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Effects of Different Levels of Immunogen on Growth Performance, Intestinal Bacteria Colonization and Survival Rate in *Rutilus kutum* Larvae

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Abstract: Administration of prebiotics as an alternative technique to replace antibiotics can stimulate the growth of beneficial bacteria and contribute to feed efficiency in fish.This studywas to determine the effect of Immunogenprebiotic on growth related parameters and gut micro flora in *Rutilus kutum*larvae.A basal diet was formulated by the use of common feed ingredients and the inclusion of Immunogen at the ratesof 0, 1, 1.5 and 2 g kg^{-1} leading to four experimental diets. Experimental diets were randomly assigned to one of 12 500L tanks, having three replicates per diet.Inclusion of 1g kg^{-1} Immunogen improved final weight, feed conversion ratio (FCR) and specific growth ratio (SGR) ($p<0.01$) in the larvae. The fishshowed a highersurvival rate at 1 g/kg prebiotic inclusion level ($p<0.01$). However, body composition was not influenced by prebiotic inclusion. Addition of prebiotic increased bacterial number at 1 g kg^{-1} ($P<0.05$), but further inclusion did not change bacterial number.In conclusion, administration of the prebiotic Immunogen is capable to improve the nutrients efficiency and fish performance of *Rutilus kutum* through growth stimulation by beneficial bacteria.

Key words: *Rutilus kutum* · Prebiotic · Feed Conversion · Survivalrate · Bacterial Number

INTRODUCTION

The shortage of natural resources such as fresh water and land has led to intensification of aquaculture system [1]. However, this condition may also increase the incidences of disease in fish farms due to deteriorated water quality [2]. Intensification can also increase stress level in farmed fish which may threat immune system and increase the incidences of bacterial infections [3]. Antibiotics have long been introduced as a solution to treat these infections [4]. But long term administration of antibiotics to treat bacterial diseases of farmed fish has been widely criticized because of bacterial resistance to antibiotic, elimination of gut microbial flora, high cost of these drugs and potential side effects [5, 6].

Public concerns about the negative impacts of antibiotics have reduced sharply their usesforaquaculture industry in the United States and Europe [4]. The increasing economic and social concerns about decreasing the use of antibiotics and other chemicals used in fish farming have encouraged more environmentally friendly approaches for increasing growth [7]. Therefore, the introduction of alternative techniques to replace the chemical drug and 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 [3]. Prebioticsgenerally include nutrients such as non-digestible carbohydrate, resistant starch, nutrient fiber, sugars, some peptides and proteins as

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well as some certain lipids that enter the intestine [8]. Xylo-oligosaccharides, fructo-oligosaccharides, inulin and other carbohydrates substrate have received considerable attention because oftheir health benefits to host [9, 10]. Several studies have demonstrated that prebiotics can improve growth parameters, disease resistance, gut morphology and modulate the intestinal microbiota in various aquatic species [4, 10-15]. Dietary supplementation of polysaccharide prebiotic seems to reform bacterial community in fish intestine, which improves health and feed efficacy in host [13, 16]. However, there is still little information on beneficial bacteria and the by-products produced by dietary non-digestible carbohydrate.

Commercial product of Immunogen is composed of several stimulating compounds such as β-glucan andmanan-oligosaccharide which have been used as feed additives in various animals [17] reported toaffect fish growth rate, feed efficiency, digestibility, immune responses andsurvival [14, 18-20]. In addition, supplementation of ß-glucan was stated to beefficient stimulator of non-specific immune functions in fish [19, 21].

While some literature is available on the effect of prebiotic on fish performance and microflora characteristics, there is little information related to Immunogen on *Rutilus kutum*. Therefore, the main objective of the current study is to identify the effects of Immunogen on growth related parameters and gut microflorain *R.kutum.*

MATERIALS AND METHODS

Experimental System and Animal: This study was carried out at theexperimental facility of Shahid Rajaee Fish Farm located in northern part of Iran. *R.kutum* larvae were breed at the reproduction facility of the fish farm and adapted to the experimental conditions a few days beforethestart of the experiment. Afterward, the fish with an average weight of 300 mgwere divided randomly into twelve 500L tanks with an initial stocking density of 500 individual per tank. The experiment lastedfor 8 weeks. Prebiotic used in this study, Immunogen, is composed ofmannan-oligosaccharide (18%) and β-glucans (1-3, 1-6) (30%). (Provided by Soroush Radian Co., Tehran, Iran).

A basal diet was formulated by the use of common feed ingredients in the region including fishmeal, soybean meal, meat meal, wheatmeal and so on (Table 1). The inclusion of Immunogen into the basal diet at the rates of0, 1, 1.5 and 2 g $kg^{-1}$was led to four experimental diets.

