Studies on the nucleotide nutrition and development of functional feeds for cultured marine species

(海産養殖種におけるヌクレオチドの効果と機能性飼料開発に関する研究)

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2016

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Major Subject: Aquatic Animal Nutrition

The United Graduate School of Agricultural Sciences

Kagoshima University, Faculty of Fisheries

Kagoshima, Japan

September 2016

Dedicated to

My late father, mother, sister, brothers & my beloved wife

And all those who inspired me during my entire study life

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ABSTRACT

Although nucleotides (NT) have long been implicated as feed attractants in both vertebrate and invertebrate species, research into potential growth and health benefits of dietary NT in aquaculture species has just been implemented in early 2000s. To date, research pertaining to NT nutrition in fishes has shown rather consistent and encouraging, beneficial results in fish health management. Research to date on dietary NT has focused mainly on a mixture of NT, rather than individual types of NT. Therefore, additional research to explore the effects of various individual NT along with mixed NT is necessary to gain a better understanding of NT nutrition in fish. Red sea bream, *Pagrus major*, and amberjack, *Seriolla dumerili*, are the two most important aquaculture species in East Asia. Intensive aquaculture of these species often caused stressful conditions which negatively affected their growth and health performances. Under these circumstances, research on dietary modulation for potential growth and health management is necessary for sustainable culture of these species. In this regards, development of functional feed through supplementing functional nutrients such as NT could be an effective alternative strategy. In the present study, both individual and mixed NT have been evaluated as functional nutrients and individual NT/nucleoside has been supplemented to develop functional feeds for these two species.

The efficacy of NT related products was evaluated initially in a feeding trial through supplementing a basal diet with NT related products such as nucleoside by- products (NBP) and inosine at 1, 3 and 0.03, 0.1%, respectively to formulate five experimental diets, including a control diet. After feeding the experimental diets to juvenile red sea breams for 60 days, results suggested that inosine and low concentration of NBP (1%) could be effectively used as dietary supplements for better growth and health performance.

In the second stage of research, six separate studies were conducted to optimize the supplementation of important purine and pyrimidine NT, *viz*. inosine 5′ monophosphate (IMP), adenosine 5′ monophosphate (AMP), guanosine 5′ monophosphate (GMP), uridine 5′ monophosphate (UMP) and cytidine 5′ monophosphate (CMP) for juvenile red sea bream and inosine nucleoside for juvenile amberjack. Simultaneously, functional effects of these supplements have been evaluated. In red sea bream, their optimal levels of IMP, inosine, UMP were about 0.4%. The optimal supplementation levels of GMP and CMP ranged from 0.45 to 0.48% and from 0.44 to 0.50%, respectively. In contrast, relatively low and a wide range of optimum AMP supplementation level were observed based on the regression analysis (0.16% for growth and 0.41 % for immunity). While, in the case of amberjack supplementation of inosine at 0.6% showed the optimum performances of growth and health parameters. Overall, the growth, feed utilization, stress resistance, intestinal health and immune responses of both species were enhanced by dietary supplementations.

To observe the functional effects of mixed purified NT, a feeding trial on juvenile red sea bream was also conducted for 56 days. Five dietary levels of purified NT mixture containing equal portion of IMP, AMP, GMP, UMP and CMP were supplemented to the basal diet (control) at 0.5, 1.0, 1.5, 2.0 and 2.5 g kg^{-1} to formulate a total of six experimental diets. The results showed that dietary administration of 1.0-1.5 g kg^{-1} mixed NT was able to promote growth, immune responses and stress resistance.

In the final step of this study, inosine and IMP were supplemented in alternative protein (dehhulled soybean meal and soyprotein concentrate, SPC) based diet as an initial step of low/non fishmeal (FM) based functional feed development for amberjack and red sea bream. Results indicated that amberjack can effectively utilize at least half of the replaced FM protein with soybean meal protein without any compromising growth performances. Moreover, inosine supplementation in 50 to 75% FM replacement groups could be helpful to improve the digestibility, immune responses, stress resistance and gut health condition of amberjack. In the case of red sea bream trial, results showed that supplementation of IMP increased the efficiency of utilizing SPC ($\leq$75%) as a sole protein source in the diet. Fish growth, feed utilization performances and blood chemical parameters were not significantly different up to 75% FM replacement groups in comparison with complete FM based control diet. In terms of non specific immune responses, all replacement diet groups showed improved immune responses when compared with control diet group. Based on these studies, NT can be used as a potential functional supplement for the development of functional feeds for amberjack and red sea bream.

学 位 論 文 要 旨

氏　名	**Md. Sakhawat Hossain**
題　目	海産養殖種におけるヌクレオチドの効果と機能性飼料開発に関する研究 **(Studies on the nucleotide nutrition and development of functional feeds for cultured marine species)**

ヌクレオチド（NT）が脊椎動物や無脊椎動物に対する誘引効果を有することは、以前から知られていたが、2000年以降は養殖種の成長や健康状態に及ぼすNTの添加効果について研究が進み、現在では魚類の健康改善効果が着目されている。しかし、その研究の多くが数種のNT混合物を用いたもので、個々のNTの効果に関する研究例は少なく、魚類におけるNTの効果を明らかにするためには、各種NTを単独で評価する必要がある。マダイとカンパチは東アジアにおける重要な養殖種であり、集約的な養殖がなされていが、環境の悪化などにより成長や健康状態の低下がしばしばみられる。これら魚種の持続的養殖には成長や健康を改善する飼料が必要であり、機能性物質を添加した機能性飼料の開発が望まれている。本研究では、各種NTのマダイ及びカンパチに対する添加効果を検討するとともに、NTを用いた機能性配合飼料の開発を目的として行った。

まず、マダイに対する核酸関連物質の効果を検討するために、ヌクレオシド副産物（NBP）を1%, 3%、またはイノシンを0.03%、0.1%添加した試験飼料を用いて60日間の飼育試験を行い、NBP1%区及びイノシン添加区で成長と健康状態が改善されることを明らかにした。

次に、マダイに対するイノシンーリン酸（IMP）,アデノシンーリン酸（AMP）,グアノシンーリン酸（GMP）,シチジンーリン酸（CMP）,ウリジンーリン酸（UMP）の添加効果とカンパチにおけるイノシンの添加効果と至適添加量を調べるために6つの飼育実験を行った。飼育実験の結果、マダイにおけるIMP,イノシン、UMPの至適添加量は、0.4%、GMPは0.45%、CMPは0.44%であった。また、AMPの至適添加量は、成長を指標とした場合0.16%、免疫応答を指標とした場合0.41%であった。いずれの試験でも、NTまたはイノシンの添加は、成長、飼料効率、環境ストレス耐性、消化管構造及び免疫応答に影響を与えることが明らかにされた。

次に、マダイに対するNT混合物の添加効果を調べるために、IMP,AMP,GMP,UMP及びCMPを等量混合した混合物を用い、飼料中に混合物を0%、0.5%、1.0%、1.5%、2.0%及び2.5%添加した6種の試験試料を用いて、56日間の飼育実験を行った。飼育実験の結果、NT混合物1.0%添加により成長、免疫応答及び環境ストレス耐性に改善が見られた。

最後に、マダイ・カンパチ用無魚粉・低魚粉飼料の開発を目的として、魚粉を脱脂大豆粕または大豆タンパク濃縮物で代替した飼料に対するイノシンまたはIMPの添加効果を、マダイ及びカンパチを用いて調べた。カンパチでは、魚粉の50%を大豆タンパクで代替しても、イノシン添加区では対照区と同等の成長を示した。また、魚粉50%及び70%代替区では、イノシン添加によって消化吸収率、免疫応答、環境ストレス耐性及び消化管の形状に改善が見られた。マダイでは、IMP添加により、魚粉75%添加までは、成長、飼料効率及び血液化学性状に関して対照区とほぼ同等の値を示した。また、自然免疫応答では、IMP添加区は対照区に比べ改善される傾向を示した。

本研究の結果、ヌクレオチド及び核酸関連物質は、マダイの成長や健康状態を改善する機能性を有し、機能性飼料の素材として有望であることが示された。

LIST OF PUBLICATIONS, SYMPOSIUM PARTICIPATION, PRESENTATIONS AND ACADEMIC AWARDS

A. Publications in Peer Reviewed Journals:

1. HOSSAIN, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M . Effects of dietary administration of guanosine monophosphate on the growth, digestibility, innate immune responses and stress resistance of juvenile red sea bream, *Pagrus major*. Fish and Shellfish Immunology, Vol.57, pp. 96-106 , August 2016.

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3. HOSSAIN MS, Koshio S, Ishikawa M, Yokoyama S, Sony NM, Dawood MAO, et al. Efficacy of nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of juvenile red sea bream, Pagrus major. Aquaculture , vol. 464, pp. 8-16. June 2016.

4. Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M., Usami, M., Ono, S., Fujieda, T. Inosine supplementation effectively provokes the growth, immune response, oxidative stress resistance and intestinal morphology of juvenile red sea bream, Pagrus major Aquaculture Nutrition (in press; doi:10.1111/anu.12463), online published: May, **2016**

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7. Dawood, M. A.O., Koshio, S., Ishikawa, M.,Yokoyama, S., El-Basuini, M.F., **Hossain, M.S.,** Nhu, T.H.,.S., Dossou, S., Moss, A. 2016. Effects of dietary supplementation of Lactobacillus rhamnosus or/and Lactococcus lactis on the growth, gut microbiota and

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9. **Hossain, M.S.,** Kader, M.A., Dey, T., Sony, N.M., Bulbul, M., Koshio, S., Effect of high inclusion of rendered animal by-product ingredients on growth, digestibility and economic performances in climbing perch *Anabas testudineus*. Aquaculture Research, doi:10.1111/are.12936, pp 1-10, August, 2015.

10**.** Dawood, M. A.O., Koshio, S., Ishikawa, M.,Yokoyama, S., El-Basuini, M.F., **Hossain, M.S.,** Nhu, T.H., Moss, A.S., Dossou, S., Wei, H.. Dietary supplementation of β-glucan improves growth performances, the innate immune response and stress resistance of red sea bream, Pagrus major. Aquaculture Nutrition, doi: 10.1111/anu.12376, August, 2015

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19. Mamun, M.A.A., Hossain M.A., **Hossain, M.S** and Ali, M. L. Effects of different types of artificial substrates on nursery production of freshwater prawn, *Macrobrachium rosenbergii* (de Man) in recirculatory system. Journal of the Bangladesh Agricultural University. Vol. 8, No. 2, pp. 333-340, 2010.
20. Ahmed, G.U., Hossain, M.M. and **Hossain, M.S**. Histopathology of diseases of an air breathing teleost, *Anabas testudineus* (Bloch) from freshwater fisheries of Bangladesh. International Journal of Sustainable Agricultural Technology. Vol. 5, No. 4, pp.75-81, July 2009.
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22. Ferdous, M.J., **Hossain, M.S.,** Mian, S. and Hossain, M.A.R. Effect of feed deprivation on growth, survival and economics of freshwater prawn, *Macrobrachium rosenbergii* and Indian major carp rohu, *Labeo rohita* polyculture. International Journal of Animal and Fisheries science, Vol. 2, No. 1, pp. 157-163, 2009.
23. Khan, S., **Hossain, M.S.,** Hossain, M.M. Production and economics of GIFT strain of tilapia (*Oreochromis niloticus*) in small seasonal ponds. Progressive Agriculture. Vol.19, No.1, pp. 97-104, 2008.

B. Publications in Contribution

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Ono, S. and Fujieda, T. Effects of dietary administration of inosine on growth, immune response, oxidative stress and gut morphology of juvenile amberjack, *Seriola dumerili* (Under review)Manuscript Number: AQUA-D-16-01141

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M. Nucleoside by products dietary supplementation influences blood chemistry, immune response, oxidative stress resistance and intestinal morphology of juvenile amberjack, *Seriola dumerili*. (Under review) manuscript number ANU-16-209 R1 **.**

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M., Maekawa, M., Fujieda, T. Inosine supplementation in low fishmeal based diets for juvenile amberjack: effects on growth, digestibility, immune response, stress resistance and gut morphology

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M. Supplemental effects of uridine monophospahte on growth, blood chemistry, oxidative stress, innate and adaptive immune responses of juvenile red sea bream, *Pagrus major*

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M. Supplementation of cytidine monophosphate enhanced growth, immune response and stresss resistance of red sea bream, *Pagrus major* juvenile.

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M. Effects of complete replacement of fishmeal by soya protein concentrate with inosine monophosphate supplementation in diets for juvenile red sea bream, *Pagrus major*

C. Participation in Conference and Symposium:

i. The 17th International Symposium on Fish Nutrition and Feeding, 5-10 June 2016, Sunvalley, Idaho, USA.
ii. Asia pacific Aquaculture, April 26-29, 2016; Grand city, Surabaya, Indonesia
iii. World Aquaculture 2015, May 26-30, Jeju, Korea.
iv. Aquaculture America 2015, February 19-22, 2015; New Orleans, Louisiana, USA
v. The 16th International Symposium on Fish Nutrition and Feeding, 25-30 May 2014, Cairns , Australia.

vi. High value Aquaculture Fin Fish Symposium, 15-18 October 2013, Kagoshima, Japan

vii. Asia Pacific Aquaculture, December 10-13th , 2013, Ho Chi Minh City , Vietnam.

viii. 5th Fisheries Conference & Research Fair, 18 – 19 January 2012 , Dhaka, Bangladesh

D. Symposium Abstracts and Presentation:

There were several abstracts published in several book of abstracts which are listed below:

HOSSAIN, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M. Dietary effects of adenosine monophosphate to enhance growth, digestibility, innate immune responses and stress resistance of juvenile red sea bream, *Pagrus major*. Presented in The 17th International Symposium on Fish Nutrition and Feeding, 5-10 June 2016, Sunvalley, Idaho, USA.

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HOSSAIN, M.S*., Koshio, S., Ishikawa, M., Yokoyama, S. Dietary nucleotide administration influences growth, immune responses and stress resistance of juvenile red sea bream (*Pagrus major*). Presented in World Aquaculture 2015, May 26-30h , 2015, Jeju, Korea.

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HOSSAIN, M.S*., Kader, M.A. Dey, T., Yokoyama, S., Ishikawa, M. and Koshio, S. Effect of replacing dietary fishmeal on growth digestibility and economic performances of Climbing perch *Anabas testudineus*. Presented in Asia Pacific Aquaculture, December 10-13th , 2013, Ho Chi Minh City , Vietnam.

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Kader, M.A*., Bulbul, M., Ahmed, G.U., **HOSSAIN, M.S.,** Hossain, M.A., Yokoyama, S., Ishikawa, M. and Koshio, S., 2009. Effects of animal proteins either alone or in combination in practical diets on growth and economic benefit of climbing perch *Anabastestudineus*(bloch). Book of Abstract- Asian-Pacific Aquaculture 2009, page 261, 3-6 November, 2009, Kualalampur, Malaysia.

Kader, M.A*.,Bulbul, M**., HOSSAIN, M.S.,** Hossain, M.M., Ahmed, G.U., Hossain, M.A., Yokoyama, S., Ishikawa, M. and Koshio, S., 2009. Effect of total replacement of fishmeal with fermented soybean and squid by-product blend in practical diets for climbing perch *Anabas testudineus*. Book of Abstract- Aquaculture America 2009, page 169, 15-18th February, 2009, Seattle, Washington, USA.

Kader, M.A*. Bulbul, M., **HOSSAIN, M.S.,** Hossain, M.A., Ahmed, G.U., Komilus, C.F., Yokoyama, S., Ishikawa, M. and Koshio, S., **2008**. Evaluation of meat and bone meal in the practical diet for sutchi catfish, *Pangasius hypophthalmus* (sauvage) reared under pond condition. Presented in the "World Aquaculture, 2008" May 19-23, 2008 Busan, Korea and awarded **First Place** of "**Aquaculture CRSP Award 2008**'.

*Indicates presenting author

E. Thesis Titles

Hossain, M.S. Evaluation of rice bran and wheat bran as supplemental feed compared to a commercial feed for the monoculture of gift strain (*oreochromis niloticus*). Master of Science, Deapartment of Aquaculture, Bangladesh Agricultural University, Bangladesh, p 76, May **2007**.

ACKNOWLEDGEMENT

All praises are due to the Almighty Allah who enables me to perform higher education and to complete as well as to submit the dissertation.

I am greatly acknowledged to the Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho) of Japan for financial support which had given me the opportunity to carry out my Doctoral course in Japan.

I would like to express deepest sense of gratitude, gratefulness, sincere appreciation and profound regards to my mentor DR. SHUNSUKE KOSHIO, Professor, Faculty of Fisheries, Kagoshima University, Japan for his affectionate encouragement, worthy inspiration, scholastic guidance and constructive criticism throughout the research work and during producing this dissertation.

With profound regard, I also would like to thank DR. MANABU ISHIKAWA, Associate Professor and DR. SAICHIRO YOKOYAMA, Assistant Professor for their sincere encouragement, valuable suggestions and hands-on training on laboratory procedures and analytical equipment operations.

I am greatly indebted to Dr. Md. Abdul Kader and Dr. Mahbuba Bulbul for introducing me to my advisor in Kagoshima University and encouraging appreciation during my research.

A Thanks goes to Dr. Atsushi Yamamoto, Professor, Faculty of Fisheries, Kagoshima University, who helped me by sharing his knowledge to do acquired immunity test. And also special thanks to Mr. MasaakiTaniguchi for his kind co-operation during conducting research in wet laboratory.

I indebted to my ex-supervisor Dr. Arshad Hossain , ex-professor of Faculty of Fisheries, Bangladesh Agricultural University and my senior Dr. Md. Shah Alam, Associate professor, North Carolina University, USA, for their kind cooperation and encouragement.

I would like to say thanks to Ajinomoto, Co., inc., Kanagawa, Japan for their analytical support (Amino acid anlysis, nucleotide analysis and histology) and providing inoisne nucleoside and nucleotide (IMP, GMP) at the same time to provide partial fund to do my research. I would like to give special thanks to Dr. Takeshi Fujieda, Maekawa Mayumi San and Sayoko Ono San, Ajinomoto Co., Inc., Japan for their kind cooperation during this research.

My research smoothly advanced with the help and cooperation of my foreign and Japanese lab-mates (masters and Ph.D students) like Dr. Lin (Myanmar), Dawood (Egypt), Nhu (Vietnam), Wang (China), Amina Moss (Bahama), Dossou (Benin), Ssatoyama, Kokubu, Nishimura, Usami and many other Japanese students who by this time already graduated. Thanks everybody for your direct and indirect cooperation.

Special thanks to the members of the “Bangladesh Association in Kagoshima, Japan” (BAK-Japan) who offered me a “Mini Bangladesh” in Japan. I always waited for the day of party when I could enjoy my native food and culture organized by BAK-Japan.

I feel proud to express my heartfelt indebtedness to my parents, brothers and sister for their sacrifice, patience, and immense love which has always served as encouragement, source of inspiration and guidance throughout this study.

It gives me a great pleasure in expressing my deepest sense of love to my sweetheart NADIA MAHJABIN SONY for her mental, physical & social support, encouragement and appreciation over the period of my research.

Md. Sakhawat Hossain

ABBREVIATIONS AND ACRONYMS

AMP	:	Adinosine monophosphate	INO	:	Inosine
ADC	:	Apparent digestibility co-efficient	IMP	:	Inosine monophosphate
BAP	:	Biological anti-oxidant potential	LA	:	Lysozyme activity
BUN	:	Blood urea nitrogen	NT	:	Nucleotide
BA	:	Bactericidal activity	NBP	:	Nucleoside by product
B agglu	:	Bacterial agglutination	NBT	:	Nitro blue tetrazolium activity
CAT	:	Catalase activity	PER	:	Protein efficiency ratio
CF	:	Condition factor	PA	:	Peroxidase activity
CMP	:	Cytidine monophosphate	S.E.M.	:	Standard error of mean
CORT	:	Relative value of cortisol	SBM	:	Soybean meal
d-ROMs	:	Reactive oxygen metabolites	SGR	:	Specific growth rate
DSM	:	Dehulled soybean meal	SOD	:	Superoxide dismutase
EAA	:	Essential amino acid	SPC	:	Soy protein concentrate
FAA	:	Free amino acid	Sur	:	Survival (%)
FCE	:	Feed conversion efficiency	T-Bil	:	Total bilirubin
FCR	:	Feed conversion ratio	T-Cho	:	Total cholesterol
Glu	:	Glucose	TG	:	Triglyceride
GOT	:	Glutamyl oxaloacetic transaminase	TSP	:	Total serum protein
GPT	:	Glutamic-pyruvate transaminase	UMP	:	Uridine monophosphate
GMP	:	Guanosine monophosphate	VSI	:	Viscerotopic index
hE	:	Enterocyte height	WG	:	Weight gain
hMV	:	Microvillus height			
hF	:	Fold height			
HSI	:	Hepatosomatic index			
	:				

CHAPTER I

General introduction

1. Literature review

1.1. General aspects of aquaculture

Aquaculture, probably the fastest growing food-producing sector, now accounts for nearly 50 percent of the world's food fish. According to the latest available statistics collected globally by FAO, world aquaculture production attained another all-time high of 90.4 million tonnes (live weight equivalent) in 2012 , including 66.6 million tonnes of food fish and 23.8 million tonnes of aquatic algae (mostly seaweeds) (FAO,2014). The global trend of aquaculture development gaining importance in total fish supply has remained uninterrupted. Farmed food fish contributed a record 42.2 percent of the total 158 million tonnes of fish produced by capture fisheries (including for nonfood uses) and aquaculture in 2012 This compares with just 13.4 percent in 1990 and 25.7 percent in 2000.

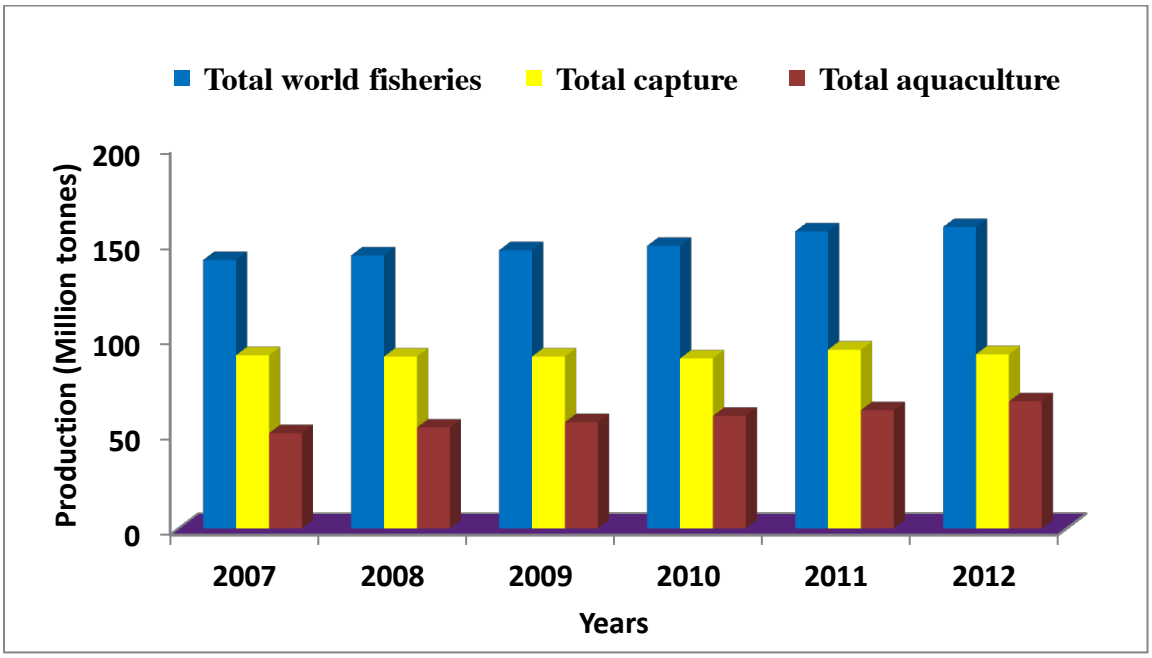


Fig. 1.1 World aquaculture, capture fisheries and total fisheries production.

Asia as a whole has been producing more farmed fish than wild catch since 2008, and its aquaculture share in total production reached 54 percent in 2012, with Europe at 18 percent and other continents at less than15 percent, whereas the capture fisheries remained in

a stable production of about 90 million MT in all over the world (FAO, 2014). Therefore, it is important to ensure the sustainable aquaculture practices in order to meet the demands of the increasing world population for animal protein.

1.2. Aquaculture and environment friendly functional aquafeeds

The world aquaculture has become intensified to keep up with the increasing demand. Intensified aquaculture not only increased the production but also increased the demand of more artificial feed and susceptibility to diseases of the cultured organisms due to deterioration of water quality and elevation of stress. For the treatment of various disease (Bacterial, viral or parasite infections) different types of drugs and chemicals are often used, which has several drawback. In case of the occurrence of bacterial infections in aquaculture the most common means of treating was the administration of antibiotics. However, these drugs have been criticized for potential development of antibiotic resistant bacteria and destruction of environmental microbial flora as well as being cost intensive and in some cases with marginal effects only. Certain antibiotics have also been shown to suppress the immune system, making organisms in aquaculture more susceptible to viral and parasite infections. This has led to the ban of sub- therapeutic use of antibiotics in Europe and more stringent regulations on application of antibiotics in lots of other countries. So, the development of alternative strategies for disease control turned out to be more and more interesting for livestock and aquaculture. One of the most promising alternative strategies in this regards might be the development of functional aqua feeds.

The concept of functional aquafeeds represents an emerging new paradigm to develop diets for fish and crustaceans. The term “Functional feeds” is used to describe fish feeds extend beyond the satisfying basic nutritional requirements of the cultured organism to improve growth and feed utilization also the support of general health and stress resistance of

the animals. Thus enabling a shift away from chemotherapeutic and antibiotic treatments of cultured organisms. A large number of additives or feed materials (prebiotics, probiotics, immunostimulants, nucleotides, vitamins, minerals and plant or algal extracts) are available for inclusion in the functional feeds (Gomez and Balcazar, 2008). Recently, in aquaculture nucleotide and its related products received highest attention as potential functional materials in aquafeed.

1.3. Dietary nucleotides

Nucleotides (NT) are low-molecular weight intracellular compounds which play key roles in nearly all biochemical processes (Gil, 2002) and it's the building block of nucleic acid. For years, nucleotides were not considered an essential nutrient for use in any dietary programs in animals and humans. It was thought that animals were able to produce sufficient nucleotides to meet their physiological demands via a *de novo* synthesis or a salvage pathway (Hoffmann, 2007). The salvage pathway is the re-synthesis of nucleotides from nucleosides that originate from nucleotide catabolism or dietary sources. In other words, the salvage pathway is recycling nucleotides from dead cells. However, over the past several years, studies have indicated that both mechanisms may not always provide enough nucleotides under certain conditions. In addition, dietary nucleotides may become essential when the endogenous supply is insufficient during biologically active situations: rapid growth, reproduction, environmental change (high stocking density and used litter conditions), protein-energy malnutrition and nutritional deficiency, combating disease, and recovery from injury. Thus, nucleotides are considered"semi" or "conditionally" essential nutrients in animals (Yu. 1998).

1.3.1. Structure of nucleotides

Nucleotides contain three major components: a sugar (either ribose or deoxyribose), a nitrogen base (either a purine or a pyrimidine) and one or more phosphate groups (Figure

1.2). A nucleotide consists of a nitrogenous base, a sugar (ribose or deoxyribose) and one to three phosphate groups. When phosphate group of nucleotide is removed by hydrolysis, the structure remaining is nucleoside. Thus, a nucleoside is composed of the base linked to a sugar in N-glycoside linkage (Figure 1.3). Nucleic acids are polymers made up of nucleotides. The base of a nucleotide is joined covalently (at N-1 of pyrimidines and N-9 of purines) in an N-glycosyl bond to the 1‟ carbon of the pentose. The nucleic acids are covalently linked through the phosphate group, in which the 5′ phosphate group of one nucleotide unit is joined to the 3′ hydroxyl group of the next nucleotide, creating a phosphodiester linkage. Nucleic acids have two kinds of pentose. If the sugar is ribose, they are components of ribonucleic acid (RNA), and if the sugar is deoxyribose, they are components of deoxyribonucleic acid (DNA). DNA contains two major purine bases, adenine (A) and guanine (G) and two major pyrimidines, cytosine (C) and thymidine (T). RNA contains adenine (A) and guanine (G), cytosine (C), and uradine (U). A nucleoprotein is any protein that is linked to a nucleic acid and found in the nuclei and cytoplasm of all living cells, as in chromatin and ribosomes (Voet and Voet, 2004).

1.3.2. Biosynthesis of nucloeotides

Nucleotides and nucleic acids are continuously being formed and degraded. There are two major pathways for a formation of nucleotide or nucleic acids: *de novo* synthesis and a salvage pathway. Nucleotides can be synthesized within cells by a *de novo* pathway from amino acid precursors, such as glutamine, formate, glycine, and aspartic acid. Also, they can be formed through a salvage pathway. In the salvage pathway, purine and pyrimidine are formed from intermediates from the degradative pathway of nucleotides. Salvage pathways are also used to recover bases and nucleosides are synthesized from degradation of RNA and DNA. This salvage pathway is simpler and more energy efficient than the *de novo* synthesis

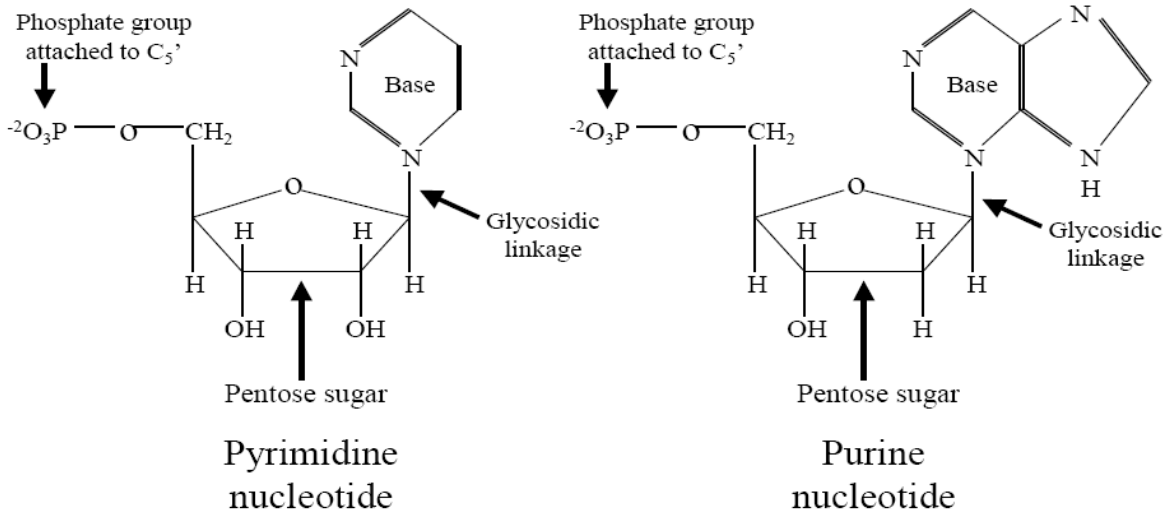


Figure 1.2. Generalized structure of a nucleotide (Adapted from Stein and Mateo, 2005). C = carbon atom, H = hydrogen atom, O = oxygen atom, and N = nitrogen atom

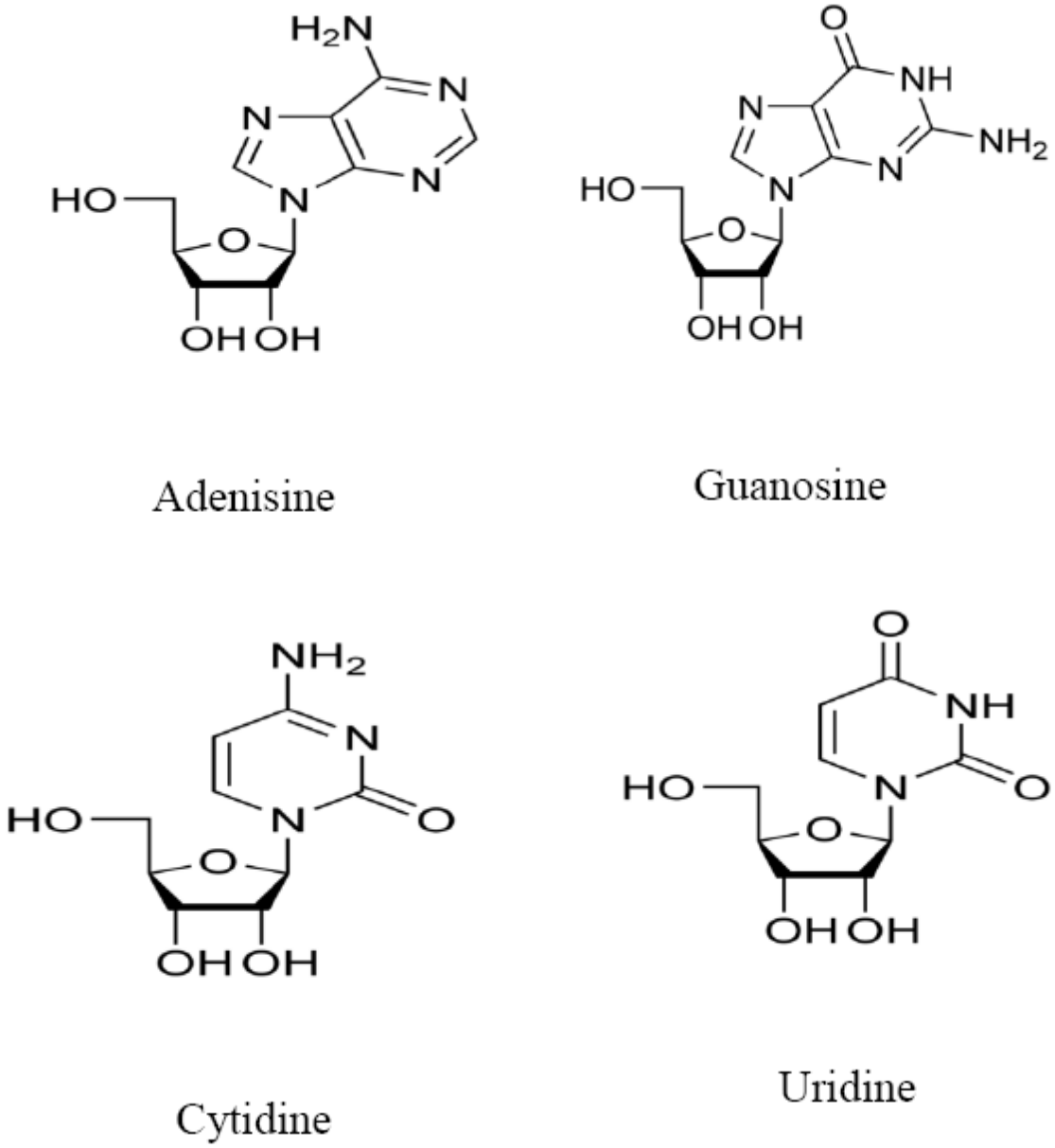


Figure 1.3. Purine (adenosine and guanosine) and pyrimidine (uridine and cytidine) bases of nucleosides.

of nucleotides and is modulated by the availability of free bases. The salvage pathway is especially important in some organs such as the intestinal tract and bone marrow, where nucleotides synthesis capacity is limited. However, nucleotides supplied via the aforementioned mechanisms may not be sufficient to maintain needs during rapid growth, nutritional deficiency, combating diseases and recovery from injuries status (Cosgrove, 1998). Thus, dietary nucleotides may become an essential nutrient for optimal function in the body when the endogenous supply is insufficient (Yu, 1998; Carver, 1999; Jung, 2011).

1.3.3. Sources of nucleotides

Most feed ingredients contain nucleoproteins (proteins conjugated to nucleic acids), which serve as dietary sources of nucleotides (Mateo, 2005). The nucleotide content is particularly high in ingredients such as fish soluble, animal protein soulubles, fishmeal, legumes (adenine contentis particularly high in black eyed peas) east extracts and unicellular organisms such as yeast and bacteria that are reach in DNA or RNA (Deveresse, 2000). Mateo (2005) measured the nucleotide concentrations in some commonly used feed ingredients (Table 1.1). The content proportion and the availability of nucleotides differ between ingredients. The major ingredients for fish feeds, which are fishmeal and soybean meal, contained 75 and 38 ppm total nucleotides, respectively (Table 1.1). Among fresh seafood sources, guanine level in anchovies and sardines was much higher than its level in squid (Table 1.2). Carver and Walker (1995) mentioned that the level of nucleotides, especially inosine monophosphate (IMP), is related to protein level on the foods. That is, foods containing high levels of protein have high nucleotide content with the exception of soybean meal. Soybean meal and corn, oil, oilseeds, and muscle protein, which is mainly composed of actin-myosin protein molecules, contain low levels of nucleotides (Deveresse, 2000). Fruits, vegetables, and dairy products also are poor sources of dietary nucleotides (Barness, 1994). Human milk especially

during early lactation contains relatively high nucleotide levels. The concentrations of nucleic acid, nucleotides, and nucleosides was 68 ± 55μmol/L, 84 ± 25μmol/L, and 10 ± 2μmol/L, respectively in human milk (Johke, 1963). Human milk contains approximately 25% of its nitrogen as non-protein nitrogen including substances such as urea, free amino acids, nucleic acids, creatine, nucleotides and carnitine. Free and cellular nucleotides, mainly cytidine 5′monophosphate (CMP) and adenosine 5′monophosphate (AMP), account for 2 - 5% of the non-protein nitrogen in human milk (Cosgrove, 1998).

1.3.4. Nucleotide absorption and metabolism

Dietary nucleoproteins and nucleic acids are broken down by proteases and nucleases to nucleotides. The phosphate in nucleotides is cleaved by alkaline phosphatases and nucleotidases in the small intestine to form nucleosides (Carver and Walker, 1995) (Figure 1.4). Nucleosides are absorbed well in the intestinal lumen than nucleotides because nucleotides have negatively charged phosphate groups that blocked absorption (Yu, 1998). Nucleotides also have a limited capacity to pass through cell membranes under physiological conditions because of the lack of a transporter system (Sanderson and He, 1994). Nucleosides uptake is the major pathway for the entering of bases (purines and pyrimidines) into the epithelial cells in the intestine and then partially metabolized. More than 90% of dietary and endogenous nucleosides and purine and pyrimidine bases are absorbed into the enterocyte (Uauy *et al*., 1990). Most of these absorbed nucleosides and purine and pyrimidine bases are rapidly degraded into uric acid and allantoin within the enterocytes (Sonoda and Tatibana 1978). In particular, purine nucleotides are degraded into uric acid, while pyrimidine nucleotides are degraded to β-alanine and β-amino iso-butyric acid (Carver and Walker, 1995).

Table 1.1. Nucleotide concentration[1] (mg/kg) in some commonly used feed ingredients (as-is basis)

Ingredients	AMP*	CMP*	GMP*	IMP*	UMP*	Total nucleotides
	---------- mg/kg ----------					
Non-fat dried milk	0	65	0	195	106	366
Dried whey	19	270	0	4	1	294
Whey protein concentrate	0	34	0	159	89	282
Corn	2	3	3	1	0	9
Soybean meal, 44%	8	16	3	2	9	38
Casein	0	1	0	0	0	1
Barley	1	2	1	1	0	5
Fish meal	11	26	2	35	1	75
Naked oats	3	3	3	1	1	11
Plasma protein, spray dried	2	2	2	1	0	7
Red blood cells, spray dried	44	0	3	6	2	55
Soy protein concentrate	1	0	2	1	0	4

[1]Adapted from Mateo (2005).

*AMP = Adenosine 5′ monophosphate; CMP = Cytidine 5′ monophosphate; GMP = Guanosine 5′ monophosphate; IMP = Inosine 5′ monophosphate; UMP = Uridine 5′ monophosphate.

Table 1.2. Purine and Pyrimidine bases content of some important feedstuffs[1].

Ingredient	Nucleotide bases, %					
	Cytosine	Uracil	Guanine	Thymine	Adenine	Total bases
Complete fish meal	0.12	0.12	0.94	0.05	0.17	1.41
Press cake fish meal	0.08	0.08	0.13	0.02	0.10	0.42
Fish soluble	0.11	0.12	2.33	0.08	0.19	2.83
Yeast	0.18	0.19	0.25	0.02	0.28	0.90
Yeast extract	0.14	0.73	0.62	0.03	0.73	2.30
Single cell protein	0.10	0.55	0.88	0.37	0.17	2.07

[1] Adapted from Devresse (2000).

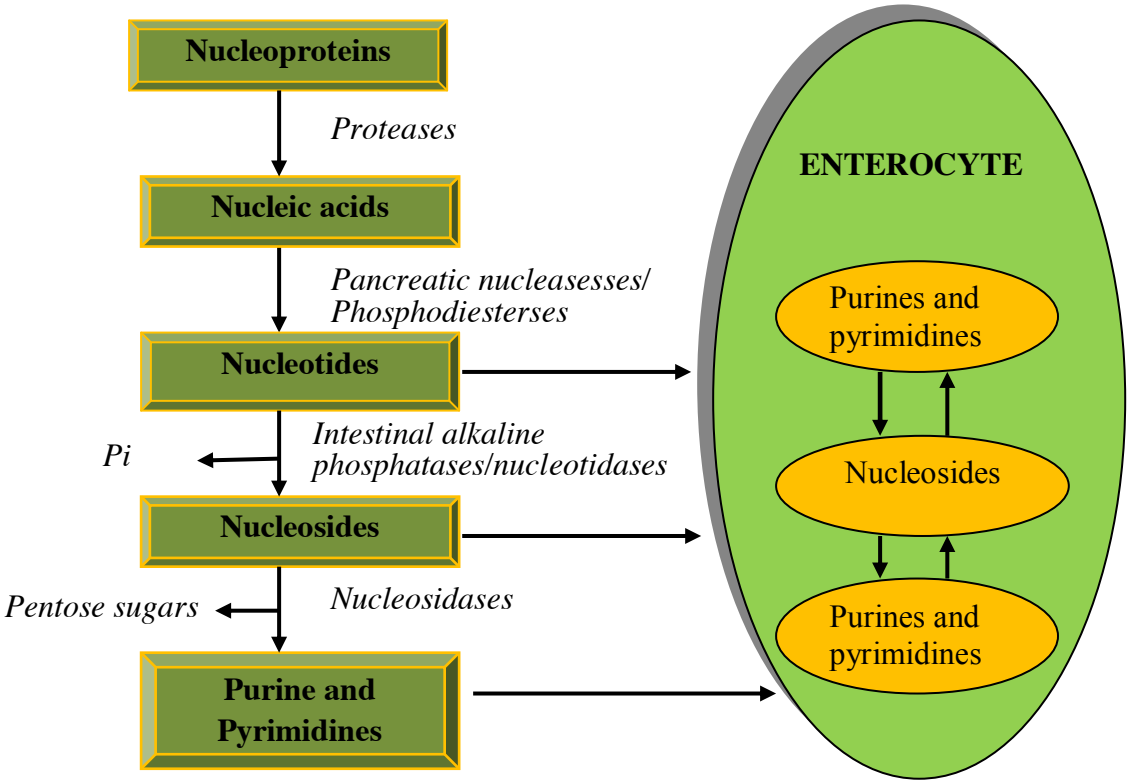


Fig. 1.4. Absorption and metabolism of nucleic acids and their related products; Adapted from Quan and Uauy (1991).

1.3.5. Functioning of nucleotides

- Nucleotides are the basic units of nucleic acids (DNA and RNA). Though, nucleoside monophosphates found in nucleic acids, actually nucleoside triphosphates are the raw materials for their synthesis.
- Cyclic Nucleotides Act as Regulatory Chemicals. Cyclic AMP (cAMP) functions as second messengers in many hormone actions, while cyclic GMP (cGMP) functions in Ca^{++} or calmodulin mediated responses.
- Nucleotides of B-Complex Vitamins Function as Coenzymes. For example, NAD^{+}, $NADP^{+}$, FMN, FAD are coenzymes useful in oxidation-reduction reactions.
- Higher nucleotides function as energy carriers, e.g. ATP, GTP, UTP and TTP. Out of these ATP is the universal energy carrier of the cell.
- Higher nucleotides involved in the synthesis of polysaccharides (e.g. UDP-glucose, ADP-glucose) and phospholipids (e.g. CDP and CTP).

More recent research has indicated that, they are important elements in mammalian nutrition especially during period of rapid growth or physiological stress and they appear to play a key role in enhancing the immune system, hence their description as "conditionally essential":

1.3.6.Nucleotide nutrition in fish

The roles of nucleotides and metabolites in fish diets have been sparingly studied for over 25 years. Beside possible involvement in diet palatability, fish feeding behavior and biosynthesis of non-essential amino acids, exogenous nucleotides have shown promise most recently as dietary supplements to enhance immunity and disease resistance of fish produced in aquaculture. Research on dietary nucleotides in fishes has shown they may improve growth in early stages of development, enhance larval quality via broodstock fortification, alter intestinal structure, increase stress tolerance as well as modulate innate and adaptive immune responses. Fishes fed nucleotide-supplemented diets generally have shown enhanced

resistance to viral, bacterial and parasitic infection. Despite occasional inconsistency in physiological responses, dietary supplementation of nucleotides has shown rather consistent beneficial influences on various fish species (Li and Gatlin, 2006).

Research to date on dietary nucleotides has focused on a mixture of nucleotides, rather than specific types of nucleotides except inosine and IMP for feeding stimulation research. (Li and Gatlin, 2006). Recently, Song et al. (2012) evaluated individual nucleotide, inosine monophosphate (IMP) and found promising results for enhancing growth, immunity and disease resistance of olive folounder. Untill now, there are no studies have been reported on other individual nucleotide or nucleoside for potential growth and health benefit of aquatic species. So, aditional research to explore specific effects of various individual nucleotides is needed to gain a better understanding of nucleotide nutrition in fish. However, information on dietary supplementation of nucleotides with fishes considered relevant to the present study are also reviewed below

Table 1.3; Research on dietary supplementation of nucleotides with fishes

Authors	Nucleotide form	Dose and/or feeding regime	Length of administration	Species	Initial size	Effects
Ramadan and Atef (1991)	Ascogen S, (Chemoforma, Augst, Switzerland)	2 and 5gkg^{-1} diet	16 weeks	Hybrid tilapia	21 days old	Growth↑, Survival↑
Ramadan et al. (1994)	Ascogen (Chemoforma, Augst, Switzerland)	5g kg^{-1} diet	120 days	Hybrid tilapia	30 days old	Antibody titer after vaccination↑, Mitogenic response of lymphocyte↑
Adamek et al., (1996)	Ascogen (Chemoforma, Augst, Switzerland)	0.62, 2.5 and 5 g kg^{-1} diet at 1% bw day^{-1}	37 days	Rainbow trout	163.4 – 169.7 g fish^{-1}	Growth ↑
Burrells et al. (2001a)	Optimuˆn, (Chemoforma, Augst, Switzerland)	2g kg^{-1} diet, containing 0.03% NT, 2% bw day^{-1}	3 weeks	Rainbow trout	217± 62 g	Survival after challenge with *V. anguillarum*↑
		2 g kg^{-1} diet, containing 0.03% NT, 1% bw day^{-1}	2 weeks	Rainbow trout	53 – 55 g	Survival after challenge with infectious salmon anaemia virus↑
		2g kg^{-1} diet, containing 0.03% NT, 2% bw day^{-1}	3 weeks	coho salmon	100 g	Survival after challenge with *Piscirickettsia salmonis*↑
		2g kg^{-1} diet, containing 0.03% NT, 2% bw day^{-1}	3 weeks	Atlantic salmon	60 g	Sea lice infection↓

Burrells et al. (2001b)	Optimuˆn, (Chemoforma, Augst, Switzerland)	2g kg^{-1} diet, containing 0.03% NT at1.5% bw day^{-1}	3 weeks before vaccination and 5 weeks post-vaccination	Atlantic salmon	34.7±9.6 g	Antibody titer↑, Mortality↓
		2g kg^{-1} diet, containing 0.03% NT at1.5% bw day^{-1}	8 weeks	Atlantic salmon	43F3.0 g	Plasma chloride↓, Growth↑
		2g kg^{-1} diet, containing 0.03% NT	10 weeks	Atlantic salmon	205 g	Intestinal fold↑
Sakai et al. (2001)	ribonuclease-digested yeast RNA (Amano Seiyaku Co-op, Tokyo, Japan)	15 mg $fish^{-1}$, by intubation	3 days	Common carp	100 g	Phagocytosis↑, Respiratory burst↑, Complement↑, lysozyme↑ , *A. hydrophila* infection↓
Leonardi et al., (2003)	Optimuˆn, (Chemoforma Augst, Switzerland)	NA	120 days	all-female rainbow trout	80 –100 g	B lymphocytes↑, Resistance to IPN virus↑, Plasma cortisol↓
Low et al. (2003)	Optimuˆn, (Chemoforma, Augst, Switzerland)	2g kg^{-1} diet, containing 0.03% NT to hand satiation daily	15 weeks	Turbot *Scophthalmus maximus*	120.9±5.1 g	Altered immunogene expression in various tissues
Li et al. (2004a,b)	Ascogen P (Canadian Biosystem Inc. Calgary , Canada)	5g kg^{-1} diet, fixed ration approaching satiation daily	7 weeks	Hybrid striped bass	7.1, 9.1 g	Neutrophil oxidative radical production↑, Survival after challenge with *Streptoccus iniae*↑.

Oliva-Teles et al., 2006	Nucleic acid	5.8 and 11.5 %	10 weeks	Sea bream	12.7 g	growth↑, ornithine carbamyltransferase activity → body composition, hepatic glutamate dehydrogenase and uricase activities
Li et al., 2007	Nucleotide mixtures	4 g kg^{-1} diet	4 weeks	Red drum	10.2 ± 0.2 g	growth↑, neutrophil oxidative radical production and survival after challenge with *V. harveyi*↑
Lin et al., 2009	Nucleotide mixtures	0.5 – 2 g kg^{-1} diet	8 weeks	Grouper	5.90 ± 0.01 g, Experiment 1; 10.33 ± 0.01 g, Experiment 2	growth and immune responses↑
Cheng et al., 2011	Ascogen	0.5% and 1% of diet	6 weeks	Red drum	7.1g	Intestinal structure↑ the non-specific immune response↑, Growth↑
Tahmasebi-Kohyani et al., 2011	Nucleotide mixtures	0.5 - 2 g kg^{-1} diet	8 weeks	Rainbow trout	23 g	Growth↑, immunity↑, resistance against *S. iniae*.↑
Tahmasebi-Kohyani et al., 2012	Nucleotide mixtures	0.5 - 2 g kg^{-1} diet	8 weeks	Rainbow trout	23g	growth and resistance against handling and crowding stress↑
Song et al., 2012	IMP,	0.0 -10.0 g kg^{-1} diet	14 weeks	olive flounder	7.5 ± 0.02 g	Growth↑,innate immunity and disease resistance↑.

Symbols represent an increase (↑) in the specified response; no change (→); decrease (↓).

2. Experimental fish, Red sea bream and amberjack, the two important aquaculture species

2.1. Red sea bream, *Pagrus major*

Red seabream, *Pagrus major*, is a fish species in the *Sparidae* family. In Japan, *Pagrus major* is known as madai (真鯛, or "genuine tai") and is prized both for its flavor and for its traditional use as an auspicious food, often served at New Year's and at festive occasions such as weddings. In Korea, it is known as *Ch'amdom* (참돔). Pagrus major is also the most commonly eaten fish in Taiwan. This fish is grilled over charcoal or wood fire in Spain and known as *besugo*. It is widespread in the Northwest Pacific from the northeastern part of South China Sea (Philippines excluded) northward to Japan. It is a marine subtropical oceanodromous demersal fish (https://en.wikipedia.org/wiki/Pagrus_major). *P. major* are commercially cultured in japan and its production reaches the second largest in Japan (Koshio, 2002). Lately, the aquaculture of this species has also developed rapidly and widely in China, and Korea.

Fig. 1.5 Experimental fish red sea bream, *Pagrus major*

2. 2 Experimental fish Amberjack, *Seriola dumerili*

Amberjack (*Seriola dumerili*) is a jack of the genus *Seriola*. It is found in the Mediterranean Sea, the Atlantic Ocean, the Pacific Ocean and the Indian coasts, living usually between 20

and 70 m of depth (with a maximum of 360 m). It is the largest genus in the Carangidae family, with a maximum length of 200 cm. It is a fast-swimming pelagic fish with similar habits to the kingfish. They are silver-blue with a golden side line, with a brown band crossing over the eye area.Amberjack is a powerful hunter which feeds on other fish and invertebrates (https://en.wikipedia.org/wiki/Greater_amberjack). The amberjack, *S*. *dumerili*, has a great potential for the global aquaculture industry owing to its rapid-growing and adaptive characteristics (Thompson et al., 1999; Mazzola et al., 2000). It is distributed throughout the tropical and subtropical seas. Recently, the aquaculture of this species is intensified in the Mediterranean region and Japan (Takakuwa et al., 2006).

Fig. 1.6. Experimental fish, amberjack (*Seriolla dumerili*)

2. 3. Nutritional studies on red sea bream and amberjack

Investigation on quantitative nutritional requirements of red sea bream for some macro and micronutrients (Forster & Ogata 1998; Ren *et al*. 2008; Kader & Koshio 2012; Rahimnejad & Lee 2013, 2014) and development of alternative protein based diet (Kader *et al*. 2010, 2012; Kader & Koshio 2012) have been defined for this fish. However, research on dietary modulation for potential growth and health management of this species (*P*. *major*) is very scarce. On the contrary; very few data are available in the literature for the specie *S*. *dumerili*. Although many papers have been published regarding the nutrient requirement of the close

related species *S. quinqueradiata* (Shimeno, 1991; Takeuchi et al., 1992a,b; Furuita et al., 1996; Shimeno et al.,1996) Compared with other fish species, *S. quinqueradiata* has a low ability to utilise carbohydrates and a high ability to utilise protein and lipid because of their carnivorous nature; whilst the vitamin and mineral requirement of the fish are also slightly different (Shimeno, 1991). In general, a diet containing about 50% of protein and 15-20% of lipid (including 1.6 to 3.1% HUFA) results in a good feed efficiency (Shimeno, 1991; Takeuchi et al., 1992 a,b) of , *S. quinqueradiata*. Jover et al. (1999) conducted research on *S. dumerili* and found almost similar protein and lipid requiremts of *S dumerili*. He tested two levels of protein (45 and 50% crude protein, CP) and two levels of lipid (14 and 17%) and the effect of lipid level was not significant, but growth of fish feed with 50% CP was higher than 45% CP. Nutritional parameters likes food intake rate, food conversion ratio and protein efficiency ratio were not significantly affected by the diets. Body composition and nutrient retention of fish were similar for all diets. However, until now in most of the cases the findings related to nutritional requirements studies of maco and microinutrients for *S. quinqueradiata* has been considering as the requirement of *S. dumerili*. So, there are huge gaps existed about current knowledge of nutritional studies of *S. dumerili*. Simulteneously, Until now, studies on nucleotide nutrition of these species are not reported elsewhere.

3. Objectives of the study

The increasing concerns about residues of antibiotic or anabolic agents in food production lead to a restrictive use of these substances to stimulate growth and treat bacterial diseases in most animal industries. The alterations in drug-use policies and consumer attitudes have already impacted aquaculture and thus have prompted interests in developing alternative strategies for growth and health management. Development of fuctional aquafeed through supplementing functional nutrients might be one of the most promising alternative

strategies in this regards. Recently, in aquaculture research nucleotides and its related product has been paid attention promisingly as functional nutrients. In aquatic animals although nucleotides has long been implicated as feed attractants in both vertebrate and invertebrate species but research into potential growth and health benefits of dietary nucleotides in aquaculture species instigated in early 2000s. However, currently there were numerous gaps in existing knowledge about exogenous nucleotide application to fish including various aspects of functional effects, doses and time of administration, digestion, influences on various physiological responses, oxidative stress response, various gene expression, absorption and metabolism etc. Moreover, supplementation of nucleotide/nucleosides in alternative protein based diet to develop low/non fishmeal based functional aquafeeds is still in its infancy and many fundamental questions remain unanswered. The selected two model experimental fish (Red sea bream and amberjack) are the most important commercially cultured marine species in japan as well as in East Asia. Untill now, However, research on dietary modulation for potential growth and health management of this species is very scarce. Effects of nucleotide or nucleoside as a functional nutrient of this species are not reported elsewhere. Therefore, the present research was conducted to evaluate nucleotides/ nucleoside as functional nutrient and supplementation of these nucleotide/nucleosides in alternative protein based diet to develop eco-friendly practical functional aquafeeds for marine species like, red sea bream and amberjack. The specific objectives of the research are –

1. To evaluate the efficacy of nucleotide related products on growth performance, immune response and gene expression of red sea bream (Chapter, III).
2. To evaluate the supplemental effects of individual nucleotide/ nucleoside on growth performance immune response, stress resistance and intestinal health condition of red sea bream and amberjack (Chapter, IV; part 1,2,3,4,5, and 6).

4. To investigate the combined effects of using mixed nucleotide on growth performance immune response, oxidative stress resistance of juvenile red sea bream (Chapter, V; part 1).
5. To develop alternative protein based eco-friedly functional practical aquafeeds through supplementation of nucleotide/nucleoside for red sea bream and amberjack (Chapter, V; Part 1 and 2).

CHAPTER II

General materials and methods

2. General materials and methods

2.1 Preparation of test diets

Dietary ingredients were first ground to a small particle size in a hammer mill and passed through a 100μm mesh sieve. For proper mixing of nucleotides with other ingredients, initially nucleotide/nucleoside and weighted amino acids (eg. Arginine, methionine, lysine etc.) of the respective diets were mixed properly in a glass beaker by hands with the help of a stainless laboratory spatula. Then this mixture was thoroughly mixed with other dry ingredients in a food mixer for 10 min. The lipid sources were premixed with a sonicator (CA - 4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Then required amount of water (35 -40% of the dry ingredients), were added to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to 7.0-7.5 with 4N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2 to 2.2 mm) to prepare pellets, which were then dried in a dry -air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 60°C for 120 min. The test diets were stored at – 28 °C in a freezer till use.

2.2 Experimental system

All the experiments were conducted in Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The feeding trials were carried out in 100/200 -L polycarbonate tanks (filled with 80/170L of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. Flow rates of 1.5 L min-1 were maintained throughout the experimental periods.

2.3 Feeding protocol

After stocking, fish were fed the respective test diets to the satiation level by hand twice daily, 7 days per week during the feeding trials. Special care was taken to collect uneaten diets which was freeze dried and finally subtracted from the total amount of supplied test diets to calculate the actual feed intake. All fish were weighted in bulk at certain (10-14 days) interval to determine growth and visually check their health condition. The water quality parameters such as water temperature, pH and salinity were measured and recorded during entire feeding trials.

2.4 Sample collection

The initial samples of 10-15 fish were stored at −20^{o}C for initial whole body analysis. At the end of the feeding trials, all fish were fasted for 24 h prior to final sampling. The total number, individual body weight and length of fish in each tank were measured. Three to Five fish from each replicate tank were randomly collected and stored at −20^{o}C for final whole body analysis. Blood was drawn by puncture of the caudal vein of individual fish. Plasma samples were collected after spinning down the heparinized blood at 3000- 4000 × g for 10-15 min at 4^{o}C. Serum was collected after clotting whole blood (non-heparinized) centrifuged at 3000-4000 ×g for 10- 15 min at 4^{o}C. All the blood samples were kept at −80°C until analysis. Liver was dissected out from three fish in each replicate tank, weighted individually to get heapatosomatic index (HIS), and viscerasomatic index (VSI) finally pooled together and stored at −80^{o}C.

2.5 Analytical procedure

Commonly used methodologies for biochemical analysis of ingredients, diets and fish samples are briefly described below.

2.5.1 Proximate composition

The ingredients, diets or fish whole body were analyzed for moisture, crude protein, crude lipid and ash, in triplicate, using standard AOAC methods (AOAC, 1990; 1995).

2.5.2 Moisture

Fish whole body were dried to a constant weight using the freeze dryer (Eyela freeze dryer FD -1, Tokyo Rikakikai Co. Ltd., Japan). Diet samples were also dried to a constant weight using mechanical convection oven at 1350C. Loss in weight represented moisture content.

Moisture (%) = {(weight before drying - weight after drying/weight before drying)} × 100

2.5.3 Ash

Samples were taken in crucibles with cover slightly ajar and placed in a muffle furnace at 550 ^{o}C until sample weight became constant. Ash contents were calculated as follow:

Ash (%) = (weight of ash/ sample weight) × 100

2.5.4 Crude protein

Approximately 0.2 g of sample and 2 g of catalizer (K_2SO_4:$CuSO_4$; 9:1) were digested with 10 ml of concentrated $H_2SO_4$and 5 ml of 30% $H_2O_2$for 90 min at 460^{o}C. Then digested samples were distilled in 50 ml of 30-40% NaOH using Kjeldahl distilling apparatus (Kjeltec System 1002, Tecator, Sweden). Approximately 150 ml distillate in H_3BO_3 solution mixed with methylene blue and methyl red indicators in ethanol was titrated with 0.1N H_2SO_4 to neutral pH. Percent of N was calculated for obtaining crude protein (%) using the following formula: Nitrogen (%) = {14.008 × (A - B) × 0.1 ×F}/{Sample weight (g) × 10}

Where, A = Amount (ml) of H_2SO_4 solution titrated for sample B = Amount (ml) of H_2SO_4 solution titrated for blank F = Factor of 0.1 N sulfuric acid solution

Crude protein (%) = % N × 6.25

2.5.5 Total lipid

Total lipids were analyzed according to Bligh and Dyer (1959) method. Approximately 0.2 g samples were homogenized with 1 ml of chloroform and 2 ml of methanol by a polytron homogenizer for 1 minute. After that, another 1 ml of chloroform were added and homogenized for 1 minute. The homogenate were filtered by a Buchner filtration apparatus. The residue on the filter papers was washed with a mixture of chloroform: methanol (1:1 v/v). After two times filtration, the homogenate were transferred to a separatory funnel, added few drops of water, shaken vigorously and stood for complete separation. The volume of chloroform, methanol and water were maintained in the proportions 1:1:0.8, respectively. The lower layer which contained chloroform and lipid fraction was collected and evaporated the chloroform completely under reduced pressure by using a rotary evaporator. Dried lipid was dissolved with chloroform: methanol (1:1 v/v) and transferred to the tarred vial. The total lipid was dried again by flashing with nitrogen and weighed to a constant weight. Total lipid is calculated as follows:

Total lipid (%) = {lipid weight (g) / dried sample weight (g)} × 100

2.5.6 . Amino acids

Total amino acid (TAA) and free amino acid (FAA) concentrations were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) according to Teshima et al . (1986a). To determine TAA, samples were prepared as follows: 2 mg samples were spiked with known amount of norleucine as an internal standard and hydrolyzded with 4 N methanesulfonic acid at 110°C for 22 h. The pH of the hydorlysate was adjusted to 2.2, filtered and stored at 4°C. To quantify the free amino acids 100 mg sample was mixed with 0.9 ml cold deionized water, 0.1 ml internal standard (norleucine, 0.6 mg DL-norleucine 0.1 ml-1 deionized water) and 5 ml 10% trichloroacetic acid (TCA), homogenized using a polytron homogenizer (Kinematica, Gmbh LITTAU, Lucerne, Switzerland). Samples were

then centrifuged at 4°C, 3000 × g for 15 min and supernatant was repeatedly washed with diethyl ether to remove TCA from homogenate. Finally, pH was adjusted to 2.2 and filtered samples were stored in 4°C. Cystine content was determined by a cysteic acid procedure (Moore, 1963). The chromatographic separation and analysis of the amino acids were performed with the HPLC unit with an ion exchange resin column.

2.5.7. Nucleotides (inosine and inosine monophosphate)

Dietary nucleotides (inosine and IMP) content of feeds were also analyzed by High Performance Liquid Chromatography (HPLC). The inosine and IMP content of the samples was determined by acidic extraction of ~1 g dry weight samples. In brief, for extraction process 25 ml of 5% percholoric acid was added to the feed sample (1g), shaking for 10 mins. After that deionized water was added to make constant volume upto 50 ml. Samples were sonicate for 10 mins, filtered and 5 ml sample solution were prepared. Finally, 0.4 ml potassium hydroxide (3 Mol/l) was added to neutralize the sample solution and stored in 4 ^{o}C. After extraction, for inosine quantification samples were loaded on an CAP CELL PAK C-18 column (Shisheido Co., Ltd. Japan), column temperature was 40 ^{o}C. The mobile phase was the mixture of 200 mmol/l phosphate buffer solution (pH 2.5) and methanol (95:5 v/v) contained 0.6% sodium heptanes sulphonate. Measurement wavelength was 260 nm. For IMP quantification samples were loaded on a MCI CDR-10 column (Mitsubishi Chemical Co. Ltd.). Colum temperature was 40 ^{o}C .The mobile phase was 1 mol/l acetate buffer (pH 3.3). Measurement wavelength was 260 nm. The minimum amount of nucleotides detectable by HPLC is ~3 ng (in 50 µL injection volume).

2.5.8. Blood parameters

Heparinized disposable syringes were used to collect blood for measuring hematocrit level. Plasma chemical parameters were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan).Biological

antioxidant potential (BAP) and reactive oxygen metabolites (d -ROMs) were measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l.,Grosseto, Italy by following Morganti et al. (2002). For the analysis of plasma cortisol, an enzyme-linked immunosorbent assay (Cortisol EIA Kit, product number EA65, Oxford Biomedical Research Inc., Oxford, MI) were used.

2.5.9. Immune responses

Several immune response parameters were measured according to several standard protocols. Serum lysozyme activity was measured with turbidimetric assays (Lygren et al., 1999). Oxidative radical production by phagocytes during respiratory burst was measured through the NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995). Serum bactericidal activity (BA) was performed according to Yamamoto and Iida (1995). The total peroxidase activity (PA) in serum was measured according to Salinas et al. (2008), The catalase activity (CAT) assay was performed According to Goth, (1991). The agglutination antibody titer was conducted accoding to the methods described by Swain et al. (2007). For the measurement of adaptive humoral immunity the methods described by Yamamoto, (1995) was followed.

