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Short communication

Effects of dietary supplementation of inosine monophosphate on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

This study was investigated to examine the effects of dietary inosine monophosphate (IMP) supplementation on growth performance, feed utilization, innate immunity, hematological parameters and disease resistance of juvenile olive flounder. Five experimental diets were formulated to contain IMP at levels of 0, 0.1, 0.2, 0.4 and 1.0%. All diets were maintained isonitrogenous (48% crude protein) and isocaloric (20.7 MJ/kg diet). Triplicate groups of olive flounder (initial body weight, 7.5 ± 0.02 g) were fed one of the experimental diets to apparent satiation (twice a day) for 14 weeks. Final body weight of fish fed 0.1–0.2% IMP were significantly higher than that of fish fed the 1.0% IMP. Groups of fish fed 0.2 or 0.4% IMP diet had significantly higher myeloperoxidase and lysozyme activities than fish fed the control diet. However, nitro-blue-tetrazolium and superoxide dismutase activities were not significantly different among all treatments. In the challenge test against *Streptococcus iniae*, cumulative mortality of fish fed IMP supplemented diets was significantly lower (15%, 4%, 4% and 9% for 0.1%, 0.2%, 0.4% and 1.0% IMP, respectively) than that of fish fed the control group (87%). The results suggest that IMP supplementation of 0.46–1.84 g into a kg of fish meal based diet (0.1–0.4% IMP product) can enhance innate immunity and disease resistance of olive flounder.

1. Introduction

A wide array of antibiotics including ampicillin, doxycycline, streptomycin, nitrofurantoin, furazolidone and oxytetracycline have been used to treat infectious bacterial diseases in aquaculture productions [1,2]. However, the efficacy of these treatments depends on many factors such as drug concentration, infection intensity, time of treatment and a range of water parameters [3,4]. Extensive antibiotic usage can lead to environment pollution and drug resistance against pathogenic agents [5,6]. The accumulation of chemicals in aquaculture products has also been noted as a concern by consumers [7]. Utilization of natural compounds to improve non-specific immune response of fish and to prevent outbreak of diseases has been considered as a feasible option for sustainable and antibiotic-free aquaculture systems.

Nucleotides (NT) have been regarded as non-essential nutrients. However, this accepted notion has been challenged by various studies which have suggested that deficiency of dietary NT may damage important tissues such as liver, heart and intestine, and immune functions [8–10]. NT has been reported to be beneficial for infants since they positively affect intestinal and hepatic functions, lipid metabolism, immune responses, and tissue development and repair under normal conditions [11–13]. Studies with terrestrial animals have indicated that NT can influence phagocyte activity [14], interleukin-2 production [15] and natural killer cell activity [16]. In fish, current research on NT have observed growth improvement in juvenile stages, enhancement of larval quality via broodstock fortification, alteration of intestinal structure and enhancement of stress tolerance [17–19]. NT has also received attention as potential immunostimulants in many fish species. Common carp *Cyprinus carpio* fed NT isolated from yeast RNA was significantly improved in terms of serum complement activity, lysozyme activity and superoxide anion production of head kidney [20]. Murthy et al. [21] also reported that Pacific white shrimp *Litopenaeus vannamei* fed a commercial product containing NT (Optimun, Chemoforma, Switzerland) had enhanced respiratory burst and total hemocyte count. However, information pertaining

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to the synthesis and metabolism of NT remains limited in fish species.

NT consists of a nitrogenous base, a pentose sugar, and one or more phosphate groups. Inosine monophosphate (IMP) is the ribonucleotide and is the first compound formed during the synthesis of purine. Also, IMP becomes adenine monophosphate (AMP) and guanine monophosphate (GMP) within several steps. Amino acids, glycinbetaine, oligopeptides, nucleosides and NT have been known to stimulate taste receptors of many fish species [22]. IMP is used as an additive to enhance the taste of food [23] and the synergistic effects between IMP and other stimulating compounds including amino acids or nucleosides influence the intensity of the taste [24,25].

Olive flounder is currently the most important marine aquaculture species in Korea. Recently, the finfish aquaculture in Korea has been facing many problems related to disease. *Streptococcus iniae* is the main etiological cause of streptococcosis in wild and farmed fish. This bacterial infection has been thought to be the primary reason for poor growth and declining fish productivity [26,27]. Research on IMP in fish is needed to offer insights into interactions between physiological responses and practical solutions in order to prevent infectious diseases [28]. Therefore, this study was designed to investigate the effects of dietary supplementation of IMP on growth performance, feed utilization, innate immunity, hematology and disease resistance against *S. iniae* in olive flounder.