Table 1: The percentage of ingredient 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
Premix	2
Nutrient composition of the basaldiet in g/kg	
Dry matter	915.2
Crude protein	375.3
Crudefat	107.4
Carbohydrate	334.6
Crude ash	98.2

Experimental Procedure: On the 1st dayof the experiment, fish were weighed and returned to the same tank. Experimental diets were given by hand to the fish at a rate of 10% of biomass, three times per day (8.00, 13.00 and 18.00hrs). Experimental diets were randomly assigned to one of 12 tanks, having three replicates per diet. Water quality parameters were checked daily to keep appropriate ranges for the fish. The parameters were temperature, salinity, ammonium, dissolved oxygen and pH.

On day 56, all fish were weighed. Afterward, 30 fish were randomly selected from each tank and sacrificed using overdosed clove essence solution for analysis of body composition.

Chemical Analysis and Bacteria Count: Feed samples were collected and pooled at regular intervals during the experimental period and ground using a 1-mm screen before analyses. Feed and fish body were analysed for dry matter through drying samples for 24 h at 103°C until constant weight [22]. Ash content was determined by incineration in a muffle furnace for 4 h at 550°C [23]. Crude protein (N×6.25) was measured by the Kjeldahl method after acid digestion according to [20]. Lipid was extracted by petroleum ether extraction in a Soxhlet apparatus. Carbohydrate fraction was determined as dry matter minus fat, protein and ash in the feed.

At the end of the experiment, further 10larvae werecollected from each treatmentand their intestinesamples were tested for bacteria counts. Priorto dissection and homogenization, the larvae were rinsed with sterilized distilled water,cleaned with ethanol (70.0%) and then washedagain with sterilized distilled water to eliminateallexterior bacteria. The intestineswere dissected outin sterile conditions. Three intestine samples fromeach fish were taken from the middle part of the intestine for microbiological analysis. Allsampleswere diluted successively using sterilizednormal saline solution (0.85% NaCl w/v) and thenplaced onto nutrient agar plates forbacteria counts.

Fish Performance: Weight gain was determined by the difference between total initial and final body weights. FCR was calculated per tank from feed intake data and weight gain. SGR was calculated from the natural logarithm of the mean final weight minus the natural logarithm of the mean initial weight and divided by the total number of experimental days expressed as a percentage per day. The calculations were based on the wet weight of the diet.

Statistical Analysis: Data are presented as means of each treatment with standard deviation. All data were verified for normality after transformation (ASIN). One -way ANOVA was used to determine the effectsofImmunogen levels on fish performance and bacteriacounts. 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 *Rutilus kutum*are presented in Table 2. Inclusion of 1g kg^{-1} Immunogen improved fish growth, FCR and SGR ($p<0.01$), but further increase did not have an impact on growth parameters in comparison to control diet.Along the growth parameters, fish displayed higher survivalrates at 1 g kg^{-1} prebiotic in comparison to other treatments ($p<0.01$).

Body composition was not influenced by prebiotic inclusion and all measured parameters at different administration levelswere almost the same (Table 3; $P>0.05$).

Total count of bacteriainthe *R.kutum*was increased by addition of Immunogen (Table 4). Addition of the prebiotic (1g kg^{-1}) also increased Gram positive bacteriacounts but future inclusion did not change bacterial numbers ($P<0.05$).

DISCUSSION

The results of this experiment showed that the addition of 1 g kg^{-1}Immunogen as a prebioticimproves growth performance and survival rate of the fish. This is similar to results ofStaykov*et al.* andLi and Gatlin [14, 24] who observed a higher feed efficiency in hybrid striped bass and rainbow trout fed Grobiotic® prebiotic and manan-oligosacchraide. Although the main cause of prebiotic effect on *R.kutum* is not fully understood, colonization of beneficial bacteria induced by dietary Immunogenmayincrease the synthesis of essential

Table 2: Growth performance in *Rutilus kutum* fry feeding on different levels of Immunogen (IMU) over a60-day experimental period All values are means of three replicates (tanks)/treatment ±standard deviation.