2.6 Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Turkey Kramer test. Quadratic regression analysis and broken line regression analysis were also performed to quantify optimum dietary nucleotides supplementation level.

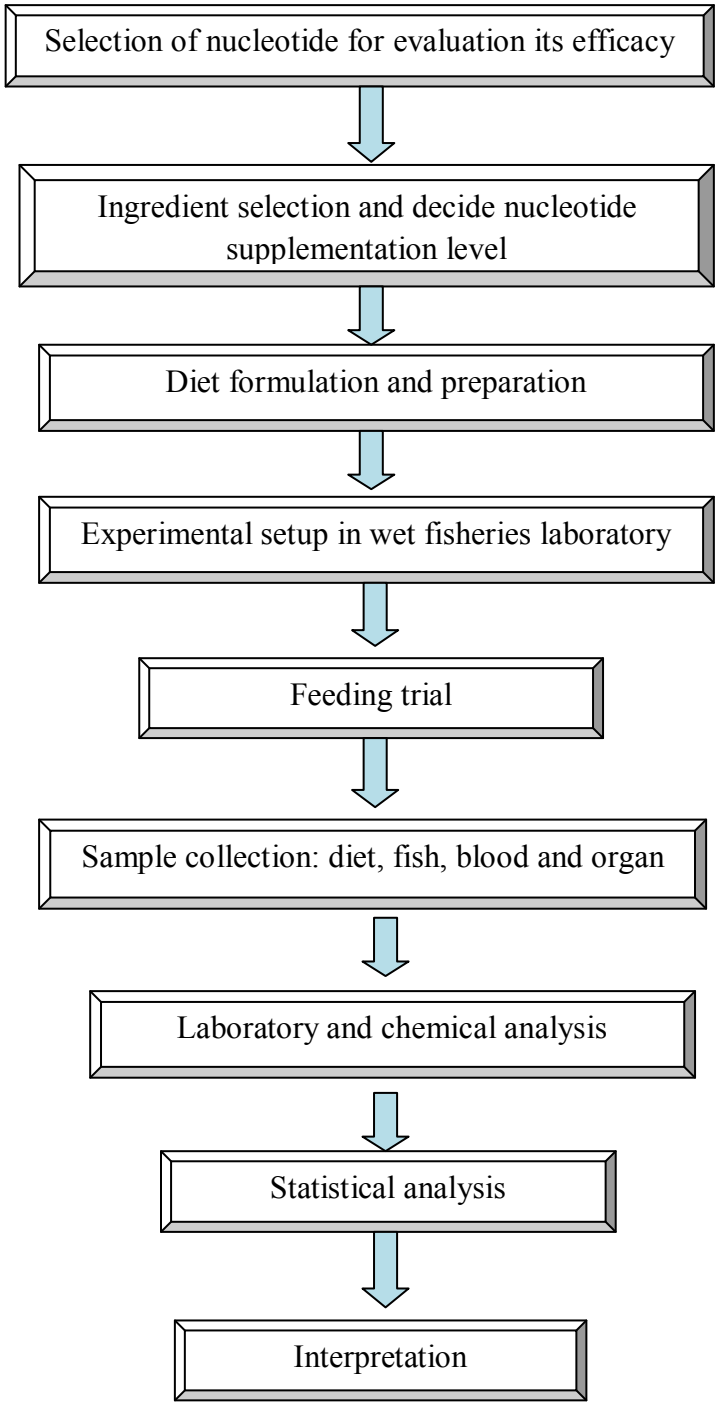


Fig. 2.1: Diagrammatic presentation of the general methodologies applied in the experiments

CHAPTER III

Efficacy of nucleotide related products

Part-1

Efficacy of nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of juvenile red sea bream, *Pagrus major*

ABSTRACT

A feeding trial was conducted to determine the efficacy of nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of juvenile red sea bream. Five experimental diets were formulated to contain 49% protein, 11% lipid and the diet without nucleotide related products supplementation was the control (D1). Nucleotide related products like; nucleoside by- products (NBP) and inosine were supplemented at 1, 3 and 0.03, 0.1% consecutively with basal ingredients of D1 and named as D2, D3 & D4 and D5 respectively. Experimental diets were fed to triplicate groups of fish for 60 days. Fish fed diet D5 showed significantly highest final body weight and % weight gain followed by the diet groups D2 and D4. Fish fed control group showed lowest growth performance and were not differed significantly with diet group D3. Feed conversion ratio and protein efficiency ratio were also significantly higher in diet group D5, whereas the other supplemented group showed intermediate value. A wide variation in some of the blood parameters observed. In case of oxidative stress parameters, fish fed inosine supplemented diets showed best conditions because of they performed better under oxidative stress conditions as well as had highest tolerance against oxidation. Among NBP supplemented groups, diet group D2 also showed acceptable condition of oxidative stress tolerance. Stress resistance against low salinity exposure (LT_{50}) also increased with dietary supplementation and it was significantly highest in fish fed diet group D5. Total serum protein, serum lysozyme activity and total peroxidase content tended to be higher ($P>0.05$) in NBP and inosine supplemented diet groups. Numerically higher hepatic IGF-1, mRNA expression was found in diet groups D2 and D4. However, IGF-1 and IGF-2 mRNA expression were not significantly altered by dietary supplementations in the present study. Considering overall performance of the present study, we concluded that, inosine and low concentration of NBP (1%) could be effectively used as dietary supplements for better growth and health performance of *Pagrus major*.

Keywords: Nucleoside by-product, Inosine, Oxidative stress, Growth, Blood chemistry, Red sea bream

1. Introduction

Nucleotides (NT) are low molecular weight intracellular compounds which play key roles in diverse essential physiological and biochemical functions including encoding genetic information, mediating energy metabolism and signal transduction (Carver and Walker, 1995). Dietary nucleotides have been reported to be beneficial for humans and animals (Gil, 2002) since they positively influence lipid metabolism, immunity, and tissue growth, development and repair (reviewed in Gil, 2002). In aquatic animals both nucleotides and nucleosides have long been implicated as feed attractants in both vertebrate and invertebrate species (Mackie and Adron, 1978; Carr and Thompson, 1983; Person-Le Ruyet et al., 1983; Carr et al., 1984). However, world-wide heightened attention on nucleotide supplementation into potential growth and health benefits in aquaculture species instigated after 2000s. To date, research pertaining to nucleotide nutrition in fishes has shown rather consistent and encouraging beneficial results in fish health management.

Inosine, a purine nucleoside containing the base hypoxanthine and the sugar ribose, occurs in transfer RNAs, and is formed during the breakdown of adenosine by adenosine deaminase (Barankiewicz and Cohen, 1985). During the industrial preparation of inosine a liquid form of by product is produced after the separation of inosine. This liquid nucleoside by- product (NBP) contains considerable portion of inosine nucleoside and small portion of some other nucleotides. Recently, in aquaculture research nucleotides and its related product has been paid attention promisingly as functional nutrients. In aquaculture dietary nucleotide and its related products supplementation has been shown to enhance growth of certain fish species (reviewed by Li and Gatlin, 2006) immune responses and disease resistance of all

male hybrid tilapia (*Saratheradon niloticus* ♀× *Saratheradon aureus* ♂ (Ramadan et al., 1994), Atlantic salmon (*Salmo salar* L.) (Burrells et al., 2001a), common carp (*Cyprinus carpio* L.) (Sakai et al., 2001), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Li et al., 2004a), grouper (*Epinephelus malabaricus*) (Lin et al., 2009), red drums (*Sciaenops ocellatus*) (Cheng et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Tahmasebi-Kohyani et al., 2012) and Japanese flounder (*Paralichthys olivaceus*) (Song et al., 2012). Supplementation of nucleotides was also reported to increased stress tolerance in Atlantic salmon (Burrells et al., 2001b), rainbow trout (Leonardi et al., 2003), red sea bream (Hossain et al., 2016b) and even gastrointestinal physiology and morphology of tilapia (Ramadan et al., 1994), Atlantic salmon (Burrells et al., 2001b) and red drum (Cheng et al., 2011).

Red sea bream, *Pagrus major*, is one of the commercially important aquaculture species, whose production reaches the second largest in Japan (Koshio, 2002). Intensive culture of this species often exposed it to stressful conditions which impaired growth and immunity of the fish. Exposure to stress places additional demands on available nucleotides, and an additional exogenous supply of nucleotides provided by dietary supplementation may help to counter the immunosuppressive effects of stress (Low et al., 2003). Although, there are some research into potential growth and health benefits of dietary nucleotides in aquaculture species (Burrells et al., 2001a, 2001b; Li et al., 2004a; Cheng et al., 2011; Song et al., 2012, Hossain et al., 2016a,b). However, in most of cases, purified nucleotide mixtures were used and still, some gap exists about current knowledge of nucleotide supplementation in fish diets and its effects on physiology and immunity. Moreover, in aquaculture, nucleotides are more widely studied than relatively cheaper nucleosides. So far, study conducted on nucleosides has mainly focused on its feeding stimulatory properties rather than its functional properties .Until recently, there is also no research on the use of low cost industrial by-product, which can also be used as a source of nucleotide or nucleoside for red

sea bream as well as other marine species. In this circumstance, studies on the efficacy of supplementing relative low cost nucleotide related products *viz*. NBP and inosine on growth and health performance are important for the effective use of these functional supplements. So, the aim of this study was to investigate the efficacy of utilizing nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of red sea bream.

2. Materials and Methods

2.1.Test fish and experimental system

Juvenile red sea bream were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (54% crude protein, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 100 l polycarbonate tanks (filled with 80 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 3.1.1, 3.1.2, 3.1.3, 3.1.3 and 3.1.4 summarize the formulation and chemical composition, total and free amino acid composition of the experimental diets respectively. Five experimental diets were formulated to contain 49% protein, 11% lipid, without

nucleotide related products supplementation considered as control (D1). Nucleotide related products like; nucleoside by- products (NBP) and inosine were supplemented at 1, 3 and 0.03, 0.1% consecutively with basal ingredients of D1 and named as D2, D3 & D4and D5 respectively. The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 10 min. liquid NBP were also simultaneously added with dry ingredients of the respective diets. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (30–40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 55 °C for about 150 min. The test diets were stored at −28 °C in a freezer until use.

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Fifteen fish, having a mean initial body weight of approximately 2.28g were randomly allocated to previously prepare fifteen tanks. Fish were fed the experimental diets by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 1 hour after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk fortnightly to determine growth and visually check their health condition. The monitored water quality parameters (mean ± S.D.) were: water temperature 18.8±1.9 °C; pH 8.1±0.7 and salinity 34.5±0.5 during the feeding trial.

Table 3.1.1: Ingredients and formulation of the experimental diets.

Ingredients	Diet group				
	D1*	D2	D3	D4	D5
Fishmeal[a]	46.53	46.15	45	46.53	46.53
Soybean meal[b]	20	20	20	20	20
Pollack liver oil[c]	3.65	3.68	3.73	3.65	3.65
Soybean lecithin[d]	2.5	2.5	2.5	2.5	2.5
Vitamin mixture[e]	3	3	3	3	3
Mineral mixture[f]	3	3	3	3	3
Stay-C[g]	0.3	0.3	0.3	0.3	0.3
Wheat flour	11	11	11	11	11
Activated gluten[h]	5	5	5	5	5
α-cellulose	5.02	4.37	3.47	4.99	4.92
NBP[i]	0	1	3	0	0
Inosine[j]	0	0	0	0.03	0.1

*According to Kader et al. (2012)

[a]Nippon Suisan Co. Ltd., Tokyo, Japan.

[b] J. Oil Mills, Japan

[c]Riken Vitamin, Tokyo, Japan.

[d]Kanto Chemical Co., Inc. Tokyo, Japan.

[e]Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[f]Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[g]Stay-C 35.

[h]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name “A-glu SS”.

[i]NBP (Nucleoside by products)Ajinomoto Co., Inc., Tokyo, Japan.

[j]Tokyo Chemical Industry Co., Ltd. Tokyo, Japan

2.4. Sample collection

The initial sample of 40 fish for whole body analysis was stored at −20 °C. At the end of the feeding trial, fish were starved for 24 h prior to final sampling. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then the total number, individual body weight and length of fish from each tank were measured. Three fish from each replicate tank were randomly collected and stored at −20 °C for final whole body analysis. Using heparinized syringes, blood was collected from the caudal vein of five fish in each replicate tank and pooled for plasma analysis. In addition non-heparinized disposable syringes were used to collect blood for serum analysis. A small fraction of the heparinized blood was used to analyze the hematocrit level. Plasma samples were obtained by centrifugation at 4000 × g for 15 min using a high-speed refrigerated microcentrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80 °C. Liver and viscera were dissected out from three fish in each replicate tank, weighed individually to get hepatosomatic index and viscerasomatic index.

2.5. Biochemical analysis

The ingredients, diets and fish whole body were analyzed for moisture, crude protein, crude lipid and ash, in triplicate, using standard methods (AOAC, 1995). Total amino acids (TAA) and free amino acids (FAA) in diets were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) as described previously (Kader et al., 2012) . Briefly the samples were prepared as follows: 2 mg samples were spiked with known amount of norleucine as an internal standard and hydrolyzed with 4 N methanesulfonic acid at 110 °C for 22 h. The pH of the hydrolysate was adjusted to 2.2, filtered and stored at 4 °C. To quantify the FAA, 100 mg sample was mixed with 0.9 ml cold deionized water, 0.1 ml internal standard (norleucine, 0.6 mg DL-norleucine 0.1 ml^{-1}

deionized water) and 5 ml 10% trichloroacetic acid (TCA), homogenized using a polytron homogenizer (Kinematica, Gmbh LITTAU, Lucerne, Switzerland). Samples were then centrifuged at 4°C, 3000 × g for 15 min and supernatant was repeatedly washed with diethyl ether to remove TCA from homogenate. Finally, pH was adjusted to 2.2 and filtered samples were stored in 4°C. The chromatographic separation and analysis of the amino acids were performed with the HPLC unit with an ion exchange resin column. Inosine and IMP content of feeds were also analyzed by High Performance Liquid Chromatography (HPLC). The inosine and IMP content of the samples was determined by acidic extraction of ~1 g dry weight samples. In brief, for extraction process 25 ml of 5% percholoric acid was added to the feed sample (1g), shaking for 10 mins. After that deionized water was added to make constant volume upto 50 ml. Samples were sonicate for 10 mins, filtered and 5 ml sample solution were prepared. Finally, 0.4 ml potassium hydroxide (3 Mol/l) was added to neutralize the sample solution and stored in 4 ^{o}C. After extraction, for inosine quantification samples were loaded on an CAP CELL PAK C-18 column (Shisheido Co., Ltd. Japan), column temperature was 40 ^{o}C The mobile phase was the mixture of 200 mmol/l phosphate buffer solution (pH 2.5) and methanol (95:5 v/v) contained 0.6% sodium heptanes sulphonate. Measurement wavelength was 260 nm. For IMP quantification samples were loaded on a MCI CDR-10 column (Mitsubishi Chemical Co. Ltd.). Colum temperature was 40 ^{o}C .The mobile phase was 1 mol/l acetate buffer (pH 3.3). Measurement wavelength was 260 nm. The minimum amount of nucleotides detectable by HPLC is ~3 ng (in 50 µL injection volume). Total serum protein and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also

measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma sample was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg. For the analysis of plasma cortisol, 100 µl plasma was mixed with 1 ml diethylether by using a vortex mixture and allow to separate the organic phase. The diethylether was evaporated under a gentle stream of nitrogen. The extract was then analyzed for cortisol concentration using an enzyme-linked immunosorbent assay (Cortisol EIA Kit, product number EA65, Oxford Biomedical Research Inc., Oxford, MI).

2.6. Evaluation of non specific immune parameters

Serum lysozyme activity was measured with turbidimetric assays (Lygren et al., 1999). Ten microliters of samples was put into well of microplate, then added 190 µl of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4,their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

Table 3.1.2: Chemical composition of the nucleoside by products (NBP), and formulated experimental diets (% dry matter basis).

Parameters	NBP	Diet groups				
		D1	D2	D3	D4	D5
Moisture	66.2	7.3	6.2	6.3	6.7	6.5
Protein	25.4	48.5	49.1	49.0	48.9	49.2
Lipid	0.2	11.57	10.78	11.10	10.40	10.30
Ash	6.8	11.22	11.20	11.37	11.18	11.25
5'-IMP(%)	0.01	0.04	0.05	0.05	0.02	0.02
Inosine(%)	3.46	0.16	0.19	0.25	0.21	0.27
Gross energy (KJg^{-1})*	-	20.96	20.82	20.85	20.72	20.71

*Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ kg^{-1}, respectively.

Carbohydrate was calculated by the difference: 100-(protein + lipid + ash).

Table 3.1.3: Total amino acid content of the experimental diets (g , $100g^{-1}$, dry matter basis)*

	Diet group				
Amino acids	D1	D2	D3	D4	D5
Aspartic acid +Asparagine	4.13	4.17	4.21	4.24	4.20
Threonine	1.86	1.87	1.88	1.89	1.87
Serine	2.06	2.07	2.06	2.05	2.03
Glutamic acid +Glutamine	8.09	8.23	8.28	8.34	8.20
Glycine	2.48	2.55	2.58	2.57	2.56
Alanine	2.57	2.62	2.62	2.62	2.59
Valine	2.06	2.15	2.20	2.26	2.24
Isoleucine	1.79	1.88	1.91	1.98	1.97
Leucine	3.41	3.49	3.49	3.56	3.50
Tyrosine	1.51	1.56	1.56	1.55	1.55
Phenylalanine	2.01	2.06	2.07	2.08	2.07
Histidine	1.43	1.47	1.47	1.50	1.50
Lysine	3.04	3.11	3.12	3.14	3.12
Arginine	2.66	2.72	2.76	2.78	2.74
Proline	2.32	2.38	2.43	2.42	2.38
Cystine	0.54	0.55	0.54	0.54	0.54
Methionine	1.04	1.02	1.07	1.09	1.09
Tryptophan	0.599	0.603	0.602	0.602	0.590

*Values are means of triplicate measurements.

Table 3.1.4: Free amino acid content of the experimental diet (g , 100 g^{-1})*.

Free amino acids	Diet groups				
	D1	D2	D3	D4	D5
Taurine	0.28	0.29	0.28	0.28	0.29
Aspartic acid	0.03	0.03	0.03	0.03	0.03
Threonine	0.03	0.03	0.03	0.03	0.03
Serine	0.02	0.02	0.02	0.02	0.02
Glutamic acid	0.06	0.06	0.06	0.06	0.06
Glycine	0.03	0.03	0.03	0.03	0.03
Alanine	0.10	0.10	0.10	0.10	0.10
Valine	0.03	0.03	0.03	0.03	0.03
Isoleucine	0.02	0.02	0.02	0.02	0.02
Leucine	0.05	0.05	0.05	0.05	0.05
Tyrosine	0.02	0.03	0.03	0.02	0.02
Phenylalanine	0.03	0.03	0.04	0.03	0.03
Tryptophan	0.01	0.01	0.01	0.01	0.01
Lysine	0.05	0.06	0.07	0.05	0.05
Histidine	0.33	0.33	0.33	0.33	0.33
Arginine	0.07	0.07	0.07	0.07	0.07
Asparagine	0.01	0.01	0.02	0.01	0.01
Proline	0.03	0.03	0.03	0.03	0.03
Total Free AA	1.21	1.24	1.24	1.21	1.23

*Values are means of triplicate measurements.

The total peroxidase content in serum was measured according to Salinas *et al*. (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

2.7. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, four fish from each rearing tank (total 12 fish per treatment) were randomly selected and transferred as duplicate groups in to a blue colored 20 L rectangular glass aquarium with 15 L tap water which was aerated for 24 h. The glass aquaria for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of red sea bream juvenile was calculated according to Moe et al. (2004) as follows: time to death (min) of a juvenile was converted to log10 values. When the juvenile were transferred to the low salinity water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was log10 100 = 2). The calculation was then conducted every 5 minutes after the first exposure. The values of log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

$Y = aX + b$ where Y = log10 (survival), X = time for individual juvenile death (min).

LT_{50} (X) was obtained when Y = 1.7 since log10 (50) = 1.7. Each value obtained from the equation above was compared statistically.

2.8. Growth factor gene expression (IGF-1 and IGF-2)

After the completion of the feeding trials, three fish from each tank were removed and immediately anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Liver samples were obtained, placed in four volumes of RNAlater (Ambion; Applied Biosystems, Foster City, CA, USA) and stored at –80°C until analysis. For the RNA extraction the RNeasy Mini Kit (Qiagen) was used. Liver samples of 15 mg inserted in a tube (SARSTEDT A.200.01S), homogenized and centrifuged at 5000×g for 15 sec. The supernatant were collected and mixed with 70% ethanol. After extraction of RNA, cDNA was synthesized using the Prime Script RT Reagent Kit (Takara, Japan) following manufacturer's protocol. Real time PCR was done using SYBR select Master Mix kit (Thermo Fisher Scientific, Japan) using following primers as shown in Table 3.1.5. Elongation factor (ef1) was used as housekeeping gene (Table 3.1.5). Amplification was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the protocol as follows: Initial 2 min denaturation at 95℃, 40 cycles of 95℃ for 15 sec, and 65℃ for 30 sec. each assay was done in triplicate.

2.9. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR, % day^{-1}) = {Ln (final weight) – Ln (initial weight) / duration} × 100

Survival (%) = 100 × (final no. of fish / initial no. of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed conversion efficiency (FCE) =) live weight gain (g)/ dry feed intake (g

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Condition factor (CF, %) = weight of fish / (length of fish)^3 × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

2.10. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Turkey Kramer test.

3. Results

3.1. Fish performance parameters

Growth performance, nutrient utilization and survival of fish are presented in Table 3.1.6. Fish fed diet D5 showed significantly highest final body weight (FBW) and % weight gain (%WG) followed by diet groups D2 and D4. Fish fed control group showed lowest growth performance and it was not differed significantly with diet group D3. Feed conversion ratio and protein efficiency ratios were also significantly higher in diet group D5, whereas the other supplemented group showed intermediate value. Feed intake (FI) was significantly higher in fish fed diet D1 followed by D5, D2, D4 and D3. Significantly lower growth performance and feed utilization was observed in control diet group (D1). Survival was not significantly influenced by dietary supplementation.

3.2. Hematological parameters

Table 3.1.7 represents the hematological parameters of red sea bream after 60 days of feeding trial. Overall, dietary treatments had no significant effect on blood chemical parameters of fish among different treatments. However, plasma glucose level showed decreasing trend

Table 3.1.5 :Forward (F) and reverse (R) primers used for real-time quantitative RT-PCR.

Name	Database name	ID	Primer sequence: 5′ - 3′	Product size
igf1(F)	miseq db	2564	TAAACCCACACCGAGTGACA	90
igf1(R)			GCGATGAAGAAAAGCTACGG	
igf2(F)	miseq db	1567	CGGCAAACTAGTGATGAGCA	97
igf2(R)			CAGTGTCAAGGGGGAAGTGT	
ef1a(F)		666	TGTGGGTGCAGTTTGACAAT	204
ef1a(R)			CTTCAACGCTCAGGTCATCA	

Table 3.1.6: Growth performance and feed utilization in red sea bream fed test diets for 60 days[1]

Parameters	Diet groups				
	D1	D2	D3	D4	D5
IBW[2]	2.29±0.00	2.28±0.02	2.29±0.01	2.29±0.0	2.29±0.01
FBW[3]	13.66±0.5[a]	14.44±0.1 [ab]	13.87±0.2[a]	14.41±0.4[ab]	15.58±0.3[b]
WG[4]	491.1± 26.4[a]	532.3±3.6[ab]	506.3±11.4 [a]	527.0±16.0[ab]	578.8±13.4[b]
SGR[5]	2.96±0.07 [a]	3.07±0.01[ab]	3.00±0.03[a]	3.06±0.04[ab]	3.19±0.03[b]
FI[6]	12.1±0.4[b]	11.2±0.5[ab]	10.2±0.3[a]	10.9±0.3[ab]	12.6±0.[ab]
FCE[7]	0.89±0.10[a]	1.04±0.05[ab]	1.04±0.02[ab]	1.06±0.01[ab]	1.10±0.02[b]
PER[8]	1.83±0.2[a]	2.11 ±0.1[ab]	2.15±0.04[ab]	2.17±0.0[ab]	2.25±0.0[b]
Sur[9]	76.7±3.4	80.0±0.0	86.7±3.8	80.0±0.0	82.2±2.2

[1]Values are means of triplicate groups ±SEM. Within a row, means with different letters are significantly different ($P < 0.05$), and means with the same letters are not significantly different ($P > 0.05$). The absence of letters indicates no significant difference between treatments.

[2] IBW: initial body weight (g).

[3] FBW: final body weight (g).

[4] WG: percent weight gain (%).

[5] SGR: specific growth rate (% day^{-1}).

[6] FI: feed intake (g $fish^{-1}$ 50 $days^{-1}$).

[7] FCE: feed conversion efficiency.

[7] PER: protein efficiency ratio.

[9] Sur: survival (%).

numerically with the dietary supplementation of NBP and inosine. Hematocrit content was significantly highest in fish fed diet group D5, whereas the other supplemented groups showed intermediate values. Supplementation free control group showed the lowest value ($P<0.05$).

3.3. Oxidative stress parameters

Oxidative status of fish was analyzed from plasma and significant differences were found in BAP and d-ROM values among different treatments (Table-3.1.8). Inosine supplemented groups showed significantly lowest d-ROM values. Whereas control and 1% NBP supplemented groups showed intermediate value. At high supplementation level of NBP (3%), there was significantly higher d-ROM value. On the other hand, inosine supplemented groups showed significantly highest BAP values and it was not differed significantly with control. NBP supplementation of 1% (D2) showed intermediate value and the 3% NBP supplemented group showed lowest BAP value. Fig. 3.1.1 shows the pattern of combined effects of d-ROMs and BAP. Fish fed diet groups D4 and D5 were located in Zone A, where it is categorized as lower intensity of oxidative stress and higher tolerance ability against oxidative stress. Diet groups D1 and D3 were located in zone B and zone C, respectively. On the other hand, fish fed diet group containing the higher level of NBP (D3) was located in Zone D, which is categorized as higher intensity of oxidative stress and lower tolerance ability against oxidative stress. Dietary supplementations of inosine and NBP also reduce the plasma cortisol levels (%) compared to control but not at a significant level.

3.4. Immunological assays

Non-specific immune parameters *viz*. total serum protein (TSP), peroxidase activity and lysozyme activity are shown in Table 3.1.8. Numerically increasing non-specific immune

Table 3.1.7 : Blood chemistry of juvenile red sea bream fed test diets for 60 days*

Parameters	Diet groups				
	D1	D2	D3	D4	D5
Hematocrit (%)	28.7±0.7[a]	31.3±0.3[ab]	31.3±0.9[ab]	31.0±2.3[ab]	35.0±0.6[b]
Glucose (mg dL^{-1})	60.7±3.8	56.7±2.9	54.0±4.0	53.0±14.0	50.7±2.7
T-Cho[1]	202.7±6.4	223.7±19.3	205.3±5.9	179.0±11.0	220.3±19.2
BUN[2]	<5	<5	<5	<5	<5
T Bill[3]	<2	2.50	3.33	<2	<2
GPT[4]	22.3±9.1	24.3±7.3	28.3±3.3	42.0±7.0	26.3±15.8
Amylase (IU L^{-1})	12.0±2.0	14.0±4.0	17.3±4.7	10.5±0.5	11.0±0.6
TG[5]	104.3±7.7	122.0±5.7	117.3±10.8	98.5±0.5	126.7±12.8

[1] T-Cho: total cholesterol (mg dL^{-1}).

[2] BUN: blood urea nitrogen (mg dL^{-1}).

[3]T- Bill: Total bilirubin (mg dL^{-1}).

[4]GPT: glutamic pyruvate transaminase (IU L^{-1}).

[5]TG: triglyceride (mg dL^{-1}).

*Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

responses were observed in dietary supplemented groups compared to control but not at a significant level.

3.5. Low salinity stress test

The results of lethal stress test against fresh water shock on LT_{50} obtained by regression analysis are shown in Fig. 3.1.2. Significantly highest LT50 was obtained in diet group D5. Although, there was no significance among the other dietary groups (D2 to D4), there was notable numerical increase in LT_{50} values compared to the control group.

3.6. Hepatic IGF-1 and IGF-2 mRNA expression

Hepatic IGF-1 and IGF-2 growth factor gene expression were presented in Fig. 3.1.3a,b. Numerically higher hepatic IGF-1 mRNA expression was found in diet groups D2 and D4. However, overall hepatic insulin like growth factor (IGF-1, IGF-2) mRNA expression was not significantly influenced by the dietary treatment.

3.7. Whole body proximate composition and biometric indices

Initial and final whole body proximate compositions of juvenile red sea bream were shown in Table 3.1.9. All the fish showed a change in the analyzed parameters compared to those of the initial values. However, whole body proximate composition and (%) and biometric indices were not influenced significantly ($P>0.05$) by the dietary treatments except in hepatosomatic index (HSI) where, diet group D3 showed significantly highest HSI value and it was not differed significantly with inosine supplemented diet groups (D4 and D5). Diet group D2 showed the intermediate value, whereas control group showed significantly lowest value.

Table 3.1.8: Innate immunity and oxidative stress response of red sea bream fed test diets for 60 days.*

Parameters	Diet groups				
	D1	D2	D3	D4	D5
T Pro[1]	2.47±0.2	2.53±0.1	2.57±0.0	2.60±0.1	2.70±0.1
LA[2]	80.0±11.6	90.0±0.0	93.3±12.0	83.3± 8.8	103.3±8.8
PA[3]	1.74±0.03	1.86±0.1	1.78±0.1	1.89± 0.2	1.91±0.02
d- ROMs[4]	19.0 ± 1.0^{ab}	18.0 ± 1.0^{ab}	35.0 ± 8.8^{b}	13.0 ± 0.0^{a}	12.0 ± 1.0^{a}
BAP[5]	3387.5 ± 130.5^{b}	3109.7 ± 48.5^{ab}	2934.0 ± 80.2^{a}	3386.5 ± 24.5^{b}	3421.7 ± 46.8^{b}
CORT [6]	61.1±0.2	58.8±0.6	60.4± 0.5	59.4±0.7	59.3±0.9

[1] T- Pro: Total protein (g dL^{-1})..

[2]d-ROMs: reactive oxygen metabolites (μ mol L^{-1})

[3] BAP: biological antioxidant potential (U. Carr.)

[4]LA: Lysozyme activity of serum (unit mL^{-1})

[5] PA: Peroxidase activity of serum (OD 450 nm)

[6]CORT: relative value of cortisol (%).

*Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

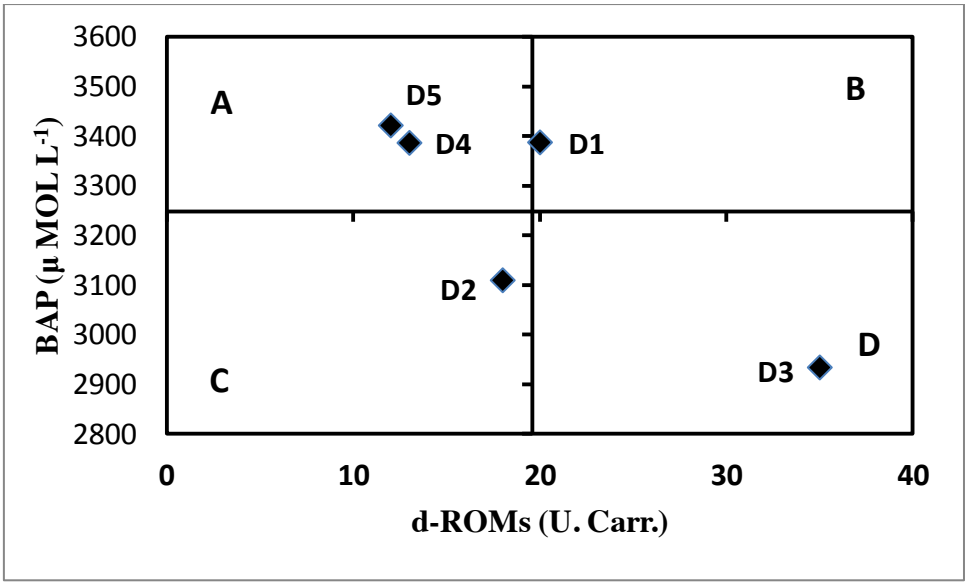


Fig. 3.1.1: Oxidative stress parameters in red sea bream fed test diets for 60 days. (Values are means of triplicate groups. Central axis based on mean values of d-ROM and BAP from each treatment) Zone A: High antioxidant potential and low reactive oxygen metabolites (good condition).Zone B: High antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone C: Low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone D: Low antioxidant potential and high reactive oxygen metabolites (stressed condition).

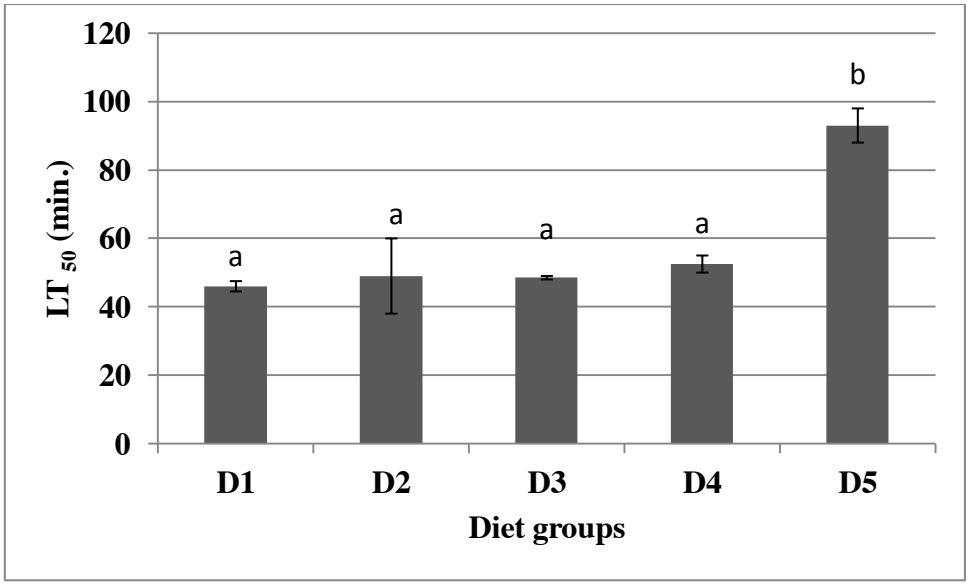


Fig. 3.1.2. LT_{50} (min) calculated from the lethal time of red sea bream exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from duplicate groups. Values with different letters are significantly different ($P<0.05$).

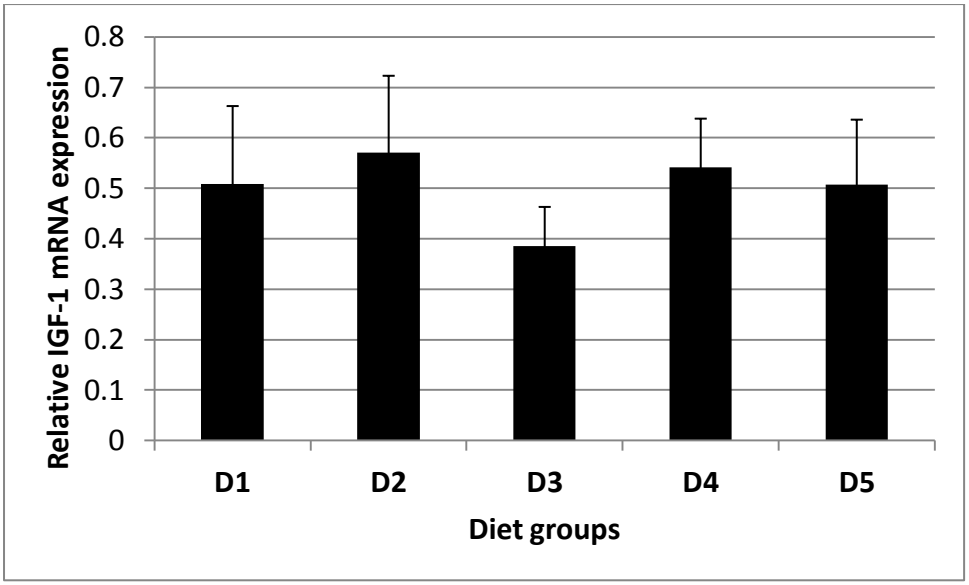


Fig. 3 (a)

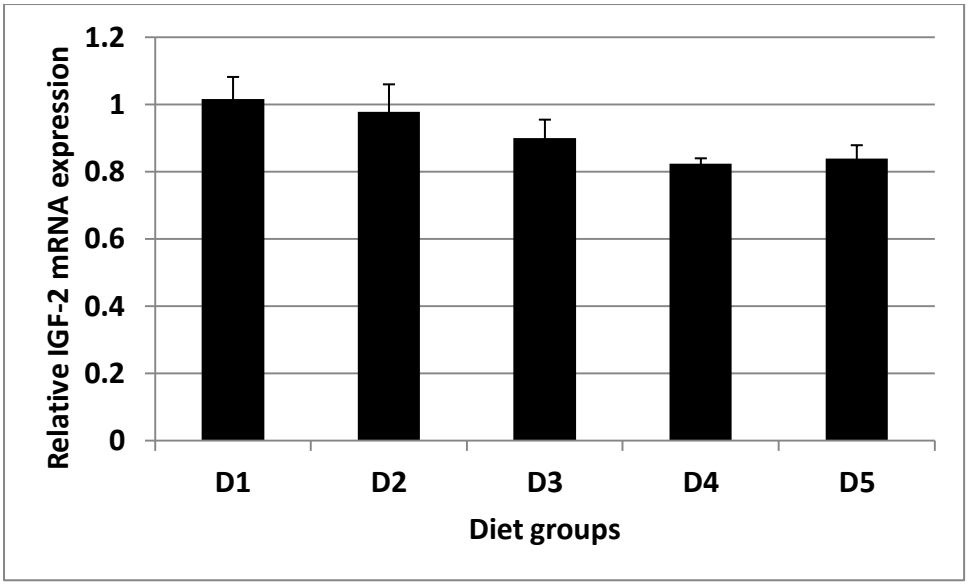


Fig. 3 (b)

Fig. 3.1.3 (a,b): Hepatic insulin like growth factor gene expression (IGF-1 and IGF-2) of red sea bream fed experimental diet for 60 days.

Table 3.1.9: Whole body proximate analysis (%) and biometric indices in juvenile red sea bream fed test diets for 60 days*

Parameters	Initial[1]	Diet group				
		D1	D2	D3	D4	D5
Moisture	19.6	75.9±0.3	76.0±0.5	76.2±0.5	76.1±0.4	76.3±0.4
Crude protein	12.7	13.7±0.2	14.0±0.4	13.6±0.2	13.9±0.3	13.9±0.1
Crude lipid	3.18	6.06±0.1	5.92±0.3	5.80±0.1	6.02±0.1	6.01±0.0
Crude ash	3.42	3.63±0.1	3.73±0.1	3.74±0.1	3.79±0.1	3.41±0.1
CF[2]	-	1.76±0.0	1.79±0.0	1.74±0.1	1.84±0.1	1.85±0.0
HSI[3]	-	1.19±0.5[a]	1.23±0.02[ab]	1.50±0.08[c]	1.47±0.07[bc]	1.47±0.05[bc]

[1] Initial values are not included in the statistical analysis.

[2]CF: condition factor (%).

[3]HSI: hepatosomatic index (%).

* Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05). Crude protein, crude lipid and ash are expressed on a wet weight basis.

4. Discussion

Increasing feed cost is the major limitation of the nucleotide as well as the immunostimulant administration in aquaculture diets. One of the most important alternative to reducing feed cost in nucleotide administration in fish feed is to search for relatively low cost industrial by-products obtained through recycling of industrial waste which contain considerable portion of nucleotides or nucleosides. In the present study a nucleoside inosine and a liquid NBP obtained through the production process of inosine were evaluated on growth, blood chemistry, oxidative stress and hepatic IGF-1 and IGF-2 mRNA expression of juvenile red sea bream.

In the present study, significantly highest FBW, %WG and SGR (% day^{-1}) of juvenile red sea bream were observed in fish fed diet D5 followed by fish fed the diets with D2, D4, D3 and D1. The highest growth performance in diet D5 and improved growth performance in diet D2 to D4 might be due to supplementation of nucleoside inosine and NBP, which contains considerable portion of inosine and small portion of some other nucleotides. Alike our present results, the growth enhancing and feeding stimulating properties of nucleosides inosine have been reported earlier in some studies (Ishida and Hidaka, 1987; Kumazawa and Kurihara, 1990; Yamaguchi, 1991). Person-Le Ruyet et al. (1983) reported dietary inosine enhanced growth and survival of turbot larvae. In subsequent research, this group showed that 10 or 20 days of feeding a diet supplemented with 0.77% inosine also significantly increased weight gain of turbot larvae. So far, there is no exact explanation how dietary nucleotide and nucleosides work to enhance growth. However, it is assumed that the growth-enhancing effect of inosine and other nucleotide in the present study resulted from improved feed intake at the beginning of weaning, promoting more rapid food intake that reduced nutrient leaching into the water or possibly playing roles in metabolism (Metailler et al., 1983, Hossain et al., 2016a,b). It is also well known that growth response was strongly

related to improve feed utilization. Our results suggested that among the tested fish that utilized experimental diets effectively, there were significantly higher feed efficiency ratio (FER) and protein efficiency ratio (PER) in fish fed diet D5. Other supplemented diet groups also showed moderately improved feed utilization (FCE, PER) values. The improved FER and PER would explain the increased growth performance in supplemented groups in the present study.

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish (Hossain et al., 2016a,b). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to those of the previous findings (Aoki et al., 1998; Hossain et al. 2016a,b). Hematocrit, which is used as an assessement for general health in fish based on different nutritional strategies, was enhanced with supplementation of dietary NBP and inosine in juvenile red sea bream. In the present experiment, hematocrit content was significantly highest in fish fed diet group D5 and the other supplemented groups showed intermediate values, whereas control group (D1) showed significantly lowest value. This indicated that dietary NBP and inosine elevated the health status of fish. Similarly, Song et al. (2012) and Hossain et al. (2016b) reported the enhanced hematocrit level with the supplementation of nucleotides in Japanese olive flounder and red sea bream diets, respectively. Interestingly, dietary NBP and inosine affected glucose levels in red sea bream ($P>0.05$). Plasma glucose is commonly considered to be one stress indicator in fish, high glucose levels often indicate the higher stress status of fish (Eslamloo et al., 2012). The lower glucose contents in fish fed supplemented diet groups, indicated that red sea bream fed supplemented diets displayed optimal physiological conditions compared to the control group. Blood glucose levels of the present study (50.7–60.7 mg dl^{-1}) were comparable to the

values (56.50–83.50 mg dl^{-1}) reported previously for juvenile red sea bream (Hossain et al., 2016b) fed nucleotide supplemented diet.

Stress is one of the emerging factors in aquaculture activities which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization (Li et al., 2009). Plasma or serum cortisol concentration is a reliable biological indicator of stress response in fish and terrestrial animals (Li et al., 2009; Small and Davis, 2002). In the present study, the relative values of plasma cortisol concentrations were decreased compared with control, with the supplementation of inosine and NBP but not at a significant level. Reduced plasma cortisol levels of red sea bream fed inosine and NBP supplemented diet in the present study are in agreement with what has been previously reported in rainbow trout (Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2012) and beluga sturgeon (*Huso huso*) Yousefi et al. (2011) . Oxidative stress can be generated at high level of reactive oxygen species (ROS) and/or decreased efficacy of antioxidant system, which is another health risk factor in humans or other mammals (Pasquini et al., 2008). Oxidative stress was measured using the free radical analytical system assessing the derivatives of oxidative stress by measuring reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) in plasma samples (Gao et al., 2012). Hossain et al. (2016a,b) reported that, fish with higher value of d-ROMs confirm a more oxidative condition, therefore, higher BAP value indicate they have stronger tolerance against oxidation. Using these parameters, our study showed that fish fed diets D4 and D5 were more tolerant of oxidative stress indicating a higher health status, which indicated high BAP and low d-ROMs values. On the contrary, fish fed diet D3 were located in Zone D, which is categorized as higher intensity of oxidative stress and lower tolerance ability against oxidative stress. To date, there is a lack of explanation about how these additives work to affect these parameters, so more studies are needed.

The lethal stress test is used to assess health status by measuring the lethal time of 50% mortality (LT50) in fresh water of the fish (Hossain et al., 2016a,b). It is well known that stress affects the survival and growth of fish, since stress responses tend to increase the energy demand at the expense of anabolic processes (Kubilay and Ulukoy, 2002). The higher value of LT50 in fish fed diet group D5 indicated a higher tolerance of the red sea bream against low-salinity stress. Fish antioxidant status is strongly related to its immune system, contributing to enhanced resistance towards different stressors (Tovar-Ramirez et al., 2010). In the light of the previous findings, results of the current study confirmed a higher tolerance against low-salinity stress in fish under less oxidative stress conditions.

In the present study, whole-body proximate composition and biometric indices were not significantly influenced by dietary inosine supplementation except HSI (Table 3.1.9). However, whole body compositions were within the normal ranges that have been reported previously (Hossain et al., 2016a,b). No significant effects of dietary nucleotides on red sea bream and red drum whole body and biometric indices were also observed by Hossain et al. (2016) and Li *et al*. (2007), respectively which is similar to our study. The liver size is relative to the nutritional status of the fish (Shoemaker *et al*., 2003; Sridee & Boonanuntanasarn, 2012). In the present study, increased HSI with NBP and inosine supplementation indicates proper storage of macro and micronutrients, healthy condition of liver and clinically healthy signs of fish.

Molecular tools have been increasingly utilized in aquaculture research to complement existing husbandry techniques and to examine the responses of fish to culture stress and environmental changes (Stone et al., 2008; Panserat and Kaushik, 2010). Molecular technology (gene expression of insulin-like growth factors IGF- I and II) has been used as the most promising molecular marker to date as a rapid indicator of growth in teleost fish (Picha et al., 2008) and to evaluate the efficacy of diets for commercial purpose within very short

time. Previous research investigating hepatic IGF-I in relation to nutritional status and growth in cultured finfish has focused on feed deprivation or feed restriction, rather than on the manipulation of a selected dietary component such as protein or lipid (Picha et al., 2006; Bower et al., 2008; Hagen et al., 2009). In the present study, an attempt has been made to investigate hepatic IGF-1, IGF-2 in relation to supplementation of functional nutrients (nucleotide related products) in red sea bream diets. IGF-1 and IGF-2 mRNA expression were not significantly altered by dietary supplementations in the present study. However, numerically higher hepatic IGF-1, mRNA expression was found in diet groups D2 and D4 and with some small exception, in most of the dietary treatments, gene expressions correlates with the growth rate of red sea bream.The positive relationship between IGF-I, IGF-2 and growth rate were further confirmed in other fishes (Uchida et al., 2003; Dyer et al., 2004; Picha et al., 2006). This unexpected correlation might be due to large variation in gene expression between individual fish. Pedroso et al. (2009) demonstrated substantial variation in hepatic IGFBP-1 mRNA in the congeneric Japanese yellowtail over a 21-day sampling period in response to feed restriction, with sampling intervals of 3 days. Another important cause of this unexpected correlation of IGF-1 with some dietary treatments might be due to the lower temperature (less than 18 ^{0}C) during the rearing period which could disturb the IGF-1 and IGF-2 expression of red sea bream. Similar temperature related drawbacks of IGFs study were also reported by Beckman et al. (2004 a,b).

According to the result of the study, it is clear that low concentration of NBP (1%) supplemented group (D2) and inosine supplemented group improves the growth, feed utilization and some of the hematological parameters of red sea bream. Further, supplementation also improves non specific immune response like, total serum protein, lysozyme activity and peroxidase activity ($P>0.05$). Blood chemical parameters and growth factor gene expression (IGF1 and IGF-2) were not significantly affected by dietary treatment.

This might be due to the inappropriate dosages or other causes. Interestingly, inosine supplementation showed best oxidative stress condition compared with NBP. Finally, it can be concluded that, inosine and low dosage of NBP (1%) could be effectively used in red sea bream diet for improved growth and health performance of this species. However, further study is needed to optimize the inclusion level of these nucleotide related products and to discover the underlying reasons for the growth-promoting effects and the improved health features found in the present study.

CHAPTER IV

Functional effects of individual nucleotides

Part-1

Effects of dietary administration of inosine on growth, immune response, oxidative stress and gut morphology of juvenile amberjack, *Seriola dumerili*

Abstract

A 50-day feeding trial was conducted to determine the effects of dietary inosine on growth performance, immune responses, oxidative stress and gut morphology of juvenile amberjack, *Seriola dumerili*. Five isonitrogenous (52% crude protein) and isolipidic (14% crude lipid) semi-purified test diets were prepared by supplementing incremental levels of dietary inosine nucleoside at 0% (D1, control), 0.1% (D2), 0.3% (D3), 0.6% (D4) and 0.9% (D5), respectively. Triplicate groups of juvenile with an initial average weight of approximately 26 g were randomly stocked in fifteen, 200L polycarbonate tanks at a stocking density of 20 fish per tank. The fish were hand-fed twice daily to apparent satiation level. At the end of the feeding trial, final body weight (g), weight gain (%) and specific growth rate (% day^{-1}) were significantly (P<0.05) higher in fish fed with D4 and D5 diets compared to those in D1 group. Significantly higher survival rate was found in D4, lowest in D1 and no differences were found between D4 and D5, respectively. Inosine supplemented groups showed significantly higher values of whole body lipid and hepatosomatic index compared to those in D1 group. Dietary inosine reduced the oxidative stress of fish. Lysozyme activity, bactericidal activity and peroxidase activity had increasing trends with the increasing levels of inosine in diets. On intestinal health condition, anterior enterocyte height was higher in D3, and anterior as well as posterior fold heights were also higher in inosine supplemented groups than D1 group. The quadratic regression analysis of weight gain and lysozyme activity revealed that the optimal levels of dietary inosine were 0.54 and 0.67 %, respectively, for juvenile amberjack, which is also inline with the most of the growth performance and health parameters of fish under present experimental condition.

Keywords: Inosine, Growth, Immune responses, Oxidative stress, Gut morphology, *Seriola dumerili*

1. Introduction

Aquaculture practices often place a great deal of physiological stress on the animals, which can result in immune suppression, reduced growth rate and increased susceptibility to disease (Anderson, 1996). In aquaculture practices, antibiotics, vaccination and other chemicals are currently used in varying degree to control these diseases. However, each of these treatment methods has its drawbacks, including suppression of aquatic animal's immune system, environmental hazards and food safety problem. Moreover, the development of antibiotic resistance in humans has led to a growing interest in antibiotic-free animal production worldwide (Bager, 2000). In this regards the addition of immunostimulants or immunomodulators to fish diets has been suggested as an effective alternative for prophylactic treatment against disease outbreaks in intensive aquaculture (Sakai, 1999). Recently, nucleotide and its related products have received attention commercially and scientifically as potential immunomodulators as well as functional materials in aquaculture.

Nucleotides are low molecular weight intracellular compounds which play key roles in nearly all biochemical processes (Gil, 2002). A nucleotide consists of a nitrogenous base, a sugar (ribose or deoxyribose) and one to three phosphate groups. When phosphate group of nucleotide is removed by hydrolysis, the structure remaining is nucleoside. Dietary supplementation of nucleotides or nucleosides have been shown to benefit many mammalian physiological and nutritional functions (Uauy, 1989; Quan, 1992; Carver, 1994; Haskó et al., 2000). In aquatic animals, both nucleotides and nucleosides have long been implicated as feed attractants in both vertebrate and invertebrate species Mackie and Adron, 1978; Carr and Thompson, 1983; Person-Le Ruyet et al., 1983; Carr et al., 1984). However, world-wide heightened attention on nucleotide supplementation into potential growth and health benefits in aquaculture species instigated after 2000s. To date, research pertaining to nucleotide nutrition in fishes has shown rather consistent and encouraging beneficial effects in fish

health management. Dietary nucleotide supplementation has been reported to improve growth and immune response of Atlantic salmon (*Salmo salar*) (Burrells et al., 2001a,b), grouper (*Epinephilus malabaricus*) (Lin et al., 2009), red drum (*Sciaenops ocellatus*) (Li et al., 2007) and Japanese flounder (*Paralichthys olivaceus*) (Song et al., 2012). Nucleotide supplementation also significantly increased mean fold heights of proximal, mid and distal intestine as well as total gut surface of Atlantic salmon compared to control (Burrells et al., 2001b). Inosine, a purine nucleoside containing the base hypoxanthine and the sugar ribose, which occurs in transfer RNAs. Inosine is formed by the deamination of adenosine and it is considered as a functional nutrient. In aquaculture, inosine has been studied most extensively as a specific nucleoside for feeding stimulation research rather than using as a functional nutrient for potential growth and health benefit of aquatic species. However, some studies on different aquatic species have reported that dietary inosine or inosine monophosphate (IMP), either alone or in combination with certain free amino acids can enhance growth performance, survival and feed intake of juvenile ell (Takeda et al., 1984), turbot *(Scophthalmus moximus)* (Mackie and Adron, 1978; Person-Le Ruyet et al., 1983), dover sole (*Solea vulgaris*) (Metailler et al., 1983), while it can also improve disease resistance and immune responses such as lysozyme activity, myloperoxidase activity and nitro-blue-tetrazolium activity of Japanese flounder, (*P. olivaceus*) (Song et al., 2012) and head kidney leucocyte superoxide anion (O_2^-) production ratio of grouper (*E. malabaricus*) (Lin et al., 2009) .

The amberjack, (*Seriola dumerili*), is a marine pelagic carnivorous species and has a great potential for the global aquaculture industry owing to its rapid-growing and adaptive characteristics. (Vergara et al., 1996; Jover et al., 1999; Thompson et al., 1999; Mazzola et al., 2000). It is distributed throughout the tropical and subtropical seas. Recently, the aquaculture of this species is intensified in the Mediterranean region and Japan (Nakata,

2000; Takakuwa et al., 2006). However, nutritional studies of this species are scarce. Effects of nucleotide or nucleoside as a functional nutrient of this species are not reported elsewhere. In addition, no studies are also reported on the use of inosine nucleoside for potential growth and health benefit of juvenile amberjack. Therefore, the present study was conducted to assess the effects of dietary inosine supplementation on growth performance, non-specific immune response, oxidative status and gut health condition of amberjack.

2. Materials and methods

2.1. Test fish and experimental system

Juvenile amberjack were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (50% crude protein, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 200 L polycarbonate tanks (filled with 170 L of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 4.1.1, 4.1.2 and 4.1.3 summarize the basal diet formulation, proximate composition and total amino acid composition of the experimental diets respectively. Fishmeal and casein based five semi-purified test diets were formulated to be nearly isonitrogenous (52% crude

protein), isolipidic (14% crude lipid) and isocaloric (21.9 KJ g^{-1} gross energy).The experiential diets were supplemented with inosine nucleoside (Tokyo Chemical Industry Co., Ltd. Tokyo, Japan) at the expense of α-cellulose to give a concentration of 0, 0.1, 0.3, 0.6, and 0.9 % in D1 (control), D2, D3, D4 and D5 diets, respectively. To prepare each diet, initially required amount of inosine and arginine were mixed properly in a glass beaker by hands with the help of a stainless spatula. Then this mixture was thoroughly mixed with other dry ingredients in a food mixer for 10 min. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (35 to 40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0 to 7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2 to 2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 60°C for about 120 min. The test diets were stored at −28 °C in a freezer until use. The ingredients and test diets were analyzed for moisture, crude protein, crude lipid and ash, in triplicates, using standard methods (AOAC, 1995). Total amino acids (TAA) and inosine in diets were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan). The TAA was analyzed according to Kader et al. (2010). The inosine content of the samples was determined by acidic extraction of ~1 g sample. In brief, for extraction process 25 ml of 5% perchloric acid was added to the feed sample, shaking for 10 mins. After that deionized water was added to make constant volume upto 50 ml. Samples were sonicate for 10 mins, filtered and 5 ml sample solution were prepared. Finally, 0.4 ml potassium hydroxide (3 Mol/l) was added to neutralize the sample solution and stored at 4 ^{o}C. The chromatographic separation and analysis of the inosine was performed with the HPLC unit with an ion exchange resin column. For inosine quantification,

Table 4.1.1 : Formulation of basal diet for juvenile amberjack.

Ingredients	Composition (%)
Fishmeal[a]	32.00
Casein[b]	20.00
Pollack liver oil[c]	6.00
Soybean lecithin[d]	6.00
Vitamin mixture[e]	3.00
Mineral mixture[f]	3.00
L-Arginine[g]	4.00
Stay-C[h]	0.15
Wheat flour	9.87
Activated gluten[i]	5.00
α-Cellulose	10.98
Inosine level[j]	0-0.9

[a]Nippon Suisan Co. Ltd., Tokyo, Japan

[b]Waco Pure Chemical Industries, Inc. (Osaka, Japan).

[c] Riken Vitamin, Tokyo, Japan.

[d] Kanto Chemical Co., Inc. Tokyo, Japan

[e] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline chloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[f] Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[g] Nacalai Tesque, Kyoto, Japan.

[h] Stay-C 35.

[i]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS".

[j]Tokyo Chemical Industry Co., Ltd. Tokyo, Japan (Inosine added to diets at the expense of α-cellulose).

Table 4.1.2: Proximate composition of the experimental diets (% dry matter basis).

Parameters	Diet groups				
	D1	D2	D3	D4	D5
Moisture	8.9	9.2	6.7	7.4	7.2
Crude protein	51.1	51.6	52.6	51.9	52.9
Crude lipid	14.0	14.2	14.2	14.3	13.8
Ash	10.3	10.2	10.5	10.4	10.4
Gross energy (KJg^{-1})*	21.8	21.9	21.9	21.9	21.9
Inosine (%)	0.03	0.11	0.31	0.61	0.90

*Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ kg^{-1}, respectively.

Carbohydrate was calculated by the difference: 100-(protein + lipid + ash).

Table 4.1.3: Total amino acid (AA) content of the experimental diets (g $100g^{-1}$, dry matter basis).*

Amino acids	Diet groups				
	D1	D2	D3	D4	D5
Aspartic acid +Asparagine	3.41	3.44	3.49	3.44	3.48
Threonine	1.80	1.78	1.82	1.81	1.81
Serine	2.31	2.24	2.31	2.31	2.31
Glutamic acid +Glutamine	8.71	8.86	8.87	8.81	8.94
Glycine	2.11	2.13	2.23	2.30	2.37
Alanine	1.96	1.98	2.00	1.99	1.99
Valine	2.35	2.45	2.46	2.40	2.44
Isoleucine	1.87	1.98	2.00	1.95	1.98
Leucine	3.52	3.63	3.65	3.59	3.65
Tyrosine	1.78	1.83	1.84	1.82	1.87
Phenylalanine	1.96	2.03	2.02	1.99	2.03
Histidine	1.09	1.13	1.15	1.13	1.14
Lysine	3.02	3.11	3.09	3.05	3.10
Arginine	5.59	5.59	5.80	5.71	5.69
Proline	3.54	3.60	3.66	3.60	3.63
Cystine	0.37	0.37	0.37	0.37	0.37
Methionine	1.12	1.13	1.15	1.13	1.15
Tryptophan	0.48	0.49	0.49	0.48	0.49
Total AA	47.00	47.80	48.40	47.90	48.40

*Values are means of triplicate measurements.

samples were loaded on an CAP CELL PAK C-18 column (Shisheido Co., Ltd. Japan), column temperature was 40 ^{o}C. The mobile phase was the mixture of 200 mmol/l phosphate buffer solution (pH 2.5) and methanol (95:5 v/v) contained 0.6% sodium heptanes sulphonate. Measurement wavelength was 260 nm. The minimum amount of nucleotides detectable by HPLC is ~3 ng (in 50 µL injection volume).

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Twenty fish, having a mean initial body weight of approximately 26 g were randomly allocated to previously prepared fifteen tanks. Fish were fed the experimental diets to visual satiation (twice a day, 09.00 and 16.00 h) for 50 days. The daily feed supplied was recorded, and the uneaten feed was collected 30 min after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk at ten days interval to determine growth and visually check their health condition. The monitored water quality parameters (mean ± S.D.) were: water temperature 25.8±1.9°C, pH 8.0±0.7, and salinity 33.9±0.5 ppt during the feeding trial.

2.4. Sample collection and biochemical analysis

The initial sample of 10 fish for whole body analysis was stored at −20 °C. At the end of the feeding trial, fish were starved for 24 h prior to final sampling. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then the total number, individual body weight and body length of fish from each tank were measured. Three fish from each replicate tank were randomly collected and stored at −20 °C for final whole body analysis. Using heparinized syringes, blood was

collected from the caudal vein of three fish in each replicate tank and pooled for plasma analysis. In addition non-heparinized disposable syringes were used to collect blood for serum analysis. A small fraction of the heparinized blood was used to analyze the hematocrit level. Plasma and serum samples were obtained by centrifugation at 4000 × g for 15 min using a high-speed refrigerated microcentrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80 °C. Liver and viscera were dissected out from three fish in each replicate tank, weighed individually to get hepatosomatic index and viscerasomatic index. Standard methods (AOAC, 1995) were also used for the analysis of whole body moisture, crude protein, crude lipid and ash. Total serum protein and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma sample was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg. For the analysis of plasma cortisol, 100 µl plasma was mixed with 1 ml diethylether by using a vortex mixture and allow to

separate the organic phase. The diethylether was evaporated under a gentle stream of nitrogen. The extract was then analyzed for cortisol concentration using an enzyme-linked immunosorbent assay (Cortisol EIA Kit, product number EA65, Oxford Biomedical Research Inc., Oxford, MI).

2.5. Immunological assays

Plasma lysozyme activity was measured with turbidimetric assays (Lygren et al., 1999). Ten microliters of samples was put into well of microplate, then added 190 µl of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4, their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

Serum bactericidal activity was measured according to Yamamoto and Iida, (1995). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g/ml, *Escherichia coli*, DH5α strain) and incubated at 25 °C for 24 h in micro tube rotator (MTR-103, AS ONE, Osaka, Japan). 50 ml of the reaction solutions were incubated on TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) at 25 °C for 24 h (on X-plate). Colony forming unit (CFU) was counted by the plate counting method as described by Ren et al. (2008). The bactericidal activity was defined as follows: (CFU of blank group-CFU of each group)/CFU of blank group × 100.

The total peroxidase content in serum was measured according to Salinas *et al.* (2008) , with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo

Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

2.5. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (WG, %)=(final weight-initial weight)×100/initial weight

Specific growth rate (SGR, % day^{-1})={Ln (final weight)-Ln (initial weight)/duration}×100

Survival (SUR, %)=100×(final no. of survived fish/initial no. of fish)

Feed intake (FI, g $fish^{-1}$50 $days^{-1}$)=(dry diet given-dry remaining diet recovered)/no. of fish

Feed conversion ratio (FCR)=dry feed intake (g)/live weight gain (g)

Protein efficiency ratio (PER)=live weight gain (g)/dry protein intake (g)

Condition factor (CF, %)=100×weight of fish/$(\text{body length of fish})^3$

Hepatosomatic index (HSI, %)=100×weight of liver/weight of fish

Viscerasomatic Index (VSI, %)=100×weight of viscera/weight of fish

2.6. Gut morphology study

At the end of feeding trial two fish from each replicate tank was used for intestinal morphology study. For preparation of intestinal sample firstly whole gastrointestinal tract was removed and anterior and posterior intestine were collected. Then segments (0.5-1 cm) of each part was cut out and the lumen was flushed with saline (Otsuka Normal Saline, Otsuka Pharmaceutical, Tokyo) followed by formalin fixative (10%-Formaldehyde Neutral Buffer Solution 37152-5-1, Nacalai Tesque, Kyoto) to remove intestinal content. Afterward each tissue was placed in plastic bag (HistoPack, Falma, Tokyo) filled with formalin fixative. Each tissue was embedded in paraffin, sliced, and stained with hematoxylin and eosine (H&E).

Two cross-sectional slices were prepared from each tissue. The tissue slides were examined under a light microscope (Eclipse 50i, Nikon, Tokyo) and a camera (Digital Sight DS2MV with control unit DS-L2, Nikon, Tokyo) interfaced with Sigma Scan Pro 5 software (SPSS Inc.,IL,USA). Intestinal fold height (hF), enterocyte height (hE), microvillous height (hMV) were measured with a magnification of 100x, 200x and 400x respectively. For each tissue, 10 measurements were performed.

2.7. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Tukey Kramer test. The parameters with significant differences were subjected to quadratic regression analysis with dietary inosine level in the diets.

3. Results

3.1. Fish performance parameters

Table 4.1.4 demonstrated the data on the growth performances. At the end of feeding trial, fish fed D4 showed significantly ($P<0.05$) highest final weight (FW), SGR, WG (%) and survival (%) followed by groups fed with D5 and D3 diets, respectively. While the fish fed with inosine free control diet (D1) showed the lowest value ($P<0.05$). The FCR was significantly lowest in fish fed D4, followed by D5 and the highest in control group. Similarly, the PER was also highest ($P<0.05$) in D4 and no differences were found among the rests. The FI was highest ($P<0.05$) in D4 and no differences were found among D3, D4 and D5; while significantly decreased in D1 and D2 groups. Overall, all the growth performance and feed utilization parameters were significantly lowest in fish fed control diet (D1).

Quadratic regression analysis showed that FW, WG, SGR, FI, FCR, PER and SUR were quadratic responses to the increasing dietary inosine levels (Y_{FW} = -104.45x^2 + 142.01x + 62.208, R = 0.9779; Y_{WG} = -405.84x2 + 550.09x + 140.37, R = 0.9775; Y_{SGR} = -2.4079x^2 + 3.2532x + 1.8186, R = 0.9903; Y_{FI} = -185.39x2 + 260.81x + 43.398, R = 0.9962; Y_{FCR} = 1.2058x2 - 1.4815x + 2.0974, R = 0.8804; Y_{PER} = -0.6979x2 + 0.8187x + 0.9223, R = 0.7658; Y_{SUR} = -45.401x2 + 75.09x + 57.949, R = 0.9894).