2. Materials and methods

2.1. Experimental diets

Five experimental diets were formulated to contain a commercial IMP product at levels of 0, 0.1, 0.2, 0.4 and 1.0% (designated as Con, 0.1%, 0.2%, 0.4% and 1.0%, respectively). IMP concentration of the product was 46% and provided by Department of Global Marketing, CJ CheilJedang Corp. All diets were formulated to be isonitrogenous (48% crude protein) and isocaloric (20.7 MJ/kg diet). Optimum dietary protein and lipid requirements of olive flounder are estimated to be about 46% and 13%, respectively [29], and the experimental diets were formulated to cover the nutrient requirements of olive flounder. Alanine was supplemented in the diets to balance total nitrogen. The dietary formulation and proximate composition are provided in Table 1. All dry materials were thoroughly mixed with fish oil and 20–30% double distilled water and extruded through a meat chopper machine (SMC-12, Kuposlice, Busan, Korea) at 5 mm diameter. The diets were dried in room temperature with electric fan for 48 h, crushed into desirable particle size and stored at −20 °C until use.

Table 1
Formulation and proximate composition of diets for junvenile olive flounder (% DM).

Ingredients	Experimental diets				
	Con	0.1%	0.2%	0.4%	1.0%
Basal composition[a]	97.8	97.8	97.8	97.8	97.8
IMP	0.0	0.1	0.2	0.4	1.0
Alanine	1.2	1.1	1.1	0.9	0.5
Cellulose	1.0	1.0	0.9	0.9	0.7
Proximate composition (%)					
Moisture	11.6	13.1	14.1	13.5	15.6
Crude protein	49.6	50.0	49.9	49.8	49.9
Crude lipid	15.2	14.9	14.3	15.0	14.9
Ash	9.5	9.9	9.2	9.2	9.4

[a] Basal composition (%): white fish meal, 48.0; soybean meal, 8.0; corn gluten meal, 8.0; wheat flour, 21.3; squid liver oil, 10.0; mineral premix, 1.0 [56]; vitamin premix, 1.0 [57]; choline chloride, 0.5.

2.2. Fish and feeding trial

Juvenile olive flounder was transported from a private hatchery (Jeju Island, Korea) to Marine and Environmental Research Institute, Jeju National University, Korea. The fish were fed a commercial diet for several weeks in order to be acclimated to the experimental facilities and conditions. 600 olive flounder (initial body weight, 7.5 ± 0.02 g) were randomly distributed into fifteen 150 L capacity polyvinyl circular tanks (40 fish per tank) with three replications per dietary treatment. All tanks were supplied with filtered seawater at a flow rate of 3 L/min and aeration to maintain enough dissolved oxygen. The photoperiod was scheduled by 11:13 h light/dark by fluorescent light. Water temperature ranged from 19 to 24 °C in accordance with seasonal change. Fish were fed the experimental diets to apparent satiation (twice a day, 08:00 and 17:00 h) for 14 weeks. Uneaten feed was collected 30 min after feeding and reweighed to determine feed intake. Feeding was stopped 24 h prior to weighing to minimize stress.

2.3. Sample collection

At the end of feeding trial, three fish per tank (9 fish per dietary treatment) were randomly sampled 6 h after the last feeding. The fish were anaesthetized with 2-phenoxyethanol (200 ppm) and blood samples were taken from caudal vein with heparinized syringes for determination of hematocrit, hemoglobin and respiratory burst activity. After the above mentioned measurements with whole blood, plasma was collected by centrifugation at 5000× *g* for 10 min. Another set of blood samples (9 fish per dietary treatment) were taken without heparin and allowed to clot at room temperature for 30 min. Serum was collected following centrifugation at 5000× *g* for 10 min and stored at −70 °C for analyses of non-specific immune responses. Experimental protocols followed the guidelines of the Animal Care and Use Committee of Jeju National University.

2.4. Growth measurements and hematological analysis

Following the feeding period, all fish in each tank were weighed and counted to compute final body weight, feed conversion ratio (FCR), protein efficiency ratio (PER) and survival. At the end of 8th week, instantaneous feed intake (palatability test) was determined under the same experimental tanks and conditions. To measure palatability of experimental diets, diets were weighed with 50 g and the fish fed their respective diet by hand for 3 min. Then remaining feeds were reweighed to determine the palatability activity. The test was conducted 3 times (3 days) and palatability activity was expressed as consumed feed/10 g fish. Hematocrit was determined for three fish per tank by a microhematocrit technique [30]. Hemoglobin, total protein, glucose and total cholesterol were determined in the same three fish by an automated blood analyzer (SLIM, SEAC Inc, Florence, Italy). Analyses of crude protein, moisture and ash in the diets were performed by standard methods [31]. Dietary lipid was determined by the method of Folch et al. [32].

