may be attributed to reduction of harmful bacteria in the intestine induced by Immunogen fermentation. The promotion of Lactobacillus growth induced by oligosaccharide substrate was reported to limit pathogenic bacteria colonization (Ringo and Gatesoupe, 1998; Thompson *et al.*, 1999; Verschuere *et al*., 2000).

Similarly, Mahious *et al.* (2006) showed that microbial community in the gastro-intestinal tract of turbot larvae, Psetta maxima, was changed significantly by dietary inclusion of inulin and oligosaccharide as prebiotics. Furthermore the growth of beneficial Gram positive bacterial populations such as Bifedobacteria and Lactobacilia in the intestine of Atlantic salmon, Salmo salar, (Reftsi *et al.*, 2010) and Persian sturgeon, *Acipenser persicus*, (Jafarnodeh, 2010) were increased by addition of mannan oligosaccharide and Immunogen, respectively. The current results also demonstrate that there is a threshold for prebiotic inclusion (1 g.kg^{-1}) and further inclusion may yield no impacts on the performance and health status of this species. On the other hand, Olsen *et al.*, (2001) observed negative effects of high doses on micro-villous organization (disarray, lacking in some areas, and less straight microvilli) in the hindgut of Arctic charr, Salvelinus alpinus, fed a high concentration of inulin (15% of the diet).

Therefore, it can be speculated that prebiotic addition beyond the threshold (> 2 $g.kg^{-1}$) may result in negative impacts on fish performance. In this study, administration of prebiotic Immunogen did not affect the fish proximate body composition. This is in contrast to the results observed in other species such as Atlantic salmon, Salmo salar, and rainbow trout, *Oncorhynchus mykiss*, fed diets containing 10 and 20 $g.kg^{-1}$ oligosaccharides, respectively (Grisdale-Helland *et al.* 2008; Dimitroglou *et al.*, 2009). Additionally, inclusion of Immunogen was proposed to increase protein and fat contents of the fish because of an improved feed efficiency. Yet, our results do not support this idea. The contradictory results on body composition obtained from prebiotic studies may be related to species, dosage levels, fermentability of the prebiotics, and different intestinal morphologies and microbiota (Hoseinifar *et al.*, 2010). Moreover, the efficiency of prebiotic applications in fish seems to be dependent on diet composition, especially carbohydrate fraction or even environmental conditions, which may have interactive effects with the prebiotic used. Further works are necessary focusing on bacterial community using different types and levels of carbohydrate at different environmental conditions in order to fully assess any possible effects on fish performance caused by those parameters. In conclusion, the results of the current study demonstrate that inclusion of the prebiotic Immunogen as a dietary complement is capable of improving the nutrients efficiency and growth performance of *R. frisii*. Administration of this prebiotic also increases Gram positive bacteria in the intestine thereby having beneficial effects on the improvement of the host nutrition. Based on the findings of this study, supplementation of Immunogen at a level of 1 g kg^{-1} diet is suitable for feeding *R. frisii* fry to support fish growth and health.

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اثرات پربیوتیک ایمونوژن بر رشد، جامعه باکتریایی دستگاه گوارش و میزان بقا در بچه ماهی سفید (*Rutilus frisii*)

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چکیده

این تحقیق به منظور تعیین اثرات پربیوتیک ایمونوژن بر پارامترهای رشد و جامعه میکروبی دستگاه گوارش در بچه ماهی سفید انجام شد. غذای پایه با استفاده از مواد اولیه غذایی معمول منطقه ساخته شد. با افزودن چهار سطح مکمل ایمونوژن (صفر، ۱، ۱/۵ و ۲ گرم در کیلوگرم) به جیره پایه چهار جیره آزمایشی تهیه شد. برای هر جیره آزمایشی سه تکرار که مخازن فایبرگلاس ۵۰۰ لیتریبود در نظر گرفته شد. این تحقیق به مدت هشت هفته به طول انجامید و دمای آب در این مدت بین ۲۱-۲۴ درجه سانتی گراد بود. بچه ماهی سفید با وزن اولیه ۱/۷ ± ۳۰۰ میلی گرم بصورت تصادفی در مخازن آزمایشی پخش شدند. در انتهای آزمایش، میزان رشد، کارآیی غذا، جامعه میکروبی دستگاه گوارش بررسی شدند. نتایج نشان داد که افزودن ۱ گرم بر کیلوگرم ایمونوژن موجب بهبود وزن نهایی، ضریب تبدیل غذایی و نرخ رشد ویژه در بچه ماهی سفید شد ($p<0.01$) و میزان نرخ بقای بچه ماهی سفید هم با افزودن یک گرم به کیلوگرم ایمونوژن افزایش یافت ($p<0.01$). بهرحال، پروتئین، چربی و خاکستر لاشه تحت تاثیر پربیوتیک قرار نگرفت ($p>0.05$). افزودن یک گرم به کیلوگرم پربیوتیک موجب افزایش تعداد باکتریهای دستگاه گوارش شد ($p<0.05$)، ولی میزان باکتریها با افزودن میزان بیشتر پربیوتیک (۱/۵ و ۲ گرم بر کیلوگرم) تغییر نکرد. نتایج این مطالعه نشان داد که اضافه کردن ایمونوژن در سطح یک گرم به کیلوگرم غذا می تواند با تحریک رشد باکتریهای مفید دستگاه گوارش موجب بهبود کارآیی غذا و رشد بچه ماهی سفید شود.