3.2. Hematological parameters

Table 4.1.5 represents the blood parameters of juvenile amberjack after 50 days of feeding trial. Overall, dietary treatments had no significant effect on blood chemical parameters of fish among different treatments except in blood urea nitrogen (BUN). BUN increased significantly in inosine supplemented groups compared to the control (D1) Triglycerides contents numerically decreased with the supplementation of inosine but not at a significant level. Hematocrit content was significantly highest in fish fed diet contained 0.61% inosine (D4) and then plateaued whereas control group (D1) showed significantly the lowest value. Meanwhile BUN and hematocrit level showed quadratic response with graded levels of inosine (Y_{BUN} = -45.059x2 + 57.739x + 10.256, R^2 = 0.773; $Y_{Haematocrit}$ = -20.585x2 + 28.511x + 29.88, R^2 = 0.9755).

3.3. Oxidative stress parameters

Fig. 4.1.1 indicates the status of oxidative stress conditions, in which the conditions of both stress intensity and tolerance ability were combined, of fish fed with test diets for 50 days. No significant effect was observed on d-ROMs and BAP of juvenile amberjack (no data illustrated here). Combined effects of d-ROM and BAP showed that fish fed 0.31% inosine content diet (D3) was located in zone A which was categorized as lower intensity of

Table 4.1.4 Growth performance and feed utilization of juvenile amberjack fed test diets for 50 days.*

Parameters	Diet groups				
	D1	D2	D3	D4	D5
IBW[1]	25.9±0.0	25.9±0.1	25.9±0.1	25.9±0.0	25.9±0.1
FBW[2]	69.1± 2.3^{a}	74.2±4.0^{a}	93.7±4.6^{b}	113.3±3.1^{c}	104.2±4.4^{bc}
WG[3]	167±9.2^{a}	187±16^{a}	262±18^{b}	338±12^{c}	302±16^{bc}
SGR[4]	1.96±0.07^{a}	2.10±0.11^{a}	2.57±0.10^{b}	2.95±0.05^{b}	2.78±0.08^{b}
FI [5]	52.8±2.5^{a}	66.6±5.3^{a}	108.7±9.0^{b}	132.5±3.2^{b}	128.2±5.2^{b}
FCR[6]	2.07±0.09^{c}	1.89±0.07^{bc}	1.84±0.03^{abc}	1.58±0.02^{a}	1.76±0.05^{ab}
PER[7]	0.95±0.05^{a}	1.03±0.04^{a}	1.04±0.02^{a}	1.22±0.02^{b}	1.07±0.03^{a}
Sur[8]	60.0±2.9^{a}	66.7±1.7^{ab}	75.0±2.9^{bc}	88.3±1.7^{d}	83.3±3.3^{cd}

[1] IBW: initial body weight (g).
[2] FBW: final body weight (g).
[3] WG: percent weight gain (%).
[4] SGR: specific growth rate (% day^{-1}).
[5] FI: feed intake (g fish^{-1} 50 days^{-1}).
[6] FCR: feed conversion ratio.
[7] PER: protein efficiency ratio.
[8] Sur: survival (%).
* Values are means of triplicate groups±S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

Table 4.1.5: Blood parameters of juvenile amberjack fed test diets for 50 days.[*]

Parameters	Diet groups				
	D1	D2	D3	D4	D5
Hematocrit (%)	30.3±2.7[a]	33.7±1.2[ab]	36.7±2.7[ab]	39.3±1.5[b]	39.0±2.0[ab]
Glucose (mg/dl)	124.3±24.5	204.7±36.3	152.0±11.1	193.0±15.9	246.5±37.5
T-Cho (mg/dl)[1]	163.7±16.5	197.7±33.4	186.7±25.3	188.7±28.9	198.5±4.5
BUN (mg/dl)[2]	9.67±0.33[a]	21.0±1.2[b]	19.3±1.8[b]	31.3±3.3[c]	25.0±1.0[bc]
T-Bill (mg/dl)[3]	0.77±0.23	0.60±0.10	0.77±0.37	0.77±0.03	1.00±0.50
GOT (IU/l)[4]	39.3±14.1	41.7±6.7	37.3±10.5	30.7±5.8	38.0±5.0
GPT(IU/L)[5]	34.0±7.9	22.3±7.1	30.7±18	29.3±12	40.0±30
TG (mg/dl)[6]	179.0±27.1	178.0±35.0	111.0±43.0	122.0±7.6	123.5±26.5
CORT (%)[7]	80.6±0.2	80.1±0.6	79.6±1.8	78.8±1.6	78.5±0.9

[1] T-Cho: total cholesterol.

[2] BUN: blood urea nitrogen.

[3]T- Bill: Total bilirubin

[4]GOT: glutamyl oxaloacetic transaminase.

[5]GPT: glutamic pyruvate transaminase.

[6]TG: triglyceride.

[7]CORT: relative value of cortisol.

[*]Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

oxidative stress and higher tolerance ability against oxidative stress; D4 and D5 in zone B; D1 and D2 in zone C respectively (Fig.4.1.1). Numerically lower values (P>0.05) for plasma cortisol levels (%) (Table 4.1.5) were obtained in the inosine supplemented groups compared to inosine free control (D1) group.

3.4. Immunological assays

3.4.1. Bactericidal activity

The serum bactericidal activity (BA) of amberjack fed with experimental diets is shown in Table 4.1.7. Comparatively higher BA was found in fish fed 0.3% inosine supplemented diet (D3) and it was not significantly different (P>0.05) with the diet D4. Fish fed control diet (D1) showed significantly the lower bactericidal activity. Quadratic regression analysis of BA showed quadratic response with graded level of dietary inosine (Y_{BA} = -61.183x2 + 61.785x + 51.64, R^2 = 0.6999).

3.4.2. Lysozyme activity

LA showed an increasing trend with the increasing dietary inosine levels and it was significantly highest in group D4 (0.61% inosine). Significantly lower plasma lysozyme activity observed in control diet group (Table 4.1.7). Meanwhile, regression analysis of plasma lysozyme activity showed quadratic response with graded level of inosine (Y_{LA}= -318.79x2 + 346.72x + 72.751, R^2 = 0.8359).

3.4.3. Total serum protein

After 50 days feeding, higher total serum protein contents found in D4. Total serum protein also numerically increased with the supplementation of inosine but not at a significant level (Table 4.1.7).

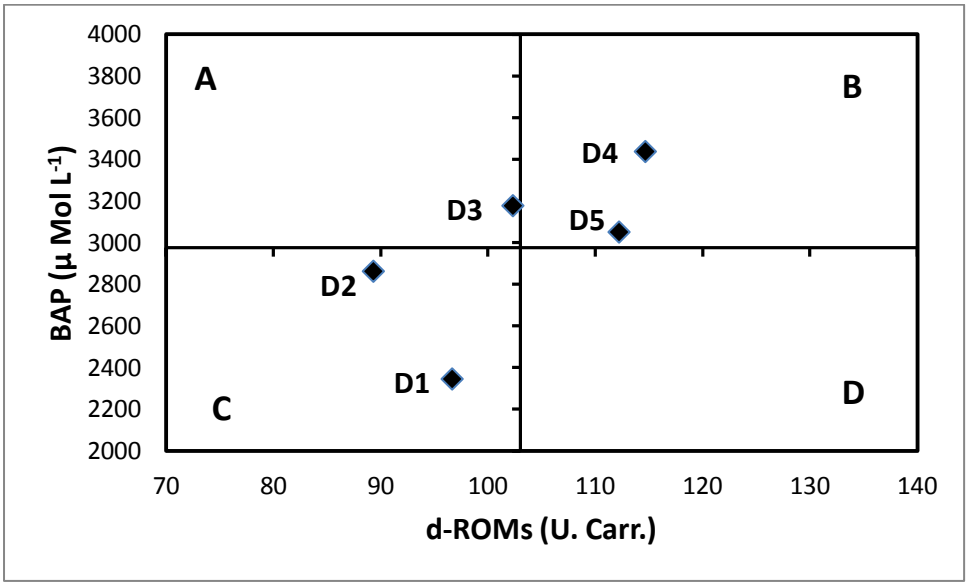


Fig. 4.1.1. Oxidative stress parameters in juvenile amberjack fed test diets for 50 days. (Values are means of triplicate groups. Central axis based on mean values of d-ROM and BAP from each treatment.) Zone A: high antioxidant potential and low reactive oxygen metabolites (good condition); Zone B: high antioxidant potential and high reactive oxygen metabolites (acceptable condition); Zone C: low antioxidant potential and low reactive oxygen metabolites (acceptable condition); Zone D: low antioxidant potential and high reactive oxygen metabolites (stressed condition).

3.4.4. Total peroxidase activity

The total peroxidase content in serum was significantly higher in diet group D3 and it was not differed significantly with other inosine supplemented diets. Significantly lower peroxidase content was measured in fish fed inosine free control group (Table 4.1.7). Regression analysis of peroxidase activity also showed quadratic response with graded level of inosine (Y_{PA} = -1.3427x2 + 1.3557x + 0.7513, R^2 = 0.3429)

3.5. Whole body proximate composition

The whole body proximate composition of juvenile amberjack at the start and end of the feeding trial is shown in Table 4.1.6. All the fish showed a change in the analyzed parameters compared to those of the initial values. Whole body proximate composition and somatic parameters were not influenced (P>0.05) by the dietary treatments except in whole body lipid and hepatosomatic index (HSI) where, inosine supplemented diets showed significantly higher values compared to those of the control group. Meanwhile, regression analysis of whole body crude lipid and HSI showed quadratic response with the graded level of inosine ($Y_{\text{whole body crude lipid}}$ = -6.3707x2 + 7.7115x + 1.5223, R^2 = 0.916; Y_{HSI} = -1.7353x2 + 1.8717x + 0.4144, R^2 = 0.8869).

3.6. Gut morphology

Table 4.1.8 shows the micromorphology of the intestine of juvenile amberjack fed diets with different concentrations of inosine for 50 days. In terms of intestinal health condition, anterior enterocyte height increased significantly in fish fed inosine supplemented groups compared to the inosine free control group (Fig. 4.1.2a, b, c). Supplementation of inosine also increased anterior and posterior fold height but not at a significant level. Anterior and posterior microvillus height, and posterior enterocyte height showed no significant difference

Table 4.1.6: Whole body proximate analysis (% wet basis) and somatic parameters in juvenile amberjack fed test diets for 50 days.*

Parameters	Initial[1]	Diet group				
		D1	D2	D3	D4	D5
Moisture	83.6	81.7±0.9	79.1±0.6	79.0±1.3	77.8±0.2	79.0±1.6
Crude protein	12.1	12.3±0.3	13.7±0.4	13.6±0.4	14.7±0.1	14.2±0.9
Crude lipid	0.7	1.5±0.2[a]	2.7±0.1[ab]	3.1±0.4[b]	3.9±0.3[b]	3.3±0.4[b]
Crude ash	3.4	3.3±0.1	3.6±0.2	3.1±0.1	3.2±0.1	3.3±0.2
CF[2]	-	1.6±0.0	1.7±0.1	1.8±0.0	1.8±0.1	1.8±0.0
HSI[3]	-	0.4±0.0[a]	0.7±0.0[b]	0.8±0.0[b]	0.9±0.0[b]	0.7±0.1[b]
VSI[4]	-	3.4±0.2	3.8±0.1	3.7±0.1	3.7±0.1	3.5±0.1

[1]Initial values are not included in the statistical analysis.

[2]CF (condition factor)=100xfish weight/(fish length)^3

[3]HSI (hepatosomatic index)=100xliver weight/fish weight

[4]VSI (viscerasomatic index)=100xviscera weight/fish weight

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05). Crude protein, crude lipid and ash are expressed on a wet weight basis

Table 4.1.7: Innate immune response of juvenile amberjack fed test diet for 50 days.*

Parameters	Diet groups				
	D1	D2	D3	D4	D5
T-Pro (g/dl)[1]	2.90±0.50	3.53±0.32	3.20±0.40	3.70±0.25	3.50±0.10
LA of plasma (unit/mL)[2]	90.0±21[a]	106.7±13[ab]	130.0±30[ab]	185.0±5[b]	120.0± 17[ab]
PA[3]	0.67±0.01[a]	0.95±0.01[ab]	1.24±0.05[b]	0.86±0.04[ab]	0.96± 0.20[ab]
BA (%)[4]	51.8±2.7[a]	57.6±2.9[ab]	69.8±0.8[c]	61.8±1.7[bc]	59.3±1.2[ab]

[1]T-Pro: Total protein

[2]LA: lysozyme activity

[3]PA: Peroxidase activity (measured at OD of 450nm).

[4]BA: Bactericidal activity=100×(CFU of blank group-CFU of each group)/CFU of blank group.

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

Table 4.1.8: Micromorphology of the intestine of juvenile amberjack fed diets with different concentrations of inosine for 50 days.*

Parameters measured	Diet groups				
	D1	D2	D3	D4	D5
Anterior intestine					
hF(μm)[1]	391±86	656±31	658±92	586±126	644±18
hE (μm)[2]	14.9±0.5[a]	17.7±1.2[ab]	22.2±2.6[b]	18.7±0.6[ab]	19.4±0.9[ab]
hMV(μm)[3]	1.5±0.1	1.4±0.1	1.6±0.1	1.6±0.1	1.6±0.1
Posterior intestine					
hF(μm)	569±91	991±198	1014±192	965±179	1107±34
hE (μm)	22.2±2.8	19.9±2.3	22.0±1.5	21.5±0.6	19.0±2.0
hMV(μm)	1.4±0.3	1.5±0.1	1.5±0.1	1.7±0.1	1.7±0.1

[1]hF: Fold height.
[2]hE: Enterocyte height.
[3]hMV: Microvillus height.
*Values are means of 10 measurements from each of three replicate groups (total measurements were 30). Within a row, values with the same letters are not significantly different (P>0.05).

among the treatments. Regression analysis of anterior enterocyte height also showed quadratic response with graded level of inosine (Y_{hE} = -19.423x2 + 21.168x + 15.298, R^2 = 0.5794).

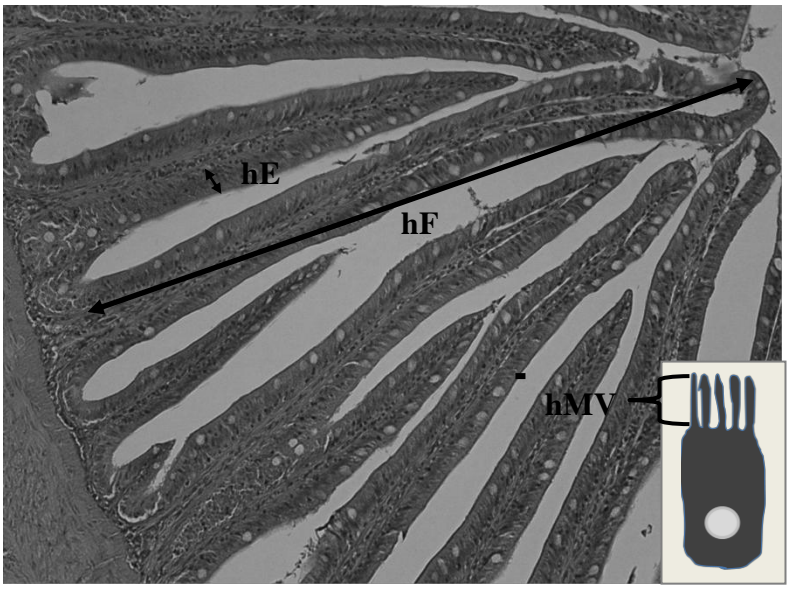
3.7. Quantification of the optimal levels of dietary inosine nucleoside for S. dumerilli

The optimal levels of dietary inosine for WG and LA were estimated by quadratic regression analysis (Fig. 4.1.3a,b). Based on WG, the optimal levels of dietary inosine was estimated to be 0.67 % in diet (Y= -405.84x^2 + 550.09x + 140.37, R^2 = 0.9775). On the other hand, the optimal levels of dietary inosine based on LA was estimated to be 0.54% in diet (Y= -318.79x^2 + 346.72x + 72.751, R^2 = 0.8359).

Discussion

Inosine is an endogenous purine nucleoside, which is formed during the breakdown of adenosine by adenosine deaminase (Barankiewicz and Cohen, 1985). Numerous studies on humans and animals have reported that inosine has been shown to exert potent effects on the immune, neural, and cardiovascular systems (Juha´sz-Nagy and Aviado, 1977; Woollard et al., 1981; Czarnecki and Herbaczynska-Cedro, 1982). In case of fish or aquatic organisms, the information regarding inosine nutrition is very scarce. In limited cases, it is used as a feeding stimulant or feed enhancer (Mackie and Adron, 1978; Kubitza et al., 1997). The present study is the first report on the dietary administration of inosine and its effects on growth, immune responses, hematological parameters and gut morphology of amberjack.

In the present study, the growth performances of amberjack were enhanced with dietary supplementations of inosine. Particularly, supplementation of 0.6% dietary inosine is considered as the best level for growth performance of juvenile amberjack. Growth enhancing and feeding stimulating properties of nucleosides like inosine have been reported



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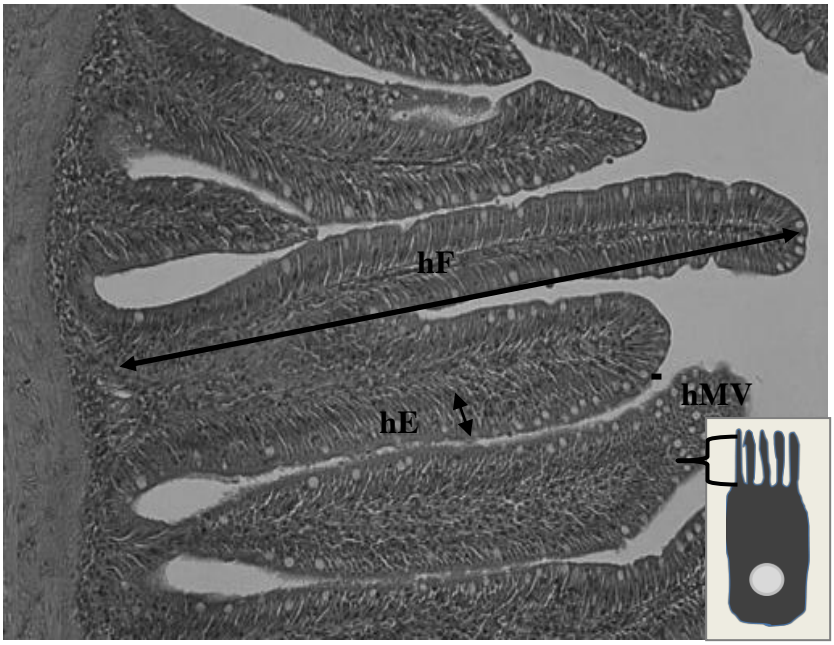

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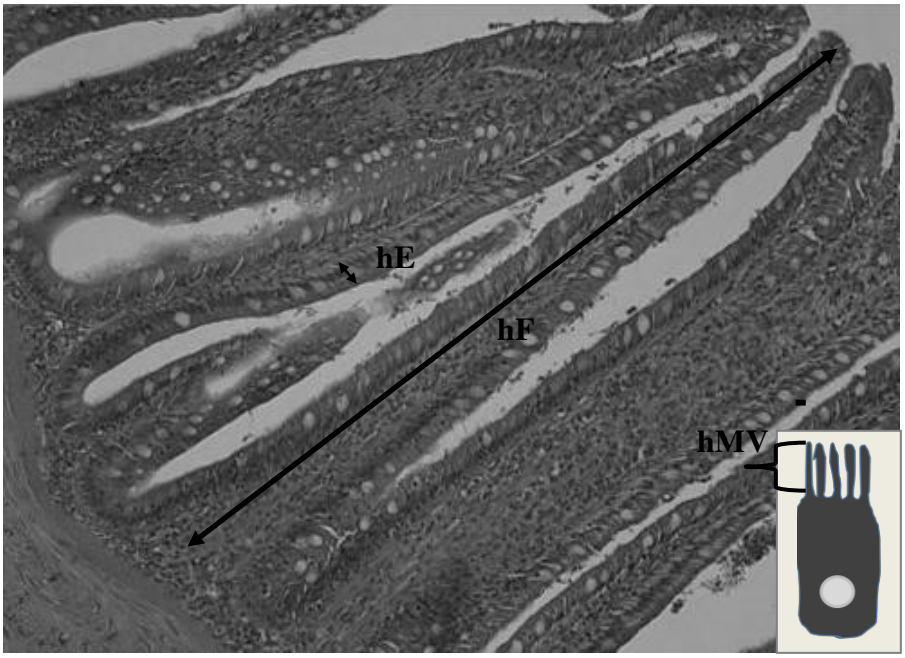
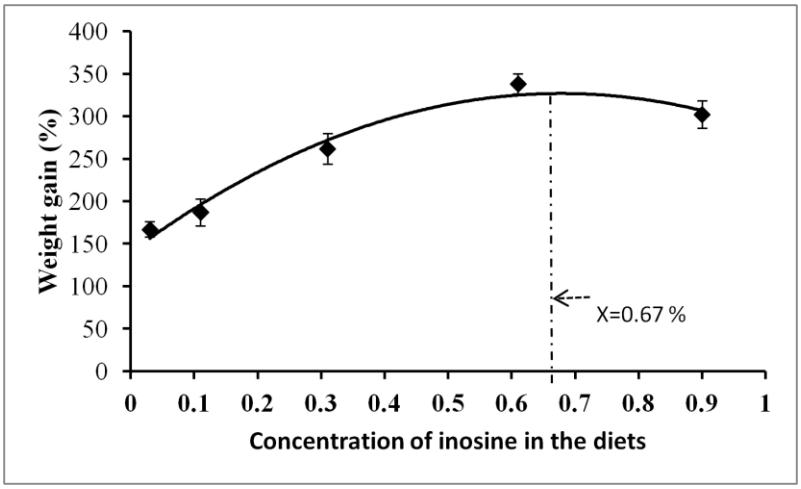
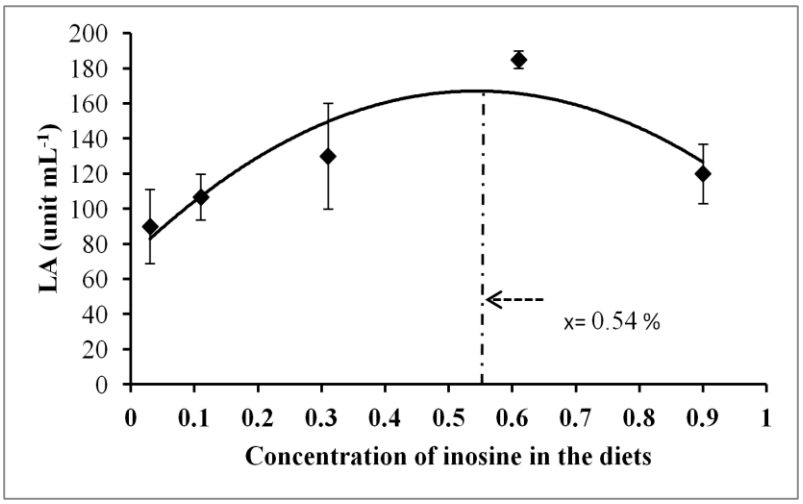


2(c)

Fig. 4.1.2. Histological appearance of the anterior intestine from fish fed (a). the control group (b). 0.3% inosine supplemented group and (c) 0.6% inosine supplemented group. Small arrow point and cartoon with bracket both indicate hMV. hE: Enterocyte height; hF: Fold height ; hMV: Microvillus height. Magnification =200, hematoxylin and eosin (H&E).



3(a)



3(b)

Fig. 4.1.3. Quadratic regression analysis of percent weight gain (% WG) (3a) and lysozyme activity (LA) (3b) for juvenile amberjack fed diets with different concentration of inosine for 50 days. Optimal dietary inosine levels derived with the quadratic regression analysis for %WG and LA were 0.67 % and 0.54% in the diet, respectively.

in some studies (Ishida and Hidaka, 1987; Kumazawa and Kurihara, 1990; Yamaguchi, 1991). Person-Le Ruyet et al., (1983) reported that the feeding of a diet supplemented with 0.77% inosine for 10 or 20 days significantly increased weight gain of turbot larvae. Hossain et al. (2016a) also found similar enhanced growth and feed utilization of red sea bream fed diets supplemented with 0.4% dietary inosine for 10 weeks. So far, there is no clear explanation of how dietary nucleotide and nucleosides works to enhance growth. However, it is assumed that one of the reasons for the faster growth indicated in fish fed inosine supplemented diets would be the improved feed intake, particularly at the beginning of weaning, and more rapid feeding might be able to reduce nutrient leaching into the water or possibly playing roles in metabolism (Metailler et al., 1983; Hossain et al., 2016a).

Supplementation of nucleotides over certain levels can results in reduced performance. It was reported that adenine was shown to be toxic to rats at concentrations in excess of 0.1% (Akintonwa et al., 1979) and to inhibit growth rate in chickens at dietary concentrations of 0.8% and above (Baker and Molitori, 1974). In case of Japanese flounder, Song et al. (2012) reported that a high dietary concentration of inosine monophosphate (IMP) (1.0%) resulted in depressed growth performance compared to lower levels of dietary IMP (0.1 to 0.2%). Adamek et al. (1996) reported that high dietary Ascogen nucleotide concentration (5%) caused growth depression of rainbow trout *(Oncorhynchus mykiss)* in a 37-day feeding trial. Hossain et al. (2016b) also found reduced growth and immune performances of red sea bream fed purified nucleotide mixtures >0.2%. Our present study concurred with the opinions of previous findings showing the effects of excessive nucleotide inclusion in diets. It found that juvenile amberjack fed diet supplemented with inosine in excess of 0.6% had numerically reduced growth and feed utilization performances.

The LA is an important index of innate immunity of fish and it varies on the sex, age and size, season, water temperature, pH, toxicants, infections and degree of stressors (Saurabh

and Sahoo, 2008). In the present study, there was a trend of increasing ($P<0.05$) LA with the supplementation of inosine compared to the control. The increased LA was also reported in common carp (*Cyprinus carpio*) (Sakai et al., 2001), rainbow trout (Tahmasebi-Kohyani et al., 2011) and Japanese flounder (Song et al., 2012) after feeding nucleotide-supplemented diets. The increased LA in inosine-supplemented groups might be due to the immunomodulating iproperties of inosine which may be acted on due to either an increase in the number of phagocytes secreting lysozyme or an increase in the amount of lysozyme synthesized per cell (Engstad et al., 1992; Kumari and Sahoo, 2006). On the other hand, inosine-free control diet (D1) showed the lowest LA in the present study indicating low nonspecific defense ability of the fish.

The anti-bacterial activity of blood serum is considered as a nonspecific response to inhibit the growth of infectious microorganisms (Yano, 1996). In the present study, *E. coli* was used in determining the bactericidal activity of fish, and that was significantly improved with inosine supplementation. Similar improved bactericidal activity were also reported in red sea bream fed diets supplemented with inosine and mixed nucleotides (Hossain et al., 2016a,b). This was also seen when bovine lactoferrin was fed to Siberian sturgeon (*Acipenser baeri*) (Eslamloo et al., 2012). At 0.9% inosine supplemented diet (D5), bactericidal activity decreased and the mean value was not significantly different from those of fish fed diets D1 and D2. This might be due to the excessive levels of inosine nucleoside in the diet D5, which supports with the findings of Burrells et al. (2001a) and Hossain et al. (2016a,b) mentioned that excessive levels of dietary nucleotides would negatively affect immunity of fish.

Proteins are the most important compounds in the serum and are essential for sustaining a healthy immune system (Kumar et al., 2005). In the present study there was a trend of increasing total proteins with the supplementation of inosine compared to the control (D1). Increased total serum proteins due to nucleotide supplementation were reported in *Catla catla*

(Jha et al., 2007), red sea bream (*P. major*) (Hossain et al., 2016a,b) and rainbow trout (Tahmasebi-Kohyani et al., 2012). These findings are in agreement with the present study. The increasing peroxidase contents with inosine supplementation in the present study show the immunomodulating properties of inosine as well as confirm other results obtained by Hossain et al. (2016 a,b) and Salinas *et al.* (2008).

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish. Blood parameters results of the current study are considered to be within the accepted values for juvenile amberjack (Kader et al., 2013; Uuyan, 2009). Dietary inosine supplementation significantly enhanced hematocrit of juvenile amberjack as a general health response towards nutritional strategies. In the present experiment, hematocrit content was significantly highest in fish fed diet group contained 0.61% inosine (D4) and then plateaued, whereas control group (D1) showed significantly lowest value. This indicated that dietary inosine elevated the health status of fish. Similarly, Song et al. (2012) and Hossain et al. (2016a,b) reported the enhanced hematocrit level by the supplementation of nucleotides in Japanese olive flounder and red sea bream diets respectively. Result of the present study also revealed that plasma GOT, GPT and TG level showed lower values ($P>0.05$) at 0.3% and 0.6% inosine supplemented diet groups compared to supplementation-free control group indicating that in amberjack, diets supplemented with 0.3 to 0.6% inosine induced an optimal physiological condition when compared with the supplementation-free control group. On the other hand, increased BUN contents with the increasing dietary supplementation level of inosine in the present study might be due to the higher protein metabolism, high protein level of diet or the fact that the kidney may not be functioning well. However, increased BUN level does not affect the growth of fish.

Stress is one of the emerging factors in aquaculture activities, which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization. Plasma or serum cortisol concentration is an available biological indicator of stress response in fish and terrestrial animals (Li et al., 2009; Small and Davis, 2002). In the present study, the relatively lower values of plasma cortisol concentration in inosine-supplemented diet groups compared to the supplementation-free control group indicated improved stress response of fish to inosine supplementation as well as the possible efficacy of dietary inosine in stress reduction. Similar observation has also been reported for other fishes in which plasma cortisol level reduced with the use of nucleotide-supplemented diet (Hossain et al., 2016ab; Tahmasebi-Kohyani et al., 2012). Oxidative stress can be generated at high level of reactive oxygen species (ROS) and/or decreased efficacy of antioxidant system, which is another health risk factor in human or other mammals (Pasquini et al., 2008). The simultaneous analysis of d-ROM and BAP provided valuable data on oxidative stress condition in humans, pig and dog (Pasquini et al., 2008; Ballerina et al., 2003). Recently, these tests have also been applied as a suitable tool for evaluating the oxidative stress of fish (Kader et al., 2010; Hossain et al., 2016a). Fish with higher d-ROM values indicate that they are under more oxidative stress conditions. On the other hand, fish with higher BAP values indicate stronger tolerance against oxidation. As shown in Fig. 4.1.1, it is interesting to note that D3 group was located in zone A, which represents the best condition with low oxidative stress and high antioxidant levels, while fish fed D4 and D5; and D1 and D2 were located in zone B and C, respectively; both of which zones are considered acceptable in the assessment of fish's health condition. Similarly, Hossain et al. (2016a,b) also illustrated that dietary supplementation of inosine and nucleotide mixtures stimulated the oxidative status of red sea bream. To date, there remains a lack of explanation about how these supplements work to affect these parameters, so further study is needed.

In the present study, only whole-body lipid content of juvenile amberjack were significantly influenced by dietary supplementation of insoine. Li et al. (2005) observed significantly increased whole body lipid content of red drum with the dietary nucleotide supplementation. Research on the effect of exogenous nucleotides and nucleosides on lipid metabolism of fishes is very limited, although it is known that dietary nucleotides can influence levels of various lipids and/or fatty acids in certain tissues, such as erythrocytes, plasma, liver or brain (Carver and Walker, 1995; Sato et al., 1995). Our observation on whole-body lipid of amberjack is likely the first report on the response of fish supplemented with dietary inosine. Information on changes in various lipids in tissues as well as HSI data in response to dietary nucleotides and nucleoside associated with physiological consequences may be important; therefore, further investigation is warranted.

The beneficial effect of dietary nucleotides on gastrointestinal tract (GIT) is well documented in human and other terrestrial animals. Reported benefits to the GIT morphology in particular, increased villus height (Uauy et al., 1990), jejunum wall thickness and villus cell number (Bueno et al., 1994), as well as reportedly augmenting surface area of the gut mucosa (Carver, 1994). However, research on gastrointestinal responses of fishes to dietary nucleotides is currently limited. Burrells et al. (2001b) first detected morphological responses of Atlantic salmon intestine to dietary nucleotides by histological examination. In that study, the mean fold heights of proximal, mid and distal intestine as well as total gut surface area of fish fed a nucleotide-supplemented diet were significantly greater than those of fish fed the control diet. Almost similar positive influence of dietary nucleotide supplementation on intestinal morphology was also reported by Borda et al. (2003) and Cheng et al. (2011) for juvenile sea bream and red drum, respectively. Recently, Hossain et al. (2016a) reported the comparative effects of inosine and IMP on red sea bream and observed that the supplementations of inosine and IMP significantly increased enterocyte

height (hE), fold height (hF) of both anterior and posterior intestine and microvillus height (hMV) of posterior intestine compared to the supplementation free-control. Moreover, with the exception of anterior hF, the inosine supplemented diet groups showed improved gut morphology compared to the IMP supplemented diet groups at the same supplementation level. In the present study, anterior enterocyte height increased significantly in fish fed inosine supplemented diets compared to the control diet. Supplementation of inosine also increased anterior and posterior fold height, but not at a significant level. The positive influence of dietary nucleoside inosine on intestinal morphology in the present study re-confirm the effects of inosine on improved intestinal morphology of fish. However, the mechanism by which dietary nucleotides or nucleosides exert their effects on the intestine is not clear at the present. Most of the ingested nucleotides are degraded to nucleosides by alkaline phosphatase and nucleotidase in the intestine, and may be further broken down by nucleosidase to produce purine and pyrimidine bases. Moreover, investigations in animals suggest that nucleosides are the primary form absorbed (Uauy, 1989). Most of the absorbed nucleosides and bases are rapidly degraded within the enterocytes (Sonoda and Tatibana, 1978), but a few may be incorporated into the tissue pools, primarily in the small intestine, liver and skeletal muscle (Burridge et al., 1976; Saviano and Clifford, 1978). Inosine as a nucleoside was absorbed directly by the intestine mostly and beneficially influenced the intestinal health of fish. The beneficial effects on intestine in the present study also might be due to the positive effects of inosine on mucosa associated lymphoid tissue (MALT) which is a very important immune organ although the knowledge on MALT of fish is very limited (Li and Gatlin, 2006).

The WG and LA activity are often used to estimate the optimum supplementation level of nutrient components in fish (Zhou et al., 2013; Wen et al., 2015). Based on the quadratic regression analysis of WG and LA, the present study illustrated that the optimal levels of

dietary inosine for juvenile amberjack might be 0.67% and 0.54 % respectively. Similarly, Hossain et al. (2016a) and Person-Le Ruyet, (1983) reported that optimum inosine supplementation level based on growth performances for turbot (*Scophthalmus maximus*) is 0.77% and 0.4% for red sea bream, respectively; while Song et al. (2012) suggested a supplementation of 0.1–0.4% IMP could enhance innate immunity and disease resistance of olive flounder.

Therefore, the present study demonstrated that the dietary supplementation of inosine positively influences the growth performances, survival rates, feed utilization, immune responses, hematological parameters, oxidative stress response and intestinal morphology of fish. Based on the present experimental condition, it can be concluded that the optimal levels of dietary inosine were 0.54 and 0.67 %, respectively, for juvenile amberjack, which is also in line with the most of the growth performance and health parameters of the fish.. However, further research is needed, including controlled disease challenges, which will be carried out in our next research, to elucidate the integrated disease defense mechanisms that may be affected by inosine supplementation in amberjack.

Part-2

Comparison of the effects of inosine and inosine monophosphate on growth, immune response, stress resistance and gut morphology of juvenile red sea bream, *Pagrus major*

ABSTRACT

Inosine and inosine monophosphate (IMP) are the most extensively studied specific nucleotide for feeding stimulation research rather than using as functional nutrients for potential growth and health benefit of aquatic species. Therefore, a 10-week feeding trial was conducted to determine comparative effects of inosine and IMP as functional nutrients on growth performance, immune responses, stress resistance and intestinal morphology of juvenile red sea bream (*Pagrus major*). Casein based semi-purified basal diet was formulated to contain 54% protein, 11% lipid, without inosine and IMP supplementation (Control) . Four levels of inosine nucleoside (0.2, 0.4, 0.6 and 0.8% for diet groups INO-0.2, INO-0.4, INO-0.6 and INO-0.8 respectively) and IMP nucleotide (0.2, 0.4, 0.6 and 0.8% for diet groups IMP-0.2, IMP-0.4, IMP-0.6 and IMP-0.8 respectively) were added to the basal diet. Each diet was randomly allocated to triplicate groups of fish with initial average weight of 6.6 g. The results indicated that dietary inosine and IMP supplementations tended to improve growth performances, in which one of the best ones was found in diet group IMP-0.6, but the values did not significantly differ from those in diet groups IMP-0.4 and INO-0.4, respectively. Superoxide dismutase, peroxidase and bactericidal activity were significantly influenced by dietary supplementation of inosine and IMP. However, the values of these parameters were not significantly different among diet groups INO-0.4, IMP-0.4 and IMP-0.6. Total serum protein, catalase and lysozyme activity were also improved ($P>0.05$) by dietary supplementation of inosine and IMP. Supplementations improved both freshwater stress and oxidative stress resistances. Fish fed diet groups INO-0.4 and IMP-0.6 showed the least oxidative stress condition. Inosine and IMP supplementations significantly increased enterocyte height (hE), fold height (hF) of both anterior and posterior intestine and microvillus height (hMV) of posterior intestine compared to those of the control. The highest hF observed in diet group IMP-0.4 and the mean value was not significantly different from

those in other IMP diets, INO-0.4 and INO-0.6, respectively. Significantly highest anterior hE and posterior hF, hE and hMV observed in diet group INO-0.4. However, no significant difference of posterior hF observed between INO-0.4 and IMP supplemented diet groups. Fish fed supplemented diets also showed improved survival compared to control (P>0.05). Considering overall performances, it can be concluded that diet groups INO-0.4 and IMP-0.4 showed relatively better performance among inosine and IMP supplemented diet groups respectively compared to control. Furthermore, diets supplemented with either inosine or IMP at 0.4% are beneficial to promote growth, immune responses, stress resistance and intestinal health condition of juvenile red sea bream.

Keywords: Inosine, Inosine monophosphate, Growth, Immunity, Oxidative stress, Gut morphology

Introduction

Nucleotides are low-molecular weight intracellular compounds which play key roles in nearly all biochemical processes (Gil, 2002) and it's the building block of nucleic acid. A nucleotide consists of a nitrogenous base, a sugar (ribose or deoxyribose) and one to three phosphate groups. When phosphate group of nucleotide is removed by hydrolysis, the structure remaining is nucleoside. Dietary supplementation of nucleotides or nucleosides has been shown to benefit many mammalian physiological and nutritional functions (Uauy, 1989; Quan, 1992; Carver, 1994; Haskó et al., 2000) and both are considered as functional nutrients. In aquatic animals both nucleotides and nucleosides have long been implicated as feed attractants in both vertebrate and invertebrate species (Mackie and Adron, 1978; Carr and Thompson, 1983; Person-Le Ruyet et al., 1983; Carr et al., 1984). However, world-wide heightened attention on nucleotide supplementation into potential growth and health benefits in aquaculture species instigated after 2000s. To date, research pertaining to nucleotide

nutrition in fishes has shown rather consistent and encouraging beneficial results in fish health management.

Research to date on dietary nucleotides has focused on a mixture of nucleotides, rather than specific types of nucleotides except inosine and IMP for the research on feed stimulants (Li and Gatlin, 2006). It also has been noted that the findings on the stimulatory effects of inosine or IMP on various fishes are not consistent due to species specificity of exogenous nucleotides or nucleosides on the behavioral or gustatory responses of fishes. Inosine, a purine nucleoside containing the base hypoxanthine and the sugar ribose, occurs in transfer RNAs, and is formed during the breakdown of adenosine by adenosine deaminase (Barankiewicz and Cohen, 1985). Inosine monophosphate (IMP) is the ribonucleotide and is the first compound formed during the synthesis of purine. Numerous studies on different aquatic species have reported that dietary inosine or IMP, either alone or in combination with certain free amino acids can enhance growth performance, survival and feed intake of juvenile eel (Takeda et al., 1984), turbot *(Scophthalmus moximus)* (Mackie and Adron, 1978; Person-Le Ruyet et al., 1983), dover sole (*Solea vulgaris*) (Metailler et al., 1983) while it can also improve immune responses and disease resistance of Japanese flounder (*Paralichthys olivaceus*) (Song et al., 2012) .

Supplementation of functional nutrients as an alternative strategy could provide potential health and development benefit beyond satisfying basic nutrition of the cultured species. Recently, in aquaculture research nucleotides and its related product has been paid attention promisingly as functional nutrients. Red sea bream (*P. major*) is one of the most economically cultured marine fish species in Japanese aquaculture, due to its economic feasibility and traditional food habits (Koshio, 2002). Effects of nucleotide as a functional nutrient of this species are not reported elsewhere. Until now, there are no studies on inosine and IMP as functional nutrients for potential growth and health benefit of red sea bream.

Therefore, the current research was conducted to evaluate the comparative effects of graded levels of inosine and IMP as functional nutrients on growth performance, immune responses, stress resistance and intestinal morphology of juvenile red sea bream.

2. Materials and methods

2.1. Test fish and experimental system

Juvenile red sea bream were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (54% crude protein, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 100 l polycarbonate tanks (filled with 80 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 4.2.1 summarize the formulation and chemical composition of the experimental diets. Nine casein based semi-purified diets were formulated to be nearly isonitrogenous (54% crude protein), isolipidic (11% crude lipid). The experiential diets were prepared by supplementing inosine (Tokyo Chemical Industry Co., Ltd. Tokyo, Japan) to give a concentration equivalent to 0.2, 0.4, 0.6, and 0.8 % of inosine in diet groups INO-0.2, INO-0.4, INO-0.6 and INO-0.8 respectively. Disodium 5'-Inosinate (5'-IMP・2Na) (Ajinomoto

Co., Inc.) also supplemented on an equal molecular weight basis of inosine to give a concentration of 0.2, 0.4, 0.6, and 0.8 % in diet groups IMP-0.2, IMP-0.4, IMP-0.6 and IMP-0.8 respectively. Basal diet without inosine and IMP supplementation was used as control. For proper mixing of inosine and IMP with other ingredients, initially inosine, IMP and weighted arginine of the respective diets were mixed properly in a glass beaker by hands with the help of a stainless laboratory spatula. Then this mixtures were thoroughly mixed with other dry ingredients in a food mixer for 10 min. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (35-40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2-2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 55 °C for about 150 min. The test diets were stored at −28 °C in a freezer until use.

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Twenty fish, having a mean initial body weight of approximately 6.6 g were randomly allocated to previously prepare twenty seven tanks. Fish were fed the experimental diets by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 1 hour after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk fortnightly to determine growth and visually check their health condition.

Table 4.2.1: Formulation and chemical composition of the experimental diets for juvenile red sea bream.

Ingredients	Diet groups								
	Control	INO-0.2	INO-0.4	INO-0.6	INO-0.8	IMP-0.2	IMP-0.4	IMP-0.6	IMP-0.8
Fishmeal[a]	20	20	20	20	20	20	20	20	20
Casein[b]	32	32	32	32	32	32	32	32	32
Pollack liver oil[c]	7	7	7	7	7	7	7	7	7
Soybean lecithin[d]	3	3	3	3	3	3	3	3	3
Vitamin mixture[e]	3	3	3	3	3	3	3	3	3
Mineral mixture[f]	3	3	3	3	3	3	3	3	3
L-arginine[g]	3	3	3	3	3	3	3	3	3
Stay-C[h]	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Wheat flour	11	11	11	11	11	11	11	11	11
Activated gluten[i]	5	5	5	5	5	5	5	5	5
ά cellulose	12.92	12.72	12.52	12.32	12.12	12.526	12.132	11.74	11.344
Inosine[j]	0	0.2	0.4	0.6	0.8	0	0	0	0
IMP[k]	0	0	0	0	0	0.394	0.788	1.18	1.576

Proximate composition (% dry matter basis)

Moisture	6.2	5.9	5.4	5.1	7.5	6.4	6.2	5.3	6.7
Crude Protein	54.5	53.9	54.5	54.7	54.5	55.2	54.8	55.6	55.1
Crude Lipid	10.9	11.0	11.1	11.3	11.4	11.4	11.2	11.5	11.4
Ash	6.2	6.1	6.2	6.2	6.0	6.5	6.5	6.7	6.7
Inosine (%)	0.06	0.26	0.45	0.63	0.75	0.18	0.26	0.44	0.47
IMP (%)	<0.01	<0.01	<0.01	<0.01	<0.01	0.09	0.22	0.24	0.43
(Inosine+IMP)	0.06	0.27	0.46	0.64	0.76	0.27	0.48	0.68	0.90

[a]Nippon Suisan Co. Ltd., Tokyo, Japan

[b]Waco Chemical Co., St. Louis, MO, USA)

[c] Riken Vitamin, Tokyo, Japan.

[d] Kanto Chemical Co., Inc. Tokyo, Japan

[e] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[f]Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[g] Nacalai Tesque, Kyoto, Japan.

[h] Stay-C 35.

[i]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name “A-glu SS”.

[j]Tokyo Chemical Industry Co., Ltd. Tokyo, Japan

[k] Ajinomoto Co., Inc., Tokyo, Japan

*****Total amino acid compositions of basal diet (g $100g^{-1}$)**:** Aspartic acid + Asparagine, 3.5; Threonine, 1.97; Serine, 2.46; Glutamic acid +Glutamine,10.41; Glycine, 1.53; Alanine,1.91; Valine, 2.83; Isoleucine, 2.29; Leucine,4.24; Tyrosine, 2.24; Phenylalanine, 2.39; Histidine, 1.38; Lysine, 3.53; Arginine, 4.54; Proline, 4.55; Cystine, 0.33; Methionine, 1.22; Tryptophan,0.62.

******Free amino acid compositions of basal diet (g $100g^{-1}$)**:** Taurine, 0.14; Aspartic acid, 0.01; Threonine, 0.02; Serine, 0.01; Glutamic acid, 0.03; Glycine, 0.02; Alanine, 0.05; Valine, 0.02; Isoleucine, 0.02; Leucine, 0.03; Tyrosine, 0.01, Phenylalanine, 0.02; Tryptophan, 0.01; Lysine, 0.03; Histidine, 0.09; Arginine, 2.61; Proline, 0.01.

The monitored water quality parameters (mean ± S.D.) were: water temperature 23.5±1.8 °C; pH 8.1±0.7 and salinity 34.5±0.5 ‰ during the feeding trial. These ranges are considered within optimal values for juvenile red sea bream.

2.4. Sample collection and biochemical analysis

The initial sample of 15 fish for whole body analysis was stored at −20 °C. At the end of the feeding trial, fish were starved for 24 h prior to final sampling. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then the total number, individual body weight and body length of fish from each tank were measured. Three fish from each replicate tank were randomly collected and stored at −20 °C for final whole body analysis. Using heparinized syringes, blood was collected from the caudal vein of three fish in each replicate tank and pooled for plasma analysis. In addition non-heparinized disposable syringes were used to collect blood (three fish per each replicate tank) for serum analysis.

A small fraction of the heparinized blood was used to analyze the hematocrit level. Plasma and serum samples were obtained by centrifugation at 4000 × g for 15 min using a high-speed refrigerated microcentrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80 °C. Hepatopancreas and viscera were dissected out from three fish in each replicate tank, weighed individually to get hepatosomatic index and viscerasomatic index. The ingredients, diets and fish whole body were analyzed for moisture, crude protein, crude lipid and ash, in triplicate, using standard methods (AOAC, 1995). Total amino acids (TAA) and free amino acids (FAA) in basal diet were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) as described previously Kader et al. (2010). Briefly the samples were prepared as follows: 2 mg samples were spiked with known amount of norleucine as an internal standard and hydrolyzed with 4 N methanesulfonic acid

at 110 °C for 22 h. The pH of the hydrolysate was adjusted to 2.2, filtered and stored at 4 °C. To quantify the FAA, 100 mg sample was mixed with 0.9 ml cold deionized water, 0.1 ml internal standard (norleucine, 0.6 mg DL-norleucine 0.1 ml^{-1} deionized water) and 5 ml 10% trichloroacetic acid (TCA), homogenized using a polytron homogenizer (Kinematica, Gmbh LITTAU, Lucerne, Switzerland). Samples were then centrifuged at 4°C, 3000 × g for 15 min and supernatant was repeatedly washed with diethyl ether to remove TCA from homogenate. Finally, pH was adjusted to 2.2 and filtered samples were stored in 4°C. Cystine content was determined by a cysteic acid procedure (Moore, 1963). The chromatographic separation and analysis of the amino acids were performed with the HPLC unit with an ion exchange resin column. Inosine and IMP content of feeds were also analyzed by High Performance Liquid Chromatography (HPLC). The inosine and IMP content of the samples was determined by acidic extraction of ~1 g dry weight samples. In brief, for extraction process 25 ml of 5% percholoric acid was added to the feed sample (1g), shaking for 10 mins. After that deionized water was added to make constant volume upto 50 ml. Samples were sonicate for 10 mins, filtered and 5 ml sample solution were prepared. Finally, 0.4 ml potassium hydroxide (3 Mol/l) was added to neutralize the sample solution and stored in 4 ^{o}C. After extraction, for inosine quantification samples were loaded on an CAP CELL PAK C-18 column (Shisheido Co., Ltd. Japan), column temperature was 40 ^{o}C. The mobile phase was the mixture of 200 mmol/l phosphate buffer solution (pH 2.5) and methanol (95:5 v/v) contained 0.6% sodium heptanes sulphonate. Measurement wavelength was 260 nm. For IMP quantification samples were loaded on a MCI CDR-10 column (Mitsubishi Chemical Co. Ltd.). Colum temperature was 40 ^{o}C .The mobile phase was 1 mol/l acetate buffer (pH 3.3). Measurement wavelength was 260 nm. The minimum amount of nucleotides detectable by HPLC is ~3 ng (in 50 µL injection volume). Total serum protein and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamyl oxaloacetic transaminase (GOT), glutamic-

pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma samples was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is the equivalent of 0.08 mg/100ml of H_2O_2. For the analysis of plasma cortisol, 100 µl plasma was mixed with 1 ml diethylether by using a vortex mixer and allow to separate the organic phase. The diethylether was evaporated under a gentle stream of nitrogen. The extract was then analyzed for cortisol concentration using an enzyme-linked immunosorbent assay (Cortisol EIA Kit, product number EA65, Oxford Biomedical Research Inc., Oxford, MI).

2.5. Immunological assays

Serum lysozyme activity was measured with turbidimetric assays (Lygren et al., 1999). Ten micro-liters of samples was put into well of microplate, then added 190 micro-liters of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4,their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System

Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

Serum bactericidal activity which was performed according to Yamamoto and Iida, (1995). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g/ml, *Escherichia coli*, IAM1239 cell line, Kagoshima, Japan) (Promega, Germany) and incubated at 25 °C for 24 h in micro tube rotator (MTR-103, AS ONE, Osaka, Japan). 50 ml of the reaction solutions were incubated on TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) at 25 °C for 24 h (on X-plate). Colony forming unit (CFU) was counted by the plate counting method as described by Ren et al. (2008). The bactericidal activity was defined as follows, (CFU of blank group-CFU of each group)/CFU of blank group × 100.

The total peroxidase content in serum was measured according to Salinas et al. (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

Superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (water-soluble tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37°C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

The catalase activity (CAT) assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate that absorbs at 405 nm (Goth, 1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol/l phosphate buffer, pH 7.4) and incubated for 60s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate ($(NH_4)_6$ Mo_7O_{24} .4 H_2O) and the yellow complex of molybdate and hydrogen peroxide is measured at 405 nm against reagent blank. One unit CAT decomposes 1 µmole of hydrogen peroxide/l minute under assay conditions. CAT activities are expressed as kilo unit per liter (kU/l)

2.6. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR, % day^{-1}) = {Ln (final weight) – Ln (initial weight) / duration} × 100

Survival (%) = 100 × (final no of fish / initial no of fish)

Feed intake (g $fish^{-1}$ 10-$week^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed efficiency ratio (FER) = live weight gain (g) /dry feed intake (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Condition factor (CF, %) = weight of fish / (body length of fish)^3 × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

Viscerasomatic Index (VSI, %) = weight of viscera/weight of fish × 100

2.7. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, six fish from each rearing tank (total 18 fish per treatment) were randomly selected and transferred in to a blue colored 20 L rectangular glass aquarium with 15 L tap water which was aerated for 24 h. The glass aquaria for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of red sea bream juvenile was calculated according to Moe et al. (2004) as follows: time to death (min) of a juvenile was converted to log10 values.

When the juvenile were transferred to the low salinity water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was log10 100 = 2). The calculation was then conducted every 5 minutes after the first exposure. The values of log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

$Y = aX + b$ where Y = log10 (survival), X = time for individual juvenile death (min).

LT_{50} (X) was obtained when Y = 1.7 since log10 (50) = 1.7. Each value obtained from the equation above was compared statistically.

2.8. Gut morphology study

At the end of feeding trial two fish from each replicate tank was used for gut morphology study. For preparation of intestinal sample firstly whole gastrointestinal tract was removed and anterior and posterior intestine was collected. Then segments (0.5-1 cm) of each part was cut out and the lumen was flushed with saline (Otsuka Normal Saline, Otsuka Pharmaceutical, Tokyo) followed by formalin fixative (10% -Formaldehyde Neutral Buffer Solution 37152-5-1, Nacalai Tesque, Kyoto) to remove intestinal content. Then each tissue

was placed in plastic bag (HistoPack, Falma, Tokyo) filled with formalin fixative. Each tissue was embedded in paraffin, sliced, and stained with hematoxylin and eosine (H&E). Two cross-sectional slices were prepared from each tissue. The tissue slides were examined under a light microscope (Eclipse 50i, Nikon, Tokyo) and a camera (Digital Sight DS2MV with control unit DS-L2, Nikon, Tokyo) interfaced with Sigma Scan Pro 5 software (SPSS Inc.,IL,USA). Intestinal fold height (hF), enterocyte height (hE), microvillous height (hMV) were measured with a magnification of 100x , 200x and 400x respectively. For each tissue, 10 measurements were performed.

2.9. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Tukey Kramer test.

Results

3.1. Proximate composition of the experimental diets

Proximate composition of the experimental diets total and free amino acid contents of the basal diet is shown in Table 4.2.1. Analyzed crude protein, lipid, ash content were more or less similar in all the experimental diets. Inosine content also more or less similar as supplemented in the diet. In case of IMP content of the diet around 30% of the supplemented IMP remained as IMP and rest of the IMP changed to inosine. However, the sums of inosine and IMP are very similar in each diet group such as INO-0.2vsIMP-0.2, INO-0.4vsIMP-0.4, INO-0.6vsIMP-0.6, and INO-0.8vsIMP-0.8, respectively.

3.2. Fish performance parameters

At the end of feeding trial, dietary inosine and IMP supplementations tended to improve growth performances (final weight, SGR, % weight gain), in which one of the best ones was found in diet group IMP-0.6, but the values did not significantly differ from those in diet groups IMP-0.4 and INO-0.4. Fish fed diet group IMP-0.6 showed significantly highest feed intake (FI) followed by the fish fed supplemented diet groups IMP-0.2, IMP-0.4, INO-0.3, IMP-0.8, INO-0.6, INO-0.2 and INO-0.8 respectively. Although inosine and IMP supplemented groups showed numerically higher feed efficiency ratio (FER) and protein efficiency ratio (PER) compared with control but they did not differ significantly among the dietary treatments. Also, survival was not significantly affected by the dietary supplementation. Fish fed inosine and IMP supplementation free control diet showed significantly lower growth and feed utilization performances in the present study.

3.3. Hematological parameters

Table 4.2.3 represents the blood parameters of juvenile red sea bream after 10 weeks feeding trial. Overall, dietary treatments had no significant effect on blood chemical parameters of fish among different treatments and one supplement could not prove the relative advantage over other, except for those of plasma glucose and triglycerides (TG). At each supplementation level, fish fed IMP supplemented diet groups showed relatively lower plasma glucose and TG values compared to inosine supplemented diet groups. However, fish fed diet groups IMP-0.2, IMP-0.4 and INO-0.8, IMP-0.2, IMP-0.6, IMP-0.8 showed significantly lower plasma glucose and TG value respectively. Supplementation-free control diet group showed significantly highest plasma glucose and TG value.

Table 4.2.2: Growth performance and feed utilization parameters of red sea bream fed experimental diets for 10 weeks.*

Diet groups	Parameters							
	IBW[1]	FBW[2]	% WG[3]	SGR[4]	FI[5]	FCE[6]	PER[7]	Sur[8]
Control	6.6±0.02	23.3±0.6^{a}	250.1±9.4^{a}	1.79±0.04^{a}	16.5±0.7^{a}	1.01±0.01	1.81±0.01	91.7±1.7
INO-0.2	6.7±0.02	25.3±0.7^{ab}	280.2±9.6^{ab}	1.91±0.04^{ab}	17.4±0.2^{ab}	1.07±0.03	1.99±0.05	98.3±1.7
INO-0.4	6.670.01	26.7±0.4^{b}	300.6±6.0^{b}	1.98±0.02^{b}	18.8±0.1^{abc}	1.07±0.01	1.96±0.03	96.7±1.7
INO-0.6	6.7±0.01	25.5±0.4^{ab}	282.2±5.8^{ab}	1.92±0.02^{ab}	17.5±0.6^{abc}	1.08±0.02	1.97±0.03	96.7±1.7
INO-0.8	6.7±0.0	25.4±0.4^{ab}	282.2±5.3^{ab}	1.92±0.02^{ab}	16.6±0.5^{a}	1.13±0.02	2.04±0.04	98.3±1.7
IMP-0.2	6.7±0.0	25.9±1.2^{ab}	289.1±17.7^{ab}	1.94±0.07^{ab}	19.2±0.4^{bc}	1.00±0.05	1.82±0.09	95.0±0.0
IMP-0.4	6.7±0.04	26.7±0.8^{b}	302.2±10.7^{b}	1.99±0.04^{b}	18.9±1.0^{abc}	1.07±0.05	1.95±0.10	93.3±3.3
IMP-0.6	6.7±0.02	28.2±0.2^{b}	323.8±4.6^{b}	2.06±0.02^{b}	20.0±0.4^{c}	1.08±0.01	1.97±0.02	100±0.0
IMP-0.8	6.7±0.01	26.3±0.6^{ab}	94.9±8.6^{ab}	1.96±0.03^{ab}	18.0±0.1^{abc}	1.09±0.03	1.99±0.06	100±0.0

[1] IBW: initial body weight (g); [2] FBW: final body weight (g);[3] DWG: percent weight gain (%);[4] SGR: specific growth rate (% day^{-1});

[5] FI: feed intake (g fish^{-1} 50 days^{-1}); [6] FCE: feed conversion efficiency;[7] PER: protein efficiency ratio;[8] Sur: survival (%).

* Values are means of triplicate group's ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

Table 4.2.3: Hematological parameters of red sea bream fed experimental diets for 10 weeks*.

Diet groups	Parameters								
	Hematocrit (%)	Glucose (mg/dl)	T-Cho (mg/dl)[1]	BUN (mg/dl)[2]	T- Bill (mg/dl)[3]	GOT (IU/L)[4]	GPT (IU/L)[5]	TG (mg/dl)[6]	CORT[7]
Control	34.3±0.9	73.0±2.5^{c}	208.0±15.1	5.7±0.67	<0.2	23.0±10.1	<10	331.0±8.0^{b}	62.4±0.3^{b}
INO-0.2	32.3±0.9	71.7±6.4^{bc}	189.0±14.0	7.0±1.16	<0.2	21.7±4.7	<10	285.3±29.0^{ab}	59.8±1.1^{ab}
INO-0.4	35.0±0.0	72.7±1.3^{bc}	192.7±23.1	7.3±1.5	<0.2	31.7±1.5	<10	267.5±12.5^{ab}	59.2±1.2^{ab}
INO-0.6	30.3±0.3	57.3±4.8^{abc}	197.3±19.1	5.3±0.3	<0.2	20.7±6.7	<10	245.7±21.2^{ab}	58.0±0.8^{a}
INO-0.8	31.7±2.9	52.7±0.9 ^{ab}	186.0±30.4	6.0±0.0	<0.2	10.7±0.3	<10	160.5±32.5^{a}	58.9±0.9^{ab}
IMP-0.2	32.7±0.3	50.7±5.6^{a}	173.0±6.8	5.7±0.3	<0.2	24.7±4.4	<10	173.7±14.0^{a}	60.4±0.4^{ab}
IMP-0.4	32.7±0.7	50.3±2.9^{a}	176.3±6.0	5.0±0.0	<0.2	19.7±1.9	<10	215.3±31.5^{ab}	61.4±0.5^{ab}
IMP-0.6	31.0±0.6	55.7±3.3 ^{abc}	159.0±12.7	5.3±0.3	<0.2	19.3±7.9	<10	172.3±21.1^{a}	58.7±0.8^{ab}
IMP-0.8	34.0±1.0	55.0±5.3 ^{abc}	170.3±8.2	7.0±1.0	<0.2	42.3±17.9	<10	185.0±18.2^{a}	59.2±0.4^{ab}

[1] T-Cho: total cholesterol; [2] BUN: blood urea nitrogen; [3]T- Bill: Total bilirubin; [4]GOT: glutamyl oxaloacetic transaminase; [5]GPT: glutamic pyruvate transaminase; [6]TG: triglyceride; [7]CORT: relative value of cortisol.

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

3.4. Oxidative stress parameters

Fig. 4.2.1 indicates the status of oxidative stress conditions, in which the conditions of both stress intensity and tolerance ability were combined, of fish fed with test diets for 10 weeks. No significant effect was observed on d-ROMs and BAP of red sea bream (no data illustrated here). Diet groups INO-0.4 and IMP-0.6 was located in Zone A, where was categorized as lower intensity of oxidative stress and higher tolerance ability against oxidative stress. On the other hand, diet groups control and INO-0.8 was located in Zone D, where was categorized as higher intensity of oxidative stress and lower tolerance ability against oxidative stress. Supplementation of inosine and IMP significantly reduce (P<0.05) the plasma cortisol levels (%) compared with control and significant lowest value obtained in 0.6% inosine supplemented diet group (INO-0.6).

3.5. Immunological assays

3.5.1. Bactericidal activity

The serum bactericidal activity of red sea bream fed with experimental diets is shown in Table 4.2.4. At the same supplementation level, both of the supplements showed relatively similar effects on increasing bactericidal activity and significantly higher bactericidal activity was found in fish fed diet groups INO-0.4, INO-0.6 and IMP-0.4, IMP-0.6 respectively. Fish fed inosine and IMP supplementationfree control diet showed significantly lower bactericidal activity.

3.5.2. Lysozyme activity

Serum lysozyme activity was increased numerically with the dietary supplementations of inosine or IMP and both of the supplements showed relatively similar effects for this

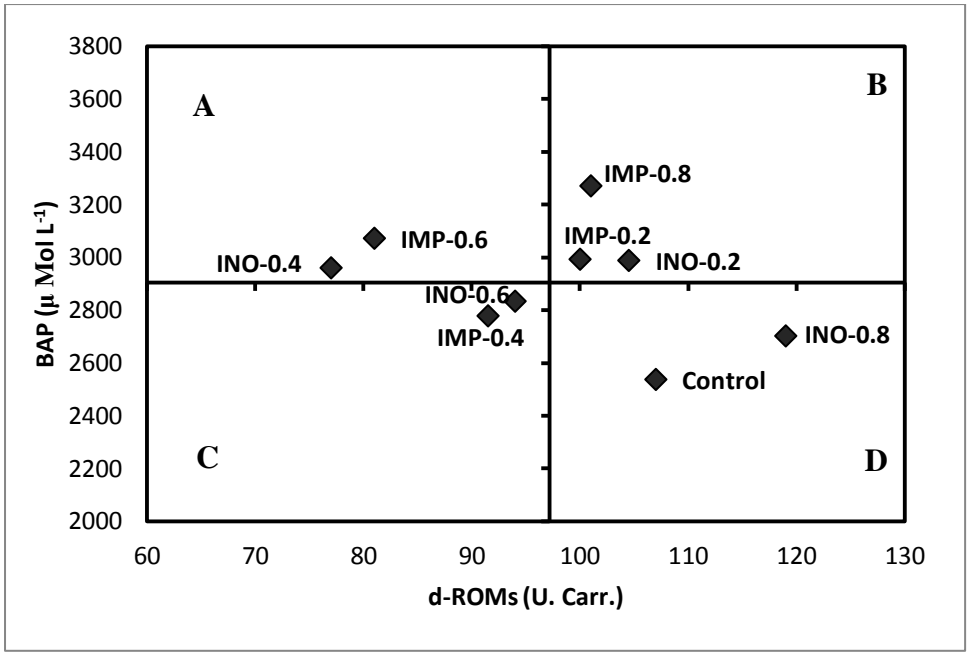


Fig. 4.2.1. Oxidative stress parameters in red sea bream (*P. major*) fed test diets for 10 weeks. (Values are means of triplicate groups. The abbreviations of experimental treatments are illustrated in the text. Central axis based on mean values of d-ROM and BAP from each treatment.) Zone A: High antioxidant potential and low reactive oxygen metabolites (good condition). Zone B: High antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone C: Low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone D: Low antioxidant potential and high reactive oxygen metabolites (stressed condition).

increase. Numerically higher lysozyme activity was observed in fish fed diet group INO-0.6 but it was not differed significantly with control group (Table 4.2.4).

3.5.3. Total serum protein

After 10 weeks feeding, higher total serum protein content was found in diet group INO-0.4. Total serum protein also numerically increased with the supplementation of inosine and IMP compared with control but not at a significant level (Table 4.2.4).

3.5.4. Total peroxidase activity

Supplementation of inosine and IMP improved the total peroxidase activity in serum of red sea bream and significantly higher value was observed in fish fed diet group IMP-0.4 followed by diet group INO-0.2. Significantly lower peroxidase activity was measured in fish fed control diet group (Table4.2. 4).

3.5.5. Superoxide dismutase (SOD) activity

At the same supplementation level of inosine and IMP, both supplements showed relatively similar SOD activity. However, significantly highest SOD activity (% inhibition) was observed in IMP 0.6% supplemented group (IMP-0.6) and not differed significantly with diet groups IMP-0.4 and INO-0.4. Control group showed significantly lowest SOD activity (Table 4.2.4).