2.5. Non-specific immune responses

The oxidative radical production by phagocytes during respiratory burst was measured by the nitro-blue-tetrazolium (NBT; Sigma, USA) assay described by Anderson and Siwicki [33]. Lysozyme activity was measured according to the turbidimetric method described by Hultmark et al. [34]. Myeloperoxidase (MPO) activity was measured according to Quade and Roth [35] with a slight modification by Kumari and Sahoo [36]. Superoxide dismutase

Table 2
Growth performance of olive flounder fed five experimental diets containing different levels of IMP for 14 weeks.

	IBW[a]	FBW[b]	IFI[c]	FCR[d]	PER[e]	Survival (%)
Con	7.5 ± 0.02	65.0 ± 0.9[ab]	0.25 ± 0.02	1.00 ± 0.02	2.23 ± 0.03	86.3 ± 8.8
0.1%	7.5 ± 0.01	68.3 ± 0.9[b]	0.26 ± 0.02	0.95 ± 0.01	2.41 ± 0.03	91.7 ± 3.8
0.2%	7.5 ± 0.04	70.2 ± 1.6[b]	0.29 ± 0.05	0.97 ± 0.04	2.40 ± 0.09	81.3 ± 7.8
0.4%	7.5 ± 0.02	65.8 ± 3.8[ab]	0.28 ± 0.03	0.98 ± 0.03	2.37 ± 0.08	87.5 ± 5.0
1.0%	7.5 ± 0.03	61.3 ± 3.2[a]	0.27 ± 0.06	1.01 ± 0.07	2.31 ± 0.16	88.3 ± 8.8

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($p < 0.05$).
[a] Initial body weight (g).
[b] Final body weight (g).
[c] Instantaneous feed intake (g) = average feed intake/total weight x 10.
[d] Feed conversion ratio = dry feed fed/wet weight gain.
[e] Protein efficiency ratio = wet weight gain/total protein given.

(SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 substrate (a water soluble tetrazolium dye) and xanthine oxidase using a SOD assay kit (Fluka, 19160) according to the manufacturer's instructions.

2.6. Challenge test

At the end of 14 weeks feeding trial, fifteen olive flounder from each tank (45 fish/treatment) were randomly collected and intraperitoneally injected with *S. iniae* (ATCC 29178) suspension (1×10^7 CFU/ml). *S. iniae* was provided by the Marine Applied Microbes and Aquatic Organism Disease Control Laboratory at the Department of Aquatic Biomedical Sciences, Jeju National University [37]. Injected fish were distributed into fifteen 60 L plastic tank for a challenge test. Fish behavior and mortality were monitored and recorded for 21 days. The liver and kidney of dead fish were streaked on modified selective agar to confirm death from *S. iniae*.

2.7. Statistical analysis

All diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in means values were compared using Tukey's HSD at the 5% level of significance ($P < 0.05$). Data are presented as mean ± SD. Percentage data were arcsine transformed before statistical analysis.

3. Results

At the end of 14-week feeding trial, growth rate of fish fed the diets supplemented with 0.1–0.2% IMP were significantly higher than those of fish fed the high dietary concentration (1.0%) (Table 2). However, no significant difference was observed in FCR, PER and survival among all the fish groups. Instantaneous feed intake test and hematological parameters of fish were not significantly affected by the supplementation of IMP (Tables 2 and 3). Fish fed 0.2–0.4% IMP diets had significantly higher MPO and lysozyme activities than fish fed the control diet (Table 4). The NBT and SOD activities were not significantly different in all fish groups. Symptoms such as dark skin, hemorrhages on dorsal fin, erratic swimming in addition to mortalities began to appear in the control groups three days after *S. iniae* injection. Cumulative mortality in the control group recorded to approximately 87% by 21 days after the challenge test with *S. iniae* (Fig. 1). Interestingly, however, the mortalities of fish groups fed IMP supplemented diets were significantly lower (15%, 9%, 4.4% and 4.4% for 0.1%, 0.2%, 0.4% and 1.0% IMP, respectively) than that of fish fed the control diet.