	Diets			
Parameters	Control	IMU1 g/kg	IMU 1.5 g/kg	IMU 2 g/kg
Initial weight (mg)	301.1±1.3	299.2±2.1	300.3±1.8	302.4± 1.5
Final weight (mg)	706± 3.5^{b}	718.6±2.6^{a}	705.8±3.3^{b}	708.9±4.2^{b}
Final length(mm)	4.80±0.02^{b}	5.01±0.03^{a}	4.82±0.08^{b}	4.81±0.03^{b}
SGR (%)	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 (%)	87.38±2.3^{b}	92.33±1.2^{a}	87.08±2.5^{b}	87.07±2.6^{b}

Differentsuperscriptlettersat the same row showsignificantdifferences

Table 3: Bodycompositionin *Rutilus kutum* feedingondifferent levels of Immunogen (IMU) overa 60-dayexperimentalperiod All valuesaremeansofthreereplicates (tanks)/ treatment ± standarddeviation

	Diets			
Parameters	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 *Rutilus kutum* feedingon differentlevels of Immunogen (IMU) overa60-dayexperimental period Allvaluesaremeans ofthreereplicates (tanks)/ treatment ± standard deviation

	Diet			
Bacterial community	Control	IMU 1 g/kg	IMU 1.5 g/kg	IMU 2 g/kg
Total count	$6.57\times10^{4}\pm910^{b}$	$6.52\times10^{5}\pm3100^{b}$	$7.09\times10^{4}\pm953^{b}$	$7.12\times10^{4}\pm821^{b}$
Gram positive*	$1.66\times10^{4}\pm152^{b}$	$1.41\times10^{5}\pm1527^{a}$	$1.87\times10^{4}\pm1616^{b}$	$1.79\times10^{4}\pm655^{b}$
Gram negative**	$4.91\times10^{4}\pm1892^{b}$	$5.11\times10^{5}\pm4509^{a}$	$5.22\times10^{4}\pm360^{b}$	$5.33\times10^{4}\pm1178^{b}$

Differentsuperscriptlettersat the same rowshowsignificantdifferences

nutrients such as fatty acids, protein and vitamins [25]. A balanced production of essential nutrients, especially fatty acids by micro-organisms is also mentioned as a reason for a better growth performance in fish [26, 27]. The beneficial bacteria may involve in production of essential vitamins and through which improve health and reduce mortality. A lower number of dead and deformed fry in four species of ornamental fish was reported to cause by synthesize of vitamin B1 and B12 by prebiotic bacterial strain,*Bacillus subtilus* [28].

Animproved survival rate along with the growth promotion of Gram positivebacteria at 1 g kg^{-1} administration level in the current studymay beattributed to reduction of harmful bacteria in the intestineinduced by Immunogen fermentation. The promotion of Gram positive bacteria growth induced by oligo-saccharide substrateis limitingpathogenic bacteria colonization [7, 20, 28]. Similarly, [29] showed that microbial community in the gastro-intestinal of turbot larvae (*Psettamaxima*) was changed significantly by dietary inclusion of inulin and oligosaccaride as prebiotics.The growth of beneficial bacteria population such as bifedobacteria and *Lactobacilia* in the intestine of Atlantic salmon and Persian sturgeon were increased by addition of manan-oligosaccharide and Immunogen [30, 31].

The current results also demonstrate that there is a threshold for prebiotic inclusion (1 g kg^{-1}) and further inclusion may yield no impactson the performance and healthstatus of the fish [32] showed negative effects of high dosesonmicrovillous organization (disarray, lacking in some areas and lessstraight) in the hindgutof Arctic charr, *Salvelinus alpinus* fed a high concentration of inulin (15% of the diet). Therefore, we hypothesize that prebiotic additionbeyond the threshold (> 2 g kg^{-1}) may result in negative impacts on fish performance.

Body proximate analyses in this study showed that administration of prebiotic Immunogendid not affect the fish body composition (Table 3). This is in contrast to the results observed in other species such as Atlantic salmon and rainbow trout fed diets containing 10 and 20 g kg^{-1} oligosaccharides, respectively [33, 34]. Inclusion of Immunogen was proposed to increase protein and fat content ofthe fish because of an improved feed efficiency. However, the results do not support the idea. The contradictory resultsobtainedfrom prebiotic studies on body composition may be related to species, dosage levels, fermentability ofthe prebiotics and the different intestinal morphology and microbiota [35]. Moreover, the efficiency of prebiotic applications in fish seems to be dependent on diet composition, especially carbohydrate fraction or even environmental condition, which may have an interaction effect with the prebiotic used. Further works are necessary focusingon bacteria community using different types and levels of carbohydrateat different environmental conditions in order to fully assess anypossible effectson fish performance caused by those parameters.

In conclusion, the results of the current study demonstrate that inclusion of the prebiotic Immunogen as adietary complement is capable of improving the nutrients efficiency and growth performance of *Rutilus kutum*. Administration of this probiotic also increases Gram positive bacteria in the intestine, thereby having beneficial effects on the improvement of hostnutrition. Based on the findings ofthis study, supplementation of Immunogen at a level of 1 g kg^{-1} diet is suitable for feeding *R.kutum* fingerlings to support fish growth and health.

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