3.5.6. Catalase (CAT) activity

CAT activity of red sea bream serum numerically reduced with the supplementation of inosine and IMP compared with control but not at a significant level (Table 4.2.4).

Table 4.2.4: Non-specific immune responses of red sea bream fed test diets for 10 weeks.*

Diet groups	Parameters					
	TSP[1]	LA[2]	PA[3]	CAT[4]	SOD[5]	BA[6]
Control	2.7±0.1	122.2± 23.0	1.79±0.04^{a}	77.8± 16.1	45.3± 2.3^{a}	55.5±3.9^{a}
INO-0.2	2.9±0.1	126.7±3.3	2.01±0.04^{b}	69.5±16.2	48.4±1.5^{ab}	70.3±0.6^{ab}
INO-0.4	3.0±0.1	150.0±11.6	1.86±0.02^{ab}	61.4±2.1	57.8±1.2^{b}	77.0±1.2^{b}
INO-0.6	2.7±0.1	163.3±12.0	1.85±0.04^{ab}	41.8±11.4	53.8±5.2^{ab}	75.8±6.1^{b}
INO-0.8	2.8±0.1	143.3±8.8	1.97±0.01^{ab}	52.1±23.3	50.1±2.0^{ab}	67.9±3.0^{ab}
IMP-0.2	2.7±0.1	153.3±8.8	1.96±0.05^{ab}	46.5±5.5	49.8±1.5^{ab}	73.6±3.9^{ab}
IMP-0.4	2.7±0.2	140.0±0.0	2.02±0.02^{b}	53.7±5.2	57.7±1.9^{b}	78.8±3.0^{b}
IMP-0.6	2.7±0.1	140.0±20.0	1.93±0.05^{ab}	30.4±2.4	58.3±2.4^{b}	78.8±1.2^{b}
IMP-0.8	3.0±0.1	150.0± 10.0	1.90±0.02^{ab}	44.1± 9.5	51.7± 1.5^{ab}	72.7±3.0^{ab}

[1] TSP: total serum protein

[2]LA: lysozyme activity

[3]PA: Peroxidase activity (measured at OD of 450nm).

[4]CAT: Catalase activity (kU/l)

[5]SOD: Superoxide dismutase (inhibition rate %).

[6]Bactericidal activity (%) = (CFU of blank group − CFU of each group)/CFU of blank group × 100.

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

3.6. Freshwater stress tolerance

The results of lethal stress test against fresh water shock on LT_{50} obtained by regression analysis are shown in Fig. 4.2.2. Supplementation of either inosine or IMP improved the freshwater stress tolerance of red sea bream and significantly highest LT_{50} obtained (112.75 min) in fish fed diet group INO-0.6. Significantly lower LT_{50} observed in inosine and IMP supplementation free control diet group.

3.7. Whole body proximate composition

The whole body proximate composition of juvenile red sea bream at the start and end of the feeding trial is shown in Table 4.2.5. All the fish showed a change in the analyzed parameters compared to those of the initial values. Whole body proximate composition were influenced ($P<0.05$) by the dietary treatments. At the same inosine and IMP supplementation level, inosine supplemented diet groups showed lower whole body moisture and higher crude protein content compared to those of IMP supplemented diet groups. Fish fed diet group INO-0.4 showed significantly lower moisture content whereas fish fed control and IMP-0.2 diet groups showed significantly higher moisture content. Whole body protein was significantly higher in diet group INO-0.2 whereas fish fed control and IMP-0.2diet groups showed the lowest value. . Significantly higher whole body lipid content was found in fish fed diet INO-0.4 and it was not differed significantly with all other inosine and IMP supplemented groups. Fish fed control diet showed significantly lower carcass lipid content. Ash content was significantly lower in fish fed diet groups with IMP-0.2 and IMP-0.4 than those in fish fed diet groups control, INO-0.2 and INO-0.6. Whole body somatic parameters were not significantly influenced by the dietary inosine and IMP supplementations.

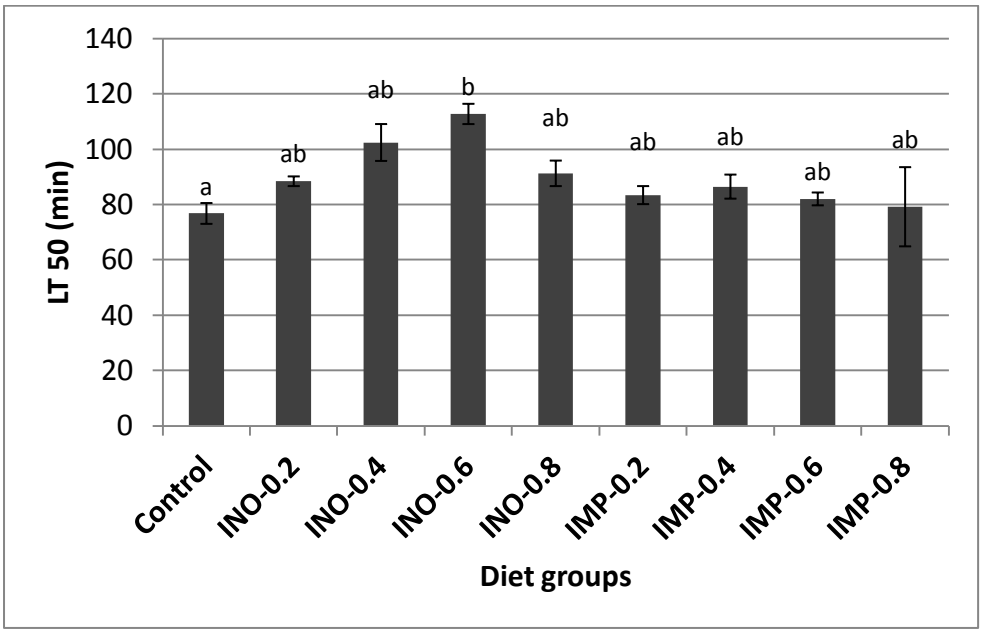


Fig. 4.2.2. LT_{50} (min) calculated from the lethal time of red sea bream exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from triplicate groups. The abbreviations of experimental diets are illustrated in the text. Values with different letters are significantly different (P<0.05).

Table 4.2.5: Whole body proximate composition and somatic parameters of red sea bream fed experimental diets for 10 weeks.*

Diet groups	Parameters						
	Moisture	protein	lipid	Ash	CF[2]	HSI[3]	VSI[4]
Initial[1]	80.3	13.2	2.0	4.6			
Control	75.5±0.6^{b}	13.4±0.3^{a}	5.8±0.6^{a}	3.9±0.1^{b}	2.10±0.1	1.70±0.2	7.51±0.5
INO-0.2	73.8±0.2 ^{ab}	14.9±0.0 ^{b}	6.6±0.2^{ab}	4.0±0.0^{b}	2.26±0.2	1.95±0.1	8.62±0.3
INO-0.4	71.9±0.1 ^{a}	14.5±0.1 ^{ab}	8.8±0.1^{b}	3.8±0.1^{ab}	2.05±0.1	1.86±0.2	8.82±0.4
INO-0.6	73.3±0.2 ^{ab}	14.2±0.1 ^{ab}	7.5±0.3^{ab}	3.9±0.0^{b}	2.01±0.1	1.53±0.0	7.63±0.1
INO-0.8	74.3±1.0 ^{ab}	13.8±0.4 ^{ab}	6.9±0.5^{ab}	3.7±0.1^{ab}	1.98±0.1	1.96±0.2	7.43±0.4
IMP-0.2	75.9±0.6 ^{b}	13.4±0.2^{a}	6.7±0.6^{ab}	3.6±0.0^{a}	1.90±0.0	1.86±0.2	7.49±0.7
IMP-0.4	74.7±0.7 ^{ab}	14.6±0.5^{ab}	6.8±0.8^{ab}	3.6±0.0^{a}	1.96±0.0	1.95±0.3	7.60±0.6
IMP-0.6	74.8±0.8^{ab}	13.8±0.2^{ab}	7.4±0.3 ^{ab}	3.7±0.1^{ab}	1.94±0.0	1.89±0.2	7.85±0.5
IMP-0.8	74.3±0.9^{ab}	13.9±0.3^{ab}	6.8±0.7^{ab}	3.8±0.0^{ab}	1.87±0.0	1.73±0.1	7.04±0.3

[1]Initial values are not included in the statistical analysis.

[2]CF (condition factor) =100 × fish weight/(fish length)$_3$

[3]HSI (hepatosomatic index) =100 × liver weight/fish weight

[4]VSI (viscerasomatic index) =100× viscera weight/fish weight

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05). Crude protein, crude lipid and ash are expressed on a wet weight basis.[1] Initial values are not included in the statistical analysis.

3.8. Gut morphology

Table 4.2.6 shows the micromorphology of the intestine of juvenile red sea bream fed diets with different concentrations of inosine and IMP for 10 weeks. In terms of intestinal health condition, supplementation of inosine and IMP increased enterocyte height (hE), fold height (hF) of both anterior and posterior intestine and microvillus height (hMV) of posterior intestine significantly compared to the control. Supplementation of inosine, IMP also increased anterior microvillus height but not at a significant level. However, with the exception of anterior hF, inosine supplemented diet groups showed improved gut morphology compared to IMP supplemented diet groups at the same supplementation level.

Discussion

In aquaculture, before 2000s, nucleotides have been implicated as feed attractants rather than as a potential functional nutrient for promoting growth and health benefit of the cultured organisms. Recently, nucleotides have received attention commercially and scientifically as functional materials in aquaculture. Research to date on dietary nucleotides has focused on nucleotides mixture and in limited cases, inosine and IMP have been used as feeding stimulants or feed enhancers (Mackie and Adron, 1978; Kubitza et al., 1997). To the best of our knowledge, there is very limited information available on dietary inosine and IMP supplementation for potential growth and health benefit of aquatic species. Under these circumstances, it is important to investigate the supplemental effects of inosine and IMP as functional materials in aquafeed.

In inosine supplemental diet groups, only a few IMP was detected, but both inosine and IMP were detected in IMP supplemental diet groups (Table 4.2.1). In fact, about 70% of the supplemented IMP changed to inosine. Although at this moment, it isnot clear exactly how and when this change occurred, a possible reason behind this may be due to the presence of

Table 4.2.6: Micromorphology of the intestine of juvenile red sea bream fed test diets for 10 weeks.*

Diet groups	Parameters					
	Anterior intestine			Posterior intestine		
	hF(μm)[1]	hE (μm)[2]	hMV(μm)[3]	hF(μm)[1]	hE (μm)[2]	hMV(μm)[3]
Control	594±20[a]	52.1±3.1[a]	7.4±0.3	297±8[a]	46.5±2.0[a]	6.0±0.1[a]
INO-0.2	79±11[ab]	59.3±1.8[ab]	7.5±0.2	300±4[ab]	48.0±1.2[ab]	7.0±0.1 [b]
INO-0.4	704±37[abc]	62.9±1.7[b]	8.4±0.7	429±40[d]	57.2±3.7[b]	8.2±0.3 [c]
INO-0.6	760±20[bc]	59.4±0.4[ab]	8.1±0.2	375±16[abcd]	52.4±1.7[ab]	6.2±0.2[ab]
INO-0.8	699±40[abc]	55.2±1.4[a]	7.3±0.2	380±7[bcd]	52.7±2.4[ab]	6.3±0.0[ab]
IMP-0.2	751±12[bc]	52.3±1.0[a]	7.2±0.2	364±15[abcd]	48.5±1.9 [ab]	6.1±0.3[ab]
IMP-0.4	817±15[c]	56.7±0.7[ab]	7.4±0.1	384±5[cd]	52.6±1.5[ab]	6.5±0.1[ab]
IMP-0.6	801±15[c]	55.5±0.7[ab]	7.5±0.1	374±8[abcd]	49.6±1.5[ab]	6.1±0.1[ab]
IMP-0.8	731±22[bc]	55.1±1.1[a]	7.4±0.3	331±6[abc]	48.5±0.6[ab]	6.9±0.2[ab]

[1] hF = fold height.

[2] hE = enterocyte height.

[3] hMV= microvillus height.

* Values are means of two fish from each of three replicate groups (10 measurements for each fish). Values in a row that do not have the same superscript are significantly different ($P < 0.05$).

phosphatase enzyme in fishmeal, wheatflour or other ingredients in basal diet, which may break down IMP into inosine. On the other hand, the sums of inosine and IMP are very similar in each diet group such as INO-0.2vsIMP-0.2, INO-0.4vsIMP-0.4, INO-0.6vsIMP-0.6, and INO-0.8vsIMP-0.8, respectively.

To the best our knowledge, our observation of individual nucleotide content (IMP, inosine) in diets compared to supplementation level would be a first such report for nucleotide based on a feeding trial of fish. Thus, the following discussion was made under the condition above. Moreover, as other consequences may be important in this change, further investigation will be warranted.

As found in the present study, the growth enhancing properties of nucleosides, inosine and nucleotide IMP have been reported earlier in some studies (Song et al., 2012; Ishida and Hidaka, 1987; Kumazawa and Kurihara, 1990; Yamaguchi, 1991). Person-Le Ruyet et al. (1983) reported dietary inosine enhanced growth and survival of turbot larvae. In subsequent research by this group, showed that 10 or 20 days of feeding a diet supplemented with 0.77% inosine also significantly increased weight gain of turbot larvae. Song et al. (2012) found improved growth performance of olive flounder fed diet supplemented with IMP at 0.1-0.2% levels. Lin et al. (2009) also reported enhanced growth of red drum fed IMP supplemented diet. So far, there is no exact explanation how dietary nucleotide and nucleosides works to enhance growth. However, it is assumed that the growth-enhancing effect of inosine and IMP in the present study resulted from significantly improved feed intake due to feeding stimulatory effects of inosine and IMP (Table 2), promoting more rapid feed intake that reduced nutrient leaching into the water or possibly playing roles in metabolism (Metailler et al., 1983). In contrast, supplementation of both inosine and IMP after a certain concentration in the present study showed depressed growth performance and feed intake. Reduced growth performance by high nucleotide levels also reported in some previous studies (Akintonwa et

al., 1979; Baker and Molitori, 1974; Adamek et al., 1996). In case of Japanese flounder Song et al. (2012) reported that a high dietary concentration of IMP (1.0%) resulted depressed growth performance compared to lower levels of dietary IMP (0.1 to 0.2%). Adamek et al. (1996) also reported that high dietary nucleotide concentration (5%) caused growth depression in rainbow trout *(Oncorhynchus mykiss)* during a 37 day feeding trial. In the present study supplementation of inosine and IMP at concentration in excess of 0.4% and 0.6% respectively caused reduced growth and feed utilization performance of juvenile red sea bream.

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish. Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea bream, compared to those of the previous findings (Aoki et al., 1998; Kader and Koshio, 2012). Plasma glucose is commonly considered to be one stress indicator in fish, high glucose levels often indicate the higher stress status of fish (Eslamloo et al., 2012). The lower blood glucose content in fish fed inosine and IMP supplement indicated that dietary IMP and inosine supplementation induced an optimal physiological condition of the fish. Simultaneously, IMP supplemented diet groups showed relatively lower plasma TG and cholesterol value compared to inosine supplemented diet groups. In a previous study, Song et al., 2012 also reported numerically reduced TG value of Japanese flounder fed IMP supplemented diets. Results showing better physiological condition of red sea bream fed inosine and IMP supplemented diets compared with supplementation-free control diet indicate that nucleotides and/or nucleosides may help to maintain low level of plasma glucose, TG and cholesterol. Additionally, they may affect the lipid metabolism somehow, but more study will be needed to clarify the details on this relationship.

Stress is one of the emerging factors in aquaculture activities, which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization (Li et al., 2009). Plasma or serum cortisol concentration is a reliable biological indicator on stress response in fish and terrestrial animals (Li et al., 2009; Small and Davis, 2002). In the present study the relative values of plasma cortisol concentrations reduced significantly with taking dietary inosine and IMP. Result of the present study supports the findings of Tahmasebi-Kohyani et al. (2012) where supplementation of nucleotide significantly reduced plasma cortisol level of rainbow trout. Oxidative stress can be generated at high level of reactive oxygen species (ROS) and/or decreased efficacy of antioxidant system, which is another health risk factor in human or other mammals (Pasquini et al., 2008). The simultaneous analysis of d-ROMs and BAP provided valuable data on oxidative stress condition in humans, pig and dog (Pasquini et al., 2008; Ballerini et al., 2003). Recently, these tests have also been applied as a suitable tool for evaluating the oxidative stress in fish (Kader et al., 2010; Han et al., 2013). It is interesting to note that fish fed diets with INO-0.4 and IMP-0.6 were located in A zone, suggesting that this fish groups hold stronger resistance against oxidative stress, and were in lower oxidative stress conditions at the same time. It would be concluded that fish fed the diet containing the optimal level of inosine and/or IMP can maintain one of the best physiological condition of the fish. However, in terms of oxidative stress, this study could not prove the relative advantages of one supplement over the other. At the same time, the mechanism that caused reduced oxidative stress by inosine and IMP supplementation should be further studied.

Evidence from animal and human studies supports the theory that dietary nucleotides are important for optimal functioning of several components of the immune system (Cosgrove, 1998). Research on fish also revealed that exogenous nucleotides can influence both humoral and cellular components of the innate immune system. A number of studies reported that fish

fed nucleotides-supplemented diets can positively affect alternative complement activity, lysozyme activity, myeloperoxidase activity, immunoglobulin M level and extracellular superoxide anion activity and some other immune functions (Metailler et al., 1983; Song et al., 2012; Sakai et al., 2001; Li et al., 2004a,b). In the present study, at the same supplementation level, both of the supplements showed relatively similar effects on enhancing immune performance of red sea bream and peroxidase activity, SOD activity were significantly improved by inosine and IMP supplementation. Song et al. (2012) reported significantly higher peroxidase activity and SOD activity of Japanese flounders fed diets supplemented with IMP, which is in agreement with the present study. Lysozyme is a mucolytic enzyme of leucocytic origin, and is considered as an important marker of the humoral innate immune response (Li et al., 2005). In the present study there was a trend of increased lysozyme activity with the supplementation of inosine and IMP compared with control. Increased lysozyme activity due to nucleotide supplementation was also reported in common carp (*Cyprinus carpio*) (Sakai et al., 2001), raibow trout (Tahmasebi-Kohyani et al., 2011) and Japanese flounder Song et al. (2012). The increased serum lysozyme activities in inosine and IMP supplemented groups were due to immunostimulating properties of inosine and IMP which may act by either an increase in the number of phagocytes secreting lysozyme or an increase in the amount of lysozyme synthesized per cell (Engstad et al., 1992; Kumari and Sahoo, 2006). Total serum proteins also showed similar increasing trend with inoisne and IMP supplementation compared to control. The increased total serum protein and lysozyme activity in inosine supplemented groups in the present study would be due to the immunostimulatory effects of dietary nucleoside, inosine. Increased total serum protein due to nucleotide supplementation was also reported in *Catla catla* (Jha et al., 2007) and rainbow trout (Tahmasebi-Kohyani et al., 2012). On the other hand control diet group showed numerically lower lysozyme activity and total serum protein in the present study indicates

low nonspecific defense ability of fish. Increased catalase activity rates were attributed to elevated levels of exogenous hydrogen peroxide (H_2O_2) which is the main cellular precursor of the hydroxyl radical (HO^-), a highly reactive and toxic form of ROS . In human increased serum catalase activity is used as a diagnostic tool in acute pancreatitis (Goth et al., 1982), hemolytic disease (Goth et al., 1983) and some liver diseases (Goth et al., 1987). The high mean catalase activity observed in fish fed control diet compared to inosine and IMP supplemented diets in the present study implies that fish in control group were in stronger oxidative stress conditions compared to supplemented groups. Results of the catalase activity as well as the BAP and d-ROMs measured in the present study reconfirmed the positive supplemental effects of inosine and IMP associated with the conditions on oxidative stress and immunity.

The anti-bacterial activity of blood serum is considered as a nonspecific response to inhibit the growth of infectious microorganisms (Yano, 1996). In the present study *E. coli* were used for determining the bactericidal activity of fish. Increased bactericidal activity with the supplementation of inosine and IMP in diets would possibly be due to the absorption of inosine either directly into the serum and their capacity for killing *E. coli* in a dose-dependent manner or the other inosine and IMP effects on the immune factors. Increased bactericidal activities in fish with the supplementation of some other immunostimulans like β- glucans, and lactoferrin were also reported in some previous studies (Eslamloo et al., 2012; Kamilya et al., 2006) and it also substantiates the immunostimulating properties of inosine and IMP at the same time. At 0.8% inosine and IMP supplemented diet groups (INO-0.8 and IMP-0.8) bactericidal activity decreased and it was not significantly different from that of fish fed control diet. This may be due to the excessive levels of inosine nucleoside and IMP in these diets, which supports the findings of Burrells et al. (2001a) mentioning excessive levels of dietary nucleotides can also negatively affect immunity in fish.

In the present study, whole-body composition of juvenile red sea bream significantly influenced by dietary inosine and IMP supplementation. However, proximate composition values were within normal ranges that have been reported previously (Kader and Koshio, 2012) for red sea bream. Significant increase in carcass protein in the present study indicates highly efficient utilization of dietary protein owing to inosine and IMP supplementation which is also supported by increased PER value of supplemented group regardless the supplementation group in the present study. Significantly highest whole-body lipid content was observed in fish 0.4% inosine supplemented group (INO-0.4) compared to control. Similarly, Li et al. (2005) observed significantly increased whole body lipid content of red drum with the dietary nucleotide supplementation. Research on the effect of exogenous nucleotides and nucleosides on lipid metabolism of fishes is very limited, although it is known that dietary nucleotides can influence levels of various lipids and/or fatty acids in certain tissues, such as erythrocytes, plasma, liver or brain (Carver and Walker, 1995; Sato et al., 1995). Our observation of whole-body lipid response in red sea bream to dietary inosine supplementation seems to be the first such report for fish. Information on changes in various lipids in tissues in response to dietary nucleotides and nucleosides associated with physiological consequences may be important; therefore, further investigation is warranted.

The beneficial effects of dietary nucleotides on gastrointestinal tract (GIT) is well documented in human and other terrestrial animals (Uauy et al., 1990; Bueno et al., 1994; Carver, 1994) but very little is known about fish and other aquatic animals (Burrells et al., 2001b; Borda et al., 2003; Cheng et al., 2011).Recently Cheng et al. (2011) reported that nucleotide supplementation significantly increased fold height of the proximal intestine, and enterocyte height of the pyloric caeca, proximal and distal enteric sections of red drum. A significantly higher microvilli height was also observed in all evaluated enteric sections of red drum fed diets supplemented with nucleotides. In the present study the supplementations

of inosine and IMP significantly increased enterocyte height (hE), fold height (hF) of both anterior and posterior intestine and microvillus height (hMV) of posterior intestine compared to the control. Supplementation of inosine, IMP also increased anterior microvillus height but not at a significant level. Moreover, with the exception of anterior hF, the inosine supplemented diet groups showed improved gut morphology compared to the IMP supplemented diet groups at the same supplementation level. Although the mechanism by which dietary nucleoside inosine exerts its positive effects over the nucleotides IMP on the intestine morphology is not clear, most of the ingested nucleotides will be degraded in the intestine to nucleosides by alkaline phosphatase and nucleotidases, and may be further broken down by nucleosidases to produce purine and pyrimidine bases. On the other hand, some investigations on land animals suggest that nucleosides are the primary form absorbed (Uauy, 1989). Most of the absorbed nucleosides and bases are rapidly degraded within the enterocytes (Sonoda and Tatibana, 1978), but certain levels may be incorporated into the tissue pools, primarily in the small intestine, liver and skeletal muscle (Burridge et al., 1976; Saviano and Clifford, 1978). Inosine as a nucleoside absorbed directly by intestine mostly and beneficially can influence intestinal health of fish. The beneficial effects on intestine in present study might be due to the positive effects of inosine on mucosa associated with lymphoid tissue (MALT) although the knowledge on MALT in fish is very limited (Li and Gatlin, 2006).

In conclusion the results of the current study demonstrated that supplementation of both inosine and inosine monophosphate can have positive influences on growth performance, survival, feed utilization, immune response, hematological parameters, and intestinal morphology of red sea bream. Even though some oxidative stress parameters, non-specific immunity and intestinal morphology improved at 0.2% supplementation level, most of the performance parameters showed relatively better condition at 0.4 % supplementation level.

Considering overall performance, it is recommended that dietary inosine and IMP supplementations at 0.4% are beneficial to promote growth, immune responses, oxidative stress and intestinal health condition of juvenile red sea bream. However, further research is needed, including controlled disease challenges, which will be carried out in our next research, to elucidate the integrated disease defense mechanisms that may be affected by inosine and IMP supplementation in red sea bream.

Part-3

Dietary effects of adenosine monophosphate to enhance growth, digestibility, innate immune responses and stress resistance of juvenile red sea bream, *Pagrus major*

ABSTRACT

Our study explored the dietary effects of adenosine monophosphate (AMP) to enhance growth, digestibility, innate immune responses and stress resistance of juvenile red sea bream. A semi-purified basal diet supplemented with 0% (Control), 0.1% (AMP-0.1), 0.2% (AMP-0.2), 0.4% (AMP-0.4) and 0.8% (AMP-0.8) purified AMP to formulate five experimental diets. Each diet was randomly allocated to triplicate groups of fish (mean initial weight 3.4g) for 56 days. The results indicated that, dietary AMP supplementations tended to improve growth performances, in which one of the best ones was found in diet group AMP-0.2 followed by diet groups AMP-0.1, AMP-0.4 and AMP-0.8. The Apparent digestibility coefficients (dry matter, protein and lipid) also improved by AMP supplementation and significantly highest dry matter digestibility observed in diet group AMP-0.2. Fish fed diet groups AMP-0.2 or AMP-0.4 had significantly higher peroxidase and bactericidal activities than fish fed the control diet. Nitro-blue-tetrazolium (NBT) activity was found to be significantly ($P < 0.05$) greater in fish fed diet group AMP-0.4 and AMP-0.8. Serum protein, lysozyme activity and agglutination antibody titer were also increased ($P>0.05$) by dietary supplementations. In contrast, catalase activity decreased with AMP supplementations. Moreover, the fish fed AMP supplemented diets had better improvement ($P<0.05$) in body lipid contents, condition factor, hematocrit content and glutamyl oxaloacetic transaminase (GOT) level than the control group. Supplementations also improved both freshwater and oxidative stress resistances. Interestingly, fish fed diet group AMP-0.2 and AMP-0.4 showed the least oxidative stress condition. Finally it is concluded that, dietary AMP supplementation had enhanced growth, digestibility, immune response and stress resistance of red sea bream. The regression analysis of weight gain and lysozyme activity revealed that the optimal levels of dietary AMP might be 0.16% and 0.41 % respectively, for red sea bream, which is also

inline with the most of the growth and health performance parameters of fish under present experimental condition.

Keywords: Adenosine monophosphate, Growth, Digestibility, Innate immune responses, Oxidative stress, *Pagrus major*

1. Introduction

Red sea bream (*Pagrus major*) is one of the most economically cultured marine fish species in East Asian countries, particularly in Japan and Korea due to its desirable taste, high market demand and traditional food habits. Intensive cultures of this species are more prone to bacterial, viral, parasitic and other environmental diseases due to deterioration of water quality and elevation of stress. In aquaculture antibiotic, vaccination and other chemicals are currently used in varying degree to control these diseases. However, each of these treatment methods has its drawbacks, including suppression of aquatic animal's immune system, environmental hazards and food safety problem. Moreover, the development of antibiotic resistance in humans has led to a growing interest in antibiotic-free animal production worldwide (Bager et al., 2000). In such circumstances, dietary supplementation of health promoting functional compounds has turned out to be more and more interesting as an effective alternative for prophylactic treatment against disease outbreaks in intensive aquaculture. Recently, in aquaculture research nucleotides and its related product has been paid attention promisingly as potential immunomodulators as well as functional nutrients.

Nucleotides are the base units for DNA and RNA synthesis during cell construction provide energy for normal cellular process and are therefore essential to growth and development (Vanburen et al., 1994). Dietary supplementation of nucleotides has been shown to benefit many mammalian physiological and nutritional functions (Uauy, 1989; Quan, 1992; Carver, 1994; Haskó et al., 2000). In aquatic animals both nucleotides and nucleosides

have long been implicated as feed attractants in both vertebrate and invertebrate species (Mackie and Adron, 1978; Carr and Thompson, 1983; Person-Le Ruyet et al., 1983; Carr et al., 1984). However, world-wide heightened attention on nucleotide supplementation into potential growth and health benefits in aquaculture species instigated after 2000s. To date, research pertaining to nucleotide nutrition in fishes has shown rather consistent and encouraging beneficial results in fish health management. Research to date on dietary nucleotides has focused on a mixture of nucleotides, rather than specific types of nucleotides except inosine and IMP for the research on feed stimulants (Li and Gatlin, 2006). Adenosine monophosphate (AMP) is a purine nucleotide which consists of a phosphate group, the sugar ribose, and the nucleobase adenine. It is an ester of phosphoric acid and the nucleoside adenosine and used as a monomer of RNA. In aquaculture AMP has been studied most extensively as a part of mixed nucleotide rather than considering as individual nucleotide for promoting growth, feed utilization and potential heath benefit. Mackie (1973) and Kiyohara et al. (1975) initially reported the chemo-attractant properties of AMP for lobster and puffer fish respectively. Lin et al. (2009) reported increased growth and non specific immune performance of red drum (*Epinephelus malabaricus)*. However, studies on nucleotide nutrition of red sea bream are very scarce. Recently, we evaluated mixed nucleotide effects on this species (Hossain et al., 2016b). However, to the best of our knowledge there is no research on individual nucleotide AMP as feeding stimulant as well as a functional nutrient for potential growth and health benefit of red sea bream. Therefore, the present study was conducted to assess the dietary effects of AMP on growth performance, feed utilization, digestibility, innate immune response and stress resistance of red sea bream juveniles.

2. Materials and Methods

2.1. Test fish and experimental system

Juvenile red sea bream were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (54% crude protein and 10% crude lipid, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 100 l polycarbonate tanks (filled with 80 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 4.3.1and 4.3.2 summarize the basal diet formulation and chemical composition of the experimental diets, respectively. Five fishmeal and casein based semi-purified diets were formulated to be nearly isonitrogenous (54% crude protein), isolipidic (11% crude lipid) and isocaloric (22 KJ g^{-1} gross energy). The experiential diets were prepared by supplementing purified AMP nucleotide as disodium salts (Sigma Aldrich Co., St. Louis, MO, USA) to the basal diet at concentrations of 0.1, 0.2, 0.4 and 0.8 % for diet group AMP-0.1, AMP-0.2, AMP-0.4 and AMP-0.8 respectively. Basal diet without AMP supplementation was used as control. For proper mixing of AMP with other ingredients, initially AMP and weighted arginine of the respective diets were mixed properly in a glass beaker by hands with the help of a stainless laboratory spatula. Then this mixture was thoroughly mixed with other dry ingredients in a food mixer for 10 min. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry

ingredients and mixed for another 10 min. Water was added gradually (35–40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 55 °C for about 150 min. The test diets were stored at −28 °C in a freezer until use.

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Twenty fish, having a mean initial body weight of approximately 3.4 g were randomly allocated to previously prepare fifteen tanks. Fish were fed the experimental diets for 56 days by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 1 hour after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk fortnightly to determine growth and visually check their health condition. The monitored water quality parameters were: water temperature 22.6±1.9 °C; pH 8.1±0.7 and salinity 34.5±0.5 during the feeding trial. These ranges are considered within optimal values for juvenile red sea bream.

2.4. Sample collection

At the end of the feeding trial, all experimental fish were fasted for 24 h. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then individual body weight of fish was measured, and the growth parameters

Table 4.3.1: Basal diet formulation of juvenile red sea bream.

Ingredients	Composition (%)
Fishmeal[a]	20
Casein[b]	32
Pollack liver oil[c]	7
Soybean lecithin[d]	3
Vitamin mixture[e]	3
Mineral mixture[f]	3
L-arginine[g]	1.5
Stay-C[h]	0.08
Dextrin	7
α-starch	6
Activated gluten[i]	5
α- cellulose	12.42
AMP[j]	0-0.8

[a]Nippon Suisan Co. Ltd., Tokyo, Japan

[b]Waco Pure Chemical Industries Inc. (Osaka, Japan)

[c] Riken Vitamin, Tokyo, Japan.

[d] Kanto Chemical Co., Inc. (Tokyo, Japan)

[e] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[f]Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[g] Nacalai Tesque, Kyoto, Japan.

[h] Stay-C 35.

[i]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS".

[j] Sigma Aldrich Co., St. Louis, MO, USA. (AMP added to diets at the expense of α-cellulose).

were calculated accordingly. Nine fish per treatment (three fish per each tank) were randomly collected, washed with distilled water and then stored at −80 °C (MDF-U383, SANYO, Tokyo, Japan) for the whole body proximate analysis. Six fish were randomly sampled from each dietary replicate tank and their blood were collected by puncture of the caudal vain using heparinized (1600 IU/ml, Nacalai Tesque, Kyoto, Japan) disposable syringes and pooled. In addition non-heparinized disposable syringes were used to collect blood for serum analysis. Partial heparinized whole blood was used to analyze the hematocrit levels while plasma and serum were obtained by centrifugation at 3000 ×g for 15 min under 4 °C, and then stored at −80 °C until the analysis. Liver and viscera were dissected out from the fish above, weight individually to calculate the hepatosomatic index (HSI) and viscerasomatic index (VSI).

2.5. Biochemical analysis

The ingredients, diets and fish whole body were analyzed in triplicate, using standard methods (AOAC, 1995). The moisture was determined by drying the sample at 105 °C to constant weight. The ash was analyzed by combustion at 550 °C for 12 h. The crude protein content was determined by measuring the nitrogen content (N × 6.25) using the Kjeldahl method with a Tecator Kjeltec System (1007 Digestion System, 1002 Distilling unit, and Titration unit; FOSS Tecator AB, Högendäs, Sweden). Crude lipid content was estimated using gravimetric method (954.02). Total serum protein (TSP) and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured

Table 4.3.2: Proximate composition of the experimental diets (% dry matter basis).

Parameters	Diet groups				
	Control	AMP-0.1	AMP-0.2	AMP-0.4	AMP-0.8
Moisture	7.9	7.5	8.2	7.5	8.3
Crude protein	54.4	54.8	54.2	54.2	54.3
Crude lipid	11.4	11.2	11.2	11.2	11.8
Ash	6.53	6.53	6.45	6.60	6.50
Gross energy ($KJg\text{-}^{1}$)*	22.1	22.1	22.1	22.0	22.2

*Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ kg^{-1}, respectively.

Carbohydrate was calculated by the difference: 100-(protein + lipid + ash).

spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously Morganti et al., 2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma samples was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg.

2.6. Evaluation of immune parameters

Serum lysozyme activity (LA) was measured with turbidimetric assays (Lygren et al., 1999). Ten micro-liters of samples was put into well of microplate, then added 190 micro-liters of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4,their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

Oxidative radical production by phagocytes during respiratory burst was measured through the NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995) with some modifications. Briefly, blood and 0.2% NBT (Nacalai Tesque, Kyoto, Japan) were mixed in equal proportion (1:1), incubated for 30 min at room temperature. Then 50 mL was taken out and dispensed into glass tubes. One milliliter of dimethylformamide (Sigma) was added and centrifuged at 2000 × g for 5 min. Finally, the optical density of supernatant was

measured at 540 nm using a spectrophotometer (Hitachi U-1000, Japan). Dimethylformamide was used as the blank.

Serum bactericidal activity (BA) was performed according to Yamamoto and Iida (1995). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g/ml, *Escherichia coli*, IAM1239 cell line, Kagoshima, Japan) (Promega, Germany) and incubated at 25 °C for 24 h in micro tube rotator (MTR-103, AS ONE, Osaka, Japan). 50 ml of the reaction solutions were incubated on TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) at 25 °C for 24 h (on X-plate). Colony forming unit (CFU) was counted by the plate counting method as described by Ren et al. (2008). The bactericidal activity was defined as follows, (CFU of blank group-CFU of each group)/CFU of blank group × 100.

The total peroxidase activity (PA) in serum was measured according to Salinas et al. (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

The catalase activity (CAT) assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate that absorbs at 405 nm Goth (1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol/l phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate ($(NH_4)_6\ Mo_7O_{24}\ .4\ H_2O$) and the yellow complex of molybdate and hydrogen peroxide is measured at 405 nm against reagent blank. One unit CAT decomposes 1 µmole of hydrogen

peroxide/l minute under assay conditions. CAT activities are expressed as kilo unit per liter (kU/l)

The agglutination antibody titer was conducted in round bottomed 'U' shaped micro titer plates after Swain et al. (2007) with slight modifications. 50 µl of serum was serially diluted in PBS (1/2, 1/4, 1/8, 1/16, 1/32and 1/64) and then equal volumes of *Vibrio anguillarum* (1×10^8 cfu ml^{-1}) was added to wells and kept for 24 h at 4 ^{0}C. The reciprocal of the highest dilution that gave agglutination was taken as the agglutination antibody titer which is expressed as log10.

2.7. Digestibility assessment

A digestibility trial was conducted at the end of the growth trial; the remaining fish from the same treatments were distributed randomly into triplicate tanks. The fish were fed a diet containing chromium oxide (Wako Pure Chemical Industries, Ltd) as the inert marker at a level of 0.5% (Cr_2O_3, 5 g kg^{-1}) to the previous formulation and fed to the fish under the same condition as the growth experiment apart from the difference in stocking density. After 5-day adaptation of the diets, feces were collected at 3-h intervals between the morning and afternoon feeds by using a siphon for two weeks. Feces of red sea bream very rapidly settled to the bottom of the tank and did not easily break up in the water so that nutrient and marker losses were minimized. Sufficient amount of feces were collected, freeze dried and immediately kept at - 20^{o}C until analysis. Concentration of chromium oxide in diets and feces was determined according to Furukawa and Tsukahara (1966).

2.8. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, five fish from each rearing tank (total 15 fish per

treatment) were randomly selected and transferred in to a blue colored 20 L rectangular glass aquarium with 15 L tap water which was aerated for 24 h. The glass aquaria for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of red sea bream juvenile was calculated according to Moe et al. (2004) as follows: time to death (min) of a juvenile was converted to log10 values. When the juvenile were transferred to the low salinity water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was log10 100 = 2). The calculation was then conducted every 5 minutes after the first exposure. The values of log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

Y= a*X*+ b where Y = log10 (survival), *X* = time for individual juvenile death (min).

LT_{50} (*X*) was obtained when Y = 1.7 since log10 (50) = 1.7. Each value obtained from the equation above was compared statistically.

2.9. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR, % day^{-1}) = {Ln (final weight) – Ln(initial weight)/duration}×100

Survival (%) = 100 × (final no. of fish / initial no. of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed conversion ratio (FCR) = dry feed intake (g)/ live weight gain (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Condition factor (CF, %) = weight of fish / $(\text{length of fish})^3$ × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

Viscerasomatic index (VSI, %)= viscera weight/fish weight × 100

2.10. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Turkey Kramer test. Weight gain (%) and lysozyme activity were subjected to broken line regression analysis with dietary AMP supplementation level.

3. Results

3.1 Growth performance and nutrient utilization

Growth performance, nutrient utilization and survival of red sea bream fed experimental diets are presented in Table 4.3.3. In general dietary AMP supplementations tended to improve growth performances (final body weight, % weight gain (WG %) and specific growth rate (SGR)), in which one of the best ones was found in diet group AMP-0.2 followed by diet groups AMP-0.1, AMP-0.4 and AMP-0.8, respectively. Feed utilization parameters of fish also improved when fed the diets containing AMP although the values were not always statistically significant. Significantly higher feed intake observed in fish fed diet group AMP-0.1 and it was not significantly different with other AMP supplemented groups. FER and PER also improved in diet groups AMP-0.1 and AMP-0.2 and then decreased the performance. The poorest growth performance and feed utilization were obtained in AMP supplementation-free control diet group. All treatments showed relatively high survival rates between 91.6 % and 93.3% and there were no significant differences among all diet groups ($P>0.05$).

Table 4.3.3: Growth performance and feed utilization parameters of red sea bream fed test diets for 56 days*

Parameters	Diet groups				
	Control	AMP-0.1	AMP-0.2	AMP-0.4	AMP-0.8
IBW	3.38±0.01	3.36±0.01	3.34±0.01	3.35±0.03	3.38±0.02
FBW	25.56±0.48[a]	28.94±1.02[ab]	29.19±0.41[b]	27.61±0.55[ab]	27.70±1.22[ab]
WG%	656.9±15.2[a]	762.6±29.12[ab]	774.8±14.8[b]	725.2±19.9[ab]	719.6±34.6[ab]
SGR	3.61±0.04[a]	3.85±0.06[ab]	3.87±0.03[b]	3.77±0.04[ab]	3.75±0.08[ab]
FI	20.14±0.37[a]	23.18±0.88 [b]	22.85±0.25[b]	22.67±044[b]	22.90±0.54[b]
FCE	1.10±0.04	1.13±0.00	1.13±0.03	1.07±0.01	1.06±0.03
PER	1.99±0.06	2.02±0.00	2.09±0.06	1.94±0.01	1.96±0.05
Sur	93.33±1.67	92.50±2.50	91.67±1.67	93.33±1.67	93.33±1.67

[a] IBW: initial body weight (g).

[b] FBW: final body weight (g).

[c] WG: percent weight gain (%).

[d] SGR: specific growth rate (% day^{-1}).

[e] FI: feed intake (g $fish^{-1}$ 50 $days^{-1}$).

[f] FCR: feed conversion ratio.

[g] PER: protein efficiency ratio.

[h] Sur: survival (%).

* Values are means of triplicate groups±S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

3.2. Hematological parameters

Table 4.3.4 represents the blood parameters of juvenile red sea bream after 56 days of feeding trial. Overall, dietary treatments had no significant effect on blood chemical parameters of fish among different treatments except in GOT. Fish fed AMP supplemented diets showed significantly reduced plasma GOT level compare to the control group. Meanwhile, plasma glucose, cholesterol, total bilirubin and GPT level also numerically reduced with fish fed AMP supplemented diets compared to control. Hematocrit content increased with fish fed AMP supplemented diets and it was significantly highest ($P<0.05$) in fish fed diet group AMP-0.4.

3.3. Oxidative stress parameters

Fig. 4.3.1 indicates the status of oxidative stress conditions, in which the conditions of both stress intensity and tolerance ability were combined, of fish fed with test diets for 56 days. Dietary AMP supplementation significantly reduce d-ROMs values ($P<0.05$) and it was lower in diet groups AMP-0.2 and AMP-0.4. In contrast, BAP values were not significantly influenced by dietary supplementation (no data illustrated here). Combined effects of d-ROM and BAP showed that fish fed diet groups AMP-0.2 and AMP-0.4 were located in zone A which was categorized as lower intensity of oxidative stress and higher tolerance ability against oxidative stress. Supplementation free-control group, lowest and highest AMP supplemented diet groups (AMP-0.1 and AMP-0.8, respectively) located in zone D (Fig. 4.3.1). Numerically lower values ($P>0.05$) for plasma cortisol levels (%) (Table 4.3.4) were obtained in the AMP supplemented groups compared to control group.

Table 4.3.4: Blood parameters of juvenile red sea bream fed test diets for 56days.[*]

Parameters	Diet groups				
	Control	AMP-0.1	AMP-0.2	AMP -0.4	AMP -0.8
Hematocrit (%)	30.3±1.7[a]	37.0±3.6[ab]	39.0±1.7[ab]	41.0±1.5[b]	37.67±1.3[ab]
Glucose (mg/dl)	73.5±9.5	51.0±4.0	56.0±11.0	57.3±7.2	50.7±12.2
T-Cho (mg/dl)[a]	149.3±5.9	146.3±9.6	141.5±10.5	132.7±12.4	142.0±10.2
BUN (mg/dl)[b]	5.33±0.3	6.0±0.6	6.67±0.9	7.5±2.5	6.33±0.9
T-Bill (mg/dl)[c]	0.23±0.03	<0.2	<0.2	<0.2	<0.2
GOT (IU/l)[d]	78.0±2.0[b]	30.5±6.5[a]	43.0±10.0[a]	36.0±5.5[a]	46.0±1.0[ab]
GPT(IU/L)[e]	78.3±7.0	36.5±16.5	44.3±3.2	45.7±18.9	28.7±3.7
TG (mg/dl)[f]	135.0±35.5	180.7±21.1	200.0±15.0	152.67±31.7	156.0±26.5

[a] T-Cho: total cholesterol.

[b] BUN: blood urea nitrogen.

[c]T- Bill: Total bilirubin

[d]GOT: glutamyl oxaloacetic transaminase.

[e]GPT: glutamic pyruvate transaminase.

[f]TG: triglyceride.

[*]Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

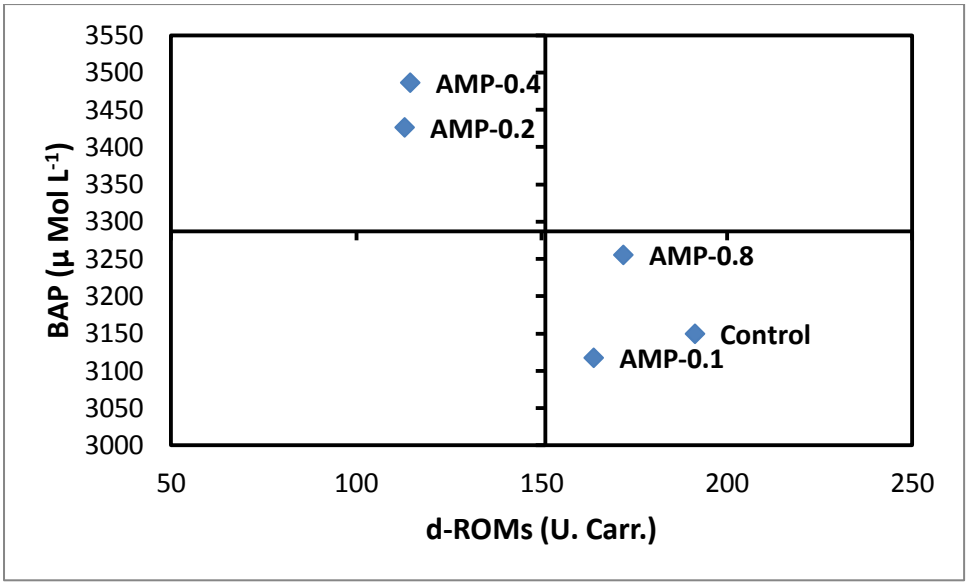


Fig. 4.3.1. Oxidative stress parameters in red sea bream (*P. major*) fed test diets for 56 days. (Values are means of triplicate groups. The abbreviations of experimental treatments are illustrated in the text. Central axis based on mean values of d-ROM and BAP from each treatment.) Zone A: High antioxidant potential and low reactive oxygen metabolites (good condition). Zone B: High antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone C: Low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone D: Low antioxidant potential and high reactive oxygen metabolites (stressed condition).

3.4. Low salinity stress test

The results of lethal stress test against fresh water shock on LT_{50} obtained by regression analysis are shown in Fig. 4.3.2. Supplementation increase the LT50 values of red sea bream compare to control and it was numerically higher in fish feed diet group AMP-0.2. However, LT50 was not significantly influenced (P>0.05) by dietary treatments.

3.5. Innate immune responses

Non-specific innate immune responses were positively affected by dietary AMP supplementations (Table 4.3.5). Fish fed diet groups AMP-0.2 or AMP-0.4 had significantly higher PA and BA than fish fed the control diet. NBT activity was found to be significantly (P < 0.05) greater in fish fed diet group AMP-0.4 and AMP-0.8. Although numerically higher TSP, LA and bacterial agglutination antibody titer were detected in AMP supplemented diet groups compared to control but there were no significant differences among treatments. In contrast, CAT activity decreased (P>0.05) with AMP supplementations.

3.6. *Digestibility coefficients*

The apparent digestibility coefficients (ADCs) of dry matter (ADC_{DM}), protein ($ADC_{Protein}$) and lipid (ADC_{Lipid}) are presented in Table 4.3.6. ADC_{DM} significantly increased by the dietary AMP supplementations and it was highest (P<0.05) in fish fed diet group AMP-0.4. Supplementation also increased $ADC_{Protein}$ and ADC_{lipid} but not a significantly level. However, AMP supplementation free control group showed lowest values of ADC_{DM}, $ADC_{Protein}$ and ADC_{Lipid} (Table 4.3.6).

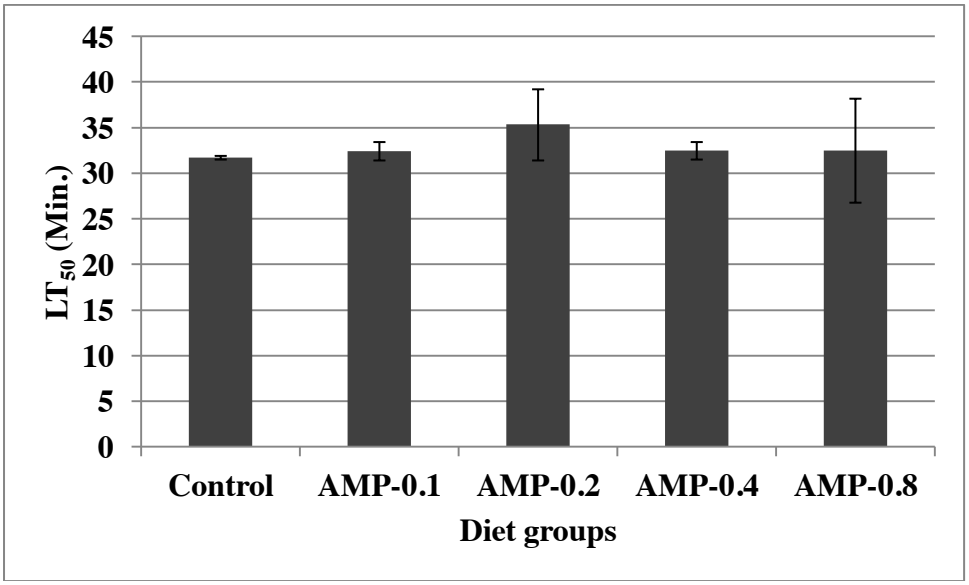


Fig. 4.3.2. LT_{50} (min) calculated from the lethal time of red sea bream exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from triplicate groups. The abbreviations of experimental diets are illustrated in the text. Values with different letters are significantly different ($P<0.05$).

Table 4.3.5: Non-specific immune response of juvenile red sea bream fed test diet for 56 days.*

Parameters	Diet groups				
	Control	AMP-0.1	AMP-0.2	AMP-0.4	AMP-0.8
TSP (g/dl)[a]	2.90±0.50	3.53±0.32	3.20±0.40	3.70±0.25	3.50±0.10
LA (unit/mL)[b]	87.5±37.5	116.7±36.3	125.0±50	183.3±16.7	141.7± 46.4
PA[c]	1.61±0.05[a]	1.65±0.05[a]	1.97±0.02[b]	1.78±0.1[ab]	1.89± 0.03[ab]
BA (%)[d]	56.6±1.8[a]	67.7±3.5[ab]	71.7±2.6[b]	75.3±2.6[b]	68.3±2.3[ab]
NBT[e]	0.35±0.02[a]	0.36±0.01[a]	0.41±0.02[ab]	0.43±0.01[b]	0.45± 0.01[b]
CAT[f]	87.3±10.4	72.3±10.5	55.8±16.7	43.68±20.2	79.4± 6.3
B-Agglu[g]	0.70±0.1	0.90±0.2	1.00±0.1	1.00±0.1	0.80± 0.1

[a]TSP: Total serum protein

[b]LA: lysozyme activity

[c]PA: Peroxidase activity (measured at OD of 450nm).

[d]BA: Bactericidal activity=100× (CFU of blank group-CFU of each group)/CFU of blank group.

[e]NBT: Nitro-blue-tetrazolium activity

[f]CAT: Catalase activity

[g]B-Agglu: Bacterial agglutination antibody titer

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

Table 4.3.6: Digestibility of juvenile red sea bream fed different experimental diets for 56 days*.

Parameters	Diet groups				
	Control	AMP-0.1	AMP-0.2	AMP-0.4	AMP-0.8
ADC_{DM}[a]	76.53±0.6[a]	80.34±0.3[b]	82.18±0.6[b]	80.95±0.7[b]	81.53±0.4[b]
$ADC_{protein}$ (%)[b]	91.82±0.3	92.98±0.01	93.04±0.1	93.13±0.01	92.60±0.6
ADC_{lipid} (%)[c]	91.97±0.6	93.10±0.3	93.97±0.1	93.66±0.6	93.79±0.2

[a] ADC_{DM} (Apparent digestibility coefficient of dry matter) = 100 – 100

$$\left(\frac{\%\,\text{marker in diet}}{\%\,\text{marker in faeces}}\right)$$

[b]$ADC_{Protein}$ (Apparent protein digestibility, %) = 100- 100

$$\left(\frac{\%\,\text{marker in diet}}{\%\,\text{marker in faeces}} \times \frac{\%\,\text{protein in faeces}}{\%\,\text{protein in diet}}\right)$$

[c]ADC_{Lipid} (Apparent lipid digestibility, %) = 100- 100

$$\left(\frac{\%\,\text{marker in diet}}{\%\,\text{marker in faeces}} \times \frac{\%\,\text{lipid in faeces}}{\%\,\text{lipid in diet}}\right)$$

*Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

3.7. Whole body proximate analysis and biometric indices

The initial and final whole body proximate compositions of juvenile red sea bream are shown in Table 3.4.7. All the fish showed a change in the analyzed parameters compared to those of the initial values. In comparison with the control group, dietary treatments had no significant influences on the whole body proximate composition and biometric indices except those of whole body crude lipid and condition factor (CF). Fish fed AMP supplemented diet showed significant increase of whole body lipid content and it was highest in diet group AMP-0.1. On the other hand supplementation-free control group showed significantly lowest value. CF also followed the similar trend of whole body lipid content of the present study.

3.8. Quantification of optimum supplementation of AMP nucleotide for P. major.

The optimum supplementation level of AMP nucleotide for % WG and LA were estimated by broken line regression analysis (Fig. 4.3.3a, b). Based on % WG, the optimal supplemental level of AMP was estimated to be 0.16 % in diet ($y = 589.5x + 672.48$; $R = 0.8267$). On the other hand, the optimal supplemental level of AMP nucleotide based on LA was estimated to be 0.41% in diet ($y = 231.34x + 87.64$; $R = 0.9756$).

4. Discussion

In aquaculture, research to date on dietary nucleotides has mainly focused on nucleotides mixture rather than individual nucleotide. In limited cases, inosine and IMP, UMP and AMP have been used as feeding stimulants or feed enhancers (Mackie and Adron, 1978; Kubitza et al., 1997, Hossain et al., 2016a). Recently, Hossain et al. (2016a) also reported inosine and IMP as potential functional nutrient materials in aquafeed. However, to the best of our knowledge, there is very limited information available on dietary AMP supplementation for potential growth and health benefit of aquatic species. Under these circumstances, it is

Table 4.3.7: Whole body proximate analysis (% wet basis) and biometric indices in juvenile red sea bream fed test diets for 56 days.*

Parameters	Initial[1]	Diet group				
		Control	AMP-0.1	AMP-0.2	AMP -0.4	AMP -0.8
Moisture	83.6	76.2±0.4	74.5±1.6	75.7±0.5	74.4±0.4	76.4±0.4
Crude protein	12.1	13.4±0.1	13.6±1.1	13.5±0.2	13.5±1.3	13.5±0.2
Crude lipid	0.7	5.4±0.2^{a}	6.6±0.2^{b}	6.2±0.2^{ab}	6.2±0.1^{ab}	5.6±0.3^{ab}
Crude ash	3.4	3.5±0.04	3.5±0.3	3.4±0.1	3.6±0.1	3.6±0.1
CF[2]	-	1.97±0.05^{a}	2.21±0.03^{b}	2.13±0.02^{ab}	2.11±0.06^{ab}	2.09±0.05^{ab}
HSI[3]	-	1.10±0.14	1.74±0.18	1.61±0.09	1.49±0.10	1.49±0.18
VSI[4]	-	7.14±0.35	7.23±0.09	7.33±0.16	7.36±0.07	6.64±0.33

[1]Initial values are not included in the statistical analysis.

[2]CF (condition factor)=100 × fish weight/(fish length)^{3}

[3]HSI (hepatosomatic index)=100 × liver weight/fish weight

[4]VSI (viscerasomatic index)=100 × viscera weight/fish weight

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05). Crude protein, crude lipid and ash are expressed on a wet weight basis.

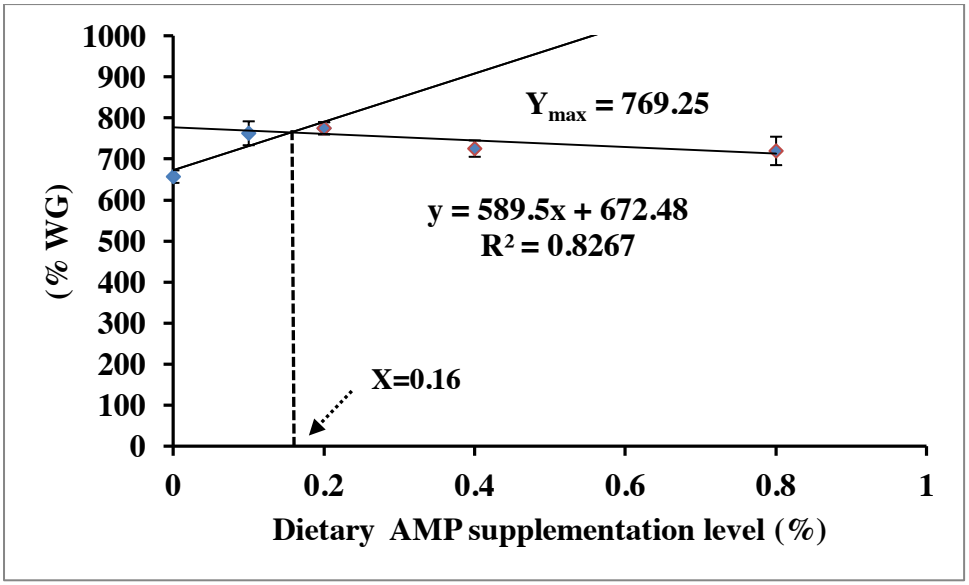


Fig. 4.3.3a. Broken line regression analysis of percent weight gain (%WG) for juvenile red sea bream (*P. major*) fed diets supplemented with graded levels of AMP for 56 days.

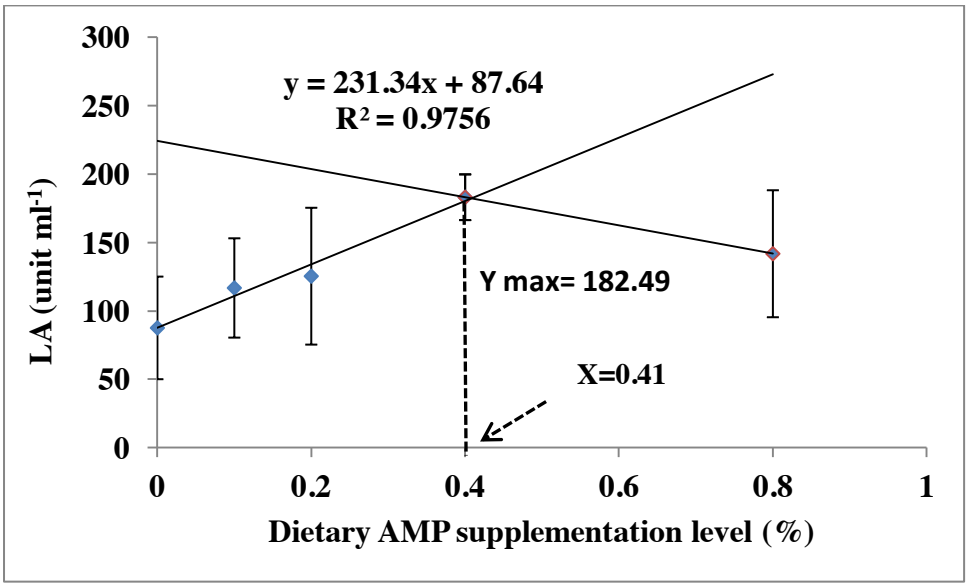


Fig. 4.3.3b. Broken line regression analysis of lysozyme activity (LA) for juvenile red sea bream (*P. major*) fed diets supplemented with graded levels of AMP for 56 days.

important to investigate the supplementation effects of AMP as functional materials in aquafeed.

The obtained results clearly demonstrated that feeding of AMP supplemented diet groups significantly improves the growth performances of red sea bream compared to supplementation-free control group after 56 days feeding trial. This observation is in agreement with the previous findings of Lin et al. (2009), reported that nucleotide AMP significantly increased the growth performance of grouper (*E. malabaricus*). Similarly, the growth enhancing effects of some other individual and mixed nucleotides have also been reported in some previous studies (Hossain et al. 2016a,b; Song et al. 2012; Cheng et al. 2011). So far, there is no exact explanation how dietary nucleotide works to enhance growth. However, it is assumed that the growth-enhancing effect of AMP in the present study resulted from improved feed intake (Table 4.3.3), promoting more rapid feed intake that reduced nutrient leaching into the water or possibly playing roles in metabolism (Metailler *et al*. 1983, Hossain et al. 2016). In the present study significantly improved feed intake in AMP supplemented diet groups in comparison to supplementation free control diet group strongly substantiates the previous hypothesis. In contrast, supplementation of AMP after a certain concentration in the present study showed reduced growth performance and feed utilization. Reduced growth performance and feed utilization by high mixed or individual nucleotide levels also reported in some previous studies (Akintonwa et al., 1979; Baker and Molitori, 1974; Adamek et al., 1996, Hossain et al. 2016 a,b). In case of Japanese flounder Song et al. (2012) reported that a high dietary concentration of IMP (1.0%) resulted depressed growth performance compared to lower levels of dietary IMP (0.1 to 0.2%). Adamek et al. (1996) also reported that high dietary nucleotide concentration (5%) caused growth depression in rainbow trout *(Oncorhynchus mykiss)* during a 37 day feeding trial. In our previous studies it was also found that, high dietary concentration of mixed nucleotides (≤0.2%), IMP and

inosine nucleoside (<0.4%) caused reduced growth and feed utilization performances of red sea bream. Similarly, in the present study supplementation of AMP at concentration in excess of 0.2% caused reduced growth and feed utilization performance of juvenile red sea bream.

The enhanced growth performance and feed utilization of fish fed dietary AMP might be also attributed to the enhanced digestibility coefficients. In the present study, the highest growth performances in AMP-0.2 diet group might be partly due to the improved $ADC_{protein}$, ADC_{lipid} and ADC_{DM} digestibility in this group. Similar enhanced digestibility were also reported by Hossain et al. (2015) for juvenile amberjack fed diet supplemented with dietary nucleoside, inosine. Increased digestibility in AMP supplemented diet groups in the present study might be due to feeding stimulatory properties of AMP. It is well documented that feeding stimulant increase the secretion of different digestive enzyme (Kofuji et al., 2006 ; Takii et al., 1986, Takii et al. 1990; Satoh 2003). These increased secretions of different digestive enzyme due to AMP supplementation might help to improve this digestibility. However, in the present study the secretion of different digestive enzyme are not studied. So, further study on secretions of different digestive enzymes due to nucleotide supplementation in fish is warranted to prove this hypothesis.

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish (Hossain et al., 2016a,b). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to those of the previous findings (Aoki et al., 1998; Hossain et al., 2016a,b). Dietary AMP supplementation significantly enhanced hematocrit of juvenile red sea bream as a general health response towards nutritional strategies. In the present experiment, hematocrit content was significantly highest in fish fed AMP-0.4 diet group, other AMP supplemented groups showed intermediate values whereas control group showed significantly lowest value. This indicated that dietary AMP elevated

the health status of fish. Similarly, Song et al. (2012) and Hossain et al. (2016a.b) reported the enhanced hematocrit level by the supplementation of nucleotides in Japanese olive flounder and red sea bream diets, respectively. Plasma glucose is commonly considered to be one stress indicator in fish, high glucose levels often indicate the higher stress status of fish (Eslamloo et al., 2012). The lower blood glucose content in fish fed AMP supplement indicated that dietary AMP supplementation induced an optimal physiological condition of the fish. Result of the present study also revealed that plasma bilirubin, GOT and GPT level showed lower values ($P>0.05$) in AMP supplemented diet groups compared to supplementation-free control group. Plasma bilirubin, GOT (or aspartate aminotransferase, AST) and GPT (or alanine aminotransferase, ALT) are often used for the evaluation of liver function as they are released into the blood during injury or damage to the liver cells (Lemaire et al., 1991). Lower values of these parameters in AMP supplemented diet groups indicated that, AMP induced an optimal physiological condition as well as better liver health condition when compared with the supplementation-free control group.

Stress is one of the emerging factors in aquaculture activities, which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization Hossain et al. (2016a,b). Dietary nucleotide increases the resistance of fish to a variety of stressors is well documented (Burrells et al., 2001b; Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2012, Hossain et al., 2016a,b). In our study, fish fed AMP supplemented diets showed increase in stress resistance of red sea bream after the exposure of low salinity stress test. In the present study highest LT_{50} value obtained in diet group AMP-0.2 (35.32 min) on the other hand supplementation free control group showed the lowest value of LT_{50} (31.21 min) indicated a lower fresh water tolerance of the red sea bream. Increased ($P>0.05$) LT_{50} of red sea bream fed diet group AMP-0.2 indicates healthy status of red sea bream (Yokoyama et al., 2005, Hossain et al. 2016b). Oxidative stress is considered to involve in plenty of diseases and

pathological status in fish (Martinez-Alvarez et al., 2005), and is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants (Celi et al., 2010). Simultaneous measurements of d-ROMs with BAP can provide a suitable tool for measuring the oxidative stress in humans, pig, rabbit and dog (Oriani et al., 2001; Ballerini et al., 2003; Pasquini et al., 2008). Recently, d-ROMs and BAP were reported to be reliable parameters for determining the oxidative stress condition of fish (Hossain et al., 2016ab). Fish with higher d-ROM values indicate that they are under more oxidative stress conditions. On the other hand, fish with higher BAP values indicate stronger tolerance against oxidation. As shown in Fig. 4.3.1, it is interesting to note that diet groups AMP-0.2 and AMP-0.4 was located in zone A, which represents the best condition with low oxidative stress and high antioxidant levels, while the other diet groups located in zone D is considered as stressed condition of fish. Similarly, Hossain et al. (2016ab) also illustrated that dietary supplementations of inosine and nucleotide mixtures stimulated the oxidative status of red sea bream. To date, there remains a lack of explanation about how these supplements work to affect these parameters, so further study is needed.