4. Discussion

Numerous studies on humans and animals have reported that dietary supplementation of NT has positive influence on growth performance, immune responses and disease resistance [11,16,38]. The present study showed that growth performance of olive flounder might be improved by the dietary supplementation of IMP at 0.1–0.2% levels. The results are in agreement with previous studies [17,39]. Adamek et al. [40] observed that optimum dietary concentration (0.06–0.25%) of a commercial NT product, Ascogen (Chemoforma, Augst, Switzerland), increased growth rate and feed efficiency while high dietary concentration (5%) caused growth depression in rainbow trout *Oncorhynchus mykiss* (170 g size) during a 37 day feeding trial. Similarly, a high dietary concentration of IMP (1.0%) in the present study resulted in depressed growth performance compared to lower levels of dietary IMP (0.1–0.2%). Reduced growth performance by high NT levels might be explained by the toxicity of serum uric acid derived from purine base [41]. In monogastric animals, high levels

Table 3
Hematological parameters of olive flounder fed five experimental diets containing different levels of IMP for 14 weeks.

	Hematocrit (%)	Hemoglobin (g/dL)	Total protein (g/dL)	Glucose (mg/dL)	Total cholesterol (mg/dL)
Con	36.3 ± 7.1	3.9 ± 0.9	4.6 ± 0.3	19.1 ± 2.9	268 ± 35
0.1%	38.2 ± 4.1	4.6 ± 0.6	4.3 ± 0.1	23.3 ± 2.4	264 ± 32
0.2%	36.2 ± 0.7	4.3 ± 0.1	4.4 ± 0.1	21.2 ± 9.9	256 ± 1
0.4%	37.4 ± 4.3	4.7 ± 0.8	4.5 ± 0.2	20.1 ± 2.0	264 ± 23
1.0%	39.2 ± 2.5	4.6 ± 0.1	4.3 ± 0.1	22.0 ± 5.7	260 ± 8

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($p < 0.05$).

Table 4
Nitro blue tetrazolium (NBT), myeloperoxidase (MPO), lysozyme and superoxide dismutase (SOD) activities of olive flounder fed five experimental diets containing different levels of IMP for 14 weeks.

	NBT (absorbance)	MPO (absorbance)	Lysozyme (U/ml)	SOD (% inhibition)
Con	0.89 ± 0.05	2.93 ± 0.12[a]	3.23 ± 0.12[a]	65.2 ± 11.9
0.1%	0.94 ± 0.02	3.18 ± 0.07[ab]	4.94 ± 1.26[ab]	72.6 ± 9.1
0.2%	0.92 ± 0.13	3.30 ± 0.18[b]	5.74 ± 0.98[b]	75.9 ± 7.2
0.4%	0.91 ± 0.11	3.23 ± 0.21[b]	4.33 ± 1.89[ab]	72.9 ± 9.2
1.0%	0.89 ± 0.11	3.04 ± 0.04[ab]	3.35 ± 0.35[ab]	66.0 ± 12.1

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($p < 0.05$).

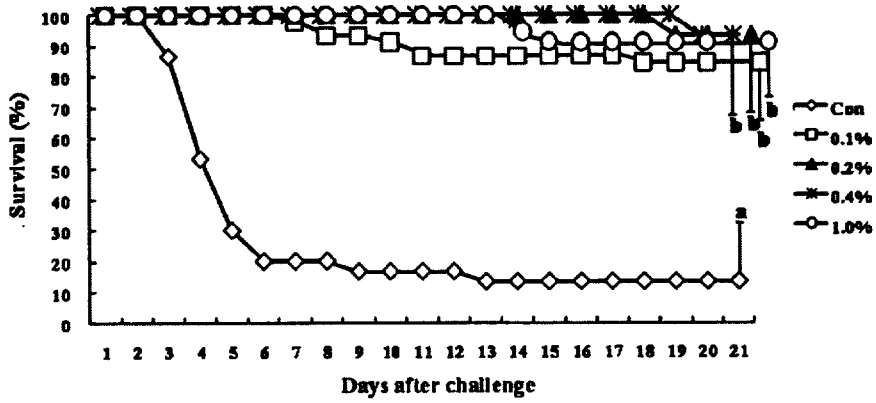

Fig. 1. Cumulative mortality of olive flounder fed five experimental diets containing different levels of IMP after challenge with *Streptococcus iniae* by intraperitoneal injection. Data represents the average ± SD from a triplicate.