In the present study, among whole-body proximate composition and biometric indices whole body lipid and condition factor (CF) were significantly influenced by dietary AMP supplementation. Similarly, Hossain et al. (2016a) and Li et al. (2005) observed significantly increased whole body lipid content of red sea bream and red drum, respectively with the dietary nucleotide supplementations. Research on the effect of exogenous nucleotides and nucleosides on lipid metabolism of fishes is very limited, although it is known that dietary nucleotides can influence levels of various lipids and/or fatty acids in certain tissues, such as erythrocytes, plasma, liver or brain (Carver and Walker, 1995: Sato et al., 1995). Our observation on whole-body lipid of red sea bream is likely the first report on the response of fish supplemented with dietary AMP. Information on changes in various lipids in tissues as

well as HSI data in response to dietary nucleotides and nucleoside associated with physiological consequences may be important; therefore, further investigation is warranted. Significant alternation of CF in the present study also reflects the improvement in body weight together with body length of fish fed AMP supplemented diets when compared with supplementation-free control diet.

Innate immune system in fishes plays a key role in preservation of fish against infectious diseases (Trichet, 2010). Lysozyme, being an enzyme with antimicrobial activity, can split peptidoglycan in bacterial cell walls especially of the Gram positive species and can cause lysis of the cells (Chipman and Sharon, 1969). In the present study, red sea bream fed diets supplemented with AMP showed increased LA. Similar increase LA with fish fed nucleoside and nucleotide supplemented diet were also reported previously in common carp (*Cyprinus carpio*) (Sakai et al., 2001), rainbow trout (Tahmasebi-Kohyani et al., 2011), Japanese flounder (Song et al., 2012) and red sea bream (Hossain et al., 2016a,b). BA is one of the most important factors in host resistance against pathogenic bacteria. Our results revealed that, supplementations of AMP increased the BA and it was significantly higher in diet group AMP-0.2 which is not differed significantly with diet group AMP-0.4. Similarly, Hossain et al. (2016a,b) reported that dietary nucleotide, nucleoside supplementations significantly increased the BA of red sea bream. Agglutination antibody titer against different bacteria is another mechanism of innate immune response which has high activity in fish Oriol Sunyer and Tort (1995). Fish fed AMP supplemented diet groups showed higher agglutination antibody titer compared with supplementation-free control diet group. PA and NBT activity in the present study also significantly increased by dietary AMP supplementation and it was highest in diet group AMP-0.2 and AMP-0.8, respectively. Similar increased PA and NBT was also observed by Song et al. (2012) in Japanese olive flounder fed diet supplemented with IMP. Our previous studies on red sea bream fed diets

with supplemented nucleotides (inosine, IMP, mixed nucleotides) also showed increased PA which are in agreement with the present study. Proteins are the most important compounds in the serum and are essential for sustaining a healthy immune system (Kumar et al., 2005). In the present study supplementation of AMP significantly increased TSP compared to the control. Increased TSP due to nucleotide supplementation were also reported in *Catla catla* (Jha et al., 2007), red sea bream (*P. major*) (Hossain et al., 2016a,b) and rainbow trout (Tahmasebi Kohyani et al. 2012). These findings are in agreement with the present study. Overall, all the non specific immune response parameters measured in the present study showed improved value in AMP supplemented diet groups compared to non supplemented control group confirming the benefits of AMP nucleotide for the non-specific innate immunity of red sea bream.

Increased CAT activity rates were attributed to elevated levels of exogenous hydrogen peroxide (H_2O_2) which is the main cellular precursor of the hydroxyl radical (HO^-), a highly reactive and toxic form of ROS (Hossain et al., 2016a,b). In human increased serum CAT activity is used as a diagnostic tool in acute pancreatitis (Goth, 1982), hemolytic disease (Goth, 1983) and some liver diseases (Goth, 1987). The high mean CAT activity observed in fish fed control diet compared to AMP supplemented diets in the present study implies that fish in control group were in stronger oxidative stress conditions compared to supplemented groups. Similar observation was also reported for red sea bream (Hossain et al., 2016a,b) where nucleotide supplementation reduces the CAT activity of red sea bream. Results of the CAT activity as well as the BAP and d-ROMs measured in the present study reconfirmed the positive supplemental effects of AMP associated with the conditions on oxidative stress and immunity.

The WG and LA activity are often used to estimate the optimum supplementation level of nutrient components in fish (Zhou et al., 2013; Wen et al., 2015). Based on the broken line

regression analysis of % WG and LA, the present study illustrated that the optimal levels of dietary AMP for juvenile red sea bream might be 0.16% and 0.41 % respectively. Results of the present study regarding optimum AMP supplementation level indicated that, in red sea bream diets optimum dietary AMP supplementation for enhance growth is lower (0.16%) than that for enhanced immune responses (0.41%). Similarly, Hossain et al. (2016a) reported that optimum inosine and IMP supplementation level based on growth performances and immunity and intestinal health 0.4% for red sea bream; while Song et al. (2012) suggested a supplementation of 0.1–0.4% IMP could enhance innate immunity and disease resistance of olive flounder.

Therefore, the present study demonstrated that the dietary supplementation of AMP positively influences the growth performances, feed utilization, innate immune responses, hematological parameters and oxidative stress response of fish. Based on the present experimental condition, it can be concluded that the optimal levels of dietary AMP were 0.16 and 0.41 %, respectively, for juvenile red sea bream, which is also in line with the most of the growth performance and health parameters of the fish.

Part-4

Effects of dietary supplementation of guanosine monophosphate on the growth, digestibility, innate immune responses and stress resistance of juvenile red sea bream, *Pagrus major*

Abstract

This experiment was conducted to investigate the effects of guanosine monophosphate (GMP) on growth, digestibility, innate immune responses and stress resistance of juvenile red sea bream, *Pagrus major*. A semi-purified basal diet supplemented with 0% (Control), 0.1% (GMP-0.1), 0.2% (GMP-0.2), 0.4% (GMP-0.4) and 0.8% (GMP-0.8) purified GMP to formulate five experimental diets. Each diet was randomly allocated to triplicate groups of fish (mean initial weight 3.4g) for 56 days. The obtained results clearly indicated that, growth performace of red sea bream postively influenced by dietary GMP supplementations and significaly higher final weight was found in fish fed diet group GMP-0.4. Specific growth rate (SGR) and pecent weight gain (%WG) also significantly higher in diet group GMP-0.4 and it was not differed ($P>0.05$) with diet group GMP-0.4 and GMP-0.8. Feed intake significantly increased with the supplementation of GMP. Feed conversion efficiency (FCE) and protein efficiency ratio (PER) also improved ($P<0.05$) when fish fed the diets containing GMP and it was significantly highest in diet group GMP-0.4. The Apparent digestibility coefficients (dry matter, protein and lipid) also improved by GMP supplementation and significantly highest protein digestibility observed in fish fed diet groups GMP-0.2, GMP-0.4 and GMP-0.8. The capabilities of enhancing immune response of fish fed GMP supplemented diet were detected by non specific immune parameters measured in this study, among these parameters paroxidase activity (PA), respiratory burst activity (NBT), Bactericidal activity (BA) were significantly affected and highest valaue obtained in GMP-0.4 diet groups. Serum lysozyme activity (LA), toal serum protein and agglutination antibody titer also increased by GMP supplementation but not at a significant level. In contrast, catalase activity decreased with GMP supplementations. In terms of oxidative stress GMP-0.2 showed best condition. Simulteneousloy, diet groups GMP-0.4 and GMP-0.2 and GMP-0.8 were also in acceptable condition. Moreover, the fish fed GMP supplemented diets

had better improvement ($P<0.05$) in body protein contents, hepatosomatic index, hematocrit content and glutamyl oxaloacetic transaminase (GOT) and glutamic-pyruvate transaminase (GPT) level than the control group. Supplementations also improved ($P<0.05$) freshwater stress resistances. Quadratic regression analysis of WG and LA reaveled that, the optimal levels of dietary GMP were 0.45 and 0.48 %, respectively, for juvenile red sea bream, which is also in line with the most of the growth performance and health parameters of the fish.

Keywords: Guanosine monophosphate, Growth, Digestibility, Innate immune responses, Oxidative stress, *Pagrus major*

1. Introduction

Over the last several decades, the world aquaculture has become intensified to meet up the increasing demand of fish. The downside of intensification of the farming operations has been economic losses, primarily due to infectious diseases, particularly during the early production stages (Kiron, 2012). An increase in the susceptibility of fish to disease is a direct consequence of immunosuppression induced by several factors pertaining to the rearing methods and environmental variations (Burrells et al. 2001b). Many studies have looked into the modulation of the immune system in fish as a means to prevent disease outbreaks and the possibility of altering nutrition to favor normal growth and enhance fish health (Li and Gatlin, 2004). Immunostimulants comprise a group of biological or synthetic compounds that activate non-specific defense mechanisms. In recent years, nucleotides received heightened attention as potential immunomodulators.

Nucleotides comprise a group of biomolecules that have diverse essential physiological and biochemical functions including encoding genetic information, mediating energy metabolism, and signal transduction (Carver and Walker 1995). Dietary supplementation of nucleotides has been shown to benefit many mammalian physiological

and nutritional functions (Uauy, 1989; Quan, 1992; Carver, 1994; Haskó et al., 2000). In aquatic animals both nucleotides and nucleosides have long been implicated as feed attractants in both vertebrate and invertebrate species (Mackie and Adron, 1978; Carr and Thompson, 1983; Person-Le Ruyet et al., 1983; Carr et al., 1984). However, world-wide heightened attention on nucleotide supplementation into potential growth and health benefits in aquaculture species instigated after 2000s. To date, research pertaining to nucleotide nutrition in fishes has shown rather consistent and encouraging beneficial results in fish health management. Research to date on dietary nucleotides has focused on a mixture of nucleotides, rather than specific types of nucleotides except inosine and IMP for the research on feed stimulants (Hossain et al. 2016a; Li and Gatlin, 2006). Guanosine monophosphate (GMP) is a purine nucleotide which consists of the phosphate group, the pentose sugar ribose, and the nucleobase guanine; hence it is a ribonucleoside monophosphate and used as a monomer of RNA. In aquaculture GMP has been studied mostly as a part of mixed nucleotide rather than considering as individual nucleotide for promoting growth, feed utilization and potential health benefit. Ikeda et al. (1991) initially reported the feeding stimulatory properties of GMP for jack mackerel. Lin et al. (2009) reported increased growth performances and some non specific immune parameters like, head kidney leucocyte superoxide anion (O2) production ratio and plasma total immunoglobulin (Ig) concentration of red drum (*Epinephelus malabaricus)*.

Red sea bream is one of the most commercially important marine fish species grown in Japan, China and Korea. Due to their desirable taste, hardiness in a crowded environment and rapid growth has made red sea bream as good candidates for intensive aquaculture. However, intensive aquaculture of this species often exposed to stressful conditions which is immunosuppressive to fish. Investigation on quantitative nutritional requirements of red sea bream for some macro and micronutrients (Forster & Ogata 1998; Ren *et al*. 2008; Kader &

Koshio 2012; Rahimnejad & Lee 2013, 2014) and development of alternative protein based diet (Kader *et al*. 2010, 2012; Kader & Koshio 2012) have been defined for this fish. However, research on dietary modulation for potential growth and health management of this species is very scarce. Recently, we evaluated the functional effects of inosine monophosphate (IMP), inosine and mixed nucleotide on this species (Hossain et al., 2016a,b). However, to the best of our knowledge there is no research on individual nucleotide GMP as feeding stimulant as well as a functional nutrient for potential growth and health benefit of red sea bream Therefore, the present study was conducted to assess the effects of dietary supplementation of GMP on the growth, digestibility, immune responses and stress resistance of juvenile red sea bream, *Pagrus major*

2. Materials and Methods

2.1. Test fish and experimental system

Juvenile red sea bream were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (54% crude protein and 10% crude lipid, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 100 l polycarbonate tanks (filled with 80 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 4.4.1and 4.4.2 summarize the basal diet formulation and chemical composition of the experimental diets respectively. Five fishmeal and casein based semi-purified diets were formulated to be nearly isonitrogenous (54% crude protein), isolipidic (11.4% crude lipid) and isocaloric (22.1 KJ g^{-1} gross energy). The experiential diets were prepared by supplementing purified GMP nucleotide as disodium salts (Sigma Aldrich Co., St. Louis, MO, USA) to the basal diet at concentrations of 0.1, 0.2, 0.4 and 0.8 % for diet group GMP-0.1, GMP-0.2, GMP-0.4 and GMP-0.8 respectively. Basal diet without GMP supplementation was used as control (GMP-0). For proper mixing of GMP with other ingredients, initially GMP and weighted arginine of the respective diets were mixed properly in a glass beaker by hands with the help of a stainless laboratory spatula. Then these mixtures were thoroughly mixed with other dry ingredients in a food mixer for 10 min. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (35–40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 55 °C for about 150 min. The test diets were stored at −28 °C in a freezer until use.

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Eighteen fish, having a mean initial body weight of approximately 3.4 g were randomly allocated to

Table 4.4.1: Basal diet formulation of juvenile red sea bream.

Ingredients	Composition (%)
Fishmeal[a]	20
Casein[b]	32
Pollack liver oil[c]	7
Soybean lecithin[d]	3
Vitamin mixture[e]	3
Mineral mixture[f]	3
L-arginine[g]	1.5
Stay-C[h]	0.08
Dextrin	7
α-starch	6
Activated gluten[i]	5
α- cellulose	12.42
GMP[j]	0-0.8

[a]Nippon Suisan Co. Ltd., Tokyo, Japan

[b]Waco Pure Chemical Industries Inc. (Osaka, Japan)

[c] Riken Vitamin, Tokyo, Japan.

[d] Kanto Chemical Co., Inc. (Tokyo, Japan)

[e] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[f]Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[g] Nacalai Tesque, Kyoto, Japan.

[h] Stay-C 35.

[i]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS".

[j] Sigma Aldrich Co., St. Louis, MO, USA. (AMP added to diets at the expense of α-cellulose).

previously prepare fifteen tanks. Fish were fed the experimental diets for 56 days by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 1 hour after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk fortnightly to determine growth and visually check their health condition. The monitored water quality parameters were: water temperature 23.8±1.9 °C; pH 8.1±0.7 and salinity 34.5±0.5 during the feeding trial. These ranges are considered within optimal values for juvenile red sea bream.

2.4. Sample collection

At the end of the feeding trial, all experimental fish were fasted for 24 h. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then individual body weight of fish was measured, and the growth parameters were calculated accordingly. Nine fish per treatment (three fish per each tank) were randomly collected, washed with distilled water and then stored at −80 °C (MDF-U383, SANYO, Tokyo, Japan) for the whole body proximate analysis. Six fish were randomly sampled from each dietary replicate tank and their blood were collected by puncture of the caudal vain using heparinized (1600 IU/ml, Nacalai Tesque, Kyoto, Japan) disposable syringes and pooled. In addition non-heparinized disposable syringes were used to collect blood for serum analysis. Partial heparinized whole blood was used to analyze the hematocrit levels while plasma and serum were obtained by centrifugation at 3000 ×g for 15 min under 4 °C, and then stored at −80 °C until the analysis. Liver was dissected out from the fish above, weight individually to calculate the hepatosomatic index (HSI).

2.5. Biochemical analysis

The ingredients, diets and fish whole body were analyzed in triplicate, using standard methods (AOAC, 1995). The moisture was determined by drying the sample at 105 °C to constant weight. The ash was analyzed by combustion at 550 °C for 12 h. The crude protein content was determined by measuring the nitrogen content (N × 6.25) using the Kjeldahl method with a Tecator Kjeltec System (1007 Digestion System, 1002 Distilling unit, and Titration unit; FOSS Tecator AB, Högendäs, Sweden). Crude lipid content was estimated using gravimetric method (954.02). Total serum protein and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma samples was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg.

Table 4.4.2: Proximate composition of the experimental diets (% dry matter basis).

Parameters	Diet groups				
	Control	GMP-0.1	GMP-0.2	GMP-0.4	GMP-0.8
Moisture	7.9	8.6	7.5	7.4	6.8
Crude protein	54.4	54.3	55.2	55.0	55.1
Crude lipid	11.4	11.6	11.3	11.4	11.4
Ash	6.5	6.5	6.5	6.7	6.8
Gross energy (KJg^{-1})*	22.1	22.1	22.1	22.1	22.1

*Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ kg^{-1}, respectively.

Carbohydrate was calculated by the difference: 100-(protein + lipid + ash).

2.6. Evaluation of non specific immune parameters

Serum lysozyme activity (LA) was measured with turbidimetric assays (Lygren et al., 1999). Ten micro-liters of samples was put into well of microplate, then added 190 micro-liters of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4,their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

Oxidative radical production by phagocytes during respiratory burst was measured through the NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995) with some modifications. Briefly, blood and 0.2% NBT (Nacalai Tesque, Kyoto, Japan) were mixed in equal proportion (1:1), incubated for 30 min at room temperature. Then 50 mL was taken out and dispensed into glass tubes. One milliliter of dimethylformamide (Sigma) was added and centrifuged at 2000 × g for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Hitachi U-1000, Japan). Dimethylformamide was used as the blank.

Serum bactericidal activity (BA) was performed according to Yamamoto and Iida (1995). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g/ml, *Escherichia coli*, IAM1239 cell line, Kagoshima, Japan) (Promega, Germany) and incubated at 25 °C for 24 h in micro tube rotator (MTR-103, AS ONE, Osaka, Japan). 50 ml of the reaction solutions were incubated on TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) at 25 °C for 24 h (on X-plate). Colony forming unit (CFU) was counted by the plate counting method as described by Ren et al. (2008). The bactericidal activity was defined as follows, (CFU of blank group-CFU of each group)/CFU of blank group × 100.

The total peroxidase activity (PA) in serum was measured according to Salinas et al. (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

The catalase activity (CAT) assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate that absorbs at 405 nm (Goth, 1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol/l phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate ($(NH_4)_6$ Mo_7O_{24} .4 H_2O) and the yellow complex of molybdate and hydrogen peroxide is measured at 405 nm against reagent blank. One unit CAT decomposes 1 µmole of hydrogen peroxide/l minute under assay conditions. CAT activities are expressed as kilo unit per liter (kU/l)

The agglutination antibody titer was conducted in round bottomed 'U' shaped micro titer plates after Swain et al. (2007) with slight modifications. 50 µl of serum was serially diluted in PBS (1/2, 1/4, 1/8, 1/16, 1/32and 1/64) and then equal volumes of *Vibrio anguillarum* (1×10^8 cfu ml^{-1}) was added to wells and kept for 24 h at 4 ^{0}C. The reciprocal of the highest dilution that gave agglutination was taken as the agglutination antibody titer which is expressed as log10.

2.7. Digestibility assessment

A digestibility trial was conducted at the end of the growth trial; the remaining fish from the same treatments were distributed randomly into triplicate tanks. The fish were fed a diet containing chromium oxide (Wako Pure Chemical Industries, Ltd) as the inert marker at a level of 0.5% (Cr_2O_3, 5 g kg^{-1}) to the previous formulation and fed to the fish under the same condition as the growth experiment apart from the difference in stocking density. After 5-day adaptation of the diets, feces were collected at 3-h intervals between the morning and afternoon feeds by using a siphon for two weeks. Feces of red sea bream very rapidly settled to the bottom of the tank and did not easily break up in the water so that nutrient and marker losses were minimized. Sufficient amount of feces were collected, freeze dried and immediately kept at - 20^{o}C until analysis. Concentration of chromium oxide in diets and feces was determined according to Furukawa and Tsukahara (1966).

2.8. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, five fish from each rearing tank (total 15 fish per treatment) were randomly selected and transferred in to a blue colored 20 L rectangular glass aquarium with 15 L tap water which was aerated for 24 h. The glass aquaria for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of red sea bream juvenile was calculated according to Moe et al. (2004) as follows: time to death (min) of a juvenile was converted to log10 values. When the juvenile were transferred to the low salinity water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was log10 100 = 2). The calculation was then conducted every 5 minutes after the first exposure. The values of

log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

Y= a*X*+ b where Y = log10 (survival), *X* = time for individual juvenile death (min).

LT_{50} (*X*) was obtained when Y = 1.7 since log10 (50) = 1.7. Each value obtained from the equation above was compared statistically.

2.9. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR, % day^{-1}) = {Ln (final weight) – Ln(initial weight)/duration}× 100

Survival (%) = 100 × (final no. of fish / initial no. of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed conversion ratio (FCR) = dry feed intake (g)/ live weight gain (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Condition factor (CF, %) = weight of fish / $(\text{length of fish})^3$ × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

Viscerasomatic index (VSI, %)= viscera weight/fish weight × 100

2.10. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Turkey Kramer test. Parameters with significant differences were subjected to quadratic regression analysis with dietary GMP supplementation level.

3. Results

3.1 Growth performance and feed utilization

Growth performance, feed utilization and survival of red sea bream fed experimental diets are presented in Table 4.4.2. In general, the growth performance and feed utilization of fish improved when fed the diets containing GMP although the values were not always statistically significant. The highest values of final body weight were obtained in fish fed a diet group GMP-0.4 followed by diet groups GMP-0.2 and GMP-0.8. Fish fed diets supplemented with ≤0.1% supplemented GMP showed significantly lowest values. Percent weight gain (%WG) and specific growth rate (SGR) also significantly highest in diet group GMP-0.4. But the values were not significantly different from those of fish fed diet groups GMP-0.2 and GMP-0.8 ($P<0.05$). Feed conversion efficiency (FCE) and protein efficiency ratio (PER) of fish improved when fed the diets containing GMP and it was significantly highest in diet group GMP-0.4. Feed intake significantly increased with the supplementation of GMP. The poorest growth performance and feed utilization were obtained in GMP supplementation-free control diet group. All treatments showed relatively high survival rates between 91.7 % and 96.7% and there were no significant differences among all groups ($P>0.05$). Quadratic regression analysis showed that FW, WG, SGR, FI, FCR and PER were quadratic responses to the increasing dietary GMP levels ($Y_{FW} = -23.767x^2 + 23.158x + 25.061$, $R = 0.9257$; $Y_{WG} = -792.25x^2 + 761.58x + 641.43$, $R = 0.9252$; $Y_{SGR} = -1.6824x^2 + 1.638x + 3.5786$, $R = 0.9288$; $Y_{FI} = -12.216x^2 + 10.913x + 21.043$, $R = 0.5212$; $Y_{FCE} = -0.9057x^2 + 0.8922x + 0.9943$, $R = 0.9636$; $Y_{PER} = -1.6873x^2 + 1.5921x + 1.8152$, $R = 0.9793$).

Table 4.4.3: Growth performance and feed utilization parameters of red sea bream fed test diets for 56 days*.

Parameters	Diet groups				
	Control	GMP-0.1	GMP-0.2	GMP-0.4	GMP-0.8
IBW	3.38±0.01	3.35±0.02	3.35±0.01	3.37±0.03	3.37±0.02
FBW	25.56±0.48[a]	26.18±1.36[a]	29.09±0.85[ab]	30.68±0.57[b]	28.33±0.02[ab]
WG%	656.9±15.2[a]	678.5±41.7[ab]	775.3±23.4[bc]	823.0±14.6[c]	742.4±2.6[abc]
SGR	3.61±0.04[a]	3.66±0.09[ab]	3.87±0.05[bc]	3.97±0.03[c]	3.81±0.01[abc]
FI	20.14±0.37[a]	23.34±0.2 [b]	22.82±0.7[b]	22.81±0.0[b]	22.09±0.4[b]
FCE	0.98±0.05[a]	1.10±0.04[ab]	1.13±0.05[ab]	1.20±0.03[b]	1.13±0.02[ab]
PER	1.80±0.1[a]	1.99±0.06[ab]	2.05±0.09[ab]	2.18±0.05[b]	2.01±0.03[ab]
Sur	93.33±1.67	91.67±1.7	95.0±2.9	95.0±0.0	96.67±1.67

[a] IBW: initial body weight (g).

[b] FBW: final body weight (g).

[c] WG: percent weight gain (%).

[d] SGR: specific growth rate (% day^{-1}).

[e] FI: feed intake (g $fish^{-1}$ 50 $days^{-1}$).

[f] FCR: feed conversion ratio.

[g] PER: protein efficiency ratio.

[h] Sur: survival (%).

[*] Values are means of triplicate groups±S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

3.2. Hematological parameters

Table 4.4.5 represents the blood parameters of juvenile red sea bream after 56 days of feeding trial. Overall, dietary treatments had no significant effect on blood chemical parameters of fish among different treatments except those of GOT and GPT. Significantly lowest GOT value obtained in diet group GMP-0.4; meanwhile, other GMP supplemented groups showed intermediate value. GPT value was significantly lowest in diet group GMP-0.8 and not differed ($P>0.05$) with diet groups GMP-0.2 and GMP-0.4. Supplementation free control group showed significantly higher GOT and GPT values. Supplementation of GMP also reduced ($P>0.05$) plasma glucose and total bilurubin values compared to control. Fish fed GMP supplemented diet showed increased hematocrit content and it was significantly highest in diet group GMP-0.2. But the values were not significantly different from those of fish fed diet groups GMP-0.4 and GMP-0.8. Meanwhile, GOT, GPT and hematocrit level showed quadratic response with graded levels of GMP ($Y_{GOT} = 129.04x^2 - 153.39x + 72.018$, $R^2 = 0.788$; $Y_{GPT} = 180.32x^2 - 224.32x + 75.84$, $R^2 = 0.8269$; $Y_{Haematocrit} = = -28.213x^2 + 30.926x + 33.378$, $R^2 = 0.799$).

3.3. Oxidative stress parameters

Fig. 4.4.1 indicates the status of oxidative stress conditions, in which the conditions of both stress intensity and tolerance ability were combined, of fish fed with test diets for 56 days. Dietary supplementation had no significant effect on d-ROMs and BAP of red sea bream (no data illustrated here). Combined effects of d-ROM and BAP showed that fish fed diet groups GMP-0.2 was located in zone A which was categorized as lower intensity of oxidative stress and higher tolerance ability against oxidative stress. Meanwhile, diet groups GMP-0.4 and GMP-0.8 located in zone B and C respectively, considered acceptable condition. Fish fed diet

Table 4.4.4: Blood parameters of juvenile red sea bream fed test diets for 56days.[*]

Parameters	Diet groups				
	Control	GMP-0.1	GMP-0.2	GMP -0.4	GMP -0.8
Hematocrit (%)	33.0±1.0[a]	35.3±1.2[ab]	41.0±1.2[c]	39.7±0.7[bc]	40.3±1.2[c]
Glucose (mg/dl)	63.7±11.3	61.3±9.8	53.3±3.9	46.0±1.7	44.0±8.2
T-Cho (mg/dl)[a]	149.3±5.9	147.0±8.5	167.3±16.8	169.7±13.3	155.0±3.6
BUN (mg/dl)[b]	5.33±0.3	8.0±0.0	7.67±0.7	8.0±0.6	8.0±1.2
T-Bill (mg/dl)[c]	0.23±0.03	0.23±0.03	<0.2	<0.2	<0.2
GOT (IU/l)[d]	78.0±2.0[b]	43.6±5.6[ab]	55.7±13.4[ab]	30.7±7.3[a]	31.7±9.1[ab]
GPT(IU/L)[e]	78.3±7.0[c]	63.0±11.4[bc]	18.0±4.2[a]	26.7±13.2[ab]	10.0±0.0[a]
TG (mg/dl)[f]	135.0±35.5	319.7±78.8	256.7±86.7	262.3±81.2	223.0±30.1

[a] T-Cho: total cholesterol.

[b] BUN: blood urea nitrogen.

[c]T- Bill: Total bilirubin

[d]GOT: glutamyl oxaloacetic transaminase.

[e]GPT: glutamic pyruvate transaminase.

[f]TG: triglyceride.

[*]Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

groups (control and GMP-0.1) located in zone D which were categorized as higher intensity of oxidative stress and lower tolerance ability against oxidative stress (Fig.4.4.1).

3.4. Freshwater stress tolerance

The results of lethal stress test against fresh water shock on LT_{50} obtained by regression analysis are shown in Fig. 4.4.2. Supplementation of GMP improved the freshwater stress tolerance of red sea bream and significantly highest LT_{50} obtained (38.69 min) in fish fed diet group GMP-0.4. Other GMP supplemented groups showed intermediate values. In contrast, GMP supplementation free control diet group showed significantly lowest LT_{50} value. Meanwhile, freshwater stress tolerance (LT_{50}) also showed quadratic responses to the increasing dietary GMP levels $Y_{LT50} = -32.752x^2 + 32.295x + 30.947$; $R^2 = 0.9869$.

3.5 Immune responses

Innate immune responses were positively affected by dietary GMP supplementation (Table 4.4.5). When compare with control GMP supplemented diet groups showed significant enhancement of BA, PA and NBT and it was highest in fish fed diet group GMP-0.4. Total serum protein (TSP), LA and bacterial agglutination antibody titer were also increased numerically with GMP supplementation but not a significant level. Moreover, dietary supplementation also significantly reduced CAT activity in GMP supplemented diet groups and it was lowest in diet group GMP-0.2. Overall, significantly reduced non specific response observed in GMP supplementation free control group. Meanwhile, non specific immune responses (BA, PA, LA, NBT and CAT) showed quadratic response with graded levels of GMP ($Y_{PA} = -1.5285x^2 + 1.481x + 1.6035$, $R^2 = 0.9934$; $Y_{NBT} = -0.3697x^2 + 0.33x + 0.3698$, $R^2 = 0.6647$; $Y_{CAT} = 178.74x^2 - 168.31x + 77.381$, $R^2 = 0.684$; $Y_{LA} = -565.94x^2 + 503.78x + 94.977$, $R^2 = 0.9719$). $Y_{BA} = -96.365x^2 + 103.58x + 54.229$; $R = 0.8995$

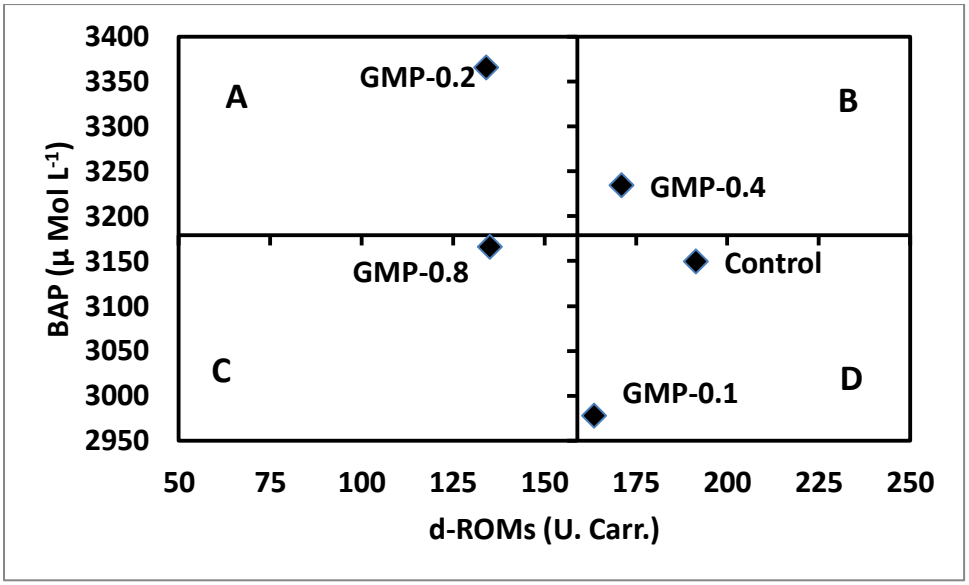


Fig. 4.4.1. Oxidative stress parameters in red sea bream (*P. major*) fed test diets for 56 days. (Values are means of triplicate groups. The abbreviations of experimental treatments are illustrated in the text. Central axis based on mean values of d-ROM and BAP from each treatment.) Zone A: High antioxidant potential and low reactive oxygen metabolites (good condition). Zone B: High antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone C: Low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone D: Low antioxidant potential and high reactive oxygen metabolites (stressed condition).

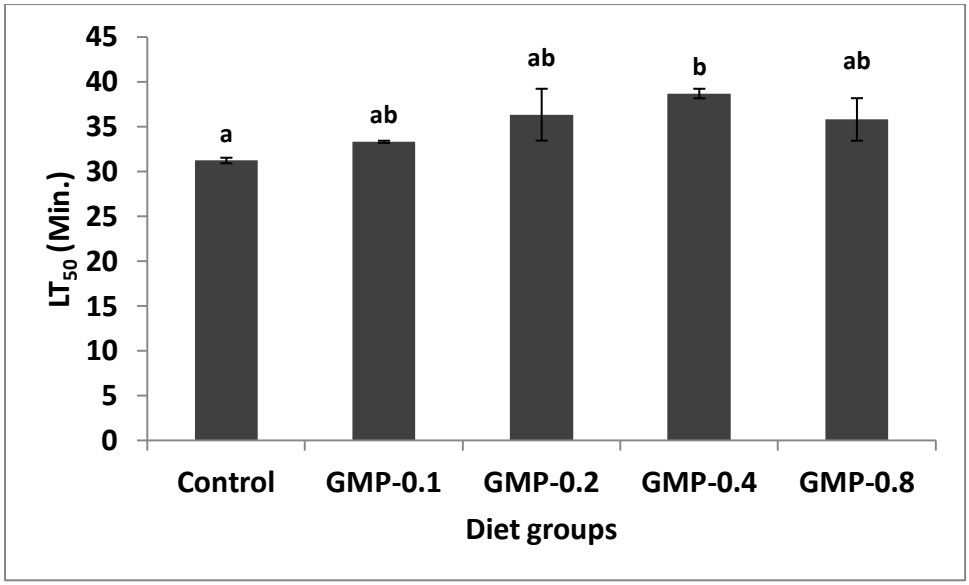


Fig. 4.4.2. LT_{50} (min) calculated from the lethal time of red sea bream exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from triplicate groups. The abbreviations of experimental diets are illustrated in the text. Values with different letters are significantly different (P<0.05).

Table 4.4.5: Non-specific immune response of juvenile red sea bream fed test diet for 50 days.*

Parameters	Diet groups				
	Control	GMP-0.1	GMP-0.2	GMP-0.4	GMP-0.8
T-Pro (g/dl)[a]	2.90±0.1	2.95±0.05	3.10±0.2	3.05±0.5	3.75±0.35
LA (unit/mL)[b]	87.5±37.5	150.0±50.0	175.0±14.4	200.0±25.0	137.5± 62.5
PA[c]	1.61±0.05[a]	1.72±0.02[ab]	1.85±0.02[bc]	1.95±0.1[c]	1.81± 0.02[bc]
BA (%)[d]	54.4±4.3[a]	66.5±4.0[ab]	65.6±4.4[ab]	83.1±4.3[b]	75.0±6.3[ab]
NBT[e]	0.35±0.02[a]	0.43±0.02[b]	0.42±0.02[b]	0.43±0.02[b]	0.40± 0.0[ab]
CAT[f]	87.3±10.4[b]	51.26±1.7[a]	43.8±4.8[a]	48.8±1.8[a]	55.2± 10.2[ab]
B-Agglu[g]	0.70±0.1	0.80±0.1	0.90±0.2	1.0±0.1	1.0± 0.1

[a]TSP: Total serum protein

[b]LA: lysozyme activity

[c]PA: Peroxidase activity (measured at OD of 450nm).

[d]BA: Bactericidal activity=100× (CFU of blank group-CFU of each group)/CFU of blank group.

[e]NBT: Nitro-blue-tetrazolium activity

[f]CAT: Catalase activity

[g]B-Agglu: Bacterial agglutination antibody titer

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

3.7. Whole body proximate analysis and biometric indices

The initial and final whole body proximate compositions of juvenile red sea bream are shown in Table 4.4.6. All the fish showed a change in the analyzed parameters compared to those of the initial values. In comparison with the control group, dietary treatments had no significant influences on the whole body proximate composition and biometric indices except those of whole body crude protein and hepatosomatic index (HSI). Fish fed GMP supplemented diet showed significant increase of whole body protein content and it was highest in diet group GMP-0.1. On the other hand supplementation-free control group showed significantly lowest value. GMP supplementation increased HSI ($P<0.05$) and it was highest in GMP-0.2 diet group. Regression analysis of whole body protein and HSI also showed quadratic response with graded level of GMP ($Y_{\text{whole body protein}} = -2.7171x^2 + 1.6166x + 13.797$, $R = 0.3151$; $Y_{HSI} = -2.567x^2 + 2.2803x + 1.0943$, $R = 0.6836$).

3.8. *Digestibility coefficients*

The apparent digestibility coefficient (ADC) of dry matter (ADC DM), protein (ADC Protein) and lipid (ADC Lipid) is presented in Table 4.4.7. $ADC_{protein}$ significantly increased the by the dietary GMP supplementation and it was highest ($P<0.05$) in fish fed diet group GMP-0.4. ADC_{DM} and ADC_{lipid} also increased with GMP supplementation but not a significantly level. However, GMP supplementation free control group showed lowest values of ADC_{DM}, $ADC_{Protein}$ and ADC_{Lipid} (Table 6). Meanwhile, regression analysis of $ADC_{Protein}$ also showed quadratic response with graded level of GMP ($Y_{ADC\ protein} = -4.6886x^2 + 5.2256x + 91.839$, $R = 0.9237$).

Table 4.4.6: Whole body proximate analysis (% wet basis) and somatic parameters in juvenile red sea bream fed test diets for 56 days.*

Parameters	Initial[1]	Diet group				
		Control	GMP-0.1	GMP-0.2	GMP -0.4	GMP -0.8
Moisture	83.6	76.2±0.4	74.9±0.2	74.6±0.4	74.9±0.9	75.3±0.3
Crude protein	12.1	13.4±0.1[a]	14.6±0.6[b]	13.9±0.2[ab]	13.8±0.1[ab]	13.4±0.1[a]
Crude lipid	0.7	5.4±0.2	5.8±0.1	6.5±0.2	6.3±0.4	6.0±0.4
Crude ash	3.4	3.5±0.04	3.6±0.1	3.7±0.1	3.6±0.1	3.7±0.1
CF[2]	-	1.97±0.05	2.05±0.06	1.96±0.03	2.07±0.02	1.93±0.02
HSI[3]	-	0.96±0.2[a]	1.46±0.1[b]	1.52±0.1[b]	1.47±0.1[b]	1.30±0.0[ab]
VSI[4]	-	7.14±0.35	7.32±0.04	7.29±0.23	7.15±0.22	6.67±0.23

[1]Initial values are not included in the statistical analysis.

[2]CF (condition factor)=100xfish weight/(fish length)^3

[3]HSI (hepatosomatic index)=100xliver weight/fish weight

[4]VSI (viscerasomatic index)=100xviscera weight/fish weight

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05). Crude protein, crude lipid and ash are expressed on a wet weight basis.

Table 4.4.7: Digestibility of juvenile red sea bream fed different experimental diets for 56 days*.

Parameters	Diet groups				
	Control	GMP-0.1	GMP-0.2	GMP-0.4	GMP-0.8
ADC_{DM}[a]	76.53±0.6	79.61±1.8	79.6±1.4	80.4±1.2	80.93±0.3
$ADC_{protein}$ (%)[b]	91.82±0.3[a]	92.20±0.11[ab]	92.95±0.2[b]	93.04±0.12[b]	93.04±0.1[b]
ADC_{lipid} (%)[c]	91.97±0.6	91.46±1.0	92.93±0.2	93.12±0.4	93.31±0.2

[a] ADC_{DM} (Apparent digestibility coefficient of dry matter) = 100 – 100

$$(\frac{\%\,\text{marker in diet}}{\%\,\text{marker in faeces}})$$

[b]$ADC_{Protein}$ (Apparent protein digestibility, %) = 100- 100

$$(\frac{\%\,\text{marker in diet}}{\%\,\text{marker in faeces}} \times \frac{\%\,\text{protein in faeces}}{\%\,\text{protein in diet}})$$

[c]ADC_{Lipid} (Apparent lipid digestibility, %) = 100- 100

$$(\frac{\%\,\text{marker in diet}}{\%\,\text{marker in faeces}} \times \frac{\%\,\text{lipid in faeces}}{\%\,\text{lipid in diet}})$$

*Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

3.7. Quantification of optimum supplementation of GMP nucleotide for P. major.

The optimum supplementation level of GMP for WG and LA were estimated by quadratic regression analysis. Based on WG, the optimal supplemental level of GMP was estimated to be 0.48 % in diet ($y = -792.25x^2 + 761.58x + 641.43$; $R = 0.9252$). On the other hand, the optimal supplemental level of GMP based on LA was estimated to be 0.45% in diet ($Y = -565.94x^2 + 503.78x + 94.977$; $R = 0.9719$)

4. Discussion

Since the first use of nucleotide in aquaculture, a growing number of studies have demonstrated that nucleotides act as an immunomodulators as well as a functional nutrient in fish and shrimp (Hossain et al., 2016 a,b; Song et al., 2012; Cheng et al., 2011), in which mixed nucleotides were commonly used in those studies. To our knowledge, this study was the first study to investigate the effects of GMP as individual nucleotide as well as a potential functional nutrient for red sea bream juveniles.

The present study demonstrated that supplementation of GMP improves the growth performances of red sea bream and significantly highest final body weight (FBW) observed in diet group GMP-0.4. This observation is in agreement with the previous findings of Lin et al. (2009), reported that nucleotide GMP significantly increased the growth performance of grouper (*E. malabaricus*). Similarly the growth enhancing effects of some other individual and mixed nucleotides have also been reported in some previous studies (Hossain et al. 2016a,b; Song et al. 2012; Cheng et al. 2011). So far, there is no exact explanation how dietary nucleotide works to enhance growth. Borda et al. (2003) presumed that an exogenous supply of nucleotides may promote growth of fish and crustaceans in early stages to meet their high rate of cell replication. However, it is assumed that the growth-enhancing effect of GMP in the present study resulted from improved feed intake (Table 4.4.3), promoting more

rapid feed intake that reduced nutrient leaching into the water or possibly playing roles in metabolism (Metailler *et al*., 1983, Hossain et al., 2016a,b). In the present study significantly improved feed intake in GMP supplemented diet groups in comparison to supplementation free control diet group strongly substantiates the previous hypothesis. In contrast, supplementation of GMP after a certain concentration in the present study showed reduced growth performance and feed utilization. Reduced growth performance and feed utilization by high mixed or individual nucleotide levels also reported in some previous studies (Akintonwa et al., 1979; Baker and Molitori, 1974; Adamek et al., 1996, Hossain et al., 2016 a,b). In case of Japanese flounder Song et al. (2012) reported that a high dietary concentration of IMP (1.0%) resulted depressed growth performance compared to lower levels of dietary IMP (0.1 to 0.2%). Adamek et al. (1996) also reported that high dietary nucleotide concentration (5%) caused growth depression in rainbow trout *(Oncorhynchus mykiss)* during a 37 day feeding trial. In our previous studies it was also found that high dietary concentration of mixed nucleotides (≤0.2%), IMP (0.4%) and inosine nucleoside (0.4%) caused reduced growth and feed utilization performances of red sea bream. Similarly, in the present study supplementation of GMP at concentration of 0.8% caused reduced growth and feed utilization performance of juvenile red sea bream.

The enhanced growth performance and feed utilization of fish fed dietary GMP might be also attributed to the enhanced digestibility coefficients. In the present study, the highest growth performances in GMP-0.4 diet group might be partly due to the improved crude protein, lipid and dry matter digestibility coefficients in this group. Similar enhanced digestibility were also reported by Hossain et al. (2015) for juvenile amberjack fed diet supplemented with dietary nucleoside, inoisne. Increased digestibility in GMP supplemented diet groups in the present study might be due to feeding stimulatory properties of GMP. It is well documented

that feeding stimulant increase the secretion of different digestive enzyme (Kofuji et al. (2006). Takii et al., 1986, Takii et al., 1990; Satoh, 2003). These increased secretion of different digestive enzyme due to GMP supplementation might help to improve these digestibility. However, in the present study the secretion of different digestive enzymes were not studied. So, further study is warranted regarding digestive enzyme activity of fish due to nucleotide supplementation to prove this hypothesis.

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish (Hossain et al., 2016a,b). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to those of the previous findings (Aoki et al., 1998; Kader and Koshio, 2012). Dietary GMP supplementation significantly enhanced hematocrit of juvenile red sea bream as a general health response towards nutritional strategies. In the present experiment, hematocrit content was significantly highest in fish fed diet group GMP-0.2 and it was not differed significantly with diet groups GMP-0.4 and GMP-0.8; whereas control group showed significantly lowest value. This indicated that dietary GMP elevated the health status of fish. Similarly, Song et al. (2012) and Hossain et al. (2016a,b) reported the enhanced hematocrit level by the supplementation of nucleotides in Japanese olive flounder and red sea bream diets respectively. Plasma glucose is commonly considered to be one stress indicator in fish, high glucose levels often indicate the higher stress status of fish (Eslamloo et al., 2012). The gradual decrease in blood glucose level ($P>0.05$) in response to GMP supplementation indicated that dietary GMP supplementation induced an optimal physiological condition of the fish. Result of the present study also revealed that, significantly lowest GOT value obtained in diet group GMP-0.4; meanwhile, other GMP supplemented groups showed intermediate value. GPT value was significantly lowest in diet group GMP-0.8 and not differed ($P>0.05$) with diet groups GMP-0.2 and GMP-

0.4. Supplementation free control group showed significantly higher GOT and GPT values. Supplementation of GMP also reduced (P>0.05) plasma glucose and total bilurubin values compared to control. Plasma bilirubin, GOT (or aspartate aminotransferase, AST) and GPT (or alanine aminotransferase, ALT) are often used for the evaluation of liver function as they are released into the blood during injury or damage to the liver cells (Lemaire et al., 1991). Lower values of these parameters in GMP supplemented diet groups indicated that, GMP induced an optimal physiological condition as well as better liver health condition when compared with the supplementation-free control group.

Stress is one of the emerging factors in aquaculture activities, which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization (Hossain et al., 2016a,b). Dietary nucleotide increases the resistance of fish to a variety of stressors is well documented (Burrells et al., 2001b; Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2012). In our study, fish fed GMP supplemented diet showed increase in stress resistance of red sea bream after the exposure of low salinity stress test. In the present study highest LT_{50} value obtained in diet group GMP-0.4 (38.16 min) on the other hand supplementation free control group showed the lowest value of LT_{50} (31.21 min) indicated a lower fresh water tolerance of the red sea bream. Increased (P>0.05) LT_{50} of red sea bream fed diet group GMP-0.4 indicates healthy status of red sea bream (Yokoyama et al., 2005, Hossain et al., 2016). Oxidative stress is considered to involve in plenty of diseases and pathological status in fish (Martinez-Alvarez et al., 2005), and is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants (Celi et al., 2010). Simultaneous measurements of d-ROMs with BAP can provide a suitable tool for measuring the oxidative stress in humans, pig, rabbit and dog (Oriani et al., 2001; Ballerini et al., 2003; Pasquini et al., 2008). Recently, d-ROMs and BAP were reported to be reliable parameters for determining the oxidative stress condition of fish (Hossain et al.,

2016a, b). Fish with higher d-ROM values indicate that they are under more oxidative stress conditions. On the other hand, fish with higher BAP values indicate stronger tolerance against oxidation. As shown in Fig. 4.4.1, it is interesting to note that diet group GMP-0.2 was located in zone A, which represents the best condition with low oxidative stress and high antioxidant levels, while fish fed diet group GMP-0.4 and GMP-0.8 were located in zone B and C, respectively; both of which zones are considered acceptable in the assessment of fish's health condition. In contrast, GMP-0.1 and supplementation-free control group located in zone D which were categorized as stressed condition due to higher intensity of oxidative stress and lower tolerance ability against oxidative stress. Supplemental effects of dietary nucleotide to reduce oxidative stress also reported previously by Hossain et al. (2016a,b) where mentioned that dietary supplementation of inosine and nucleotide mixtures stimulated the oxidative status of red sea bream. To date, there remains a lack of explanation about how nucleotides work to affect these parameters, so further study is needed.

In the present study, among whole-body proximate composition and Biometric indices whole body crude protein and hepatosomatic index (HSI) were significantly influenced by dietary GMP supplementation. Similarly, Hossain et al. (2016) observed significantly increased whole body crude protein content of red sea bream with the dietary nucleotide (IMP and inosine) supplementation. Significant increase in carcass protein in the present study indicates highly efficient utilization of dietary protein owing to GMP supplementation which is also supported by increased PER value of supplemented group regardless the supplementation group in the present study. The liver size is relative to the nutritional status of the fish (Shoemaker *et al*. 2003; Sridee & Boonanuntanasarn 2012). Significantly increased HIS in the present study with fish fed GMP supplemented diet groups GMP-0.1, GMP-0.2 and GMP-0.4 indicates proper storage of macro and micronutrients and healthy condition of liver as well as clinical healthy signs of fish.

It is well-documented that the nutritional status of fish influences its innate immune system which is considered as an essential component in combating infections thereby modulating the resistance to disease (Kiron, 2012). The results of the present study indicated that dietary supplementation of functional nutrient GMP caused an improvement in the innate immune responses of red sea bream. Proteins are the most important compounds in the serum and are essential for sustaining a healthy immune system (Kumar et al., 2005). In the present study there was a trend of increased total serum proteins (TSP) with the supplementation of GMP compared with control. The increased TSP in GMP supplemented groups indicates the immune-stimulatory competence of the dietary GMP. Similar increased TSP due to nucleotide supplementation were also reported in *Catla catla* (Jha et al. 2007), red sea bream (*P. major*) (Hossin et al., 2016a,b) and rainbow trout (Tahmasebi-Kohyani et al. 2012). Lysozyme, being an enzyme with antimicrobial activity, can split peptidoglycan in bacterial cell walls especially of the Gram positive species and can cause lysis of the cells (Chipman, 1969). In the present study, red sea bream fed diets supplemented with GMP showed increased lysozyme activity. Similar increase lysozyme active with fish fed nucleoside and nucleotide supplemented diets were also reported previously in common carp (*Cyprinus carpio*) (Sakai et al., 2001), rainbow trout (Tahmasebi-Kohyani et al., 2011), Japanese flounder (Song et al. 2012) and red sea bream (Hossain et al., 2016a,b). Bactericidal activity (BA) is one of the most important factors in host resistance against pathogenic bacteria. Our results revealed that, the highest serum BA was found in fish GMP supplemented diets. Similarly, Hossain et al. (2016a,b) reported that dietary nucleotide, nucleoside supplementation significantly increased the BA of red sea bream. Agglutination antibody titer against different bacteria is another mechanism of innate immune response which has high activity in fish (Oriol Sunyer and Tort, 1995). Fish fed GMP supplemented diet groups showed higher agglutination antibody titer compared with supplementation-free control diet

group. Peroxidase activity (PA) and NBT activity in the present study also significantly increased by dietary GMP supplementation and it was highest in diet group GMP-0.4. Similar increased PA and NBT was also observed by Song et al. (2012) in Japanese olive flounder fed diet supplemented with IMP. Our previous studies on red sea bream fed diets with supplemented nucleotides (inosine, IMP, mixed nucleotides) also showed increased PA which are in agreement with the present study. Overall, all the non specific immune response parameters measured in the present study showed improved value in GMP supplemented diet groups compared to non supplemented control group confirming the benefits of GMP nucleotide for the non-specific innate immunity of red sea bream.

Increased CAT activities were attributed to elevated levels of exogenous hydrogen peroxide (H_2O_2) which is the main cellular precursor of the hydroxyl radical (HO^-), a highly reactive and toxic form of ROS (Hossain et al. 2016a,b) . In human increased serum catalase activity is used as a diagnostic tool in acute pancreatitis (Goth et al., 1982), hemolytic disease (Goth et al., 1983) and some liver diseases (Goth et al., 1987). Significantly higher mean CAT activity observed in fish fed control diet compared to GMP supplemented diets in the present study implies that fish in control group were in stronger oxidative stress conditions compared to supplemented groups. Similar observation was also reported for red sea bream (Hossain et al., 2016a,b) where nucleotide supplementation reduce the CAT activity of red sea bream. Results of the CAT activity as well as the BAP and d-ROMs measured in the present study reconfirmed the positive supplemental effects of GMP associated with the conditions on oxidative stress and immunity.

The WG and LA activity are often used to estimate the optimum supplementation level of nutrient components in fish (Zhou et al., 2013; Wen et al., 2015). Based on the quadratic regression analysis of WG and LA, the present study illustrated that the optimal levels of dietary GMP for juvenile red sea bream might be 0.48% and 0.45 % respectively which is

also inline with the most of the growth performance and health parameters of fish under present experimental condition. Similarly, Hossain et al. (2016a) reported that optimum inosine and IMP supplementation level based on growth performances and immunity and intestinal health 0.4% for red sea bream; while Song et al. (2012) suggested a supplementation of 0.1–0.4% IMP could enhance innate immunity and disease resistance of olive flounder.

Therefore, the present study demonstrated that the dietary supplementation of GMP positively influences the growth performances, survival rates, feed utilization, innate immune responses, hematological parameters and oxidative stress response of fish. Based on the present experimental condition, it can be concluded that the optimal levels of dietary GMP were 0.45 and 0.48 %, respectively, for juvenile red sea bream, which is also in line with the most of the growth performance and health parameters of the fish.

Part-5

Dietary cytidine monophosphate enhances the growth, blood constituents, innate immune functions and stress resistance of juvenile red sea bream, *Pagrus major*

Abstract

The present study was designed to evaluate the effects of dietary cytidine monophospahte on growth, blood constituents, innate immune functions and stress resistance of juvenile red sea bream, *Pagrus major*. A semi-purified basal diet supplemented with 0% (Control), 0.1% (CMP-0.1), 0.2% (CMP-0.2), 0.4% (CMP-0.4) and 0.8% (CMP-0.8) purified CMP to formulate five experimental diets. Each diet was randomly allocated to triplicate groups of fish (mean initial weight 2.9 g) for 56 days. In general dietary CMP supplementations tended to improve growth performances (final body weight, % weight gain and specific growth rate), in which significantly highest performances were found in diet group CMP-0.4 followed by diet groups CMP-0.8 and CMP-0.2. CMP supplementation resulting in increased feed conversion efficiency and protein efficiency ratio and highest value ($P<0.05$) obtained in diet group CMP-0.8. Diet groups with CMP supplementation ≤0.1% (Control, CMP-0.1) showed significantly lowest growth and feed utilization performances. The capabilities enhancing immune response of fish fed CMP supplemented diet were detected by innate and acquired immune parameters measured in this study. Fish fed diet groups CMP-0.2 and CMP-0.4 had significantly higher NBT and TSP than fish fed the control diet, respectively. LA, PA also showed increasing trend with the supplementation of CMP and it was numerically higher in diet group CMP-0.4. In contrast, CAT activity decreased ($P>0.05$) with dietary CMP supplementations. Dietary supplementation of CMP had an increase ($P>0.05$) aquired immune response (antibody titer) after 21 days of vaccination of formalin killed V*ibrio anguillarum*. In terms of Oxidative stress diet groups CMP-0.2 and CMP-0.4 showed best condition with low oxidative stress and high antioxidant levels. Moreover, the fish fed CMP supplemented diets had better improvement ($P<0.05$) in body ash contents, glucose and total cholesterol level than the control group. The quadratic regression analysis of weight gain and lysozyme activity revealed that the optimal levels of dietary CMP were 0.44 and 0.50%,

respectively for juvenile red sea bream, which is also inline with the most of the growth performance and health parameters of fish under present experimental condition. Finally, it could be concluded that CMP can be used as a functional supplements for the diets of red sea bream.

Keywords: Cytidine monophosphate, Growth, adaptive immunity, Innate immune responses, Oxidative stress, *Pagrus major*

1. Introduction

Over the last several decades, aquaculture has developed rapidly and become a major food producing sector in the world. With the expansion and intensification of aquaculture, cultural organisms are often exposed to stressful conditions due to deterioration of water quality and elevation of stress. This consequence also increased the susceptibility to bacterial, viral, parasitic and other environmental diseases of the cultured organisms which are the most growth constricting factors and has lead to increase interest in fish health management. Now-a-days, the role of nutrition on health management through the modulation of immune response and disease resistance has turned into a research area of top priority with aims to lessen the dependence on chemotherapeutics and reduce disease-related economic losses (Kiron 2012; Oliva-Teles 2012). In this regards currently, one preferred approach is the use of immunomodulators or immunostimulants which can be supplemented to the fish diets. Hence, immunomodulation has been proposed as a potential method to protect the cultured fish from infectious pathogens by increasing their innate immune system (Aoki 1992). Recently, nucleotide and its related products have received attention commercially and scientifically as potential immunomodulators as well as functional materials in aquaculture.

Nucleotides refer to a group of biochemical substances (a purine or a pyrimidine base, a ribose or 2-deoxyribose sugar and one or more phosphate groups) with different physiological roles such as coding and translation of genetic information, contribution to energy metabolism, participation in the structure of coenzymes (allosteric effectors and cellular agonists), regulator of lymphocyte maturation, activation of and increase in phagocytosis and macrophages and immunological responses (Gatlin & Li 2007). In aquatic animals both nucleotides and its related products have long been implicated as feed attractants in both vertebrate and invertebrate species (Mackie and Adron, 1978; Carr and Thompson, 1983; Person-Le Ruyet et al., 1983; Carr et al., 1984). However, world-wide heightened attention on nucleotide supplementation into potential growth and health benefits in aquaculture species instigated after 2000s. To date, research pertaining to nucleotide nutrition in fishes has shown rather consistent and encouraging beneficial results in fish health management. Research to date on dietary nucleotides has focused on a mixture of nucleotides, rather than specific types of nucleotides except inosine and IMP for the research on feed stimulants (Li and Gatlin, 2006). Cytidine monophosphate (CMP), also known as 5'-cytidylic acid or simply cytidylate, is a nucleotide that is used as a monomer in RNA (Pascal, 2008). It is an ester of phosphoric acid with the nucleoside cytidine. CMP consists of the phosphate group, the pentose sugar ribose, and the nucleobase cytosine; hence, a ribonucleoside monophosphate. In aquaculture CMP has been studied mostly as a part of mixed nucleotide rather than considering as individual nucleotide for promoting growth, feed utilization and potential health benefit. To the best of our knowledge Lin et al., (2009) first evaluated CMP as individual NT and reported that CMP supplementation increased growth and non specific immune performance of red drum (*Epinephelus malabaricus)* .

Red sea bream are high-quality seafood in East Asia and around the world. They are also good candidates for intensive aquaculture because of their desirable taste, hardiness in a

crowded environment and rapid growth. However, intensive aquaculture of this species often faced stressful conditions which negatively affect their growth and health performances. Research on dietary modulation for potential growth and health management of this species is very scarce. Recently several studies have demonstrated that mixed and several individual purine nucleotides (IMP, Inosine, AMP, GMP etc.) as functional nutrients can improve growth and health performance of red sea bream. Until now, there are no studies on individual CMP nucleoside as functional nutrients for potential growth and health benefit of red sea bream. Therefore, the present study was conducted to assess the dietary supplementation of CMP on growth performance, blood constituents, innate and acquired immune functions and stress resistance of juvenile red sea bream (*P. major*).

2. Materials and Methods

2.1. Test fish and experimental system

Juvenile red sea bream were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (54% crude protein and 10% crude lipid, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 100 l polycarbonate tanks (filled with 80 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 4.5.1and 4.5.2 summarize the basal diet formulation and chemical composition of the experimental diets, respectively. Five fishmeal and casein based semi-purified diets were formulated to be nearly isonitrogenous (48.5% crude protein), isolipidic (11.5% crude lipid) and isocaloric (21.5 KJ g^{-1} gross energy). The experiential diets were prepared by supplementing purified CMP nucleotide as disodium salts (Sigma Aldrich Co., St. Louis, MO, USA) to the basal diet at concentrations of 0.1, 0.2, 0.4 and 0.8 % for diet group CMP-0.1, CMP-0.2, CMP-0.4 and CMP-0.8 respectively. Basal diet without CMP supplementation was used as control. For proper mixing of CMP with other ingredients, initially CMP and weighted arginine of the respective diets were mixed properly in a glass beaker by hands with the help of a stainless laboratory spatula. Then this mixture was thoroughly mixed with other dry ingredients in a food mixer for 10 min. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (35–40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 55 °C for about 150 min. The test diets were stored at −28 °C in a freezer until use.

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Eighteen fish, having a mean initial body weight of approximately 2.9 g were randomly allocated to

Table 4.5.1: Basal diet formulation of juvenile red sea bream.

Ingredients	Composition (%)
Fishmeal[a]	20
Casein[b]	32
Pollack liver oil[c]	7
Soybean lecithin[d]	3
Vitamin mixture[e]	3
Mineral mixture[f]	3
L-arginine[g]	1
Stay-C[h]	0.08
Dextrin	7
α-starch	6
Activated gluten[i]	5
α- cellulose	12.92
CMP[j]	0-0.8

[a]Nippon Suisan Co. Ltd., Tokyo, Japan

[b]Waco Pure Chemical Industries Inc. (Osaka, Japan)

[c] Riken Vitamin, Tokyo, Japan.

[d] Kanto Chemical Co., Inc. (Tokyo, Japan)

[e] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[f]Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[g] Nacalai Tesque, Kyoto, Japan.

[h] Stay-C 35.

[i]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS".

[j] Sigma Aldrich Co., St. Louis, MO, USA. (AMP added to diets at the expense of α-cellulose).

previously prepare fifteen tanks. Fish were fed the experimental diets for 56 days by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 1 hour after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk fortnightly to determine growth and visually check their health condition. The monitored water quality parameters were: water temperature 21.6±1.8 °C; pH 8.1±0.7 and salinity 34.5±0.5 during the feeding trial. These ranges are considered within optimal values for juvenile red sea bream.

2.4. Sample collection

At the end of the feeding trial, all experimental fish were fasted for 24 h. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then individual body weight of fish was measured, and the growth parameters were calculated accordingly. Nine fish per treatment (three fish per each tank) were randomly collected, washed with distilled water and then stored at −80 °C (MDF-U383, SANYO, Tokyo, Japan) for the whole body proximate analysis. Six fish were randomly sampled from each dietary replicate tank and their blood were collected by puncture of the caudal vain using heparinized (1600 IU/ml, Nacalai Tesque, Kyoto, Japan) disposable syringes and pooled. In addition non-heparinized disposable syringes were used to collect blood for serum analysis. Partial heparinized whole blood was used to analyze the hematocrit levels while plasma and serum were obtained by centrifugation at 3000 ×g for 15 min under 4 °C, and then stored at −80 °C until the analysis. Liver and viscera were dissected out from the fish above, weight individually to calculate the hepatosomatic index (HSI) and viscerasomatic index (VSI).

2.5. Biochemical analysis

The ingredients, diets and fish whole body were analyzed in triplicate, using standard methods (AOAC, 1995). The moisture was determined by drying the sample at 105 °C to constant weight. The ash was analyzed by combustion at 550 °C for 12 h. The crude protein content was determined by measuring the nitrogen content (N × 6.25) using the Kjeldahl method with a Tecator Kjeltec System (1007 Digestion System, 1002 Distilling unit, and Titration unit; FOSS Tecator AB, Högendäs, Sweden). Crude lipid content was estimated using gravimetric method (954.02). Total serum protein (TSP) and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma samples was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg.

Table 4.5.2: Proximate composition of the experimental diets (% dry matter basis).

Parameters	Diet groups				
	Control	CMP-0.1	CMP-0.2	CMP-0.4	CMP-0.8
Moisture	6.77	6.39	7.97	6.82	6.97
Crude protein	48.74	48.34	49.76	47.95	48.16
Crude lipid	11.60	11.78	11.60	11.40	11.70
Ash	7.53	7.53	7.45	7.60	7.50
Gross energy ($KJg\text{-}^{1}$)*	21.6	21.6	21.7	21.5	21.6

*Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ kg^{-1}, respectively.

Carbohydrate was calculated by the difference: 100-(protein + lipid + ash).

2.6. Evaluation of immune parameters

2.6.1. Non specific innate immune responses

Serum lysozyme activity (LA) was measured with turbidimetric assays (Lygren et al., 1999). Ten micro-liters of samples was put into well of microplate, then added 190 micro-liters of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4,their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

Oxidative radical production by phagocytes during respiratory burst was measured through the NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995) with some modifications. Briefly, blood and 0.2% NBT (Nacalai Tesque, Kyoto, Japan) were mixed in equal proportion (1:1), incubated for 30 min at room temperature. Then 50 mL was taken out and dispensed into glass tubes. One milliliter of dimethylformamide (Sigma) was added and centrifuged at 2000 × g for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Hitachi U-1000, Japan). Dimethylformamide was used as the blank.

The total peroxidase activity (PA) in serum was measured according to Salinas et al. (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

The catalase activity (CAT) assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate that absorbs at 405 nm (Goth, 1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol/l phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate ($(NH_4)_6$ Mo_7O_{24} .4 H_2O) and the yellow complex of molybdate and hydrogen peroxide is measured at 405 nm against reagent blank. One unit CAT decomposes 1 µmole of hydrogen peroxide/l minute under assay conditions. CAT activities are expressed as kilo unit per liter (kU/l)

2.6.2 Aquired immune response and agglutination antibody titer

Aquired immune response and agglutination antibody titer was measured according to Yamamoto (1995). Briefly, formalin killed *Vibrio anguillarum* was used for this study. Frozen stock-culture of *V. anguillarum* (Kagoshima University) was grown in Thiosulfate-citrate-bile salts-sucrose agar (TCBS agar *"Nissui"*) at 25◦C with for 48 h. Harvested cells were transferred in sterilized physiological saline. To kill the bacteria formalin at the concentration of 0.3% was added and kept it for 2 days at 4^{o}C. Afterwards, the bacterial cells were washed with sterilized saline for 3 times and finally killed bacterial cells were resuspended in the saline 10 mg ml^{-1}. Five fish from each replicate tank (Fifteen fish, $treatment^{-1}$) were vaccinated by intra-peritoneal (IP) injection with100 µl of formalin killed *V. anguillarum* cells (10 mg/ml). Each group of fish continued to be fed twice daily with the same experimental diet that was assigned in the growth trial. After 21 days of vaccination, blood samples were collected from randomly chosen fish. Serum samples were collected and stored as described above until assayed. Agglutinating antibody titers against *V. anguillarum* was conducted in round bottomed 'U' shaped micro titer. 50 µl of serum was serially diluted

in PBS (1/2, 1/4, 1/8, 1/16, 1/32and 1/64) and then equal volumes of *Vibrio anguillarum* (10mg ml^{-1}) was added to wells and kept for 24 h at 4 ^{0}C. The reciprocal of the highest dilution that gave agglutination was taken as the agglutination antibody titer which is expressed as log10.

2.7. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, five fish from each rearing tank (total 15 fish per treatment) were randomly selected and transferred in to a blue colored 20 L rectangular glass aquarium with 15 L tap water which was aerated for 24 h. The glass aquaria for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of red sea bream juvenile was calculated according to Moe et al. (2004) as follows: time to death (min) of a juvenile was converted to log10 values. When the juvenile were transferred to the low salinity water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was log10 100 = 2). The calculation was then conducted every 5 minutes after the first exposure. The values of log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

$Y = aX + b$ where Y = log10 (survival), X = time for individual juvenile death (min).

LT_{50} (X) was obtained when Y = 1.7 since log10 (50) = 1.7. Each value obtained from the equation above was compared statistically.

2.8. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR, % day^{-1}) = {Ln (final weight) – Ln(initial weight)/duration}× 100

Survival (%) = 100 × (final no. of fish / initial no. of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed conversion ratio (FCR) = dry feed intake (g)/ live weight gain (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Condition factor (CF, %) = weight of fish / $(\text{length of fish})^3$ × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

Viscerasomatic index (VSI, %)= viscera weight/fish weight × 100

2.9. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Turkey Kramer test. Weight gain (%) and lysozyme activity, NBT and TSP were subjected to quadratic regression analysis with dietary CMP supplementation level.

Results

3.1 Growth performance and nutrient utilization

Growth performance, nutrient utilization and survival of red sea bream fed experimental diets are presented in Table 4.5.3. All treatments showed relatively high survival rates between 90.7 % and 98.1% and there were no significant differences among all diet groups ($P>0.05$). In general dietary CMP supplementations tended to improve growth performances (final body weight, % weight gain (WG %) and specific growth rate (SGR)), in which significantly highest performances were found in diet group CMP-0.4 followed by diet groups CMP-0.8 and CMP-0.2. Diet groups with CMP supplementation ≤0.1% (Control,

CMP-0.1) showed significantly lowest growth performances. Among feed utilization parameters FCE was significantly higher in diet group CMP-0.8 and it was not differed significantly with diet groups CMP-0.2 and CMP-0.8 followed by diet group CMP-0.1. PER also significantly higher in diet group CMP-0.8 and not significantly differed with diet group CMP-0.4. Diet groups CMP-0.1 and CMP-0.2 showed intermediate values. Significantly lowest FCE and PER observed in supplementation free control group. Feed intake was not significantly influenced by dietary supplementations.

3.2. Hematological parameters

Table 4.5.4 represents the blood parameters of juvenile red sea bream after 56 days of feeding trial. Overall, dietary treatments had no significant effect on blood chemical parameters of fish among different treatments except in blood glucose and total cholesterol. Fish fed CMP supplemented diet groups CMP-0.1 and CMP-0.2 showed significantly lower values followed by diet groups CMP-0.8 and CMP-0.4. Supplementation free control groups showed significantly higher values. Supplementation of CMP significantly increased the total cholesterol level and it was highest in diet group CMP-0.8 whereas control group showed significantly lowest cholesterol value. Supplementations also numerically increase hematocrit content and decreased plasma bilirubin, GOT and GPT level, respectively compare to the control group.

3.3. Oxidative stress parameters

Fig. 4.5.1 indicates the status of oxidative stress conditions, in which the conditions of both stress intensity and tolerance ability were combined, of fish fed with test diets for 56 days. Dietary CMP supplementation did not significantly influence the d-ROMs and BAP

Table 4.5.3: Growth performance and feed utilization parameters of red sea bream fed test diets for 56 days*

Parameters	Diet groups				
	Control	CMP-0.1	CMP-0.2	CMP-0.4	CMP-0.8
IBW	2.87±0.01	2.88±0.0	2.87±0.0	2.87±0.01	2.87±0.0
FBW	17.62±0.09^{a}	18.17±0.35^{a}	18.77±0.29^{ab}	19.82±0.34^{b}	18.91±0.43^{ab}
WG%	514.03±3.1^{a}	532.0±12.07^{a}	553.6±10.0^{ab}	590.1±11.7^{b}	557.9±14.6^{ab}
SGR	3.24±0.01^{a}	3.29±0.03^{a}	3.35±0.03^{ab}	3.45±0.03^{b}	3.36±0.04^{ab}
FI	11.63±0.06	11.35±0.34	11.48±0.34	11.95±0.34	11.08±0.13
FCE	1.27±0.01^{a}	1.35±0.01^{ab}	1.38±0.02^{bc}	1.42±0.03^{bc}	1.45±0.03^{c}
PER	2.60±0.02^{a}	2.79±0.02^{ab}	2.79±0.04^{ab}	2.96±0.06^{bc}	3.0±0.06^{c}
Sur	90.7±1.8	92.6±1.8	92.6±1.8	98.1±1.9	92.6±1.8

[a] IBW: initial body weight (g).

[b] FBW: final body weight (g).

[c] WG: percent weight gain (%).

[d] SGR: specific growth rate (% day^{-1}).

[e] FI: feed intake (g fish^{-1} 50 days^{-1}).

[f] FCR: feed conversion ratio.

[g] PER: protein efficiency ratio.

[h] Sur: survival (%).

* Values are means of triplicate groups±S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

Table 4.5.4: Blood parameters of juvenile red sea bream fed test diets for 56days.[*]

Parameters	Diet groups				
	Control	CMP-0.1	CMP-0.2	CMP -0.4	CMP -0.8
Hematocrit (%)	28.3±1.2	32.7±1.5	32.0±1.5	32.3±1.9	32.3±0.33
Glucose (mg/dl)	77.7±4.9[b]	49.7±1.5[a]	51.3±6.1[a]	66.7±6.6[ab]	56.7±7.13[ab]
T-Cho (mg/dl)[a]	187.0±11.1[a]	217.0±4.2[ab]	235.7±13.5[ab]	212.0±13.1[ab]	242.3±8.4[b]
BUN (mg/dl)[b]	<5	<5	<5	<5	<5
T-Bill (mg/dl)[c]	0.27±0.07	<0.2	<0.2	<0.2	<0.2
GOT (IU/l)[d]	46.0±19.1	29.7±7.5	24.0±1.0	32.0±4.2	26.0±15.5
GPT(IU/L)[e]	14.3±4.3	<10	<10	12.7±2.2	<10
TG (mg/dl)[f]	300.7±58.7	>500	>500	418.0±49.5	>500

[a] T-Cho: total cholesterol.

[b] BUN: blood urea nitrogen.

[c]T- Bill: Total bilirubin

[d]GOT: glutamyl oxaloacetic transaminase.

[e]GPT: glutamic pyruvate transaminase.

[f]TG: triglyceride.

[*]Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

vlaues ($P<0.05$) in the present study (no data illustrated here). However, combined effects of d-ROM and BAP showed that fish fed diet groups CMP-0.2 and CMP-0.4 were located in zone A which was categorized as lower intensity of oxidative stress and higher tolerance ability against oxidative stress. Supplementation free-control group and lowest CMP supplemented diet groups (CMP-0.1) located in zone B and C respectively. Highest supplemented group (CMP-0.8) located in zone D indicates higher intensity of oxidative stress and lower tolerance ability against oxidative stress.

3.4. Low salinity stress test

The results of lethal stress test against fresh water shock on LT_{50} obtained by regression analysis are shown in Fig. 4.5.2. Supplementation increase the LT50 values of red sea bream compare to control (57.5 mins.) and it was numerically higher in fish feed diet group CMP-0.2 (72.5 min). However, LT50 was not significantly influenced ($P>0.05$) by dietary treatments.

3.5. Innate immune responses

Non-specific innate immune responses were positively affected by dietary CMP supplementations (Table 4.5.5) but always not statistically significant different among treatments. Fish fed diet groups CMP-0.2 and CMP-0.4 had significantly higher NBT and TSP than fish fed the control diet, respectively. LA, PA also showed increasing trend with the supplementation of CMP and it was numerically higher in diet group CMP-0.4. In contrast, CAT activity decreased ($P>0.05$) with dietary CMP supplementations and it was numerically lower in diet group CMP-0.1.

Table 4.5.5: Non-specific immune response of juvenile red sea bream fed test diet for 50 days.*

Parameters	Diet groups				
	Control	CMP-0.1	CMP-0.2	CMP-0.4	CMP-0.8
TSP (g/dl)[a]	2.2±0.0[a]	2.7±0.0[ab]	2.7±0.2[ab]	3.1±0.3[b]	2.7±0.2[ab]
LA (unit/mL)[b]	300.0±34.2	320.0±100.0	376.7±123.3	456.7±236.7	204.4±8.0
PA[c]	1.88±0.05	1.96±0.07	2.87±1.0	2.95±0.9	2.06± 0.1
NBT[d]	0.42±0.02[a]	0.54±0.04[ab]	0.55±0.04[ab]	0.63±0.05[b]	0.50± 0.03[ab]
CAT[e]	32.3± 1.1	22.2±8.4	25.8±3.2	29.04±2.4	27.9±2.6

[a]TSP: Total serum protein

[b]LA: lysozyme activity

[c]PA: Peroxidase activity (measured at OD of 450nm).

[d]NBT: Nitro-blue-tetrazolium activity

[e]CAT: Catalase activity

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

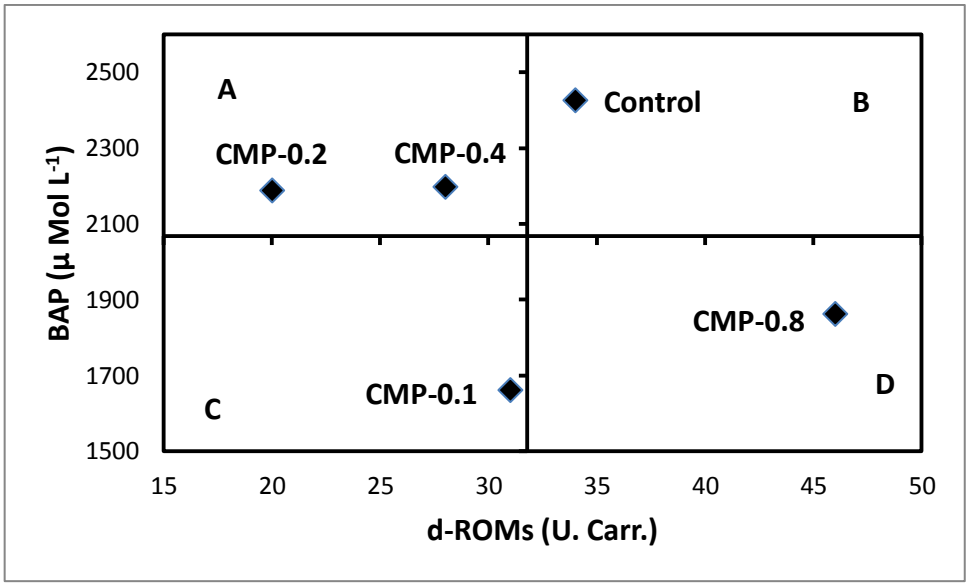


Fig. 4.5.1. Oxidative stress parameters in red sea bream fed test diets for 56 days. (Values are means of triplicate groups. The abbreviations of experimental treatments are illustrated in the text. Central axis based on mean values of d-ROM and BAP from each treatment.) Zone A: High antioxidant potential and low reactive oxygen metabolites (good condition). Zone B: High antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone C: Low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone D: Low antioxidant potential and high reactive oxygen metabolites (stressed condition).

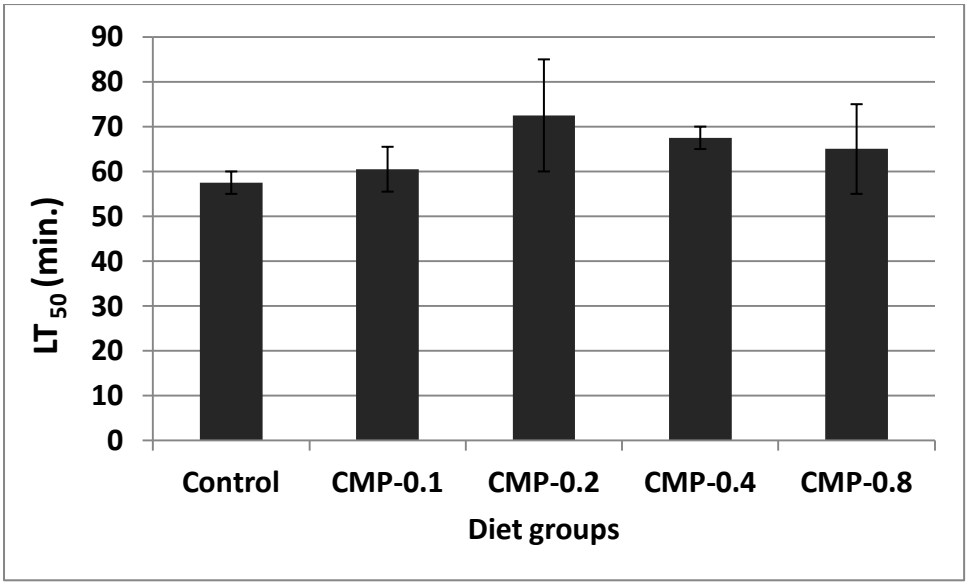


Fig. 4.5.2. LT_{50} (min) calculated from the lethal time of red sea bream exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from triplicate groups. Values with different letters are significantly different (P<0.05)

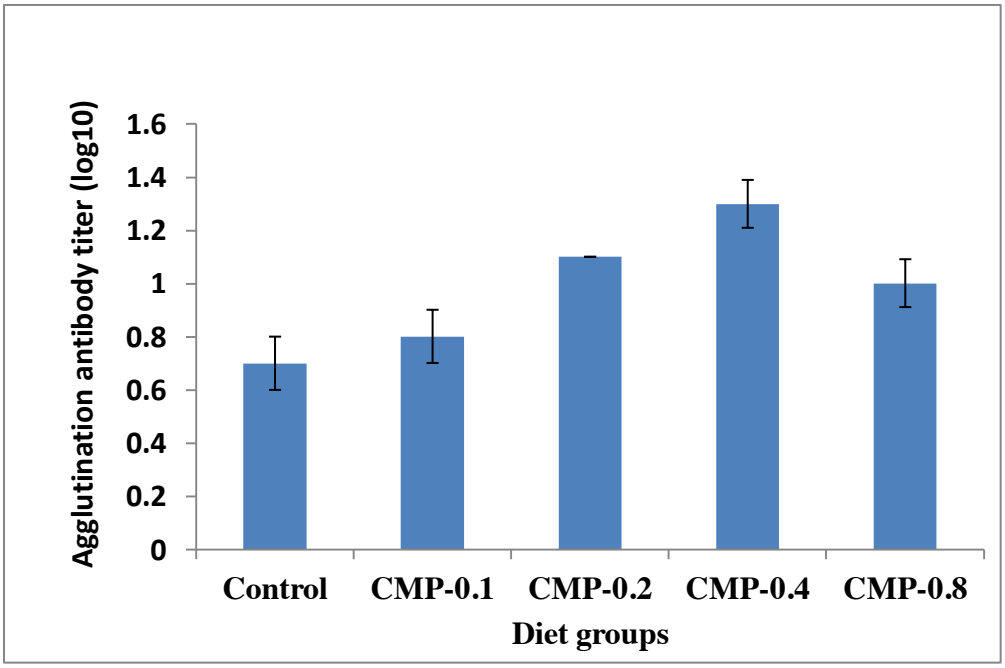


Fig.4.5.3. Aquired immune response (agglutination antibody titer) of red sea bream after vaccination of 21 days with formalin killed *V. aguillarum*. Data were expressed as mean ± S.E.M. from triplicate groups.

3.5.1. Aquired humoral immune response

Agglutination antibody titer was evaluated as aparameter of aquired humoral immune response is presented in Fig. 4.5.3. In comparison with control dietary supplementation showed increased antibody titer and it was numerically higher in CMP-0.4 diet group. However, antibody titer was not significnaly different among treatments.

3.6. Whole body proximate analysis and biometric indices

The initial and final whole body proximate compositions of juvenile red sea bream are shown in Table 4.5.6. All the fish showed a change in the analyzed parameters compared to those of the initial values. In comparison with the control group, dietary treatments had no significant influences on the whole body proximate composition and biometric indices with exception of whole body ash content. Fish fed CMP supplemented diet showed significant increase of whole body ash content and it was highest in diet group CMP-0.1. However, the other supplemented groups were not significantly differed with diet groups CMP-0.1. On the other hand supplementation-free control group showed significantly lowest value.

3.7. Quantification of optimum supplementation of CMP nucleotide for P. major.

The optimum supplementation level of CMP nucleotide for % WG, TSP and NBT were estimated by quadratic regression analysis .Based on % WG, the optimal supplemental level of CMP was estimated to be 0.50 % in diet ($Y_{WG} = -312.84x^2 + 313.07x + 508.79$ R = 0.9639) (Fig. 4.5.4). On the other hand, the optimal supplemental level of CMP nucleotide based on TSP and NBT were estimated to be 0.48% in diet ($Y_{TSP} = -3.7593x^2 + 3.5764x + 2.2462$; R = 0.9192) and 0.44% ($Y_{NBT} = -1.0124x^2 + 0.8952x + 0.4315$; R = 0.9409), respectively.

Table 4.5.6: Whole body proximate analysis (% wet basis) and biometric indices in juvenile red sea bream fed test diets for 56 days.*

Parameters	Initial[1]	Diet group				
		Control	CMP-0.1	CMP-0.2	CMP -0.4	CMP -0.8
Moisture	82.6	78.5±0.1	76.6±0.5	76.2±1.1	77.3±0.2	77.5±0.9
Crude protein	10.9	12.1±0.1	13.0±0.1	12.7±0.7	12.2±0.1	12.7±0.3
Crude lipid	2.4	5.50±0.0	6.04±0.6	6.82±0.4	6.24±0.2	5.63±0.5
Crude ash	3.4	2.84±0.1^{a}	3.28±0.0^{b}	3.26±0.1^{b}	3.11±0.0^{ab}	3.26±0.0^{b}
CF[2]	-	1.58±0.07	1.59±0.07	1.62±0.02	1.65±0.06	1.59±0.03
HSI[3]	-	1.06±0.1	1.34±0.1	1.27±0.1	1.23±0.0	1.30±0.1
VSI[4]	-	5.31±0.2	5.29±0.1	5.46±0.2	5.32±0.2	5.36±0.1

[1]Initial values are not included in the statistical analysis.

[2]CF (condition factor)=100 × fish weight/(fish length)^{3}

[3]HSI (hepatosomatic index)=100 × liver weight/fish weight

[4]VSI (viscerasomatic index)=100 × viscera weight/fish weight

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05). Crude protein, crude lipid and ash are expressed on a wet weight basis.

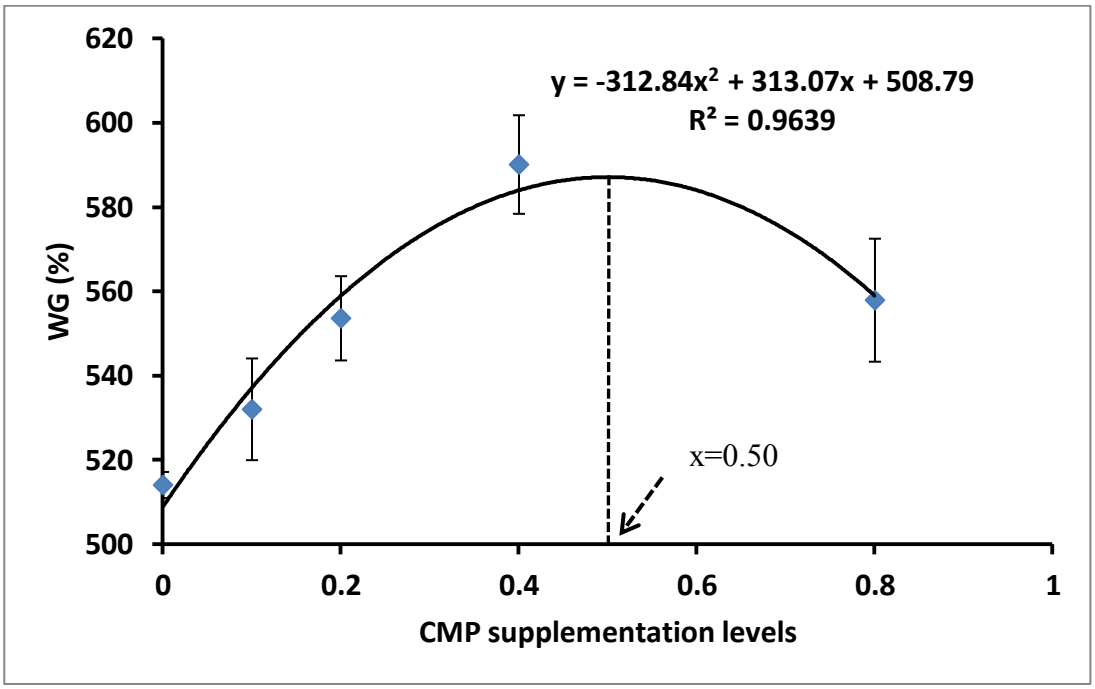


Fig. 4.5.4. Quadratic regression analysis of percent weight gain (%WG) for juvenile red sea bream (*P. major*) fed diets supplemented with graded levels of CMP for 56 days.

4. Discussion

During the intensification of aquaculture culture organisms are often exposed to suboptimal culture condition due to elevation of stress and deterioration of water quality which ultimately caused diseases. Although prophylactic treatments using antibiotics have been used with some success in controlling such diseases, these chemotherapeutic remedies were widely criticized for their negative impacts (Anderson, 1992).). In this regards currently, one preferred approach is to supplement immunomodulators or immunostimulants in fish diets which will help to protect the cultured fish from infectious pathogens by increasing their innate immune system. Recently, nucleotide and its related products have received attention commercially and scientifically as potential immunomodulators as well as functional materials in aquaculture. Our results showed that CMP when fed at a medium dose (0.4%) for 56 days could enhance immunity, growth and survival of *P. major* fingerlings.

Result of the study demonstrated that CMP increased the growth performance of red sea bream and it was significantly higher in diet group CMP-0.4. Growth-enhancing effects of nucleotide mixtures have been reported earlier in other fish species such as tilapia (Ramadan et al., 1991), red drum (Li et al., 2007), grouper (Lin et al., 2009). Red drum (Li et al., 2007), rainbow trout (Tahmasebi-Kohyani et al. 2011) ,and red sea bream (Hossain et al. 2016a,b) fed diets supplemented with various levels of nucleotides (0.3-3 g kg^{-1}) showed significantly enhanced weight gain and feed efficiency compared to fish fed the basal diet. Meanwhile, Li et al. (2007), observed transient growth-enhancing effect of dietary nucleotides in their study and explained the conventional controversy about nucleotide effects on fish growth. To date, there is no exact explanation on how nucleotides work to enhance growth rate. Borda et al. (2003) presumed that an exogenous supply of nucleotides may promote growth of fish and crustaceans in early stages to meet their high rate of cell replication. However, the mechanism of growth-promotion by dietary nucleotides remains to

be identified in fish. The results of feed utilization parameters in the present study suggest that the tested fish utilized experimental diets effectively by CMP supplementation resulting in increased feed conversion efficiency and protein efficiency ratio. Hossian et al. (2016 a,b) also observed similar improved feed utilization performances of red sean bream fed diets supplemented with mixed and individual nucleotides (inosine, inosine monophosphate).

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish (Hossain et al., 2016a,b). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to those of the previous findings (Aoki et al., 1998; Hossain et al., 2016a,b). Plasma glucose is commonly considered to be one stress indicator in fish, high glucose levels often indicate the higher stress status of fish (Eslamloo et al., 2012). The significantly lower blood glucose content in fish fed diet groups CMP-0.1, CMP-0.2 followed by CMP-0.8 and CMP-0.4 indicated that dietary CMP supplementation induced an optimal physiological condition of the fish. Result of the present study also revealed that plasma bilirubin, GOT and GPT level showed lower values ($P>0.05$) in CMP supplemented diet groups compared to supplementation-free control group. Plasma bilirubin, GOT (or aspartate aminotransferase, AST) and GPT (or alanine aminotransferase, ALT) are often used for the evaluation of liver function as they are released into the blood during injury or damage to the liver cells (Lemaire et al., 1991). Lower values of these parameters in CMP supplemented diet groups indicated that, CMP induced an optimal physiological condition as well as better liver health condition when compared with the supplementation-free control group. Dietary CMP supplementation also enhanced hematocrit content of juvenile red sea bream as a general health response towards nutritional strategies. Similarly, Song et al. (2012) and Hossain et al. (2016a,b) reported the enhanced hematocrit level by the supplementation of nucleotides in Japanese olive flounder and red sea bream diets,

respectively. Improved hematocrit content in the supplemented groups of the present study indicated that dietary CMP elevated the health status of fish

Stress is one of the emerging factors in aquaculture activities, which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization (Hossain et al., 2016a,b). Dietary nucleotide increases the resistance of fish to a variety of stressors is well documented (Burrells et al., 2001b; Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2012, Hossain et al., 2016a,b). In our study, fish fed CMP supplemented diets showed increase in stress resistance of red sea bream after the exposure of low salinity stress test. In the present study highest LT_{50} value obtained in diet group CMP-0.2 (72.5 min) on the other hand supplementation free control group showed the lowest value of LT_{50} (57.5 min) indicated a lower fresh water tolerance of the red sea bream. Increased ($P>0.05$) LT_{50} of red sea bream fed CMP supplemented diet groups indicates healthy status of red sea bream (Yokoyama et al., 2005, Hossain et al. 2016b). Oxidative stress is considered to involve in plenty of diseases and pathological status in fish (Martinez-Alvarez et al., 2005), and is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants (Celi et al., 2010). Simultaneous measurements of d-ROMs with BAP can provide a suitable tool for measuring the oxidative stress in humans, pig, rabbit and dog (Oriani et al., 2001; Ballerini et al., 2003; Pasquini et al., 2008). Fish with higher d-ROM values indicate that they are under more oxidative stress conditions. On the other hand, fish with higher BAP values indicate stronger tolerance against oxidation. In the present study oxidative status of fish was determined by measuring plasma d-ROMs and BAP values. As shown in Fig. 4.5.1, it is interesting to note that diet groups CMP-0.2 and CMP-0.4 was located in zone A, which represents the best condition with low oxidative stress and high antioxidant levels. Control diet group and lowest CMP supplemented group (CMP-0.1) were located in zone B and Zone C, respectively which are

also considered acceptable. On the contrary, higher level of CMP supplemented group CMP-0.8 located in zone D, considered as stressed condition of fish. Similarly, Hossain et al. (2016a,b) also illustrated that dietary supplementation of inosine and nucleotide mixtures stimulated the oxidative status of red sea bream. To date, there remains a lack of explanation about how these supplements work to affect these parameters, so further study is needed.

In the present study whole-body composition and biometric indices of juvenile red sea bream were not influenced significantly by dietary supplementation except whole body ash. However, whole body proximate composition values were within the normal ranges of red sea bream that have been reported previously (Hossain et al. 2016a,b). Significantly increased whole body ash content in the supplemented groups indicated increased mineral retention through supplementation of CMP which ultimately increased the whole body ash content in red sea bream. Research on the effect of exogenous nucleotides and nucleosides on mineral deposition of fishes is scarce. Our observation on whole-body ash of red sea bream is likely the first report on the response of fish supplemented with dietary CMP. Information on changes in whole body ash data in response to dietary nucleotides and nucleoside associated with physiological consequences may be important; therefore, further investigation is warranted.

The enhancement of various immune functions in response to nucleotide supplementation has been reported in several fish species (Sakai et al., 2001; Li et al., 2004a,b; Cheng et al. 2011). In the present study, innate and adaptive immune responses were positively influences by dietary CMP supplementations, although all the parameters are not always significantly different among treatments. Proteins which are the most important compounds in the serum and are essential for sustaining a healthy immune system (Kumar et al., 2005) showed significantly increased value in CMP supplemented diet groups in the present study compare to the control. Increased total serum proteins due to nucleotide

supplementation were also reported in *Catla catla* (Jha et al., 2007), red sea bream (*P. major*) (Hossain et al., 2016a,b) and rainbow trout (Tahmasebi Kohyani et al. 2012). In the present study, neutrophil oxidative production (NBT) of red sea bream tended to be significantly influenced by dietary CMP supplementations. (Table 4.5.5), which is in agreement with some findings (Li and Gatlin, 2007). Similar observations also were reported in red drum (Cheng et al., 2011), hybrid striped bass (Li et al., 2004) and common carp (Sakai et al., 2001). Supplementations of CMP also increased (P>0.05) LA and PA compared to control. Similar increase LA and PA with fish fed nucleoside and nucleotide supplemented diet were also reported previously in common carp (*Cyprinus carpio*) (Sakai et al., 2001), rainbow trout (Tahmasebi-Kohyani et al., 2011) , Japanese flounder Song et al. (2012) red sea bream (Hossain et al. 2016a,b) and red drum , *Sciaenops ocellatus* (Cheng et al. 2011). Findings of the present study are in agreement with the mentioned previous findings indicates immune-stimulatory properties of nucleotides. Increased catalase activity rates were attributed to elevated levels of exogenous hydrogen peroxide (H_2O_2) which is the main cellular precursor of the hydroxyl radical (HO^-), a highly reactive and toxic form of ROS (Hossain et al. 2016a,b). In human increased serum catalase activity is used as a diagnostic tool in acute pancreatitis (Goth et al., 1982), hemolytic disease (Goth et al., 1983) and some liver diseases (Goth et al., 1987). The high mean catalase activity observed in fish fed control diet compared to CMP supplemented diets in the present study implies that fish in control group were in stronger oxidative stress conditions compared to supplemented groups. Similar observation was also reported for red sea bream (Hossain et al., 2016a,b) where nucleotide supplementation reduce the CAT activity of red sea bream. Results of the catalase activity as well as the BAP and d-ROMs measured in the present study reconfirmed the positive supplemental effects of CMP associated with the conditions on oxidative stress and immunity.

Apart of increased non specific immune responses, adaptive humoral immunity also positively influenced by dietary CMP supplementation (P>0.05) in the present study. Bacterial agglutination antibody titer were increased with CMP supplementation and it was numerically higher (P>0.05) in diet group CMP-0.4 after 21th days of vaccination of formalin killed *V. anguillarum*. Ramadan et al. (1994) first observed that dietary supplementation of nucleotides (Ascogen, Chemoforma Basel, Switzerland) had a marked immune-potentiating effect on both humoral and cell-mediated immune responses of tilapia after intramuscular injection or direct immersion with formalin-killed *Aeromonas hydrophila*. Antibody titers after vaccination as well as mitogenic responses of lymphocytes from fish fed the ascogen-supplemented diet were significantly and tremendously higher than those of fish fed the basal diet. Similar phenomena were reported on other species such as rainbow trout (Burrells et al., 2001b; Leonardi et al., 2003) and hybrid striped bass (Li et al., 2004a). The antibody titer of hybrid striped bass fed an oligonucleotide-supplemented diet after vaccination with formalin-killed *Streptococcus iniae* was three times higher than that of fish fed the basal diet (Li et al., 2004a). Result of the present study strongly substinate the previous above findings at the same time reconfirm the capabilities of dietary nucleotide to enhancing innate and adaptive immune response of fish.

The significantly influenced WG and innate immune responses (TSP and NBT) were used for regression analysis to estimate the optimum supplementation level of CMP in the present study. Based on the quadratic regression analysis of WG, TSP and NBT the present study illustrated that the optimal levels of dietary CMP for juvenile red sea bream might be 0.50% and 0.48%and 0.44% respectively. Similarly, Hossain et al. (2016a) reported that optimum inosine and IMP supplementation level based on growth performances and immunity and intestinal health 0.4% for red sea bream; while Song et al. (2012) suggested a

supplementation of 0.1–0.4% IMP could enhance innate immunity and disease resistance of olive flounder.

Therefore, the present study demonstrated that the dietary supplementation of CMP positively influences the growth performances, feed utilization, innate and adaptive immune responses, hematological parameters and oxidative stress response of fish. Based on the present experimental condition, it can be concluded that the optimal levels of dietary CMP were 0.44 and 0.50%, respectively, for juvenile red sea bream, which is also in line with the most of the growth and health performance parameters of the fish.

Part-6

Dietary administration of uridine monophosphate enhances the growth, innate and aquired immune functions and stress resistance of juvenile red sea bream, *Pagrus major*

Abstract

In the present study we investigate the administration effects of dietary uridine monophosphate to enhance the growth, innate and adaptive immune functions and stress resistance of juvenile red sea bream, *Pagrus major*. A casein fismeal based semi-purified basal diet supplemented with 0% (Control), 0.1% (UMP-0.1), 0.2% (UMP-0.2), 0.4% (UMP-0.4) and 0.8% (UMP-0.8) purified UMP to formulate five experimental diets. Each diet was randomly allocated to triplicate groups of fish (mean initial weight 2.9 g) for 56 days. In general dietary UMP supplementation tended to improve growth performances (final body weight, % weight gain and specific growth rate), in which significantly highest performances were found in diet group UMP-0.4 followed by diet groups UMP-0.8 and UMP-0.1 and UMP-0.2. UMP supplementation resulting in increased feed conversion efficiency and protein efficiency ratio and significantly highest value ($P<0.05$) obtained in diet groups UMP-0.4 and UMP-0.8. Feed intake also increased numerically ($P>0.05$) with dietary UMP supplementation. Supplementation free control groups showed significantly lowest growth and feed utilization performances. The capabilities enhancing immune response of fish fed UMP supplemented diet were detected by non specific and acquired immune parameters measured in this study. PA was significnaly higher in fish fed diet groups UMP-0.8 and higher ($P<0.05$) TSP values obtained in diet groups UMP-0.4 and UMP-0.8. Other measured non specific immune parameters (LA, NBT, CA) also positively influenced by dietaryUMP supplementation but not at a significant level. Dietary supplementation of UMP had an increase aquired immune response (antibody titer) after 15 and 21 days of vaccination of formalin killed V*ibrio anguillarum*. In the 15th day after vaccination, significanly higher agglutination antibody titer was found in diet group UMP-0.4 and simultaneously other supplemetnted diet groups showed intermediate values. In terms of oxidative stress, diet group UMP-0.4 showed best condition with low oxidative stress and high antioxidant levels.

Moreover, the fish fed UMP supplemented diets had better improvement ($P<0.05$) in body Lipid contents, hepatosomatic index, glucose and hematocrit content than the control group. The quadratic regression analysis of weight gain and agglutination antibody titer revealed that the optimal levels of dietary UMP were 0.48 and 0.59 %, respectively, for juvenile red sea bream, which is also in line with the most of the growth performance and health parameters of the fish.

Keywords: Uridine monophosphate, Growth, adaptive immunity, Innate immune responses, Oxidative stress, *Pagrus major*

1. Introduction

Infectious diseases and fish feeds management are probably the major limiting factors for the successful production of fish species. Proper nutrition is critical not only to achieve optimal growth rates but also to maintain the health of cultured fish (Sealey and Gatlin, 2001). For a number of years, the fish nutrition field focused mainly on establishing the minimum nutrient requirements for normal growth of different fish species (NRC, 2011). However, nowadays, the role of nutrition on health management through the modulation of immune response and disease resistance has turned into a research area of top priority with aims to lessen the dependence on chemotherapeutics and reduce disease-related economic losses (Kiron, 2012; Oliva-Teles, 2012). In this regards currently, one preferred approach is the use of immunomodulators or immunostimulants which can be supplemented to the fish diets. Recently, nucleotide and its related products have received attention commercially and scientifically as potential immunomodulators as well as functional materials in aquaculture.

Nucleotides are low molecular weight intracellular compounds which play key roles in nearly all biochemical processes (Gil, 2002) and it is the building block of nucleic acids. Dietary supplementation of nucleotides or its related products (nucleosides, nucleic acids) have been shown to benefit many mammalian physiological and nutritional functions Uauy, 1989; Quan, 1992; Carver, 1994; Haskó et al., 2000) . In aquatic animals both nucleotides and its related products have long been implicated as feed attractants in both vertebrate and invertebrate species (Mackie and Adron, 1978; Carr and Thompson, 1983; Person-Le Ruyet et al., 1983; Carr et al., 1984). However, world-wide heightened attention on nucleotide supplementation into potential growth and health benefits in aquaculture species instigated after 2000s. To date, research pertaining to nucleotide nutrition in fishes has shown rather consistent and encouraging beneficial results in fish health management. Research to date on dietary nucleotides has focused on a mixture of nucleotides, rather than specific types of nucleotides except inosine and IMP for the research on feed stimulants (Li and Gatlin, 2006).

Uridine monophosphate (UMP), also known as 5'-uridylic acid is a pyrimidine nucleotide and it is used as a monomer in RNA. It is an ester of phosphoric acid with the nucleoside uridine. UMP consists of the phosphate group, the pentose sugar ribose, and the nucleobase uracil; hence, it is a ribonucleoside monophosphate. In aquaculture UMP has been studied mostly as a part of mixed nucleotide rather than considering as individual nucleotide for promoting growth, feed utilization and potential health benefit. Kiyohara et al. (1975) initially reported the chemo-attractant properties of UMP for puffer fish. Afterward, Ishida and Hidaka (1987) also reported UMP as the most effective gustatory stimulant of various marine teleosts including aigo rabbitfish, isaki grunt, kampachi amberjack, maaji jack mackerel and masaba chub mackerel. Lin et al. (2009) also reported increased growth and non specific immune performance of red drum (*Epinephelus malabaricus)* fed diet supplemented with dietary UMP.

Red sea bream (*Pagrus major*) is one of the most economically cultured marine fish species in East Asian countries, particularly in Japan and Korea due to its desirable taste, high market demand and traditional food habits. Investigation on quantitative nutritional requirements of red sea bream for some macro and micronutrients (Forster & Ogata 1998; Ren et al., 2008; Kader & Koshio, 2012; Rahimnejad & Lee, 2013; 2014) and development of alternative protein based diet (Kader *et al*. 2010, 2012; Kader & Koshio 2012) have been defined for this fish. However, research on dietary modulation for potential growth and health management of this species is very scarce. Recently, we evaluated the effects of mixed NT and individual NT (IMP, inosine) on this species (Hossain et al., 2016a,b). However, to the best of our knowledge there is no research on individual nucleotide UMP as feeding stimulant as well as a functional nutrient for potential growth and health benefit of red sea bream. Therefore, the present study was conducted to assess the dietary effects of UMP on growth performance, feed utilization, digestibility, innate immune response and stress resistance of red sea bream juveniles.

2. Materials and Methods

2.1. Test fish and experimental system

Juvenile red sea bream were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (54% crude protein and 10% crude lipid, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 100 l polycarbonate tanks (filled with 80 l of water) in a flow through sea water system where each tank was equipped

with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 4.6.1and 4.6.2 summarize the basal diet formulation and chemical composition of the experimental diets, respectively. Five fishmeal and casein based semi-purified diets were formulated to be nearly isonitrogenous (48.5% crude protein), isolipidic (11.5% crude lipid) and isocaloric (22 KJ g^{-1} gross energy). The experiential diets were prepared by supplementing purified UMP nucleotide as disodium salts (Sigma Aldrich Co., St. Louis, MO, USA) to the basal diet at concentrations of 0.1, 0.2, 0.4 and 0.8 % for diet group UMP-0.1, UMP-0.2, UMP-0.4 and UMP-0.8 respectively. Basal diet without UMP supplementation was used as control. For proper mixing of UMP with other ingredients, initially UMP and weighted arginine of the respective diets were mixed properly in a glass beaker by hands with the help of a stainless laboratory spatula. Then this mixture was thoroughly mixed with other dry ingredients in a food mixer for 10 min. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (35–40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 55 °C for about 150 min. The test diets were stored at −28 °C in a freezer until use.

Table 4.6.1: Basal diet formulation of juvenile red sea bream.

Ingredients	Composition (%)
Fishmeal[a]	20
Casein[b]	32
Pollack liver oil[c]	7
Soybean lecithin[d]	3
Vitamin mixture[e]	3
Mineral mixture[f]	3
L-arginine[g]	1
Stay-C[h]	0.08
Dextrin	7
α-starch	6
Activated gluten[i]	5
α- cellulose	12.92
UMP[j]	0-0.8

[a]Nippon Suisan Co. Ltd., Tokyo, Japan

[b]Waco Pure Chemical Industries Inc. (Osaka, Japan)

[c] Riken Vitamin, Tokyo, Japan.

[d] Kanto Chemical Co., Inc. (Tokyo, Japan)

[e] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[f]Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[g] Nacalai Tesque, Kyoto, Japan.

[h] Stay-C 35.

[i]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS".

[j] Sigma Aldrich Co., St. Louis, MO, USA. (UMP added to diets at the expense of α-cellulose).

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Eighteen fish, having a mean initial body weight of approximately 2.9 g were randomly allocated to previously prepare fifteen tanks. Fish were fed the experimental diets for 56 days by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 1 hour after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk fortnightly to determine growth and visually check their health condition. The monitored water quality parameters were: water temperature 21.6±1.8 °C; pH 8.1±0.7 and salinity 34.5±0.5 during the feeding trial. These ranges are considered within optimal values for juvenile red sea bream.

2.4. Sample collection

At the end of the feeding trial, all experimental fish were fasted for 24 h. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then individual body weight of fish was measured, and the growth parameters were calculated accordingly. Nine fish per treatment (three fish per each tank) were randomly collected, washed with distilled water and then stored at −80 °C (MDF-U383, SANYO, Tokyo, Japan) for the whole body proximate analysis. Six fish were randomly sampled from each dietary replicate tank and their blood were collected by puncture of the caudal vain using heparinized (1600 IU/ml, Nacalai Tesque, Kyoto, Japan) disposable syringes and pooled. In addition non-heparinized disposable syringes were used to collect blood for serum analysis. Partial heparinized whole blood was used to analyze the hematocrit levels while plasma and serum were obtained by centrifugation at 3000 ×g for 15 min under 4

°C, and then stored at −80 °C until the analysis. Liver and viscera were dissected out from the fish above, weight individually to calculate the hepatosomatic index (HSI) and viscerasomatic index (VSI).

2.5. Biochemical analysis

The ingredients, diets and fish whole body were analyzed in triplicate, using standard methods (AOAC, 1995). The moisture was determined by drying the sample at 105 °C to constant weight. The ash was analyzed by combustion at 550 °C for 12 h. The crude protein content was determined by measuring the nitrogen content (N × 6.25) using the Kjeldahl method with a Tecator Kjeltec System (1007 Digestion System, 1002 Distilling unit, and Titration unit; FOSS Tecator AB, Högendäs, Sweden). Crude lipid content was estimated using gravimetric method (954.02). Total serum protein (TSP) and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma samples was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically

Table 4.6.2: Proximate composition of the experimental diets (% dry matter basis).

Parameters	Diet groups				
	Control	UMP-0.1	UMP-0.2	UMP-0.4	UMP-0.8
Moisture	6.77	6.80	7.01	7.87	7.62
Crude protein	48.74	48.75	48.64	48.37	49.39
Crude lipid	11.60	11.86	11.15	11.15	10.94
Ash	7.53	7.33	7.45	7.57	7.48
Gross energy (KJg^{-1})*	21.6	21.7	21.5	21.5	21.5

*Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ kg^{-1}, respectively.

Carbohydrate was calculated by the difference: 100-(protein + lipid + ash).

detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg.

2.6. Evaluation of immune parameters

2.6.1. Non specific innate immune responses

Serum lysozyme activity (LA) was measured with turbidimetric assays (Lygren et al., 1999). Ten micro-liters of samples was put into well of microplate, then added 190 micro-liters of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4, their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

Oxidative radical production by phagocytes during respiratory burst was measured through the NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995) with some modifications. Briefly, blood and 0.2% NBT (Nacalai Tesque, Kyoto, Japan) were mixed in equal proportion (1:1), incubated for 30 min at room temperature. Then 50 mL was taken out and dispensed into glass tubes. One milliliter of dimethylformamide (Sigma) was added and centrifuged at 2000 × g for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Hitachi U-1000, Japan). Dimethylformamide was used as the blank.

The total peroxidase activity (PA) in serum was measured according to Salinas et al. (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-

developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

The catalase activity (CAT) assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate that absorbs at 405 nm (Goth et al., 1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol/l phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate ($(NH_4)_6$ Mo_7O_{24} .4 H_2O) and the yellow complex of molybdate and hydrogen peroxide is measured at 405 nm against reagent blank. One unit CAT decomposes 1 µmole of hydrogen peroxide/l minute under assay conditions. CAT activities are expressed as kilo unit per liter (kU/l)

2.6.2 Aquired immune response and agglutination antibody titer

Aquired immune response and agglutination antibody titer was measured according to Yamamoto (1995). Briefly, formalin killed *Vibrio anguillarum* was used for this study. Frozen stock-culture of *V. anguillarum* was grown Thiosulfate-citrate-bile salts-sucrose agar (TCBS agar *"Nissui"*) at 25◦C with for 48 h. Harvested cells were transferred in sterilized physiological saline. To kill the bacteria formalin at the concentration of 0.3% was added and kept it for 2 days at 4^{o}C. Afterwards, the bacterial cells were washed with sterilized saline for 3 times and finally killed bacterial cells were resuspended in the saline 10 mg ml^{-1}. Five fish from each replicate tank (Fifteen fish, treatment^{-1}) were vaccinated by intra-peritoneal (IP) injection with100 µl of formalin killed *V. anguillarum* cells (10 mg/ml). Each group of fish continued to be fed twice daily with the same experimental diet that was assigned in the growth trial. After 15 days and 21 days of vaccination, blood samples were collected from two/three randomly chosen fish. Serum samples were collected and stored as described above

until assayed. Agglutinating antibody titers against *V. anguillarum* was conducted in round bottomed 'U' shaped micro titer. 50 µl of serum was serially diluted in PBS (1/2, 1/4, 1/8, 1/16, 1/32and 1/64) and then equal volumes of *Vibrio anguillarum* (10mg ml^{-1}) was added to wells and kept for 24 h at 4 ^{0}C. The reciprocal of the highest dilution that gave agglutination was taken as the agglutination antibody titer which is expressed as log10.

2.7. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, five fish from each rearing tank (total 15 fish per treatment) were randomly selected and transferred in to a blue colored 20 L rectangular glass aquarium with 15 L tap water which was aerated for 24 h. The glass aquaria for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of red sea bream juvenile was calculated according to Moe et al. (2004) as follows: time to death (min) of a juvenile was converted to log10 values. When the juvenile were transferred to the low salinity water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was log10 100 = 2). The calculation was then conducted every 5 minutes after the first exposure. The values of log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

Y= a*X*+ b where Y = log10 (survival), *X* = time for individual juvenile death (min).

LT_{50} (*X*) was obtained when Y = 1.7 since log10 (50) = 1.7. Each value obtained from the equation above was compared statistically.

2.8. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR, % day^{-1}) = {Ln(final weight) – Ln(initial weight)/duration} × 100

Survival (%) = 100 × (final no. of fish / initial no. of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed conversion ratio (FCR) = dry feed intake (g)/ live weight gain (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Condition factor (CF, %) = weight of fish / $(\text{length of fish})^3$ × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

Viscerasomatic index (VSI, %)= viscera weight/fish weight × 100

2.9. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Turkey Kramer test. Weight gain (%) and agglutination antibody titer were subjected to quadratic regression analysis with dietary UMP supplementation level.

3. Results

3.1 Growth performance and nutrient utilization

Growth performance, nutrient utilization and survival of red sea bream fed experimental diets are presented in Table 4.6.3. All treatments showed relatively high survival rates between 90.7 % and 96.27% and there were no significant differences among all diet groups (P>0.05). In general dietary UMP supplementations tended to improve growth performances (final body weight, % weight gain (WG %) and specific growth rate (SGR)), in which significantly highest performances were found in diet group UMP-0.4 followed by diet

groups UMP-0.8 and UMP-0.1 and UMP-0.2. UMP supplementation free control group showed significantly lowest growth performances. Among feed utilization parameters FCE and PER were significantly higher in diet group UMP-0.4 and it was not differed significantly with diet groups UMP-0.8 followed by diet groups UMP-0.2 and UMP-0.1. Feed intake also increased numerically with the dietary UMP supplementations but not at a significant level. Overall supplementation free control group showed lowest growth and feed utilization and survival performance in the present study.

3.2. Hematological parameters

Table 4.6.4 represents the blood parameters of juvenile red sea bream after 56 days of feeding trial. Overall, dietary treatments had no significant effect on blood chemical parameters of fish among different treatments except in blood glucose. Fish fed UMP supplemented diet groups UMP-0.1 and UMP-0.2 showed significantly lower values of plasma glucose whereas diet group UMP-0.4 and UMP-0.8 showed intermediate value. Supplementation free control groups showed significantly higher values. Supplementation of UMP also numerically reduced the plasma GOT, GPT, BUN and total bilirubin in compare with control group. Supplementations also significantly increase hematocrit content and it was significantly higher in diet group UMP-0.4 and the other supplemented groups showed interdediate value. On the contrary supplementation free control group showed significantly lower hematocrit content in the present study.

3.3. Oxidative stress parameters

Fig. 4.6.1 indicates the status of oxidative stress conditions, in which the conditions of both stress intensity and tolerance ability were combined, of fish fed with test diets for 56 days. Dietary UMP supplementation did not significantly influence the d-ROMs and BAP

Table 4.6.3: Growth performance and feed utilization parameters of red sea bream fed test diets for 56 days*

Parameters	Diet groups				
	Control	UMP-0.1	UMP-0.2	UMP-0.4	UMP-0.8
IBW	2.87±0.01	2.87±0.01	2.87±0.01	2.87±0.0	2.87±0.0
FBW	17.62±0.1[a]	19.13±0.7[ab]	19.01±0.3[ab]	20.77±1.0[b]	20.50±0.8[ab]
WG%	514.1±3.1[a]	566.6±23.7[ab]	562.2±9.2[ab]	622.3±33.8[b]	613.7±26.8[ab]
SGR	3.24±0.01[a]	3.39±0.06[ab]	3.37±0.03[ab]	3.53±0.08[b]	3.51±0.07[ab]
FI	11.63±0.06	12.44±0.4	12.35±0.1	13.41±0.8	13.0±0.6
FCE	1.27±0.01[a]	1.31±0.02[ab]	1.31±0.02[ab]	1.34±0.0[b]	1.36±0.02[b]
PER	2.60±0.02[a]	2.68±0.04[ab]	2.69±0.3[ab]	2.76±0.01[b]	2.74±0.03[b]
Sur	90.73±1.83	96.27±1.9	94.40±0.0	94.43±3.2	94.43±3.2

[a] IBW: initial body weight (g).

[b] FBW: final body weight (g).

[c] WG: percent weight gain (%).

[d] SGR: specific growth rate (% day^{-1}).

[e] FI: feed intake (g $fish^{-1}$ 50 $days^{-1}$).

[f] FCR: feed conversion ratio.

[g] PER: protein efficiency ratio.

[h] Sur: survival (%).

* Values are means of triplicate groups±S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

Table 4.6.4: Blood parameters of juvenile red sea bream fed test diets for 56days.[*]

Parameters	Diet groups				
	Control	UMP-0.1	UMP-0.2	UMP -0.4	UMP -0.8
Hematocrit (%)	28.3±1.2[a]	29.3±0.3[ab]	30.7±0.3[ab]	33.3±0.7[b]	32.67±1.7[ab]
Glucose (mg/dl)	77.6±4.8[b]	43.7±6.2[a]	43.3±2.6[a]	53.7±1.7[ab]	74.7±13.0[ab]
T-Cho (mg/dl)[a]	187.0±11.1	205.7±6.7	194.7±4.1	183.7±22.9	236.0±13.5
BUN (mg/dl)[b]	5.33±0.3	<5	<5	<5	5.33±0.3
T-Bill (mg/dl)[c]	0.27±0.07	0.30±0.0	0.20±0.0	0.23±0.03	0.27±0.07
GOT (IU/l)[d]	46.0±19.1	33.0±1.5	15.7±3.2	17.7±5.4	32.0±11.0
GPT(IU/L)[e]	14.3±4.3	13.3±3.3	<10	<10	12.3±2.3
TG (mg/dl)[f]	300.7±58.7	406.0±49.2	429.0±33.8	403.0±48.5	443.7±56.3

[a] T-Cho: total cholesterol.

[b] BUN: blood urea nitrogen.

[c]T- Bill: Total bilirubin

[d]GOT: glutamyl oxaloacetic transaminase.

[e]GPT: glutamic pyruvate transaminase.

[f]TG: triglyceride.

[*]Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

vlaues ($P<0.05$) in the present study (no data illustrated here). However, combined effects of d-ROM and BAP showed that fish fed diet groups UMP-0.4 was located in zone A which was categorized as lower intensity of oxidative stress and higher tolerance ability against oxidative stress. Simultaneously, diet group UMP-0.8 and UMP-0.2 were located in zone B and Zone C, respectively. Supplementation free-control group and lowest UMP supplemented diet groups (UMP-0.1) located in zone D indicate higher intensity of oxidative stress and lower tolerance ability against oxidative stress.

3.4. Low salinity stress test

The results of lethal stress test against fresh water shock on LT_{50} obtained by regression analysis are shown in Fig. 4.6.2. Supplementation significantly increase the LT_{50} values of red sea bream and it was significantly higher in diet group UMP-0.4 (78.81 min.), other supplemented groups showed intermediate values. In contrast, supplementation free control group showed significantly lower LT_{50} value (57.5 min.).

3.5. Innate immune responses

Innate immune responses were positively affected by dietary UMP supplementations (Table 4.6.5) but not always significantly different among treatments. In comparison with control fish fed diet groups UMP-0.4 and UMP-0.8 had significantly higher TSP and the other supplemented groups showed intermediate values. PA also significantly higher in diet group UMP-0.8 and the other supplemented groups showed intermediate values in comparison to control. LA and NBT also showed increasing trend with the dietary UMP supplementation and it was higher ($P>0.05$) in diet groups UMP-0.2 and UMP-0.4, respectively. In contrast, CAT activity decreased ($P>0.05$) with UMP supplementations and lower value was observed in diet group UMP-0.4.

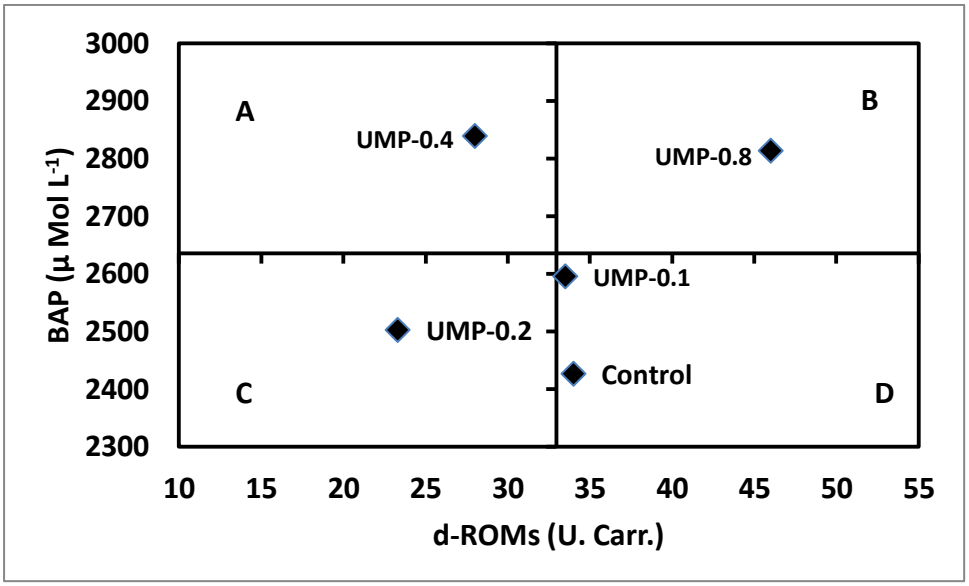


Fig. 4.6.1. Oxidative stress parameters in red sea bream fed test diets for 56 days. (Values are means of triplicate groups. The abbreviations of experimental treatments are illustrated in the text. Central axis based on mean values of d-ROM and BAP from each treatment.) Zone A: High antioxidant potential and low reactive oxygen metabolites (good condition). Zone B: High antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone C: Low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone D: Low antioxidant potential and high reactive oxygen metabolites (stressed condition).

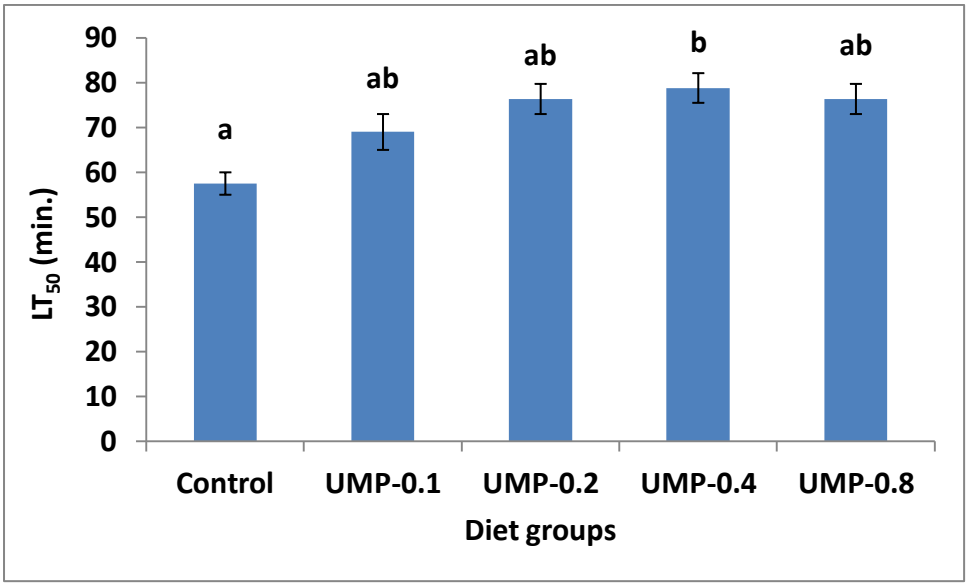


Fig.4.6.2. LT_{50} (min) calculated from the lethal time of red sea bream exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from triplicate groups. Values with different letters are significantly different (P<0.05)

Table 4.6.5: Non-specific immune response of juvenile red sea bream fed test diet for 50 days.*

Parameters	Diet groups				
	Control	UMP-0.1	UMP-0.2	UMP-0.4	UMP-0.8
TSP (g/dl)[a]	2.2±0.03[a]	2.4±0.1[ab]	2.5±0.1[ab]	2.8±0.1[b]	2.8±0.1[b]
LA (unit/mL)[b]	300.0±34.2	303.0±143.0	568.8±187.8	356.1±110.5	451.0± 204.7
PA[c]	1.88±0.05[a]	2.06±0.03[ab]	1.99±0.01[ab]	1.98±0.07[ab]	2.86± 0.5[b]
NBT[d]	0.54±0.04	0.55±0.04	0.59±0.02	0.62±0.04	0.56± 0.02
CAT[f]	27.9±2.6	26.6±2.4	28.4±1.6	25.8±2.8	26.2± 3.3

[a]TSP: Total serum protein

[b]LA: lysozyme activity

[c]PA: Peroxidase activity (measured at OD of 450nm).

[d]NBT: Nitro-blue-tetrazolium activity

[e]CAT: Catalase activity

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

3.6. Aquired immune responses

Dietary supplementation of UMP had an increase aquired immune response (agglutination antibody titer) after 15 and 21 days of vaccination of formalin killed V*ibrio anguillarum* (Fig. 4.6.3). In the 15th day after vaccination, significanly higher agglutination antibody titer was found in diet group UMP-0.4 and simultaneously other supplemetnted diet groups showed intermediate values. Almost similar trend of antibody titer value observed in 21 days of vaccination but values were not significnaly different among treatments.

3.7. Whole body proximate analysis and biometric indices

The initial and final whole body proximate compositions of juvenile red sea bream are shown in Table 7. All the fish showed a change in the analyzed parameters compared to those of the initial values. In comparison with the control group, dietary treatments had no significant influences on the whole body proximate composition and biometric indices with the exceptions of whole body moisture, crude lipid content and HSI. Fish fed UMP supplemented diet showed significant reduced whole body moisture content and it was lowest in diet group UMP-0.1 where the other supplemented groups showed intermediate values. On the contrary, in comparison to control significantly increased whole body lipid content was found in supplemented groups and it was highest in Diet group UMP-0.1. HSI was significantly higher in diet group UMP-0.4 and it was not differed significantly with diet groups UMP-0.1 and UMP-0.2 followed by diet groups UMP-0.8. Supplementation-free control group showed lowest HIS as well as other biometrics indices values.

3.8. Quantification of optimum supplementation of UMP nucleotide for P. major.

The optimum supplementation level of UMP nucleotide for % WG and adaptive immune response (agglutination antibody titer) estimated by quadratic regression analysis (Fig. 4.6.4a,

b). Based on % WG, the optimal supplemental level of UMP was estimated to be 0.59 % in diet ($Y_{WG\%} = -312.84x^2 + 371.37x + 517.55$; $R = 0.9257$). On the other hand, the optimal supplemental level of UMP nucleotide based on adaptive immune response (agglutination antibody titer) was estimated to be 0.48% in diet ($Y_{\text{antibody titer}} = -2.8087x^2 + 2.6927x + 0.6509$; $R^2 = 0.9485$).

4. Discussion

In aquaculture, before 2000s, nucleotides have been implicated as feed attractants rather than as a potential functional nutrient for promoting growth and health benefit of the cultured organisms. Recently, nucleotides have received attention commercially and scientifically as potential immunomodulators as well as functional materials in aquaculture (Hossain et al., 2016a). However, such knowledge is still scarce and most of the cases mixed nucleotides were used rather than evaluating the efficacy of individual nucleotide. Hence, it is important to investigate the dietary administration effects of individual nucleotide, UMP as functional materials in aquafeed.

The results of the present study clearly revealed that feeding of UMP supplemented diet groups significantly improves the growth performances of red sea bream compared to supplementation-free control group after 56 days feeding trial. This observation is in agreement with the previous findings of Lin et al. (2009), reported that nucleotide UMP significantly increased the growth performance of grouper (*E. malabaricus*). Similarly, the growth enhancing effects of some other individual and mixed nucleotides have also been reported in some previous studies (Hossain et al. 2016a,b; Song et al. 2012; Cheng et al. 2011). According to Li et al. (2007), transient growth-enhancing effect of dietary nucleotides observed in their study may explain the conventional controversy about nucleotide effects on fish growth. To date, there is no exact explanation on how nucleotides work to enhance

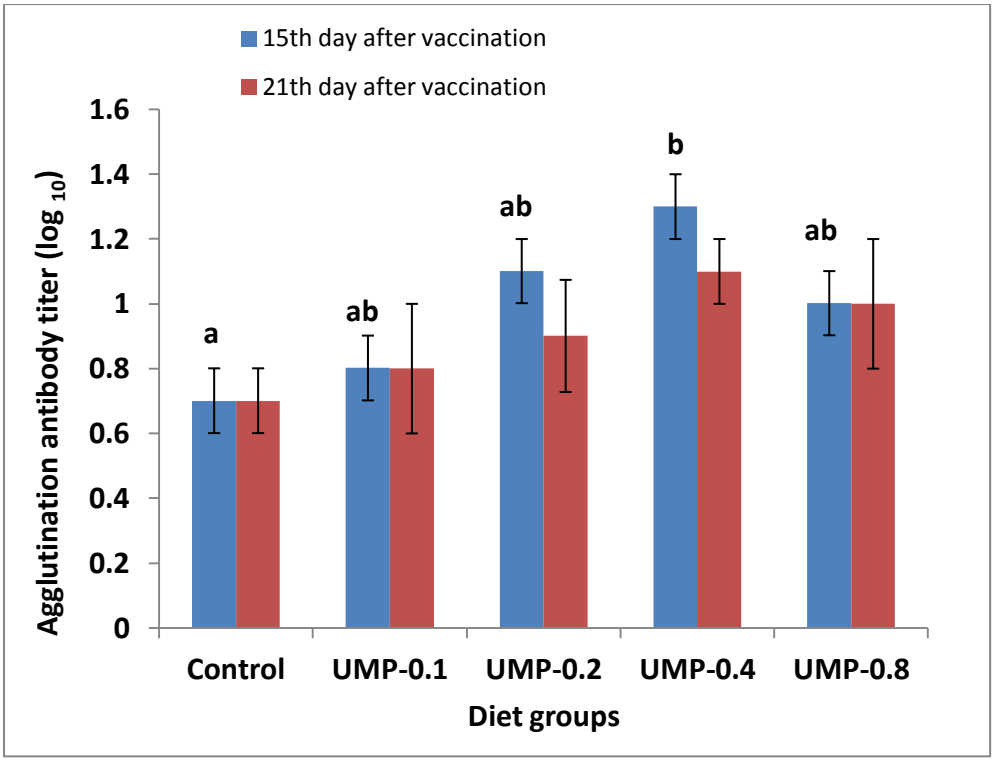


Fig.4.6.3. Aquired immune response (agglutination antibody titer) of red sea bream after vaccination of 15th and 21st days with formalin killed *V. aguillarum*. Data were expressed as mean ± S.E.M. from triplicate groups. Values with different letters are significantly different (P<0.05)

Table4.6. 6: Whole body proximate analysis (% wet basis) and biometric indices in juvenile red sea bream fed test diets for 56 days.*

Parameters	Initial[1]	Diet group				
		Control	UMP-0.1	UMP-0.2	UMP -0.4	UMP -0.8
Moisture	83.5	78.5±0.1[b]	76.1±0.3[a]	76.4±0.4[ab]	76.7±0.5[ab]	76.8±0.6[ab]
Crude protein	11.9	12.1±0.1	12.7±0.3	12.9±0.2	12.9±0.0	12.4±0.2
Crude lipid	0.7	5.5±0.01[a]	6.9±0.01[b]	6.3±0.3[ab]	6.6±0.3[ab]	6.4±0.3[ab]
Crude ash	3.3	2.84±0.07	3.18±0.2	3.08±0.1	3.0±0.1	3.03±0.1
CF[2]	-	1.58±0.07	1.74±0.04	1.73±0.07	1.75±0.04	1.76±0.02
HSI[3]	-	1.06±0.1[a]	1.41±0.1[b]	1.41±0.0[b]	1.46±0.1[b]	1.34±0.1[ab]
VSI[4]	-	5.17±0.2	5.31±0.3	5.29±0.2	5.31±0.04	5.25±0.15

[1]Initial values are not included in the statistical analysis.