of dietary NT increased plasma uric acid and produced toxicological effects and disturbances on metabolism of protein, fat and carbohydrate [42,43]. Subsequent studies, however, suggested that salmonids might be able to metabolize relatively high levels of NT by virtue of their active liver uricase [44,45]. Rumsey et al. [46,47] concluded that salmonids were not adversely affected either nutritionally or physiologically by high dietary NT concentrations. Regardless of the dietary NT level, studies have reported unaffected growth performance. Juvenile channel catfish *Ictalurus punctatus* fed diets supplemented with a purified NT mixture (IMP, AMP, GMP, UMP and CMP at ratio of 1:1:1:1:1) were not affected in terms of weight gain, feed intake, FER or survival [48]. Red drum *Sciaenops ocellatus* was not also influenced with respect to weight gain and feed efficiency [19,49]. These results indicate that growth performance of fish to exogenous NT can be variable by dietary basal NT levels from incorporated fish meal level or fish species tested [49]. Responses by the supplemented NT would be more dramatic when diets contain limited amount of basal NT [19].

Research on terrestrial animals and humans has indicated that dietary NT is crucial for optimal functioning of the immune system [50]. NT could enhance the humoral (specific) branch of immune response in both mice and humans [51,52]. A number of studies reported that fish fed NT-supplemented diets can be positively affected by increased alternative complement activity, lysozyme activity, immunoglobulin M level and extracellular superoxide anion activity [17,19]. In the present study, NBT, MPO, lysozyme and SOD activities were analyzed to verify non-specific immune responses of fish. Olive flounder fed diets supplemented with 0.2–0.4% IMP had significantly higher MPO and lysozyme activities compared to fish fed the control diet without NT. Low et al. [53] found that dietary NT improved expression of immunoglobulin M in gill and spleen of turbot fed a NT-supplemented diet. Further studies are necessary to clarify the non-specific immune responses at the gene level. Commercial NT products contain other compounds such as trace minerals, nucleosides, free amino acids and polysaccharides. Thus, information as to concentration and contents of NT is limited because most fish studies have used commercial NT products. It is, therefore, necessary to evaluate optimum levels of NT for innate immunity of several fish species. The calculated supplemental concentrations of IMP in this study was 0 (Con), 0.46 (0.1%), 0.92 (0.2%), 1.84 (0.4%) and 4.60 g (1.0%) per kg diet. Fish meal is rich in NT and contains approximately 2 g NT/kg [11]. The control diet in the present study presumably contained ~ 0.1% NT. Therefore, it is suggested that the optimum NT dietary level is approximately 0.14–0.28% for olive flounder. Lin et al. [39] reported that dietary concentration of 0.15% mixed-purified NT (mixture of IMP, AMP, GMP, UMP and CMP at ratio of 1:1:1:1:1) positively influenced on growth performance and immunity of the grouper *Epienphelus malabaricus* and that purified IMP level at 0.15% in diet also resulted in positive performances in the parameters tested. The suggested optimum NT level for grouper seems low compared to the optimum range observed in the present study. However, the discrepancy in the optimum level between the two studies is likely to be due to differences in NT purity or fish species.

The non-specific immune system is greatly important for disease resistance in fishes [54]. Dietary NT can improve disease resistance of fish to various pathogens. Li et al. [55] reported that dietary supplementation of oligonucleotides from yeast RNA can increase disease resistance of hybrid striped bass (140 g size) against *S. iniae*. Tahmasebi-Kohyani et al. [17] reported that cumulative mortality of rainbow trout (initial body wt, 23 g) fed NT-supplemented diets (1–2%) was recorded to approximately 39%, meanwhile fish group fed an unsupplemented-NT diet was observed to have 85% mortality after challenge with *S. iniae* after a 21-day feeding trial. They suggested that dietary NT can enhance immune systems in fish and thereby increase resistances to pathogens. In the present study, initial mortality of the control group began 3 days after the challenge, while IMP supplemented group began after 7 days. This result indicates that dietary supplementation of IMP can enhance resistance to bacterial infection possibly by increased or boosted immune responses found in improved MPO and lysozyme activities. The exact mechanism through which this happens needs to be further investigated.

In conclusion, although optimum levels of IMP have the potential to increase growth performance, high levels over 0.46% (1% IMP product) can cause negative effects on growth performance in olive flounder. Innate immunity and disease resistance against *S. iniae* could be improved in fish fed optimum IMP level. The optimum supplementation level into a fish meal based diet appears to be between 0.46 and 1.84 g IMP/kg (0.1–0.4% IMP product) in practical fish feed formulation for juvenile olive flounder.

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