[2]CF (condition factor)=100 × fish weight/(fish length)^3

[3]HSI (hepatosomatic index)=100 × liver weight/fish weight

[4]VSI (viscerasomatic index)=100 × viscera weight/fish weight

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05). Crude protein, crude lipid and ash are expressed on a wet weight basis.

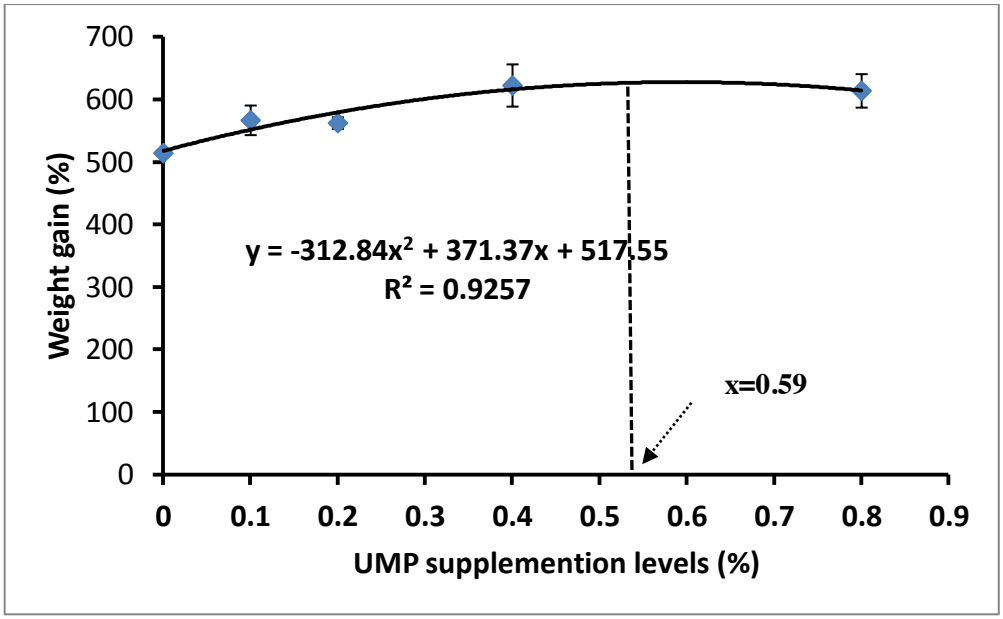


Fig. 4.6.4 (a)

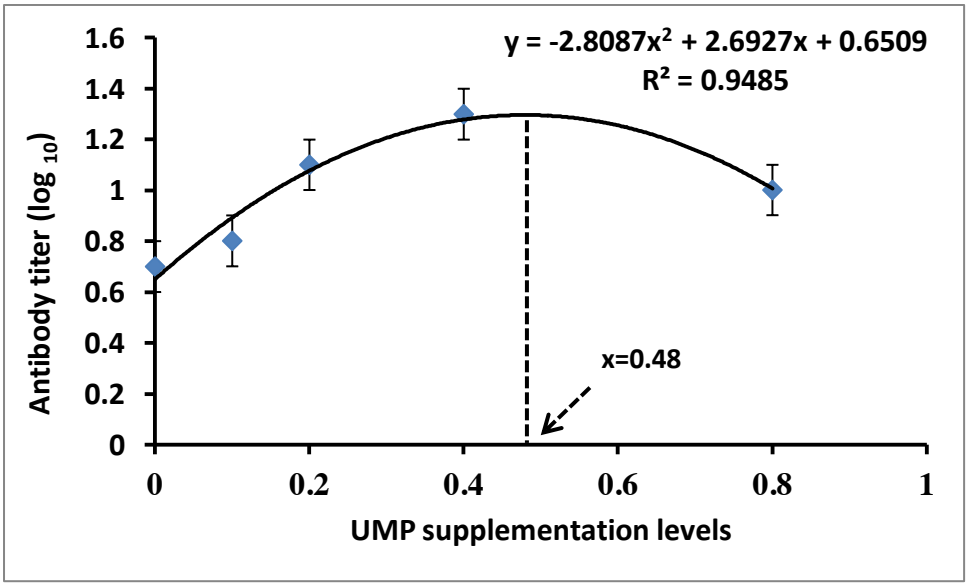


Fig. 4.6.4 (b)

Fig. 4.6.4a,b. Quadratic regression analysis of percent weight gain (%WG) and agglutination titer for juvenile red sea bream (*P. major*) fed diets supplemented with graded levels of UMP for 56 days.

growth rate. Borda et al. (2003) presumed that an exogenous supply of nucleotides may promote growth of fish and crustaceans in early stages to meet their high rate of cell replication. Another assumption regarding the growth-enhancing effect of UMP in the present study resulted from improved feed intake (Table 4.6.3), promoting more rapid feed intake that reduced nutrient leaching into the water or possibly playing roles in metabolism (Metailler *et al*. 1983, Hossain et al. 2016). In the present study improved feed intake ($P>0.05$) in UMP supplemented diet groups in comparison to supplementation free control diet group strongly substantiates the previous hypothesis. However, the exact mechanism of growth promotion by dietary nucleotides remains to be identified in fish and further study is warranted in this regards.

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish (Hossain et al., 2016a,b). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to those of the previous findings (Aoki et al., 1998; Hossain et al., 2016a,b). Dietary UMP supplementation significantly enhanced hematocrit of juvenile red sea bream as a general health response towards nutritional strategies. In the present experiment, hematocrit content was significantly highest in fish fed UMP-0.4 diet group, other UMP supplemented groups showed intermediate values whereas control group showed significantly lowest value. This indicated that dietary UMP elevated the health status of fish. Similarly, Song et al. (2012) and Hossain et al. (2016a,b) reported the enhanced hematocrit level by the supplementation of nucleotides in Japanese olive flounder and red sea bream diets, respectively. Plasma glucose is commonly considered to be one stress indicator in fish, high glucose levels often indicate the higher stress status of fish (Eslamloo et al., 2012). The significantly lower blood glucose content in fish fed diet groups UMP-0.1, UMP-0.2 followed by UMP-0.4 and UMP-0.8 indicated that dietary UMP

supplementation induced an optimal physiological condition of the fish. Result of the present study also revealed that BUN, plasma bilirubin, GOT and GPT level showed lower values ($P>0.05$) in UMP supplemented diet groups compared to supplementation-free control group. Plasma bilirubin, GOT (or aspartate aminotransferase, AST) and GPT (or alanine aminotransferase, ALT) are often used for the evaluation of liver function as they are released into the blood during injury or damage to the liver cells (Lemaire et al., 1991). Lower values of these parameters in UMP supplemented diet groups indicated that, UMP induced an optimal physiological condition as well as better liver health condition when compared with the supplementation-free control group.

Stress is one of the emerging factors in aquaculture activities, which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization (Hossain et al., 2016a,b). Dietary nucleotide increases the resistance of fish to a variety of stressors is well documented (Burrells et al., 2001b; Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2012, Hossain et al., 2016a,b). In our study, fish fed UMP supplemented diets showed increase in stress resistance of red sea bream after the exposure of low salinity stress test. In the present study highest LT_{50} value obtained in diet group UMP-0. (78.8 min) on the other hand supplementation free control group showed the lowest value of LT_{50} (57.5 min) indicated a lower fresh water tolerance of the red sea bream. Increased ($P>0.05$) LT_{50} of red sea bream fed UMP supplemented diet groups indicates healthy status of red sea bream (Yokoyama et al., 2005, Hossain et al. 2016b). Oxidative stress is considered to involve in plenty of diseases and pathological status in fish (Martinez-Alvarez et al., 2005), and is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants (Celi et al., 2010). Simultaneous measurements of d-ROMs with BAP can provide a suitable tool for measuring the oxidative stress in humans, pig, rabbit and dog (Oriani et al., 2001; Ballerini et al., 2003; Pasquini et

al., 2008). Recently, d-ROMs and BAP were reported to be reliable parameters for determining the oxidative stress condition of fish (Hossain et al. 2016a,b). Fish with higher d-ROM values indicate that they are under more oxidative stress conditions. On the other hand, fish with higher BAP values indicate stronger tolerance against oxidation. As shown in Fig. 4.6.1, it is interesting to note that diet groups UMP-0.4 was located in zone A, which represents the best condition with low oxidative stress and high antioxidant levels. Diet groups UMP-0.8 and UMP-0.2 located in zone B and Zone C, respectively which are also considered acceptable. On the contrary, diet groups UMP-0.1 and control located in zone D, considered as stressed condition of fish. Similarly, Hossain et al. (2016a,b) also illustrated that dietary supplementation of inosine and nucleotide mixtures stimulated the oxidative status of red sea bream. To date, there remains a lack of explanation about how these supplements work to affect these parameters, so further study is needed.

In the present study among whole-body composition of juvenile red sea bream whole body protein and ash were not influenced by dietary supplementation and within normal ranges that have been reported previously (Hossain et al. 2016a,b). However, whole-body lipid and moisture content of juvenile red sea bream were significantly influenced by dietary treatments. An inverse relationship between the carcass moisture and lipid content in fish observed in the present study similar type of inverse relationship of carcass composition of fish was also reported earlier by Garling and Wilson (1976); Jauncey (1982). Hossain et al. (2016b) and Li et al. (2005) observed significantly increased whole body lipid content of red sea bream and red drum, respectively with the dietary nucleotide supplementation. Research on the effect of exogenous nucleotides and nucleosides on lipid metabolism of fishes is very limited, although it is known that dietary nucleotides can influence levels of various lipids and/or fatty acids in certain tissues, such as erythrocytes, plasma, liver or brain (Carver and Walker, 1995; Sato et al., 1995). Our observation on whole-body lipid of red sea bream is

likely the first report on the response of fish supplemented with dietary UMP. Information on changes in various lipids in tissues as well as HSI data in response to dietary nucleotides and nucleoside associated with physiological consequences may be important; therefore, further investigation is warranted. In the present study, among biometric indices HSI were significantly influenced by dietary UMP supplementation. The liver size is relative to the nutritional status of the fish (Shoemaker *et al*. 2003; Sridee & Boonanuntanasarn 2012). Increased HIS in the present study with UMP supplementation indicates proper storage of macro and micronutrients and healthy condition of liver as well as clinical healthy signs of fish.

Evidence from animal and human studies supports the theory that dietary nucleotides are important for optimal functioning of several components of the immune system (Cosgrove, 1998). The enhancement of various immune functions in response to nucleotide supplementation has been reported in several fish species (Sakai et al., 2001;Li et al., 2004a,b; Cheng et al. 2011). In the present study, innate and adaptive immune response were positively influences by dietary UMP supplementations, although all the parameters are not always significantly different among treatments. Proteins which are the most important compounds in the serum and are essential for sustaining a healthy immune system (Kumar et al., 2005) showed significantly increased value in UMP supplemented diet groups in the present study compare to the control. Increased total serum proteins due to nucleotide supplementation were also reported in *Catla catla* (Jha et al., 2007), red sea bream (*P. major*) and rainbow trout (Tahmasebi Kohyani et al. 2012). PA in the present study also significantly increased by dietary UMP supplementation and it was highest in diet group UMP-0.8. Similar increased PA was also observed by Song et al. (2012) in Japanese olive flounder fed diet supplemented with IMP. Our previous studies on red sea bream fed diets with supplemented nucleotides (inosine, IMP, mixed nucleotides) also showed increased PA which are in

agreement with the present study. Supplementations of UMP also increased (P>0.05) LA and neutrophil oxidative production (NBT) compared to control. Similar increase LA and NBT with fish fed nucleoside and nucleotide supplemented diet were also reported previously in common carp (*Cyprinus carpio*) (Sakai et al., 2001), rainbow trout (Tahmasebi-Kohyani et al., 2011) , Japanese flounder Song et al. (2012) red sea bream (Hossain et al. 2016a,b) and red drum , *Sciaenops ocellatus* (Cheng et al. 2011). These findings are in agreement with the present study. Increased catalase activity rates were attributed to elevated levels of exogenous hydrogen peroxide (H_2O_2) which is the main cellular precursor of the hydroxyl radical (HO^-), a highly reactive and toxic form of ROS (Hossain et al. 2016a,b) .In human increased serum catalase activity is used as a diagnostic tool in acute pancreatitis (Goth et al., 1982), hemolytic disease (Goth et al., 1983) and some liver diseases (Goth et al., 1987). The high mean catalase activity observed in fish fed control diet compared to UMP supplemented diets in the present study implies that fish in control group were in stronger oxidative stress conditions compared to supplemented groups. Similar observation was also reported for red sea bream (Hossain et al., 2016a,b) where nucleotide supplementation reduce the CAT activity of red sea bream. Results of the catalase activity as well as the BAP and d-ROMs measured in the present study reconfirmed the positive supplemental effects of UMP associated with the conditions on oxidative stress and immunity.

In the present study a part of increased non specific immune response adaptive humoral immunity also positively influenced by dietary UMP supplementation. Bacterial agglutination antibody titer were significantly higher in supplemented groups after 15^{th} days of injecting formalin killed *V. anguillarum* . agglutination antibody titer also increased in supplemented groups after 21 days of vaccination but not at a significant level. Ramadan et al. (1994) first observed that dietarysupplementation of nucleotides (Ascogen, Chemoforma Basel, Switzerland) had a marked immune-potentiating effect on both humoral and cell-

mediated immune responses of tilapia after intramuscular injection or direct immersion with formalin-killed *Aeromonas hydrophila*. Antibody titers after vaccination as well as mitogenic responses of lymphocytes from fish fed the ascogen-supplemented diet were significantly and tremendously higher than those of fish fed the basal diet. Similar phenomena were reported on other species such as rainbow trout (Burrells et al., 2001b; Leonardi et al., 2003) and hybrid striped bass (Li et al., 2004a). The antibody titer of hybrid striped bass fed an oligonucleotide-supplemented diet after vaccination with formalin-killed *Streptococcus iniae* was three times higher than that of fish fed the basal diet (Li et al., 2004a). Result of the present study strongly substinate the previous above findings at the same time reconfirm the capabilities of dietary nucleotide to enhancing innate and adaptive immune response of fish.

The significantly influenced WG and humoral adaptive immune response (agglutination antibody titer) are used to estimate the optimum supplementation level of UMP in the present study. Based on the quadratic regression analysis of WG and agglutination antibody titer, the present study illustrated that the optimal levels of dietary UMP for juvenile red sea bream might be 0.59% and 0.48%. Similarly, Hossain et al. (2016a) reported that optimum inosine and IMP supplementation level based on growth performances and immunity and intestinal health 0.4% for red sea bream; while Song et al. (2012) suggested a supplementation of 0.1–0.4% IMP could enhance innate immunity and disease resistance of olive flounder.

Therefore, the present study demonstrated that the dietary supplementation of UMP positively influences the growth performances, feed utilization, innate immune responses, hematological parameters and oxidative stress response of fish. Based on the present experimental condition, it can be concluded that the optimal levels of dietary UMP were 0.48 and 0.59 %, respectively, for juvenile red sea bream, which is also in line with the most of the growth performance and health parameters of the fish.

CHAPTER V

Functional effects of mixed nucleotides

Part-1

Dietary nucleotide administration influences growth, immune responses and oxidative stress resistance of juvenile red sea bream (*Pagrus major*)

ABSTRACT

A 56- day feeding trial was carried out to determine effects of purified nucleotide mixture for red sea bream (*Pagrus major*). Five dietary level of purified nucleotide mixture containing equal portion of inosine 5′ monophosphate (IMP), adenosine 5′ monophosphate (AMP), guanosine 5′ monophosphate (GMP), uridine 5′ monophosphate (UMP) and cytidine 5′ monophosphate (CMP) were supplemented to the basal diet (control, D1) at 0.5 (D2), 1.0 (D3), 1.5 (D4), 2.0 (D5) and 2.5 (D6) g kg^{-1} . Triplicate groups of fish (initial weight: 2.6 g) were stocked in 100-L polycarbonate circular tanks at a density of 18 fish per tank under the flow-through system, and were fed the respective test diets twice daily to satiation level. After feeding trial, significantly higher growth performances, survival (%) and feed utilization were observed in fish fed with 1.5 % mixed nucleotide supplemented diet (D4). Growth performance parameters were not differed significantly among diet groups D3 and D4. Feed conversion ratio, protein efficiency ratio and survival were not differed among diets D2 to D5. All the growth and feed utilization parameters were significantly lower in D1 and D6. In case of oxidative stress parameters fish fed nucleotide supplemented diets showed best condition because of the least oxidative stressed condition as well as the highest tolerance against oxidation. Stress resistance against low salinity exposure (LT_{50}), significantly increased with nucleotide supplementation and was highest in D4. Total serum protein, serum lysozyme activity and total peroxidase content tended to be higher ($P>0.05$) in nucleotide supplemented group. Catalase activity reduced ($P<0.05$) with nucleotide supplementation and significantly lower value obtained in D3 and D4 diet groups. Bactericidal activity was also increased ($P<0.05$) with the supplementation of nucleotide in diets regardless of the inclusion levels. Considering overall performance of fish in a 56 days growth trial, it is therefore recommended a dietary administration of 1.0-1.5 g kg^{-1} mixed nucleotides to promote growth, immune responses and stress resistance of juvenile red sea bream.

Key words: nucleotide; growth; immune response; oxidative stress; red sea bream.

1. Introduction

Nucleotides (NT) are low molecular weight intracellular compounds which play key roles in diverse essential physiological and biochemical functions including encoding genetic information, mediating energy metabolism and signal transduction (Carver and Walker, 1995). Dietary nucleotides have been reported to be beneficial for humans and animals (Gil, 2002) since they positively influence lipid metabolism, immunity, and tissue growth, development and repair (reviewed in Gil, 2002). Nucleotides have traditionally been considered to be non-essential nutrients (Li et al., 2007). There are three ways that an animal can obtain the required nucleotides. These include recycling from dead cells known as the salvage pathway, direct de novo synthesis from amino acids, or through the diet (Quan et al., 1990). However, the processes to produce purines and pyrimidines by de novo synthesis and the salvage pathway are thought to be energetically costly. Additionally, under stressful conditions, such as infection or during fast growth and development, de novo synthesis of nucleotides may become limiting and supplementation through the diet can improve both human and animal health performance (Burrells et al., 2001a, 2001b; Carver and Walker, 1995; Cosgrove, 1998; Li et al., 2005) and in this circumstances nucleotides has been classified as ‘semi-essential’ or ‘conditional nutrition’ (Vanburen et al., 1994; Cosgrove, 1998).

In aquatic animals nucleotides have long been implicated as feed attractants in both vertebrate and invertebrate species (Mackie, 1973; Kiyohara et al., 1975; Carr and Thompson, 1983; Carr et al., 1984; Ishida and Hidaka, 1987). However, research into potential growth and health benefits of dietary nucleotides in aquaculture species did not begin until the early 2000s (Huu et al., 2012). In Aquaculture dietary nucleotide

supplementation has been shown to enhance growth of certain fish species (reviewed by Li and Gatlin, 2006) immune responses and disease resistance of all male hybrid tilapia (*Saratheradon niloticus* ♀× *Saratheradon aureus* ♂ (Ramadan et al., 1994), Atlantic salmon (*Salmo salar* L.) (Burrells et al., 2001a), common carp (*Cyprinus carpio* L.) (Sakai et al., 2001), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Li et al., 2004), grouper (*Epinephelus malabaricus*) (Lin et al., 2009), red drums (*Sciaenops ocellatus*) (Cheng et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Tahmasebi-Kohyani et al., 2012) and Japanese flounder (*Paralichthys olivaceus*) (Song et al., 2012). Supplementation of nucleotides was also reported to increased stress tolerance in Atlantic salmon (Burrells et al., 2001b), rainbow trout (Leonardi et al., 2003) and even gastrointestinal physiology and morphology of tilapia (Ramadan et al., 1994), Atlantic salmon (Burrells et al., 2001b) and red drum (Cheng et al., 2011).

Red sea bream (*Pagrus major*), is one of the most economically cultured marine fish species in Japanese aquaculture, due to its economic feasibility and traditional food habits (Koshio, 2002). Lately, the aquaculture of this species has also developed rapidly and widely in China, and Korea. Intensive culture of this species often exposed to stressful condition which impaired growth and immunity of fish. Exposure to stress places additional demands on available nucleotides and an additional exogenous supply of nucleotides provided by dietary supplementation may help to counter the immunosuppressive effects of stress (Low et al., 2003). Although there are some research into potential growth and health benefits of dietary nucleotides in aquaculture species (Burrells et al., 2001a, 2001b; Li et al., 2004; Cheng et al., 2011; Song et al., 2012) however, there are numerous gaps in existing knowledge about supplementation of nucleotides in diets of fish and its effects on physiology and immunity. Effects of nucleotide as potential immunostimulant as well as a functional nutrient of this species are not reported elsewhere. Information is not available on the NT

requirement and its effects on growth, immune responses and stress resistance of red sea bream. Therefore, a feeding study was conducted to determine effects of dietary mixture of five purified NT, i.e., inosine 5′ monophosphate (IMP), adenosine 5′ monophosphate (AMP), guanosine 5′ monophosphate (GMP), uridine 5′ monophosphate (UMP) and cytidine 5′ monophosphate (CMP) on growth performance, immune responses and stress resistance of juvenile red sea bream.

2. Materials and Methods

2.1. Test fish and experimental system

Juvenile red sea bream were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (54% crude protein and 10% crude lipid, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 100 l polycarbonate tanks (filled with 80 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 5.1.1and 5.1.2 summarize the basal diet formulation and chemical composition of the experimental diets respectively. Six casein based semi-purified diets were formulated to be nearly isonitrogenous (47% crude protein), isolipidic (12% crude lipid) and isocaloric

(20 KJ g^{-1} gross energy). The experiential diets were prepared by supplementing a mixture of purified nucleotide as disodium salts (Sigma Aldrich Co., St. Louis, MO, USA) of IMP, AMP, GMP, UMP and CMP at ratio of 1 : 1 : 1 : 1 : 1 to the basal diet at concentrations of 0.5, 1.0, 1.5 , 2.0 and 2.5 g mixed-NT kg^{-1} for diet-2 (D2), diet-3 (D3), diet-4 (D4), diet-5 (D5) and diet-6 (D6) respectively. Basal diet without NT supplementation was used as control (D1). The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 10 min. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (35–40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 55 °C for about 150 min. The test diets were stored at −28 °C in a freezer until use.

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Eighteen fish, having a mean initial body weight of approximately 2.6 g were randomly allocated to previously prepare eighteen tanks. Fish were fed the experimental diets for 56 days by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 1 hour after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk fortnightly to determine growth and visually check their health condition. The monitored water quality parameters (mean ± S.D.) were: water temperature

Table 5.1.1: Composition of basal diet for juvenile red sea bream.

Ingredients	Percent in diet
Fish meal[1]	28
Casein[2]	28
Soybean lecithin[3]	5
Pollack liver oil[4]	6
Dextrin[5]	7
ά starch[6]	6
Vitamin mixture[7]	3
Mineral mixture[8]	3
Stay-C[9]	0.06
Activated gluten[10]	5
ά cellulose	8.94
Nucleotide mixture[11]	0-0.25

[1]Nippon Suisan Co. Ltd., Tokyo, Japan.

[2]Waco Chemical Co., St. Louis, MO, USA.

[3] Riken Vitamin, Tokyo, Japan.

[4, 5] Kanto Chemical Co., Inc. Tokyo, Japan.

[6]Asahi Chemicals (Wakayama, Japan).

[7] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01; Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[8]Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[9]Stay-C 35.

[10]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS".

[11] Sigma Aldrich Co., St. Louis, MO, USA. (Nucleotide mixture added to diets at the expense of ά cellulose. Supplemented as disodium salts: inosine 5′ monophosphate (IMP), adenosine 5′ monophosphate (AMP), guanosine 5′ monophosphate (GMP), uridine 5′ monophosphate (UMP) and cytidine 5′ monophosphate (CMP)).

22.8±1.9 °C; pH 8.1±0.7 and salinity 34.5±0.5 during the feeding trial. These ranges are considered within optimal values for juvenile red sea bream.

2.4. Sample collection

At the end of the feeding trial, all experimental fish were fasted for 24 h. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then individual body weight of fish was measured, and the growth parameters were calculated accordingly. Nine fish per treatment (three fish per each tank) were randomly collected, washed with distilled water and then stored at −80 °C (MDF-U383, SANYO, Tokyo, Japan) for the whole body proximate analysis. Six fish were randomly sampled from each dietary replicate tank and their blood were collected by puncture of the caudal vain using heparinized (1600 IU/ml, Nacalai Tesque, Kyoto, Japan) disposable syringes and pooled. In addition non-heparinized disposable syringes were used to collect blood for serum analysis. Partial heparinized whole blood was used to analyze the hematocrit levels while plasma and serum were obtained by centrifugation at 3000 ×g for 15 min under 4 °C, and then stored at −80 °C until the analysis. Liver was dissected out from the fish above, weight individually to calculate the hepatosomatic index (HSI).

2.5. Biochemical analysis

The ingredients, diets and fish whole body were analyzed in triplicate, using standard methods (AOAC, 1995). The moisture was determined by drying the sample at 105 °C to constant weight. The ash was analyzed by combustion at 550 °C for 12 h. The crude protein content was determined by measuring the nitrogen content (N × 6.25) using the Kjeldahl method with a Tecator Kjeltec System (1007 Digestion System, 1002 Distilling unit, and Titration unit; FOSS Tecator AB, Högendäs, Sweden). Crude lipid content was estimated

using gravimetric method (954.02). Total serum protein and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma samples was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg. For the analysis of plasma cortisol, 100 µl plasma was mixed with 1 ml diethylether by using a vortex mixture and allow to separate the organic phase. The diethylether was evaporated under a gentle stream of nitrogen. The extract was then analyzed for cortisol concentration using an enzyme-linked immunosorbent assay (Cortisol EIA Kit, product number EA65, Oxford Biomedical Research Inc., Oxford, MI).

2.6. Evaluation of non specific immune parameters

Serum lysozyme activity was measured with turbidimetric assays (Takahashi et al., 1986). Ten microliters of samples was put into well of microplate, then added 190 µl of

Table 5.1.2: Chemical composition of the experimental diets (% dry matter basis).

Parameters	Diet group					
	D1	D2	D3	D4	D5	D6
Moisture	7.73	7.42	7.88	7.12	7.91	8.04
Crude protein	46.68	46.37	46.69	46.57	46.31	46.68
Crude lipid	12.03	12.37	12.30	12.16	12.96	12.78
Ash	8.89	8.88	9.08	9.13	8.90	9.11
Gross energy ($KJg-^{1}$)*	20.01	20.01	20.02	20.09	20.15	19.85

*Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ kg^{-1}, respectively.

Carbohydrate was calculated by the difference: 100-(protein + lipid + ash + moisture).

substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4,their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

The total peroxidase content in serum was measured according to Salinas et al. (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

The catalase (CAT) activity assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate that absorbs at 405 nm (Goth, 1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol/l phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate ($(NH_4)_6\ Mo_7O_{24}\ .4\ H_2O$) and the yellow complex of molybdate and hydrogen peroxide is measured at 405 nm against reagent blank. One unit CAT decomposes 1 µmole of hydrogen peroxide/l minute under assay conditions. CAT activities are expressed as kilo unit per liter (kU/l)

Serum bactericidal activity was performed according to Yamamoto and Iida (1995). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g/ml, *Escherichia coli*, IAM1239 cell line, Kagoshima, Japan) and incubated at 25 °C for 24 h in micro tube rotator (MTR-103, AS ONE, Osaka,

Japan). 50 ml of the reaction solutions were incubated on TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) at 25 °C for 24 h (on X-plate). Colony forming unit (CFU) was counted by the plate counting method as described by Ren et al. (2008). The bactericidal activity was defined as follows, (CFU of blank group-CFU of each group)/CFU of blank group × 100.

2.7. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, five fish from each rearing tank (total 15 fish per treatment) were randomly selected and transferred in to a blue colored 20 L rectangular glass aquarium with 15 L tap water which was aerated for 24 h. The glass aquaria for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of red sea bream juvenile was calculated according to Moe et al. (2004) as follows: time to death (min) of a juvenile was converted to log10 values. When the juvenile were transferred to the low salinity water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was log10 100 = 2). The calculation was then conducted every 5 minutes after the first exposure. The values of log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

$Y = aX + b$ where Y = log10 (survival), X = time for individual juvenile death (min).

LT_{50} (X) was obtained when Y = 1.7 since log10 (50) = 1.7. Each value obtained from the equation above was compared statistically.

2.8. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR, % day^{-1}) ={Ln (final weight) – Ln(initial weight)/duration} × 100

Survival (%) = 100 × (final no. of fish / initial no. of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed conversion ratio (FCR) = dry feed intake (g)/ live weight gain (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Condition factor (CF, %) = weight of fish / $(\text{length of fish})^3$ × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

2.9. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Turkey Kramer test.

3. Results

3.1. Fish performance parameters

Growth performance, nutrient utilization and survival of fish are presented in Table 5.1.3. Survival (%) was significantly ($P<0.05$) higher in D4 and it was not significantly differed ($P>0.05$) with fish fed diet D2 to D5. Fish fed with D4 showed the highest final weight, specific growth rate (SGR, %day^{-1}), weight gain (%) and it was not significantly differed with D3. No significant difference was also found in terms of feed conversion ratio and protein efficiency ratio between the treatments fed with diets D2 to D5. Significantly higher FI was found in fish fed diet D4 and it was not significantly differed with fish fed diet D1 to D5. All the growth and feed utilization parameters were significantly lower in D1 and D6.

Table 5.1.3: Growth performance and feed utilization in juvenile red sea bream fed test diets for 56 days.*

Parameters	Diet group					
	D1	D2	D3	D4	D5	D6
IBW [1]	2.62±0.01	2.62±0.01	2.61±0.00	2.62±0.00	2.62±0.01	2.62±0.00
FBW [2]	8.39± 0.12^{ab}	9.22±0.12^{bc}	9.90±0.15^{cd}	10.59±0.26^{d}	9.07±0.25^{bc}	7.84±0.22^{a}
WG [3]	220.1±4.9^{ab}	252.3±4.4^{bc}	279.1±5.9^{cd}	304.4±10.1^{d}	246.5±8.4^{bc}	199.7±8.0^{a}
SGR [4]	1.66±0.02^{ab}	1.80±0.02^{bc}	1.90±0.02^{cd}	1.99±0.04^{d}	1.78±0.04^{bc}	1.57±0.04^{a}
FI [5]	11.0±0.1^{ab}	11.1±0.6^{ab}	11.8±0.5^{ab}	12.2±0.1^{b}	11.2±0.2^{ab}	10.5±0.2^{a}
FCR[6]	2.24±0.05^{bc}	1.83±0.11^{a}	1.74±0.06^{a}	1.63±0.08^{a}	1.96±0.03^{ab}	2.49±0.09^{c}
PER[7]	0.96±0.02^{ab}	1.19±0.07^{bc}	1.37±0.10^{c}	1.33±0.60^{c}	1.10±0.02^{abc}	0.86±0.03^{a}
Sur[8]	75.9±1.9^{ab}	83.3±3.2^{bc}	83.3±0.0^{bc}	85.2±1.9^{c}	80.9±2.8^{abc}	72.2±0.0^{a}

[1] IBW: initial body weight (g); [2] FBW: final body weight (g); [3] WG: percent weight gain (%);[4] SGR: specific growth rate (% day^{-1}).

[5] FI: feed intake (g fish^{-1} 50 days^{-1}); [6] FCR: feed conversion ratio; [7] PER: protein efficiency ratio; [8] Sur: survival (%).

* Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

3.2. *Hematological parameters*

Table 5.1.4 represents the blood parameters of red sea bream after 8 weeks of feeding trial. Overall, dietary treatments had no significant effect on hematocrit and blood chemical parameters of fish among different treatments except for those of plasma glucose and GOT. Plasma glucose level was significantly lower in fish fed diet D4 and it was not significantly differed with other nucleotide supplemented group. Significantly higher plasma glucose level observed in fish fed nucleotide free control diet. Plasma GOT level was significantly lower in fish fed diet D5 and it was not significantly differed with fish fed diet D1 to D4. Nucleotide supplementation 0.25% (D6) showed significantly higher plasma GOT level. Plasma total bilirubin (T-bill) and GPT level showed decreasing trend numerically with the supplementation of nucleotide mixture up to 0.20% level (D5). Simultaneously fish fed diet containing NT mixture of 0.25% (D6) showed numerically increased GPT and T-bill value.

3.3. *Oxidative stress parameters*

Oxidative status of fish was analyzed from plasma and significant differences were not found in d-ROM and BAP values among the treatments. Fig. 5.1.1 indicates the status of oxidative stress conditions, in which the conditions of both stress intensity and tolerance ability were combined, of fish fed test diets for 56 days. Fish fed diet D5 was located in Zone A, where is categorized as lower intensity of oxidative stress and higher tolerance ability against oxidative stress. On the other hand, nucleotide supplementation free diet group D1 located in Zone D, where it's categorized as higher intensity of oxidative stress and lower tolerance ability against oxidative stress. Diet groups D4, D6 and D2, D3 located in zone B and C, respectively which were also acceptable condition. Numerically lower values (P>0.05) for plasma cortisol levels (%) were obtained in the nucleotide supplemented groups compared to nucleotide free control group (D1) (Table 5.1.4).

Table5.1. 4 : Hematological parameters in red sea bream fed test diet for 56 days.*

Parameters	Diet group					
	D1	D2	D3	D4	D5	D6
Hematocrit (%)	35.3±1.2	31.7±2.6	33.0±0.6	37.3±2.3	35.3±0.9	40.3±2.7
Glucose (g/dl)	83.5±1.5^{b}	69.0±9.0 ^{ab}	72.0±5.0^{ab}	56.5±6.5^{a}	58.0±8.0^{a}	68.0±4.0^{ab}
T-Cho (mg/dl)[1]	218.5±1.5	166.0±21.9	166.3±33.3	226.7±4.3	167.5±4.5	179.0±36.0
BUN (mg/dl)[2]	5.5±0.5	7.7±0.3	<5	<5	6.0±0.0	6.0±1.0
T- Bill (mg/dl)[3]	0.80±0.20	0.60±0.06	0.50±0.06	0.43±0.07	0.50±0.10	0.90±0.10
GOT (IU/L)[4]	124.5±49.5^{ab}	94.3±8.8^{ab}	102.7±9.5^{ab}	92.3±14.2^{ab}	75.0±17.0^{a}	145.5±16.5^{b}
GPT (IU/L)[5]	49.0±8.0	41.7±5.9	42.0±8.5	41.0±9.6	33.0±8.0	50.5±6.5
TG (mg/dl)[6]	185.5±29.5	200.7±25.6	175.7±24.4	229.0±1.0	134.5±30.5	127.0±23.0
CORT (%)[7]	28.0±0.1	27.2±0.3	26.4±0.5	26.9±0.6	27.9±0.2	27.3±0.4

[1] T-Cho: total cholesterol; [2] BUN: blood urea nitrogen; [3]T- Bill: Total bilirubin; [4]GOT: glutamyl oxaloacetic transaminase; [5]GPT: glutamic pyruvate transaminase; [6]TG: triglyceride;[7]CORT: relative value of cortisol.

*Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

3.4. Immunological assays

Non specific immune parameters *viz*. total serum protein (TSP), peroxidase activity, lysozyme activity, CAT activity and bactericidal activity are showing in Table 5.1.5. Nucleotide supplemented group showed numerically increased TSP value (P>0.05) compared to nucleotide supplementation free control group. Total peroxidase content also increased (P>0.05) with the supplementation of mixed NT upto 1.0 g kg^{-1} diet (D3) compared to control and then gradually decreased again. Significantly highest (P<0.05) CAT activity observed in supplementation free control group; intermediate in diet groups D2, D5 and D6; and the lowest in D3 and D4 diet groups. In the present study significantly higher bactericidal activity was also found in fish fed diet D3 and it was not significantly differed (P>0.05) with other nucleotide supplemented groups. Fish fed nucleotide supplementation free control diet showed significantly lower bactericidal activity. Serum lysozyme activity showed gradually increasing trend numerically with the supplementation of mixed NT upto 1.5 g kg^{-1} diet (D4) compared to control and then gradually decreased again. However, lysozyme activity was not influenced (P>0.05) by the dietary treatments in the present study.

3.5. Low salinity stress test

The results of lethal stress test against fresh water shock on LT_{50} obtained by regression analysis are shown in Fig. 5.1.2. LT_{50} showed increasing trend up to fish fed diet D4, from D5 LT_{50} showed again decreasing trend. Significantly higher (P<0.05) LT_{50} value obtained in D4 (27.39 min) and it was not significantly differed with other nucleotide supplemented groups. Significantly lower LT_{50} observed in nucleotide supplementation free control group.

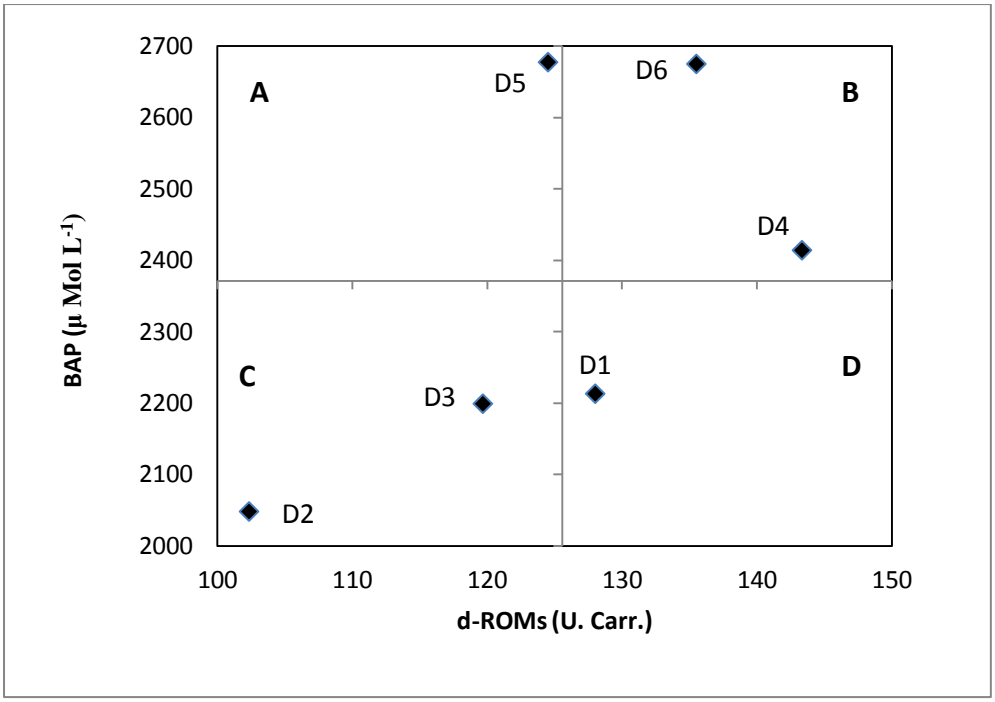


Fig. 5.1.1. Oxidative stress parameters in juvenile red sea bream fed test diets for 56 days. Values are means of triplicate groups. Zone A: high antioxidant potential and low reactive oxygen metabolites (good condition); Zone B: high antioxidant potential and high reactive oxygen metabolites (acceptable condition); Zone C: low antioxidant potential and low reactive oxygen metabolites (acceptable condition); Zone D: low antioxidant potential and high reactive oxygen metabolites (stressed condition).

Table 5.1.5 : Nonspecific immune parameters in red sea bream fed test diet for 56 days.*

Parameters	Diet group					
	D1	D2	D3	D4	D5	D6
TSP [1]	2.9±0.1	3.1±0.1	3.3±0.0	3.2±0.1	3.1±0.2	3.1±0.2
PA[2]	0.80±0.12	0.95±0.02	1.15±0.14	1.14±0.34	0.96±0.20	0.94±0.12
CAT[3]	96.4±6.1[c]	49.9±4.9[ab]	40.3±2.8[a]	42.1±7.8[a]	46.3±7.4[ab]	74.9±7.3[bc]
LA [4]	36.7±3.3	37.8±2.2	44.4±5.9	53.3±3.9	40.0±3.9	33.3±10.2
BA (%)[5]	45.7±2.9[a]	55.4±5.4[ab]	67.5±3.2[b]	60.7±3.6[ab]	57.1±1.4[ab]	61.8±2.5[ab]

[1]TSP: total serum protein (g/dL).

[2]PA: peroxidase activity (measured at OD 450 nm)

[3]CAT: catalase activity (KU/L)

[4]LA: lysozyme activity of serum (unit/ml).

[5]BA: bactericidal activity (%) = (CFU of blank group − CFU of each group)/CFU of blank group × 100.

*Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

3.6. Whole body proximate composition

The whole body proximate composition of juvenile red sea bream at the start and end of the feeding trial is shown in Table 5.1.6. All the fish showed a change in the analyzed parameters compared to those of the initial values. However, whole body proximate composition and somatic parameters were not influenced significantly ($P>0.05$) by the dietary treatments.

4. Discussion

Before 2000s nucleotides have been implicated as feed attractant rather than considering as potential functional nutrient for promoting growth and health benefits of the cultured aquatic organisms. Recently NT have received commercial and scientific interest as a functional nutrient in aquaculture (Burrells et al., 2001a,b; Li et al., 2004; Lin et al. 2009; Cheng et al., 2011; Song et al., 2012). However, information on potential growth and health benefit of NT supplementation of this species is lacking. To our knowledge, this study was the first study to investigate the effects of mixed NT as functional nutrient on different performances of red sea bream juveniles.

The obtained data showed that dietary supplementation of 1.5 g mixed-NT kg^{-1} (D4) showed higher growth performance and it was not significantly differed with fish fed diet 1.0 g mixed-NT kg^{-1} (D3). The results are in agreement with previous studies (Tahmasebi-Kohyani et al., 2011; Lin et al., 2009; Song et al., 2012). Growth performance reduced significantly with fish fed diet more than 1.5g mixed-NT kg^{-1} and it was significantly lower in fish fed diet D6 (2.5g mixed-NT kg^{-1}) which was not significantly different with control diet group . Reduced growth performance with excessive NT supplementation level was also observed in some previous studies for both fish and other terrestrial animal. For example, a commercial NT product, Ascogen (Chemoforma, Augst, Switzerland) at a high dietary

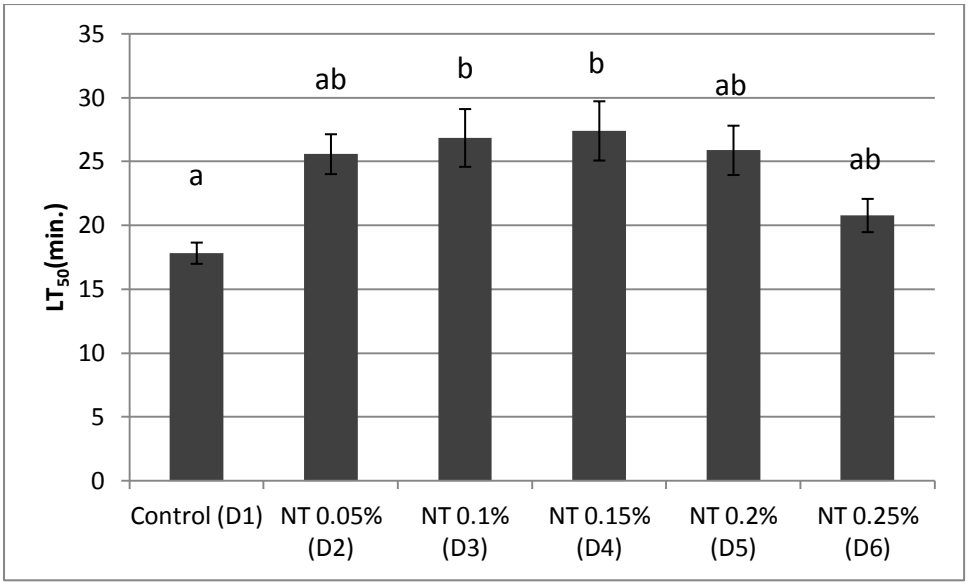


Fig.5.1.2. LT50 (min) calculated from the lethal time of red sea bream exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from triplicate groups. Values with different letters are significantly different (P<0.05).

Table 5.1.6: Whole body proximate analysis (% wet basis) and somatic parameters in juvenile red sea bream fed test diet for 56 days.*

Parameters	Initial[1]	Diet group					
		D1	D2	D3	D4	D5	D6
Moisture	81.3	76.4±1.0	77.31±1.01	77.69±0.27	77.27±0.67	77.00±0.18	76.85±0.77
Crude protein	10.7	13.6±0.4	13.5±0.5	13.1±0.1	13.0±0.2	13.1±0.2	13.3±0.4
Crude lipid	2.37	4.07±0.1	3.59±0.2	3.65±0.2	4.24±0.2	3.99±0.03	4.00±0.1
Crude ash	4.54	4.11±0.06	4.48±0.14	4.09±0.05	4.14±0.09	4.20±0.02	4.22±0.05
CF[2]	-	1.68±0.01	1.76±0.03	1.79±0.01	1.74±0.05	1.84±0.08	1.85±0.02
HSI[3]	-	1.56±0.1	1.18±0.1	1.19±0.1	1.46±0.1	1.38±0.2	1.47±0.1

[1] Initial values are not included in the statistical analysis.

[2]CF: condition factor (%).

[3]HSI: hepatosomatic index (%).

* Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different (P>0.05). Crude protein, crude lipid and ash are expressed on a wet weight basis.

concentration (5%) caused growth depression in rainbow trout, *O. mykiss* (Adamek et al., 1996); Similarly, a high dietary concentration of IMP (1.0%) resulted in depressed growth performance of olive flounder (*P. olivaceus*) compared to lower levels of dietary IMP (0.1 - 0.2%) in a 14 weeks growth study (Song et al., 2012). Adenine has been shown to be toxic to rats at concentrations in excess of 0.1% (Akintonwa et al., 1979) and to inhibit growth rate in chickens at dietary concentrations of 0.8% and above (Baker and Molitori, 1974). Reduced growth performance by high NT levels (>1. 5 g mixed-NT kg^{-1}) in the present study might be explained by the high serum uric acid from purine metabolism and associated toxicity, as well as adverse effects on the metabolism of other nutrients (Rumsey et al., 1992). In monogastric animals, high levels of dietary NT increased plasma uric acid and produced toxicological effects and disturbances on metabolism of protein, fat and carbohydrate (Kihlberg, 1972; Heaf and Davies, 1976). Subsequent studies, however suggested that salmonids might be able to metabolize relatively high levels of NT by virtue of their active liver uricase (Kinsella, 1985; Oliva-Teles and Goncalves, 2001). 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) for 8 weeks were not affected in terms of weight gain, feed intake, FER or survival (Welker et al., 2011). Red drum (*S. ocellatus*) fed diets supplemented with purified NT mixtures for 3 weeks were not also influenced with respect to weight gain and feed efficiency (Li et al., 2007). 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 (Li et al., 2007).

Haematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish. Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile

red sea beam, compared to those of the previous findings (Aoki et al., 1998; Kader and Koshio, 2012). Although, plasma glucose was significantly lower in fish fed diet D4 and it was not significantly different with other nucleotide supplemented groups. Nucleotide supplementation free control diet group showed significantly higher plasma glucose level. However, the glucose level (56.50–83.50 mg dl^{-1}) were comparable to the values (57.7–79.0 mg dl^{-1}) reported previously for juvenile red sea bream (Kader and Koshio, 2012). Plasma bilirubin gradually reduced with the supplementation of mixed NT up to 1.5 g kg^{-1} diet (D4) compared to control and then gradually increased again. GOT and GPT values also gradually reduced up to mixed NT of 2.0 g kg^{-1} diet (D5) and increased again in fish fed diet D6. Plasma bilirubin, GOT (or aspartate aminotransferase, AST) and GPT (or alanine aminotransferase, ALT) are often used for the evaluation of liver function as they are released into the blood during injury or damage to the liver cells (Lemaire et al., 1991). Higher levels of several blood parameters in fish fed diet D6 indicate that the kidneys may not have been functioning as well as they should be. However, the values of hematocrit, BUN and triglycerides, were not affected by the dietary treatments, and were found to be within values in the previous studies of juvenile red sea beam (Takagi et al., 2001; Kader and Koshio, 2012).

Plasma or serum cortisol concentration is a reliable biological indicator of stress response in fish and terrestrial animals (Li et al., 2009; Small and Davis, 2002). In the present study the relative values of plasma cortisol concentrations were decreased numerically compared with control with the supplementation of mixed NT but not at significant level. Reduced plasma cortisol level of red sea bream fed nucleotide supplemented diet in the present study are in agreement to what has been previously reported in rainbow trout (Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2012) and beluga sturgeon (*Huso huso*) Yousefi et al. (2011). Oxidative stress is considered to involve in plenty of diseases and

pathological status in fish (Martinez-Alvarez et al., 2005), and is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants (Celi et al., 2010). Simultaneous measurements of d-ROMs with BAP can provide a suitable tool for measuring the oxidative stress in humans, pig, rabbit and dog (Oriani et al., 2001; Ballerini et al., 2003; Pasquini et al., 2008). Recently, d-ROMs and BAP were reported to be reliable parameters for determining the oxidative stress condition of fish. As indicated in Fig. 5.1.1, it is interesting to note that D5 was located in zone A which represents the best condition with low oxidative stress and high antioxidant levels, while control group (D1) located in zone D exposed with higher oxidative stress. Fish fed D4, D6 and D2, D3 located in zone B and C respectively indicates comparatively lower oxidative stress than the control group. Although the mechanism that caused reduced oxidative stress for different NT supplementation level should be further studied.

Evidence from animal and human studies supports the theory that dietary nucleotides are important for optimal functioning of several components of the immune system (Cosgrove, 1998). Various peptides such as lysozymes, antibodies, complement factors and other lytic factors are present in serum acts as a first line defense to prevent adherence and colonization of microorganisms (Alexander and Ingram, 1992) leading to prevention of infection and disease. Many authors have reported the enhancement of the lysozyme activity, complement activity, total serum protein, and bactericidal activity (Saki et al., 2001; Li et al., 2004; Cheng et al., 2011; Tahmasebi-Kohyani et al., 2011; Song et al., 2012) due to administration of dietary nucleotides in several fish species. In the present study lysozyme activity tended to be increase with NT supplementation but not at significant level which is in agreement with findings of Li et al. (2004). In the present study *Escherichia coli* were used for determining the bactericidal activity of fish, and serum bactericidal activity was significantly higher in nucleotide supplemented group 1.0g kg^{-1} diet (D3) which was not

significantly different with other nucleotide supplemented groups. Bactericidal activity of present study is also an agreement with the findings of Welker et al. (2011). NT supplementation free control group showed significantly lower bactericidal activity. Increased bactericidal activity with the supplementation of mixed NT in diets regardless of the inclusion levels was possibly due to the absorption of NT into the serum and their capacity for killing *E. coli* in a dose-dependent manner or the other mixed NT effects on the immune factors of fish.

Proteins are the most important compounds in the serum and are essential for sustaining a healthy immune system (Kumar et al., 2005). In the present study there was a trend of increased total proteins with the supplementation of NT compared with control. The increased total protein in NT supplemented group indicates that dietary nucleotides may act as an immunostimulant. The probable immunostimulatory effect of dietary nucleotides in our study is in agreement with Jha et al. (2007) who reported the increase of serum total protein contents in *Catla catla* juveniles fed diet supplemented with 0.8% NT. Tahmasebi-Kohyani et al. (2012) also reported significantly increased total serum protein in rainbow trout (*O. mykiss*) fed nucleotide supplemented diet. Increasing peroxidase content with nucleotide supplementation in the present study was also the evident of immunostimulating properties of nucleotides. Salinas et al. (2008) also reported increased peroxidase content of Gilthead Seabream (*Sparus aurata)* fed immunostimulant (heat-inactivated bacteria) our present study also strongly support this finding. Increased CAT activity rates were attributed to elevated levels of exogenous hydrogen peroxide (H_2O_2) which is the main cellular precursor of the hydroxyl radical (HO^-), a highly reactive and toxic form of ROS . In human increased serum CAT activity is used as a diagnostic tool in acute pancreatitis (Goth et al., 1982), hemolytic disease (Goth et al., 1983) and some liver diseases (Goth et al., 1987). The high mean CAT activity observed in fish fed control diet compared to mixed NT supplemented

groups implies that fish in control group exposed to an oxidative stress and relatively poor immune condition compared to supplemented groups. Results of the CAT activity also strongly correlates with the results of BAP and dROMs measured in the present study which reconfirmed the positive supplemental effects of mixed NT in regards to best oxidative stress condition as well as immunity.

Dose–responses of dietary nucleotides on fish are scarcely studied. Sakai et al. (2001) reported a dose-dependent effect of exogenous nucleotides on macrophage phagocytic activity of common carp, which may suggest that excessive dietary nucleotides inhibited immune responses. In the present study non specific immune parameters responds in a dose dependent manner and showed best performance in fish fed diet supplemented with 1.0 to 1.5 g mixed NT kg^{-1} diet. Fish fed diet supplemented with excessive NT (>1.5 g mixed NT kg^{-1}) showed reduced immune performance. Findings of our present study also supports the findings of Burrells et al. (2001a) and Welker et al. (2011) who mentioned excessive levels of dietary nucleotides can also negatively affect immunity in fish.

Dietary nucleotide increases the resistance of fish to a variety of stressors is well documented (Burrells et al., 2001b; Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2012). In our study, fish fed mixed NT supplemented diet showed significant increase in stress resistance of red sea bream after the exposure of low salinity stress test. In the present study significantly higher LT_{50} value obtained in D4 (27.39 min) and it was not significantly different with other nucleotide supplemented group. Significantly lower value of LT_{50} obtained in control group (17.82 min) indicated a lower fresh water tolerance of the red sea bream. Increased ($P<0.05$) LT_{50} of red sea bream fed nucleotide supplemented diet indicates healthy status of red sea bream (Yokoyama et al., 2005). Increased LT_{50} due to nucleotide supplementation might be due to increasing osmoregulatory capacity of red sea bream which

support with the finding of Burrells et al. (2001b) where he mentioned that dietary nucleotide supplementation increased osmoregulatory competence of Atlantic salmon.

Whole-body composition of red sea bream was not significantly influenced by dietary nucleotides in the present study (Table 5.1.6). No significant effect of dietary nucleotides on red drum whole body and somatic parameters was also observed by Li et al. (2007) which similar our study. In another study Li et al. (2005) observed significantly increased whole-body lipid of red drum fed diet supplemented with dietary nucleotides (Optimun); however, the present study failed to confirm that response. Differences in the genetic background of different species and basal feed ingredients (fishmeal based basal diet versus casein and fishmeal based semi-purified basal diet) may have contributed to this inconsistency.

In conclusion, the results of this study show that significantly higher growth performances, survival (%) and feed utilization were observed in fish fed diet supplemented with 1.5 g NT kg^{-1} . Growth performance parameters were not differed significantly among 1.0 to 1.5 g mixed NT kg^{-1} . Feed conversion ratio, protein efficiency ratio and survival were not differed among fish fed diets supplemented with 0.5 to 2.0 g mixed NT kg^{-1}. Significantly lower growth and feed utilization parameters were observed in nucleotide free (control) and 2.5g NT kg^{-1} group. Fish fed nucleotide supplemented diets showed best oxidative stress condition and freshwater stress tolerance. Red sea bream fed a diet containing nucleotide showed an increase in the humoral immune response by increasing total serum protein, bactericidal activity, peroxidase activity and lysozyme activity. Considering overall performance of 56 days growth trial we therefore recommend dietary nucleotide administration at 1.0-1.5 g kg^{-1} to elevate the growth, humoral immunity and stress resistance of juvenile red sea bream (P. *major*).

CHAPTER VI

Development of low/non fishmeal based functional feed

Part-1

Inosine supplementation in low fishmeal based diets for juvenile amberjack S*eriola dumerili*: effects on growth, digestibility, immune response, stress resistance and gut morphology

Abstract

A 56 days feeding trial was conducted to determine the effects of dietary inosine in low fishmeal (FM) based diet and its effect on growth performance, digestibility, immune responses, stress resistance and gut morphology of juvenile amberjack. Seven isocaloric diets were formulated where diet 1 (D1) was FM based control diet. Diet 2 to 7 were formulated by replacing 25, 50 and 75 % of FM protein with extruded soybean meal (SBM) and 0.6% inosine supplementation in D2($FM25_{INO}$), D4 ($FM50_{INO}$) and D6($FM75_{INO}$) diets respectively; and without inosine supplementation in D3(FM25), D5(FM50) and D7(FM75) respectively. Triplicate groups of fish (24.9 g) were randomly stocked in 200L polycarbonate tanks at a rate of 24 fish per tank and fed to satiation twice daily. After feeding trial, final weight, specific growth rate (SGR, %/day) and weight gain (%) were not differed significantly ($P>0.05$) among the diet groups D1 to D4. Feed intake, feed conversion ratio followed the similar trends. All growth and feed utilization parameters significantly depressed from the diet group D5 and no difference was found among the rest (D5 to D7). The ADC's of dry matter, protein and lipid was significantly higher in D1 and it was not differed significantly with insoine supplemented groups. In case of oxidative stress parameters, fish fed inosine supplemented diets showed best condition. Stress resistance against low salinity exposure (LT_{50}), significantly increased with inosine supplementation and was highest in D4. Serum lysozyme activity, Alternative complement pathways (ACH_{50}), bactericidal activity tended to be higher ($P>0.05$) in inosine supplemented groups compared to the non supplemented groups. In terms of intestinal health, enterocyte height, fold height and microvillus height of the anterior and posterior enteric sections increased significantly in fish fed diets D1 to D4 and other inosine supplemented group regardless of the inclusion levels. Result of the study indicated that at least half of the fishmeal could be replaced with soybean meal and supplementation of inosine in the diets of juvenile amberjack. Moreover,

inosine supplementation in 50 to 75% FM replacement groups could be helpful to improve the digestibility, immune responses, stress resistance and intestinal health condition of fish.

Keywords: Inosine, low fishmeal based diet, immune responses, stress resistance, gut morphology, *Seriola dumerili*

Introduction

The amberjack, (*Seriola dumerili*), is a marine pelagic carnivorous species and has a great potential for the global aquaculture industry owing to its rapid-growing and adaptive characteristics. (Jover et al., 1999; Thompson et al., 1999; Mazzola et al., 2000). It is distributed throughout the tropical and subtropical seas except the Pacific Ocean. Now-a-days, aquaculture of this species is intensified in the Mediterranean region and Japan (Nakata, 2000; Takakuwa et al., 2006). In raising amberjack, most farmers use commercially manufactured feeds, which often contain high levels of fishmeal as dietary protein. Simultaneously worldwide increasing demand and unstable supply and high price has made FM a limiting factor of feed production in aquaculture industry. Use of alternative protein sources as FM substitute could overcome the limitations of feed production and could make aquaculture venture profitable (Hossain et al., 2015a).

Soybean proteins have been recognized as one of the most appropriate alternative protein sources for fishmeal in aquafeed because of their consistent nutritional composition, comparatively balanced amino acid profile, availability and reasonable price (Storebakken et al., 2000). Approximately 20 to 40% FM protein can be replaced by SBM protein in diets for carnivorous fish species without reducing growth performance or nutrient utilization, such as in black sea bream (20%) (*Acanthopagrus schlegelii*) (Zhou et al., 2011), European sea bass (25%) (*Dicentrarchus labrax*) (Tibaldi et al., 2006), turbot (25%) (*Scophthalmus maximus*) (Day and Plascencia-Gonzalez, 2000), parrot fish (20% or 30%, depending on the size of

fish) (Oplegnathus fasciatus) (Lim and Lee, 2009), Japanese seabass (30%) (Lateolabrax japonicus) (Li et al., 2011), Atlantic salmon (33%) (Salmo salar) (Carter and Hauler, 2000), Asian seabass (37.5%) (*Lates calcarifer*). It is also well documented that, High dietary SBM inclusion resulted lower feed intake, reduced weight gain, morphological changes of distal intestinal epithelium and abnormal health condition of fish (Kaushik et al., 1995; Krogdahl et al., 2003; Chou et al., 2004; Hernández et al., 2007; Deng et al., 2006; Li et al., 2011; Baeverfjord and Krogdahl, 1996; Burrells et al., 1999; Chen et al., 2011) due to containing several antinutritional factors and imbalance of amino acids in SBM. However, Supplementation strategies would improve the palatability and nutritional quality of the high plant protein based diets which would help to recover feed intake as well as the overall performance of fish. Recently, in aquaculture research supplementation of nucleotide or nucleoside as functional nutrients for potential growth and health benefit of aquatic species has been paid attention promisingly.

Nucleotides are low molecular weight intracellular compounds which play key roles in nearly all biochemical processes (Gill, 2002). A nucleotide consists of a nitrogenous base, a sugar (ribose or deoxyribose) and one to three phosphate groups. When phosphate group of nucleotide is removed by hydrolysis, the structure remaining is nucleoside. Inosine, widely used a purine nucleoside containing the base hypoxanthine and the sugar ribose, which occurs in transfer RNAs. Inosine is formed by the deamination of adenosine and it's considered as a functional nutrient (Hossain et al. 2016a). Dietary nucleotides or nucleosides supplementation have been shown to benefit many mammalian physiological and nutritional functions (Uauy, 1989; Quan, 1992; Carver, 1994; Haskó et al., 2000) and gained wide attention as potential immunomodulators. In aquatic animals dietary nucleotide has been reported to improve growth, feed intake, immune response, intestinal health condition (Hossain et al. 2016a). Whereas in both vertebrate and invertebrate aquatic species dietary nucleoside has long been

implicated as only feed attractants and very few is known about the supplemental effects of nucleoside on growth, immune response, stress resistance and intestinal morphology. Moreover, FM contained abundant nucleic acids and nucleotides, while little was found in SBM (Mateo et al., 2004). So supplementation of Nucleotides or nucleosides in high fish meal replaced SBM based diet could overcome the negative of high SBM based diet. However, there is no information is available on the supplemental effects of dietary individual nucleoside inosine in gradually replaced FM protein with soybean protein based diets and its effects on growth, immune response, stress resistance , digestibility and intestinal morphology of amberjack. Therefore, the purpose of the present study was to investigate the effect of dietary individual nucleoside, inosine supplementation in diets with gradually replaced FM protein with soybean protein on growth, immune responses, stress resistance, digestibility and intestinal morphology in juvenile amberjack.

2. Materials and methods

2.1. Test fish and experimental system

Juvenile amberjack were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (50% crude protein, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 200 l polycarbonate tanks (filled with 170 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 6.1.1, 6.1.2 and 6.1.3 summarize the formulation and chemical composition of the experimental diets. Seven diets were formulated to be nearly isonitrogenous (48% crude protein), isolipidic (13% crude lipid) and isocaloric (20 KJ g^{-1} gross energy) where diet 1 (D1) was FM based control diet. Diet 2 to 7 were formulated by replacing 25, 50 and 75 % of FM protein with extruded SBM and 0.6% inosine (Tokyo Chemical Industry Co., Ltd. Tokyo, Japan) supplementation in D2($FM25_{INO}$), D4 ($FM50_{INO}$) and D6($FM75_{INO}$) diets respectively; and without inosine supplementation in D3(FM25), D5(FM50) and D7(FM75) respectively. For proper mixing of inosine with other ingredients, initially inosine and weighted supplemented amino acids were mixed properly in a glass beaker by hands with the help of a stainless laboratory spatula. Then this mixtures were thoroughly mixed with all the dry ingredients in a food mixer for 10 min. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (35–40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 60 °C for about 120 min. The test diets were stored at −28 °C in a freezer until use.

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Twenty four fish, having a mean initial body weight of approximately 25 g were randomly allocated to

Table-6.1.1: Formulation and chemical composition of the experimental diets.

Ingredients	Diet groups						
	D1 (Control)	D2($FM25_{INO}$)	D3(FM25)	D4($FM50_{INO}$)	D5(FM50)	D6($FM75_{INO}$)	D7(FM75)
Fishmeal[a]	61.00	45.75	45.75	30.50	30.50	15.25	15.25
Soybean meal[b]	0.00	20.65	20.65	41.30	41.30	61.95	61.95
Pollack liver oil[c]	4.40	4.80	4.80	5.20	5.20	5.80	5.80
Soybean lecithin[d]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin mixture[e]	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Mineral mixture[f]	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Stay-C[g]	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Wheat flour	9.00	6.00	6.00	3.00	3.00	0.00	0.00
Activated gluten[h]	5.00	5.00	5.00	5.00	5.00	5.00	5.00
ȧ cellulose	11.55	7.61	8.21	4.27	4.87	0.74	1.34
FAA[i]	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Inosine[j]	0.00	0.60	0.00	0.60	0.00	0.60	0.00
Lysine[k]	0.00	0.33	0.33	0.67	0.67	1.01	1.01
Methonine[l]	0.00	0.17	0.17	0.34	0.34	0.51	0.51
Chemical composition (% dry matte basis)							
Crude Protein	49.40	49.30	49.0	48.8	48.6	48.7	48.5
Crude Lipid	13.27	13.69	13.23	13.25	12.73	12.44	12.9
Ash	12.75	11.68	11.51	10.31	10.45	9.06	9.10
Inosine (%)	0.14	0.67	0.10	0.63	0.10	0.60	0.04

[a]Nippon Suisan Co. Ltd., Tokyo, Japan

[b]Soyabean meal (dehulled soybean meal; J. Oil Mills, Japan)

[c] Riken Vitamin, Tokyo, Japan.

[d] Kanto Chemical Co., Inc. Tokyo, Japan

[e] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[f] Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[g]Stay-C 35.

[h]Glico Nutrition Company Ltd. Osaka, Japan. Commercial name “A-glu SS”.

[i]FAA: Free amino acids (alanine, betine and glycine); Nacalai Tesque, Kyoto, Japan

[j] Tokyo Chemical Industry Co., Ltd. Tokyo, Japan

[k,l]Nacalai Tesque, Kyoto, Japan

Table 6.1.2 : Total amino acid content of the experimental diets (g $100g^{-1}$).

Sample name	Diet groups						
	D1 (control)	D2($FM25_{INO}$)	D3(FM25)	D4($FM50_{INO}$)	D5(FM50)	D6($FM75_{INO}$)	D7(FM75)
Aspartic acid +Asparagine	3.96	4.10	4.15	4.32	4.20	4.37	4.37
Threonine	1.89	1.82	1.82	1.76	1.71	1.64	1.64
Serine	1.90	1.99	1.98	2.04	1.97	2.06	2.09
Glutamic acid +Glutamine	7.21	7.53	7.56	8.04	7.78	8.19	8.17
Glycine	2.96	2.91	2.77	2.74	2.52	2.46	2.30
Alanine	3.10	2.84	2.86	2.65	2.55	2.34	2.32
Valine	2.24	2.13	2.18	2.17	2.09	2.05	2.02
Isoleucine	1.90	1.86	1.92	1.97	1.88	1.92	1.88
Leucine	3.42	3.36	3.41	3.41	3.24	3.28	3.26
Tyrosine	1.52	1.52	1.55	1.56	1.49	1.56	1.56
Phenylalanine	1.94	1.99	2.02	2.09	2.01	2.13	2.11
Histidine	1.44	1.33	1.37	1.29	1.23	1.17	1.16
Lysine	3.28	3.25	3.32	3.34	3.27	3.26	3.26
Arginine	2.59	2.65	2.70	2.78	2.69	2.77	2.78
Proline	2.26	2.29	2.32	2.38	2.29	2.35	2.38
Cystine	0.47	0.51	0.50	0.54	0.51	0.53	0.55
Methionine	1.18	1.18	1.19	1.21	1.15	1.16	1.16
Tryptophan	0.608	0.606	0.602	0.596	0.591	0.590	0.590
Total AA	43.9	43.9	44.2	44.9	43.2	43.8	43.6

Values are means of triplicate measurements.

Table6.1.3: Free amino acid contents of the experimental diets (g $100g^{-1}$).

Free amino acids	Diet groups						
	D1 (control)	D2($FM25_{INO}$)	D3(FM25)	D4($FM50_{INO}$)	D5(FM50)	D6($FM75_{INO}$)	D7(FM75)
Tau	0.35	0.27	0.27	0.18	0.18	0.09	0.09
Aspartic acid	0.02	0.03	0.03	0.03	0.03	0.04	0.04
Threonine	0.04	0.04	0.04	0.03	0.03	0.02	0.02
Serine	0.02	0.02	0.02	0.01	0.01	0.01	0.01
Glutamic acid	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Glutamine	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glycine	0.34	0.35	0.36	0.34	0.35	0.35	0.35
Alanine	0.45	0.43	0.44	0.40	0.41	0.38	0.38
Valine	0.06	0.05	0.05	0.03	0.04	0.02	0.02
Cystine	0.00	0.01	0.01	0.02	0.02	0.03	0.03
Methionine	0.01	0.17	0.18	0.33	0.35	0.52	0.52
Isoleucine	0.04	0.03	0.03	0.02	0.02	0.01	0.01
Leucine	0.09	0.07	0.07	0.05	0.05	0.03	0.03
Tyrosine	0.03	0.02	0.02	0.02	0.02	0.02	0.02
Phenylalanine	0.05	0.04	0.04	0.03	0.03	0.02	0.02
Tryptophan	0.01	0.01	0.01	0.02	0.02	0.02	0.02
Lysine	0.07	0.31	0.32	0.56	0.62	0.80	0.80
Histidine	0.33	0.26	0.26	0.17	0.18	0.09	0.09
Arginine	0.06	0.07	0.07	0.07	0.08	0.08	0.08
Asparagine	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Proline	0.03	0.03	0.03	0.02	0.02	0.01	0.02
Total Free AA	2.09	2.29	2.33	2.43	2.55	2.62	2.63

Values are means of triplicate measurements.

previously prepare twenty one tanks. Fish were fed the experimental diets by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 30 min after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk at ten days interval to determine growth and visually check their health condition. The monitored water quality parameters were: water temperature 25.8±1.9 °C; pH 8.0±0.7 and salinity 33.9±0.5 during the feeding trial.

2.4. Sample collection and biochemical analysis

The initial sample of 10 fish for whole body analysis was stored at −20 °C. At the end of the feeding trial, fish were starved for 24 h prior to final sampling. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then the total number, individual body weight and length of fish from each tank were measured. Three fish from each replicate tank were randomly collected and stored at −20 °C for final whole body analysis. Using heparinized and nonheparinized syringes, blood was collected from the caudal vein for both plasma and serum of four fish in each replicate tank and pooled. A small fraction of the heparinized blood was used to analyze the hematocrit level. Plasma samples were obtained by centrifugation at 4000 × g for 15 min using a high-speed refrigerated microcentrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80 °C. Liver and viscera were dissected out from three fish in each replicate tank, weighed individually to get hepatosomatic index and viscerasomatic index. The ingredients, diets and fish whole body were analyzed for moisture, crude protein, crude lipid and ash, in triplicate, using standard methods (AOAC, 1995). Total amino acids (TAA) and free amino acids (FAA) and inosine content in diets were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) as described previously (Hossain et

al., 2016a). Plasma chemical parameters such as total protein, total albumin, total bilirubin, glucose, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002) . Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma sample was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg. For the analysis of plasma cortisol, 100 µl plasma was mixed with 1 ml diethylether by using a vortex mixture and allow to separate the organic phase. The diethylether was evaporated under a gentle stream of nitrogen. The extract was then analyzed for cortisol concentration using an enzyme-linked immunosorbent assay (Cortisol EIA Kit, product number EA65, Oxford Biomedical Research Inc., Oxford, MI).

2.5. Immunological assays

Serum lysozyme activity was measured with turbidimetric assays (Lygren et al., 1999). Ten microliters of samples was put into well of microplate, then added 190 µl of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4,their absorbance

were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

Serum bactericidal activity which was performed according to Yamamoto and Iida, (1995). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g/ml, *Escherichia coli*, IAM1239 cell line, Kagoshima, Japan) and incubated at 25 °C for 24 h in micro tube rotator (MTR-103, AS ONE, Osaka, Japan). 50 ml of the reaction solutions were incubated on TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) at 25 °C for 24 h (on X-plate). Colony forming unit (CFU) was counted by the plate counting method as described by Ren et al. (2008). The bactericidal activity was defined as follows, (CFU of blank group-CFU of each group)/CFU of blank group × 100.

The total peroxidase content in serum was measured according to Salinas *et al*., 2008, with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

The CAT activity assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate that absorbs at 405 nm Goth (1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol/l phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate

($(NH_4)_6$ Mo_7O_{24} .4 H_2O) and the yellow complex of molybdate and hydrogen peroxide is measured at 405 nm against reagent blank. One unit CAT decomposes 1 µmole of hydrogen peroxide/l minute under assay conditions. CAT activities are expressed as kilo unit per liter (kU/l)

Serum alternative complement pathway (ACP) activity was assayed according to Yano (1996). Briefly, a series of volumes of the diluted serum ranging from 0.1 to 0.25 ml were dispensed into test tubes and the total volume made up to 0.25 ml with barbitone buffer in the presence of ethyleneglycol-bis (2-aminoethoxy)-tetra acetic acid (EGTA) and Mg^{2+}, then 0.1 ml of rabbit red blood cells (RaRBC) was added to each tube. After incubation for 90 min at 20 ^{o}C, 3.15 ml 0.9% NaCl was added. Following this, the sample was centrifuged at 1600× g for 10 min at 4 ^{o}C to eliminate unlysed RaRBC. The optical density of the supernatant was measured at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added. The volume yielding 50% haemolysis was determined and in turn used for assaying the complement activity of the sample (ACH_{50} value = units/ml) for each experimental group.

2.6. Digestibility assessment

After finished the growth trial, the remaining fish from the triplicate groups of each treatment were distributed randomly into duplicate tanks. Test diets were prepared with the addition of 0.5% chromic oxide (Cr_2O_3) as inert marker to the previous diet formulation and fed to the fish under the same condition maintained for the feeding trial. After one week acclimatization with new diets, faeces were collected from rectum and lower part of the hindgut through sacrificing the fish. Collected faeces were stored at -20°C and subsequently freeze dried and dry matter, crude protein and lipid contents were determined as described above. Chromic

oxide content in faeces and diets were determined according to Furukawa and Tsukahara (1966).

2.7. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, six fish from each rearing tank (total 18 fish per treatment) were randomly selected and transferred in to a black colored 50 L polycarbonated tanks with 40 L tap water which was aerated for 24 h. The tanks for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of amberjack juvenile was calculated according to Hossain *et al*. (2016) as follows: time to death (min) of a juvenile was converted to log10 values. When the juvenile were transferred to the low salinity water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was log10 100 = 2). The calculation was then conducted every 5 minutes after the first exposure. The values of log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

$Y = aX + b$ where Y = log10 (survival), X = time for individual juvenile death (min).

LT_{50} (X) was obtained when Y = 1.7 since log10 (50) = 1.7. Each value obtained from the equation above was compared statistically.

2.8. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR, % day^{-1}) = {Ln (final weight) –Ln (initial weight)/duration}× 100

Survival (%) = 100 × (final no of fish / initial no of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed conversion ratio (FCR) = dry feed intake (g)/ live weight gain (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Apparent digestibility coefficient (ADC, %) = 100 – (100 × (% Cr_2O_3 in diet/% Cr_2O_3 in faeces) × (% nutrient in faeces/% nutrient in diet))

Condition factor (CF, %) = weight of fish / $(\text{length of fish})^3$ × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

Viscerasomatic Index (VSI, %) = weight of viscera/weight of fish × 100

2.9. Gut morphology study

After feeding trial two fish from each replicate tank was used for gut morphology study. For preparation of intestinal sample firstly whole gastrointestinal tract was removed and anterior and posterior intestine was collected. Then segments (0.5-1 cm) of each part was cut out and the lumen was flushed with saline (Otsuka Normal Saline, Otsuka Pharmaceutical, Tokyo) followed by formalin fixative (10% -Formaldehyde Neutral Buffer Solution 37152-5-1, Nacalai Tesque, Kyoto) to remove intestinal content. Then each tissue was placed in plastic bag (HistoPack, Falma, Tokyo) filled with formalin fixative. Each tissue was embedded in paraffin, sliced, and stained with hematoxylin and eosine (H&E). Two cross-sectional slices were prepared from each tissue. The tissue slides were examined under a light microscope (Eclipse 50i, Nikon, Tokyo) and a camera (Digital Sight DS2MV with control unit DS-L2, Nikon, Tokyo) interfaced with Sigma Scan Pro 5 software (SPSS Inc.,IL,USA). Intestinal fold height (hF), enterocyte height (hE), microvillous height (hMV) were measured with a magnification of 100x , 200x and 400x respectively. For each tissue, 10 measurements were performed.

2.10. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Tukey Kramer test.

Results

3.1. Fish performance parameters

At the end of feeding trial, fish fed diet group with $FM50_{INO}$ (D4) showed the highest final weight, SGR, weight gain (%)and it was not differed significantly (P>0.05) among the fish meal based control diet groups (D1) and diet groups $FM25_{INO}$(D2) and FM25(D3) (Table 6.1.4). Significantly lower growth performance observed in 75 % FM replaced groups ($FM75_{INO}$, FM75) and it was not differed significantly with FM50 (D5) diet group. Feed intake, feed conversion ratio followed the similar trends. Protein efficiency ratio was significantly higher in FM based control diet group and it was not differed significantly with diet group D1 to D4 and D6. All growth and feed utilization parameters significantly depressed from the diet group FM50 (D5) and no difference was found among the rest (D5 to D7). During the experimental period, the survival rates of fish fed different experimental diets ranged from 79.17 to 93.06%. Numerically higher survival observed in each graded FM replacement group supplemented with inosine compared with non supplemented groups. Numerically lower survival was observed in diet group FM75 (D7).

3.2. Hematological parameters

Table 6.1.5 represents the blood parameters of juvenile amberjack after 56 days of feeding trial. Although wide variations were observed on some of the parameters, no significant alteration was identified among the treatments except for those of hematocrit, total cholesterol, BUN and Amylase activity. Hematocrit content was significantly higher in diet group $FM25_{INO}$ (D2) and it was not differed significantly among diet D1 to D6. Inosine supplemented group showed relatively higher hematocrit values compared to non supplemented one. Interestingly significantly higher total cholesterol level was observed in FM based control diet group (D1) and it was not differed significantly with other non inosine supplemented diet groups (D3, D5 and D7). Lower cholesterol values were observed fish fed inosine supplemented diet compared to non supplemented one. Significantly lower BUN value was observed in D1 and it was not differed significantly among the diet groups D1 to D4. Significantly increasing BUN content observed non inosine supplemented 50% FM replaced group (D5) to D7. Amylase activity did not affect significantly up to 50% FM replacement level. Numerically lower TG and glucose value were observed in FM based control diet group and showing increasing trend with the increasing level of FM replacement level.

3.3. Stress resistance parameters

3.3.1 Oxidative stress parameters

Oxidative status, the combined effects pattern of d-ROMs and BAP, of juvenile amberjack fed with test diets were illustrated in Fig. 6.1.1.Fish fed with diets Control (D1), $FM50_{INO}$ (D4) were located in zone A, meanwhile Fish fed with diet group $FM25_{INO}$ (D2) was in zone B; FM25(D3), $FM75_{INO}$ (D6) in zone C and Zone D was occupied by fish fed with Diet groups FM50 (D5) and FM75(D7). Numerically lower values (P>0.05) for plasma cortisol

Table 6.1.4: Growth performance and feed utilization of juvenile amberjack fed test diets for 56 days*.

Parameters	Diet groups						
	D1 (control)	D2($FM25_{INO}$)	D3(FM25)	D4($FM50_{INO}$)	D5(FM50)	D6($FM75_{INO}$)	D7(FM75)
IBW[a]	24.98±0.01	24.99±0.04	24.98±0.00	24.98±0.03	24.98±0.03	24.98±0.02	24.98±0.01
FBW[b]	142.06±4.22[b]	143.58±5.42[b]	141.85±1.51[b]	143.81±6.43[b]	110.64±8.99[a]	109.05±5.73[a]	100.28±3.18a
WG[c]	468.67±16.67[b]	474.60±21.88[b]	467.77±6.04[b]	475.64±25.90[b]	341.54±35.50[a]	336.31±22.69[a]	301.33±12.62[a]
SGR[d]	3.10±0.05[b]	3.12±0.07[b]	3.10±0.02[b]	3.12±0.8[b]	2.64±0.14[a]	2.63±0.10[a]	2.48±0.06[a]
Sur[e]	91.67±2.41	93.06±1.39	87.50±4.17	87.50±2.41	84.72±1.39	90.28±5.55	79.17±4.81
FI[f]	134.68±2.78[bc]	141.74±4.14[c]	139.64±1.51[c]	141.80±3.64[c]	118.79±6.54[ab]	119.77±3.63[ab]	101.38±2.71[a]
FCR[g]	1.18±0.02[a]	1.21±0.02[a]	1.23±0.04[ab]	1.24±0.05[ab]	1.48±0.08[bc]	1.48±0.06[bc]	1.49±0.07[c]
FCE[h]	0.85±0.02[b]	0.83±0.01[b]	0.81±0.03[b]	0.81±0.03[b]	0.68±0.04[a]	0.68±0.03[a]	0.67±0.04[a]
PER[i]	1.71±0.04[b]	1.65±0.02[ab]	1.66±0.06[ab]	1.66±0.06[ab]	1.40±0.08[a]	1.44±0.07[ab]	1.42±0.06[a]

[a] IBW: initial body weight (g);[b] FBW: final body weight (g);[c] WG: percent weight gain (%); [d] SGR: specific growth rate (% day^{-1}); [e] Sur: survival (%); [f] FI: feed intake (g $fish^{-1}$ 50 $days^{-1}$);[g]FCR: feed conversion ratio; [h]FCR: feed conversion efficiency; [i] PER: protein efficiency ratio.

* Values are means of triplicate groups±S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

Table 6.1.5: Hematological parameters in juvenile amberjack fed test diet for 56 days.[*]

Parameters	Diet group						
	D1(control)	D2($FM25_{INO}$)	D3(FM25)	D4($FM50_{INO}$)	D5(FM50)	D6($FM75_{INO}$)	D7(FM75)
Hematocrit (%)	40.33±1.45^{ab}	44.67±1.76^{b}	38.00±1.00^{ab}	43.50±1.50^{ab}	40.00±1.53^{ab}	39.33±2.19^{ab}	36.33±1.45^{a}
Glucose (g/dl)	94.00±4.04	119.00±17.21	105.00±16.00	99.00±5.00	111.00±13.50	128.33±6.01	216.67±58.36
T-Cho (mg/dl)[a]	270.0±17.21^{b}	173.3±12.81 ^{a}	288.5±26.5^{b}	166.0±10.0 ^{a}	206.7±27.4^{ab}	160.3±13.0 ^{a}	196.0±11.6 ^{ab}
BUN (mg/dl)[b]	10.67±0.88^{a}	12.67±1.76^{a}	9.00±0.00^{a}	15.50±1.50^{ab}	23.00±1.53^{bc}	27.00±2.65^{c}	23.33±1.45^{bc}
T- Bill (mg/dl)[c]	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
GOT (IU/l)[d]	17.0±7.0	20.67±7.45	<10	<10	22.67±5.46	12.0±2.0	22.0±3.0
GPT(IU/L)[e]	<10	<10	<10	<10	<10	<10	<10
TG (mg/dl)[f]	61.67±15.62	118.33±18.66	116.50±63.50	113.00±13.00	81.00±26.31	110.33±10.41	145.00±16.86
Amylase	82.00±30.24^{ab}	91.00±22.81b^{a}	159.00±17.00^{b}	85.50±6.50^{ab}	78.00±19.00^{ab}	39.67±8.74^{a}	47.00±13.11 ^{a}
CORT (%)[g]	58.35±0.28	60.28±0.10	59.91±0.45	59.25±0.11	59.23±1.40	60.70±0.93	62.12±0.49

[a] T-Cho: total cholesterol; [b] BUN: blood urea nitrogen; [c]T- Bill: Total bilirubin; [d]GOT: glutamyl oxaloacetic transaminase; [e]GPT: glutamic pyruvate transaminase; [f] TG: triglyceride; [g]CORT: relative value of cortisol.

[*]Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

levels (%) were obtained in FM based control group and inosine supplemented groups compared to non supplemented groups at each replacement level.

3.3.2 Fresh water stress test

The results of lethal stress test against fresh water shock on LT_{50} obtained by regression analysis are shown in Fig. 6.1.2. Significantly higher LT_{50} values were observed in fish fed diet $FM50_{INO}$ (D4) and it was not differed significantly among the diet groups of D1 to D4 and D6. Significantly lower LT_{50} values observed in diet group FM75 (D7).

3.4. Immunological assays

Non specific immune responses of juvenile amberjack in the present study are presented in Table 6.1.6. Significantly higher TSP and BA were found in fish fed $FM25_{INO}$ diet group. Meanwhile, peroxidase activity (PA) was also significantly higher in fish fed diet group $FM25_{INO}$ and it was not significantly different with control diet group (D1). Catalase activity, LA and ACH_{50} were also positively influenced ($P>0.05$) by dietary inosine supplementation. Simultaneously it is interesting to note that all the immune parameters in inosine supplemented groups showed improved performance compare to non supplemented groups at each replacement level. Diet group FM75 (D7) showed significantly lowest non specific immune responses.

3.5. Whole body proximate composition

The whole body proximate composition of juvenile amberjack at the start and end of the feeding trial is shown in Table 6.1.7. All the fish showed a change in the analyzed parameters compared to those of the initial values. Whole body proximate composition and somatic parameters were not influenced ($P>0.05$) by the dietary treatments except in condition factor

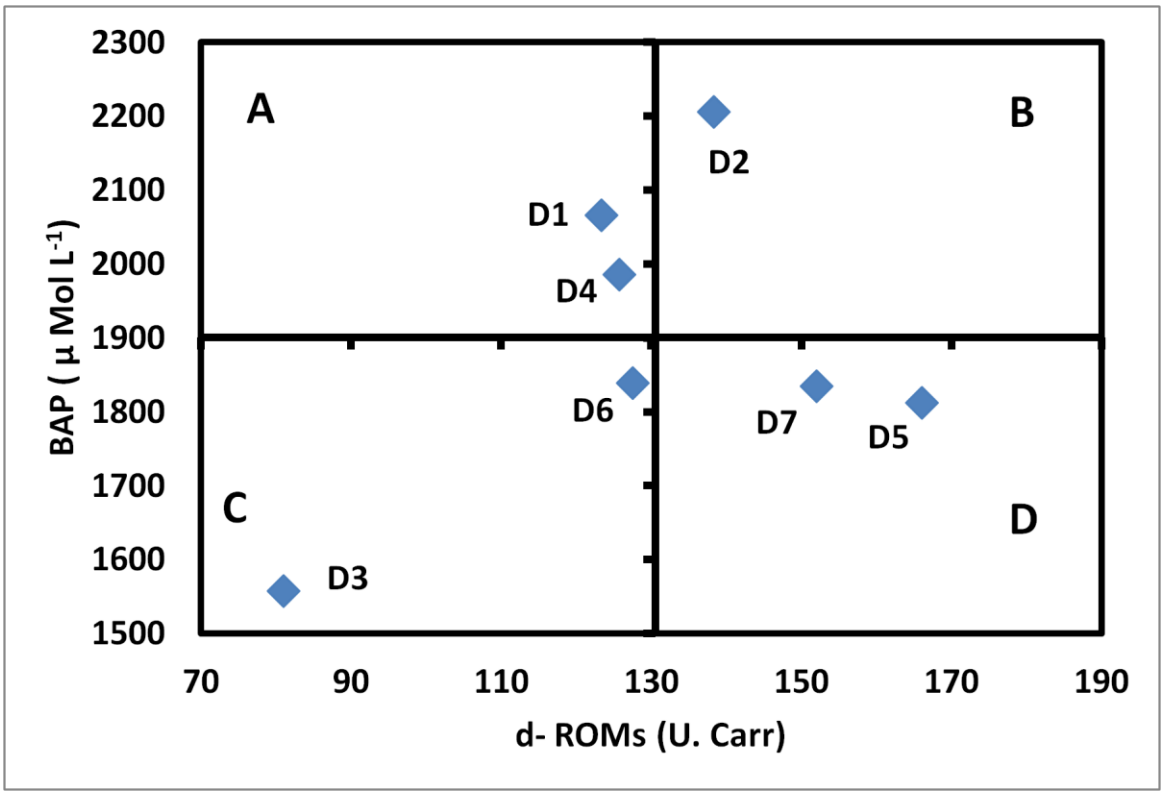


Fig. 6.1.1: Oxidative stress parameters in juvenile amberjack (*S. dumerili*) fed test diets for 56 days. Values are means of triplicate groups. Zone A: High antioxidant potential and low reactive oxygen metabolites (good condition). Zone B: High antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone C: Low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone D: Low antioxidant potential and high reactive oxygen metabolites (stressed condition)

(CF) where, significantly highest CF value observed in fish fed FM based control diet. Numerically higher carcass protein and lipid content was observed in inosine supplemented group compared to non supplemented groups in each graded replacement level.

3.6. Digestibility assessment

The ADC of dry matter, crude protein and lipid are presented in Table 6.1.8. Inosine supplementation significantly affects the digestibility of the juvenile amberjack. ADCs of dry matter and lipid was significantly higher in fish fed diet D4 and it was not differed significantly with fish fed diet D1, D2, D3 and D6. Protein digestibility was significantly higher in fish meal based control diet group (D1) and it was not differed significantly with D2, D3, D4 and D6. Significantly lower ADCs of dry matter, protein and lipid were observed in non inosine supplemented 75% FM replaced group (D7).

3.7. Gut morphology

Table 6.1.9 shows the micromorphology of the intestine of juvenile amberjack fed different experimental diets for 56 days. In terms of intestinal health condition, significantly higher anterior fold height (hF) observed in D2 and it was not differed significantly among diet groups of D2,D3,D4 and D6. Meanwhile significantly higher enterocyte height (hE) observed in D6 and it was not differed among diet groups of D1 to D6. Posterior hE and microvillus height (hMV) increased significantly in diet group D6 and D1 respectively and no significant difference were observed among diet groups D1 to D6. Fish fed diet group FM75 (D7) showed significantly lower intestinal micromorphology values of amberjack. Although anterior hMV and posterior hF were not affected significantly by the different diet groups but numerically increased values were observed in insoine supplemented groups compared to non supplemented one.

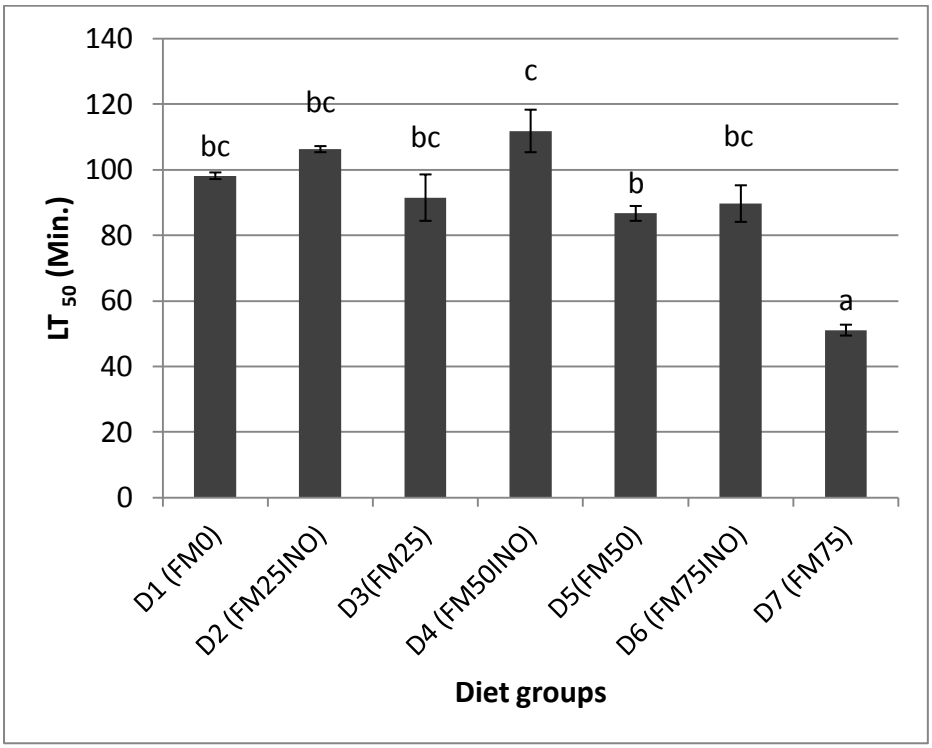


Fig.6.1.2. LT_{50} (min) calculated from the lethal time of juvenile amberjack exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from triplicate groups. Values with different letters are significantly different (P<0.05).

Table 6.1.6: Non specific immune responses of juvenile amberjack fed test diet for 56 days.[*]

Parameters	Diet group						
	D1(Control)	D2($FM25_{INO}$)	D3(FM25)	D4($FM50_{INO}$)	D5(FM50)	D6($FM75_{INO}$)	D7(FM75)
TSP[a]	2.8±0.12[ab]	3.5±0.03[b]	2.75±0.05[ab]	3.15±0. 15[ab]	3.40±0.10[ab]	3.45±0.25[ab]	2.63±0.12[a]
LA[b]	93.33±3.33	100.00±20.0	85.00±5.0	90.00±0.00	76.67± 8.82	86.67±8.82	76.67±3.33
BA[c]	55.36±5.36[ab]	67.50±3.21[b]	60.72±3.58[ab]	57.14±1.43[ab]	54.30±1.98[ab]	64.79±2.50[ab]	45.72±2.89[a]
PA[d]	1.57±0.11[b]	1.51±0.08[ab]	1.44±0.03[ab]	1.70±0. 07[b]	1.18±0.01[a]	1.37±0.11[ab]	1.14±0.10[a]
ACH_{50}[e]	67.57±20.01	69.37±11.39	61.42±1.49	66.75±7.19	53.16±3.75	55.96±6.06	50.95±1.17
CAT[f]	66.07±21.03	50.75±3.11	55.59±5.89	39.58±10.0	50.25±1.68	42.07±10.06	75.01±19.54

[a] TSP: total serum protein

[b]LA: lysozyme activity

[c]Bactericidal activity (%) = (CFU of blank group − CFU of each group)/CFU of blank group × 100.

[d]PA: Peroxidase activity(measured at OD of 450nm).

[e]ACH_{50}: Alternative complement pathways (unit ml^{-1})

[f]CAT: Catalase activity (kU/l)

[*]Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

Table 6.1.7: Whole body proximate analysis (% wet basis) and somatic parameters in juvenile amberjack fed test diets for 56 days.*

Parameters	Initial[1]	Diet group						
		D1(FM0)	D2($FM25_{INO}$)	D3(FM25)	D4($FM50_{INO}$)	D5(FM50)	D6($FM75_{INO}$)	D7(FM75)
Moisture	82.58	76.73±0.28	76.42±0.80	77.08±0.11	76.65±1.25	77.07±0.42	77.08±0.40	78.52±0.59
Crude protein	12.06	15.29±0.13	15.31±0.20	15.07±0.14	15.44±1.09	15.37±0.50	15.09±0.21	14.15±0.23
Crude lipid	0.70	4.70±0.08	4.91±0.0.62	4.76±0.16	4.82±0.33	4.10±0.02	4.34±0.22	3.77±0.69
Crude ash	3.23	2.58±0.02	2.58±0.2	2.46±0.02	2.59±0.18	2.73±0.09	2.56±0.09	2.59±0.22
CF[2]	-	$1.55±0.03^{b}$	$1.45±0.04^{ab}$	$1.46±0.01^{ab}$	$1.46±0.04^{ab}$	$1.41±0.02^{ab}$	$1.37±0.04^{a}$	$1.34±0.04^{a}$
HSI[3]	-	1.13±0.10	1.21±0.10	1.15±0.08	1.08±0.06	1.13±0.02	1.04±0.03	1.07±0.03

[1] Initial values are not included in the statistical analysis.

[2]CF: condition factor (%).

[3]HSI: hepatosomatic index (%).

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

Table 6.1.8: Digestibility of juvenile amberjack fed different experimental diets for 56 days*.

Parameters	Diet groups						
	D1(Control)	D2($FM25_{INO}$)	D3(FM25)	D4($FM50_{INO}$)	D5(FM50)	D6($FM75_{INO}$)	D7(FM75)
ADC[a]	61.95±0.9[b]	59.78±1.6[ab]	56.76±2.5[ab]	62.94±1.2[b]	52.25±1.9[a]	56.75±1.9[ab]	52.60±0.7[a]
Protein digestibility (%)[b]	83.35±0.5[c]	81.26±1.3[abc]	79.59±0.7[abc]	82.66±0.7[bc]	75.82±0.9[ab]	81.56±2.0[abc]	74.99±1.8[a]
Lipid digestibility (%)[c]	85.25±0.6[b]	83.13±2.0[b]	81.19±0.3[ab]	87.04±0.2[b]	75.05±0.7[a]	82.08±0.2[ab]	75.57±2.6[a]

[a]Apparent dry matter digestibility = $100 - 100\ \left(\frac{\%\ \text{marker in diet}}{\%\ \text{marker in faeces}}\right)$

[b]Protein digestibility (%) = $100 - 100\ \left(\frac{\%\ \text{marker in diet}}{\%\ \text{marker in faeces}} \times \frac{\%\ \text{protein in faeces}}{\%\ \text{protein in diet}}\right)$

[c]Lipid digestibility (%) = $100 - 100\ \left(\frac{\%\ \text{marker in diet}}{\%\ \text{marker in faeces}} \times \frac{\%\ \text{lipid in faeces}}{\%\ \text{lipid in diet}}\right)$

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

Table 6.1.9: Micromorphology of the intestine of juvenile amberjack fed different experimental diets for 56 days.*

Parameters	Diet group						
	D1(Control)	D2($FM25_{INO}$)	D3(FM25)	D4($FM50_{INO}$)	D5(FM50)	D6($FM75_{INO}$)	D7(FM75)
Anterior intestine							
hF(μm)[a]	755.9±44.6^{ab}	934.4±19.4^{c}	900.2±9.2^{bc}	906.5±25.8^{bc}	675.2±39.9^{ab}	810.4±56.4^{abc}	721.6±25.2^{a}
hE (μm)[b]	24.0±0.8^{ab}	24.3±0.9^{ab}	23.4±0.5^{ab}	25.8±0.9^{ab}	24.1±0.4^{ab}	26.1±0.8^{b}	22.7±0.4^{a}
hMV(μm)[c]	1.50±0.03	1.49±0.04	1.50±0.02	1.48±0.02	1.40±0.02	1.51±0.03	1.41±0.02
Posterior intestine							
hF(μm)	601.6±31.6	698.4±53.2	672.3±49.9	719.2±52.0	689.4±55.3	717.9±47.6	677.5±28.6
hE (μm)	24.2±0.7^{ab}	23.9±0.2^{ab}	22.6±0.6^{ab}	24.8±0.4^{ab}	23.4±0.5^{ab}	24.9±0.7^{b}	22.3±0.3^{a}
hMV(μm)	1.66±0.05^{b}	1.61±0.05^{ab}	1.58±0.05^{ab}	1.48±0.02^{ab}	1.44±0.08^{ab}	1.50±0.07^{ab}	1.41±0.02^{a}

[a] hF = fold height.

[b] hE = enterocyte height.

[c] hMV= microvillus height.

* Values are means of two fish from each of three replicate groups (10 measurements for each fish). Values in a row that do not have the same superscript are significantly different ($P < 0.05$).

4. Discussion

Dietary nucleotides received wide attention as potential immunomodulator. Latterly, several studies have indicated improved health, disease resistance and growth in a variety of aquaculture species when mixtures of nucleotides are included in the diet. (Burrells et al., 2001a, 2001b; Li et al., 2004, 2007, 2009; Hossain et al. 2016b). To date, most studies on potential benefits of nucleotides in aquaculture have focused on mixed nucleotides rather than individual nucleotide or nucleoside. In addition, little knowledge was found on nucleoside, inosine supplementation in low FM based diet and its effects on growth, digestibility, immune response, stress resistance and gut morphology of juvenile amberjack. Therefore the present study has got immense importance in the field of immunonutrition as well as development of functional aquafeeds.

In the present study juvenile amberjack can tolerate upto 50% of SMB protein when nucleoside insoine is supplemented along with methionine and and lysine where as only methionine and lysine supplement can tolerate 25% replacement level. Yellotail is a sensitive species to accept SBM protein in their diet. In previous study it was found that only 20-30 of the SBM protein accepted by yellowtail without compromising growth (Tomas et al 2005 Shimeno et al. 1992; Viyakarn et al. 1992; Watanabe et al. 1992). The increasing acceptance of FM replaced SBM based diet by juvenile amberjack in the present study was due to supplementation of functional nutrients inosine, which has both growth and health promoting capacity as reported in our previous study (Hossain et al. 2016a). Person-Le Ruyet et al. (1983) reported dietary inosine enhanced growth of turbot larvae. Peng et al. (2013) reported no significant growth reduction of juvenile turbot fed diet replaced upto 40% FM protein with SBM protein supplemented with mixed nucleotide and amino acids. Juvenile red drum and Japanese flounder shown to tolerate replacement of protein from FM with defatted soybean meal upto 50 % (Reigh and Eills, 1992) and 45% (kikuchi, 1999) respectively. Our present

study is consistent with the above findings. The variation of tolerance to soybean protein is species specific and this is also closely associated with SBM types, presence of antinutrients and amino acid imbalance of SBM. Significantly lower growth performance was observed in 75% FM replaced (FM75) group and it was not differed significantly with diet groups FM50 and $FM75_{INO}$). Significantly lower growth performance was associated with the reduced feed intake which also consistent with the findings of Bureau et al., 1998; Chen et al., 2011). Where they also stated that suppression of feed intake could be the main reason for reducing growth performance when FM was replaced by SBM.

In the present study significantly higher FI observed in fish fed diet group $FM50_{INO}$ and it was not differed significantly with D, D2 and D3. Feed intake significantly reduced when fish fed more than 50 % replaced FM protein with SBM without inosine supplementation was presumably because of changes in palatability (Davis et al., 1995; Meilahn et al., 1996; Reigh and Ellis, 1992) as a consequence of the replacement of FM by SBM. Addition of palatability enhancers is an effective approach when developing diets containing high plant protein in order to maintain feed attractiveness and induce adequate feed consumption rate by fish (Kissil et al., 2000; Papatryphon and Soares, 2000). Improved FI was reported with the supplementation of inosine in red sea bream fish (Hossain et al., 2016a) fed semi purified diet. chemo-attractive and feeding stimulatory effect is well documented for certain nucleotide and nucleoside (Li and Gatlin 2006). Mackie (1973) first analyzed the low-molecular weight fraction of squid and hypothesized nucleotide (AMP) and nucleoside (inosine) components as the main chemo-attractants for aquatic animals such as the lobster. inosine and inosine-5-monophosphate have been reported as specific feeding stimulants for turbot fry, *Scohthalmus maximus* (Mackie and Adron, 1978), Dover sole, *Solea solea* (with betaine and glycine; Metailler et al., 1983), and yellowtail, *Seriola quinqueradiata* (Takeda et al., 1984).

Evidence from animal and human studies supports the theory that dietary nucleotides are important for optimal functioning of several components of the immune system (Cosgrove, 1998). In the present study numerically higher lysozyme activity was found in fish fed diet group $FM25_{INO.}$ Meanwhile, inosine supplemented groups showed higher LA ($P>0.05$) compared to non supplemented groups of different replacement level. The increased serum lysozyme activity of inosine supplemented groups were due to immunostimulating properties of inosine which may be acted on due to either an increase in the number of phagocytes secreting lysozyme or due to an increase in the amount of lysozyme synthesized per cell (Engstad et al., 1992; Kumari and Sahoo, 2006; Hossain et al. 20116a,b). Increased lysozyme activity due to nucleotide and nucleoside (inosine) supplementation were also reported for common carp (*Cyprinus carpio*) (Sakai et al., 2001), raibow trout (Tahmasebi-Kohyani et al., 2011) and japanese flounder (Song et al., 2012) and red sea bream (Hossain et al., 2016a).

The anti-bacterial activity of blood serum is considered as a nonspecific response to inhibit the growth of infectious microorganisms (Yano, 1996). In the present study *E. coli* were used for determining the bactericidal activity of amberjack. Serum bactericidal activity was significantly higher in $FM25_{INO}$ diet group and it was not differed significantly with all other replacement group with inosine supplementation. Without inosine supplementation 75% FM replaced with SBM (D7) showed significantly lower bactericidal activity. Increased bactericidal activity in fish with the supplementation of inosine was also reported in our previous research (Hossain et al. 2016a) on red sea bream. In some other studies increased bactericidal activity was also reported feeding different immunostimulans like β- glucans, lactoferrin, nucleotides (Eslamloo et al., 2012; Kamilya et al., 2006; Hossain et al., 2016b) for *catla catla* and Siberian sturgeon *Acipenser baeri* and red sea bream respectively . Finding of the present study also strongly substantiate the immunostimulating properties of

inosine at the same time. Significantly lower bactericidal activity at fish fed diet group SBM75 was might be due to inflammation of distal intestine which stimulated immune responses (Baeverfjord and Krogdahl, 1996; Burrells et al., 1999; Chen et al., 2011; Krogdahl et al., 2003) negatively that resulted reduced bactericidal activity. On the other hand supplementation of inosine in the same FM replaced group (SBM 75I) showed increased bactericidal activity might be due to the immunostimulating proterties of inosine.

In the present study total serum protein was significantly higher in $FM25_{INO}$ diet group. Simultaneously supplemented groups showed improved values compared to non supplemented groups. Increasaed total serum protein due to nucleotide supplementation were also reported for *Catla catla* (Jha et al., 2007), rainbow trout (Tahmasebi-Kohyani et al., 2012) and red sea bream (Hossain et al. 2016a,b) are also in agreement with the present study. The increased total serum protein in inosine supplemented groups might be due to immunostimulatory effects of dietary inosine. Increasing peroxidase content with inosine supplementation in the present study was also the evident of immunostimulating properties of nucleoside, inosine. Salinas et al. (2008) also reported increased PA of Gilthead Seabream (*Sparus aurata)* fed immunostimulant (heat-inactivated bacteria) our present study also strongly support this finding. Increased catalase activity rates were attributed to elevated levels of exogenous hydrogen peroxide (H_2O_2) which is the main cellular precursor of the hydroxyl radical (HO^-), a highly reactive and toxic form of ROS (Hossain et al., 2016a,b). In human increased serum catalase activity is used as a diagnostic tool in acute pancreatitis (Goth et al., 1982), hemolytic disease (Goth et al., 1983) and some liver diseases (Goth et al., 1987). In the present study high mean catalase activity observed in fish fed FM75 as well as non supplemented diet groups implies that non supplemented groups exposed to an oxidative stress and relatively poor immune condition compared to supplemented groups. Results of the catalase activity also strongly correlates with the results of BAP and dROMs measured in

the present study which reconfirmed the positive supplemental effects of inosine in regards to best oxidative stress condition as well as immunity.

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish (Hossain et al., 2016a,b) In the present experiment significantly highest haematocrit value were found in $FM25_{INO}$ diet group and it was not differed significantly with upto 50% FM replacement groups (supplemented and non supplemented) and $FM75_{INO}$ diet group. Increased hematocrit values in supplemented groups indicate the efficiency of inosine, wherein iron is evenly distributed without any reduction in the synthesis of hemoglobin. Similar increased heamatocrit values were also observed in our previous research (Hossain et al., 2016a) on red sea bream fed inosine supplemented diet. Values of glucose, triglycerides, total bilirubin contents, GOT and GPT activities were not significantly affected by dietary inosine supplementation in different level of FM replaced diets. However, all the blood parameters are considered to be within the normal range for juvenile amberjack, compared to those of the previous findings (Kader et al., 2013; Uyan et al., 2009) .Blood urea level is sensitive but not specific indicator for renal dysfunction. The significant increase blood urea nitrogen in fish fed more than 50% FM replaced diet might be due to the higher protein metabolism, high protein level of diet or kidneys may not have been functioning as well as they should be. Higher BUN level at high (more than 50%) FM replaced with SBM based diet in this study agreed with the findings of Kader et al. (2010) and Takagi et al. (2001) when red sea bream was fed the high soybean protein based diet. Likewise, lower cholesterol contents ($P<0.05$) observed in fish fed $FM75_{INO}$ and other inosine supplemented diet groups indicating the capabilities of maintaining low plasma cholesterol level as well as clinical healthy signs of fish.

Oxidative stress can be generated at high level of reactive oxygen species (ROS) and/or decreased efficacy of antioxidant system, which is another health risk factor in human

or other mammals (Pasquini et al., 2008). The simultaneous analysis of d-ROMs and BAP provided valuable data on oxidative stress condition in humans, pig and dog (Pasquini et al., 2008; Ballerini et al., 2003). Recently, these tests have also been applied as a suitable tool for evaluating the oxidative stress in fish (Kader et al., 2010; Hossain et. al., 2016a,b). Fig. 3 demonstrated the oxidative stress conditions of fish fed test diets based on the data of d-ROM and BAP values. It is interesting to note that diet groups control (D1), $FM50_{INO}$(D4) located in A zone, suggesting that this fish groups hold stronger resistance against oxidative stress, and were in lower oxidative stress conditions at the same time. Fish fed D2, and D3, D6 located in zone B and C respectively also considered acceptable condition. Fish fed non inosine supplemented ≥50% FM replaced diet groups (FM50 and FM75) located in zone D, which indicates poor oxidative stress condition. However, to date there remains a lack of explanation about how inosine nucleoside works to effect on these parameters, so more studies are needed.

Dietary nucleotide increases the resistance of fish to a variety of stressors is well documented (Hossain et al. 2016a,b; Burrells et al. 2001b) but little is known about dietary nucleosides. In our study, fish fed inosine supplemented low FM based diet showed significant increase in stress resistance compared to non supplemented groups after the exposure of low salinity stress test. In the present study significantly higher LT_{50} value obtained in diet group $FM50_{INO}$ (111.83 min) and it was not significantly different with other inosine supplemented groups and FM based control group. Significantly lower value of LT_{50} obtained in FM75 diet group (51.09 min) indicated a lower fresh water tolerance of the amberjack. Increased ($P<0.05$) LT_{50} of ambrjack fed inosine supplemented diet indicates healthy status of amberjack (Hossain et al. 2016a,b ;Yokoyama et al., 2005). Increased LT_{50} due to inosine supplementation might be due to increasing osmoregulatory capacity of amberjack which support with the finding of Burrells et al. (2001b) where he mentioned that

dietary nucleotide supplementation increased osmoregulatory competence of Atlantic salmon. Fish antioxidant status is strongly related to immune system, contributing to enhance resistance towards different stressors (Tovar-Ram´ırez 2010). Results of the current study confirmed a higher tolerance against low-salinity stress in fish in less oxidative stress conditions

In the present study among whole-body composition of juvenile amberjack whole body moisture, protein and ash were not influenced significantly by dietary inosine supplementation. Although numerically increased proximate composition values were obtained in inosine supplemented diet groups compared to non supplemented groups. In our previous study inosine supplementation significantly increased the lipid content of Juvenile red sea bream (Hossain et al., 2016a). However, present study failed to confirm that response. Differences in the basal feed ingredients may have contributed to this inconsistency. Among somatic parameters no significant difference were found among treatments but CF is significantly influenced by dietary inosine supplementation.

Apparent digestibility coefficients (ADCs) of dry matter, protein and lipid were significantly affected by dietary inosine supplementation at different replacement level in the current study (Table 6.1.8). Inosine supplemented groups showed higher ($P<0.05$) digestibility values compared to non supplemented groups at different FM replacement level. Increased digestibility on inosine supplemented groups in the present study might be due to feeding stimulatory properties of inosine. It is well documented that feeding stimulant increase the secretion of different digestive enzyme. Kofuji et al. (2006) reported that supplementation of feeding stimulant (Alanine, proline and IMP mixtures) increase the pepsin, trypsin and chymotrypsin secretions in yellow tail (*Seriola quinqueradiata*) . Japanese eel fed a diet with FS exhibited increased secretion of proteolytic and amylolytic enzymes (Takii et al., 1986).Yellowtails fed diets containing FS or krill also showed higher

levels of secretion of protease from the stomach and intestine (Takii et al. 1990), or intestine only (Satoh 2003). Increased secretion of digestive enzyme might be inhibiting the functions of anti-nutritional factors which are commonly associated with SBM and improve the digestibility in the present study.

In the present study, fold height, enterocyte height and microvillus height in distal intestine of amberjack decreased significantly with increasing soybean protein level ≥50%, which paralleled with the findings of these researches on Atlantic salmon (Baeverfjord and Krogdahl, 1996), Asian seabass (Boonyaratpalin et al., 1998) and Japanese flounder (Chen et al., 2011). These results indicated that the structure of distal-intestine of turbot would be broken at high SBM level, which could be related to antinutritional factors such as lectin (Van den Ingh et al., 1991) or saponin (Chen et al., 2011). Inosine supplanted groups in the present study showed significantly increased fold height, enterocyte height in anterior intestine and microvillus height, enterocyte height in posterior intestine. Although anterior hMV and posterior hF were not affected significantly by inosine supplementation but numerically increased values were observed in insoine supplemented groups compared to non supplemented groups. The beneficial effects of dietary nucleotides on gastrointestinal tract is well documented in human and other terrestrial animals (Uauy et al., 1990; Bueno et al., 1994; Carver, 1994) but very little is known about fish and other aquatic animals (Burrells et al. (2001b); Cheng et al. 2011) . Recently Hossain et al. (2016a) reported in red sea bream, inosine increased enterocyte height (hE), fold height (hF) of both anterior and posterior intestine and microvillus height (hMV) of posterior intestine significantly compared to the control. As supplementation of nucleoside, inosine in SBM protein based diet is a very preliminary work, the mechanism by which dietary nucleosides exert their effects on the intestine is not clear. Most possible mechanism might be the direct absorption of nucleoside inosine by intestine of fish that beneficially influence intestinal health of fish. It is reported

that most of the ingested nucleotides are degraded in the intestine to nucleosides by alkaline phosphatase and nucleotidases, and may be further broken down by nucleosidases to produce purine and pyrimidine bases; however, investigations in animals suggest that nucleosides are the primary form absorbed (Uauy, 1989). Most of the absorbed nucleosides and bases are rapidly degraded within the enterocytes (Sonoda and Tatibana, 1978), but -5% may be incorporated into the tissue pools, primarily in the small intestine, liver and skeletal muscle (Burridge et al., 1976; Saviano and Clifford, 1978). The beneficial effects on intestine in present study also might be due to the positive effects of inosine on mucosa associated lymphoid tissue (MALT) which is a very important immune organ although the knowledge on MALT in fish is very limited (Li and Gatlin, 2006).

5. Conclusion

In conclusion results of the current study demonstrated that 25% of FM protein replaced by soybean meal with amino acids (methonine and lysine) supplementation did not significantly reduced growth, feed utilization, digestibility, Immunity, stress resistance and intestine morphology compared with FM based control. Supplementation of inosine increase the replacement level of FM and at least half of the fishmeal could be replaced with soybean meal in the diets of juvenile amberjack. Moreover, inosine supplementation in 50 to 75% FM replacement groups could be helpful to improve the digestibility, immune responses, stress resistance and intestinal health condition of amberjack (*S. dumerili*).

Part -2

Effects of complete replacement of fishmeal by soya protein concentrate with inosine monophosphate supplementation in diets for juvenile red sea bream, *Pagrus major*

Abstract

A feeding trial was conducted to develop low/ non fishmeal based functional feed for red sea bream, *Pagrus major* by gradually replacing fishmeal protein with Soya protein concentrate (SPC). Five isoprotenous (45%) and isocaloric (21 KJ g-1) diets were prepared by replacing 0 (FM100), 25 (FM75), 50(FM50), 75 (FM25) and 100% (FM0) of the fishmeal protein with SPC. Based on recent findings (Hossain *et al*., 2016a), all the replacement diets were supplemented with 0.4 % functional supplements, inosine monophosphate (IMP). Triplicate groups of fish (4.8 g) were stocked in 100-l polycarbonate circulate tanks at a rate of 20 fish per tank. Fish were fed the respective test diets to satiation twice daily for 8 weeks. At the end of the feeding trial results demonstrated that fish fed diet group FM50 had the significantly highest final weight, weight gain specific growth rate and feed intake and it was not differed significnaly with diet group FM75. No signifcnat difference of growth performance and feed intake was also observed among the diet group FM100 and FM25. Feed utilization parameters (Feed efficiency ratio, Protien efficiency ratio) were not also significnaly differed ($P > 0.05$) among the diet groups FM100, FM75, FM50 and FM25. All growth and feed utilization parameters were significantly lower in diet group FM0. Total cholesterol was significantly higher in diet group FM75 and not differed significantly with control, FM50 and FM25 diet groups. BUN was significantly lower in diet group FM100 and FM75, where as diet group FM50 and FM25 showed intermediate value. TG level was significantly increased with the increasing replacement level and it was significantly highest in FM0 diet group where as FM100 group showed significantly lower TG value. In case of oxidative stress parameters, fish fed diet group FM100 showed best condition, whereas FM75 FM50 and FM25 diet groups are in acceptable condition. Stress resistances against low salinity exposure (LT50) were significantly higher in FM50 diet group. Control group were not differed up to 75% FM replaced diet groups with SPC. Significantly higher peroxidase

activity (PA) and Lysozyme activity (LA) were observed in fish fed diet group FM25 compared to control. Overall, increased non specific immune response (LA, PA, NBT, agglutination antibody titier, TSP) observed in the entire IMP supplemented FM replaced diet groups in comparison to control. Considering overall performance it can be concluded that, supplementation of IMP increased the efficiency of utilizing SPC (≤75%) as a sole protein source in the diet of red sea bream and IMP can be used as a potential functional supplement for the development of functional feeds for juvenile red sea bream.

Keywords: Inosine monophosphate, Soyprotein concentrate, low fishmeal based diet, immune responses, stress resistance, *Pagrus major*

1. Introduction

Red sea bream, *Pagrus major* is one of the commercially important aquaculture species, whose production reaches the second largest in Japan (Koshio, 2002). Recently aquaculture of this species has been also rapidly extended in Korea and China. In raising red sea bream, most farmers use commercially manufactured feeds, which often contain high levels of fishmeal (FM) as dietary protein (Kader et al., 2012). Fishmeal is the superior protein source in aquafeeds and simultaneously it is a limiting factor in aquaculture industry. By using fishmeal as the only or major protein source, aquafeeds become expensive which is not cost-effective (Hossain et al., 2015a). Therefore, replacement of fishmeal with cost-effective alternative protein sources is the prerequisite for profitable aquaculture venture.

Because of relatively high content of available protein, relatively well-balanced amino acid profile, and reasonable price and steady supply of soybeans, soybean meal (SBM) has been widely used as the most cost-effective alternative for high-quality fish meal in diets for many aquaculture animals (Ai and Xie, 2005; Chen et al., 2011; Hernández et al., 2007;

Li et al., 2011; Storebakken et al., 2000). Approximately 20 to 40% FM protein can be replaced by SBM protein in diets for carnivorous fish species without reducing growth performance or nutrient utilization, such as in black sea bream (20%) (*Acanthopagrus schlegelii*) (Zhou et al., 2011), European sea bass (25%) (*Dicentrarchus labrax*) (Tibaldi et al., 2006), turbot (25%) (*Scophthalmus maximus*) (Day and Plascencia-Gonzalez, 2000), parrot fish (20% or 30%, depending on the size of fish) (*Oplegnathus fasciatus*) (Lim and Lee, 2009), Japanese seabass (30%) (*Lateolabrax japonicus*) (Li et al., 2011), Atlantic salmon (33%) (*Salmo salar*) (Carter and Hauler, 2000), Asian seabass (37.5%) (*Lates calcarifer*) (Boonyaratpalin et al., 1998), cobia (40%) (*Rachycentron canadum*) (Chou et al., 2004; Zhou et al., 2005) and sharpsnout seabream (40%) (*Diplodus puntazzo*) (Hernández et al., 2007). It is well known that, the deficiency of indispensable amino acids and the presence of antinutritional factors (e.g. oligosaccharides, b-conglycinin and trypsin inhibitors) has made soybean meal limiting for its further inclusion in the diets for carnivorous fishes (Francis et al. 2001; Gatlin et al. 2007; Glencross et al. 2007). Compared with soybean meal, soy protein concentrate (SPC), a soy protein product made by removing nitrogen-free extracts (carbohydrates,p hytic acid, oligosaccharides) from roller milled soy flakes with 80% aqueous alcohol or isolelectric leaching, has a higher content of digestible protein and lower contents of antinutritional factors (Freitas et al. 2011; Colburn et al. 2012). However, alike others soybean protein high inclusion of SPC reduced feed intake, growth rate, feed utilization efficiency, immunity and intestinal health condition (Kaushik et al., 1995; Krogdahl et al., 2003; Chou et al., 2004; Deng et al., 2006; Li et al., 2011; Berge et al. 1999; Kissil et al. 2000). In these circumstances, development of dietary supplementations that support optimum fish health and growth is of increasing interest in fish nutrition research. Addition of functional nutrients could recover the negative effects of alternative protein or

low FM based diet. Recently, in aquaculture research nucleotides and its related product has been paid attention promisingly as functional nutrients

Inosine monophosphate (IMP) is the ribonucleotide and is the first compound formed during the synthesis of purine. Numerous studies on different aquatic species have reported that dietary inosine or IMP, either alone or in combination with certain free amino acids can enhance growth performance, survival and feed intake of juvenile eel (Takeda et al., 1984), turbot *(Scophthalmus moximus)* (Mackie and Adron, 1978; Person-Le Ruyet et al., 1983), dover sole (*Solea vulgaris*) (Metailler et al., 1983) and red sea bream (*pagrus major*) (Hossain et al., 2016a) while it can also improve immune responses and disease resistance of Japanese flounder (*Paralichthys olivaceus*) (Song et al., 2012) . In our recent study on IMP, we found promising results in terms of enhancing growth, feed utilization, immunity and gut health condition of red sea bream (Hossain et al., 2016a). However, almost no information was available on the supplemental effect of IMP in complete FM replaced with SPC based diet on growth and health performance of red sea bream. So, the aim of this study was to investigate the efficacy of utilizing IMP in complete FM replaced with SPC based diet on growth, blood chemistry, oxidative stress and immune response of red sea bream.

2. Materials and Methods

2.1. Test fish and experimental system

Juvenile red sea bream were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and flow through sea water and fed a commercial formulated diet (54% crude protein and 10% crude lipid, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 100 l polycarbonate tanks

(filled with 80 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 6.1.1 summarize the formulation and chemical composition of the experimental diets. Five experimental diets were formulated to be nearly isonitrogenous (45% crude protein), isolipidic (12% crude lipid) and isocaloric (21 KJ g^{-1} gross energy) where diet 1 was 61% FM based control diet (FM100). Fish meal protein was gradually replaced at 25, 50, 75 and 100% with soya protein concentrate (SPC) and designated as FM75, FM50, FM25 and FM0, respectively. All the replacement diets were supplemented with 0.6 % Disodium 5'-Inosinate (5'-IMP・2Na) (Ajinomoto Co., Inc.) according to Hossain et al. (2016a). For proper mixing of IMP with other ingredients, initially IMP and weighted supplemented amino acids were mixed properly in a glass beaker by hands with the help of a stainless laboratory spatula. Then this mixtures were thoroughly mixed with all the dry ingredients in a food mixer for 10 min. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (35–40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 60 °C for about 120 min. The test diets were stored at −28 °C in a freezer until use.

Table 6.2.1 : Formulation and chemical compositions of the experimental diets.

Ingredients	Diet groups				
	FM100	FM75	FM50	FM25	FM0
Fishmeal[a]	61.00	45.75	30.50	15.25	0
SPC[b]	0.00	12.87	25.75	38.62	51.49
Pollack liver oil[c]	4.00	4.90	5.85	6.75	7.65
Soybean lecithin[d]	2.00	2.00	2.00	2.00	2.00
Vitamin mixture[e]	3.00	3.00	3.00	3.00	3.00
Mineral mixture[f]	3.00	3.00	3.00	3.00	3.00
Stay-C[g]	0.10	0.08	0.08	0.08	0.08
Wheat flour	10.00	10.00	10.00	10.00	10.00
Activated gluten[h]	5.00	5.00	5.00	5.00	5.00
α cellulose	11.00	11.17	12.06	13.00	13.95
FAA[i]	0.90	0.90	0.90	0.90	0.90
IMP[j]	0.00	0.79	0.79	0.79	0.79
Lysine[k]	0.00	0.36	0.71	1.07	1.42
Methonine[l]	0.00	0.18	0.36	0.54	0.72
Chemical compositions					
Moisture	7.90	7.47	8.19	7.48	8.34
Crude protein	45.44	45.88	45.18	44.87	44.77
Crude lipid	11.58	11.54	11.53	12.36	12.51
Ash	12.87	11.74	9.71	7.88	5.78
Gross energy (KJg^{-1})*	20.47	20.69	20.98	21.47	21.35

[a]Nippon Suisan Co. Ltd., Tokyo, Japan

[b]SPC: Soya protein concentrate (Seiyu stock company, Osaka, Japan)

[c] Riken Vitamin, Tokyo, Japan.

[d] Kanto Chemical Co., Inc. Tokyo, Japan

[e] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[f] Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[g] Stay-C 35.

[h] Glico Nutrition Company Ltd. Osaka, Japan. Commercial name “A-glu SS”.

[i] FAA: Free amino acids (alanine, betine and glycine); Nacalai Tesque, Kyoto, Japan

[j] Ajinomoto Co., Inc., Tokyo, Japan

[k,l] Nacalai Tesque, Kyoto, Japan

*Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ kg^{-1}, respectively.

Carbohydrate was calculated by the difference: 100-(protein + lipid + ash).

2.3. Feeding protocol

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Twenty fish, having a mean initial body weight of approximately 4.9g were randomly allocated to previously prepare fifteen tanks. Fish were fed the experimental diets for 56 days by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 1 hour after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk fortnightly to determine growth and visually check their health condition. The monitored water quality parameters were: water temperature 21.5±1.8 °C; pH 8.1±0.7 and salinity 34.5±0.5 during the feeding trial. These ranges are considered within optimal values for juvenile red sea bream.

2.4. Sample collection

At the end of the feeding trial, all experimental fish were fasted for 24 h. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}. Then individual body weight of fish was measured, and the growth parameters were calculated accordingly. Nine fish per treatment (three fish per each tank) were randomly collected, washed with distilled water and then stored at −80 °C (MDF-U383, SANYO, Tokyo, Japan) for the whole body proximate analysis. Six fish were randomly sampled from each dietary replicate tank and their blood were collected by puncture of the caudal vain using heparinized (1600 IU/ml, Nacalai Tesque, Kyoto, Japan) disposable syringes and pooled. In addition non-heparinized disposable syringes were used to collect blood for serum analysis. Partial heparinized whole blood was used to analyze the hematocrit levels while plasma and serum were obtained by centrifugation at 3000 ×g for 15 min under 4

°C, and then stored at −80 °C until the analysis. Liver and viscera were dissected out from the fish above, weight individually to calculate the hepatosomatic index (HSI) and viscerasomatic index (VSI).

2.5. Biochemical analysis

The ingredients, diets and fish whole body were analyzed in triplicate, using standard methods (AOAC, 1995). The moisture was determined by drying the sample at 105 °C to constant weight. The ash was analyzed by combustion at 550 °C for 12 h. The crude protein content was determined by measuring the nitrogen content (N × 6.25) using the Kjeldahl method with a Tecator Kjeltec System (1007 Digestion System, 1002 Distilling unit, and Titration unit; FOSS Tecator AB, Högendäs, Sweden). Crude lipid content was estimated using gravimetric method (954.02). Total serum protein (TSP) and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma samples was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically

detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg.

2.6. Evaluation of immune parameters

Serum lysozyme activity (LA) was measured with turbidimetric assays (Lygren et al., 1999). Ten micro-liters of samples was put into well of microplate, then added 190 micro-liters of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4,their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

Oxidative radical production by phagocytes during respiratory burst was measured through the NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995) with some modifications. Briefly, blood and 0.2% NBT (Nacalai Tesque, Kyoto, Japan) were mixed in equal proportion (1:1), incubated for 30 min at room temperature. Then 50 mL was taken out and dispensed into glass tubes. One milliliter of dimethylformamide (Sigma) was added and centrifuged at 2000 × g for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Hitachi U-1000, Japan). Dimethylformamide was used as the blank.

The total peroxidase activity (PA) in serum was measured according to Salinas et al. (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-

developing reaction in serum samples was stopped by adding 50 µl of 2M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

The catalase activity (CAT) assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate that absorbs at 405 nm (Goth, 1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol/l phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate ($(NH_4)_6$ Mo_7O_{24} .4 H_2O) and the yellow complex of molybdate and hydrogen peroxide is measured at 405 nm against reagent blank. One unit CAT decomposes 1 µmole of hydrogen peroxide/l minute under assay conditions. CAT activities are expressed as kilo unit per liter (kU/l)

The agglutination antibody titer was conducted in round bottomed 'U' shaped micro titer plates after Swain et al. (2007) with slight modifications. 50 µl of serum was serially diluted in PBS (1/2, 1/4, 1/8, 1/16, 1/32and 1/64) and then equal volumes of *Vibrio anguillarum* (1×10^8 cfu ml^{-1}) was added to wells and kept for 24 h at 4 ^{0}C. The reciprocal of the highest dilution that gave agglutination was taken as the agglutination antibody titer which is expressed as log10.

2.8. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, five fish from each rearing tank (total 15 fish per treatment) were randomly selected and transferred in to a blue colored 20 L rectangular glass aquarium with 15 L tap water which was aerated for 24 h. The glass aquaria for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of red sea bream juvenile was calculated according

to Moe et al., (2004) as follows: time to death (min) of a juvenile was converted to log10 values. When the juvenile were transferred to the low salinity water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was log10 100 = 2). The calculation was then conducted every 5 minutes after the first exposure. The values of log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

Y= aX+ b where Y = log10 (survival), X = time for individual juvenile death (min).

LT_{50} (X) was obtained when Y = 1.7 since log10 (50) = 1.7. Each value obtained from the equation above was compared statistically.

2.9. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR, % day^{-1}) = {Ln(final weight) – Ln (initial weight)/duration}× 100

Survival (%) = 100 × (final no. of fish / initial no. of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed conversion ratio (FCR) = dry feed intake (g)/ live weight gain (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Condition factor (CF, %) = weight of fish / (length of fish)^3 × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

Viscerasomatic index (VSI, %)= viscera weight/fish weight × 100

2.10. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of P<0.05

were considered significant. Significance differences between means were evaluated using the Turkey Kramer test.

3. Results

3.1 Growth performance and nutrient utilization

Growth performance, nutrient utilization and survival of red sea bream fed experimental diets are presented in Table 6.2.2. Survival (%) was not significantly ($P > 0.05$) different at the end of the feeding trial. Fish fed diet with 75 % FM replaced group (FM25) supported growth equivalent to that of the 100% FM based diet group (FM100) and there was no significant differences in final weight, weight gain and SGR of fish fed diet groups FM100, FM75, FM50 and FM25. However, the growth parameters were significantly ($P<0.05$) higher in fish fed FM50 and FM75 respectively compared to FM100 and FM0. Among the treatment significantly lowest growth performance was found in diet group FM0. Similarly, significantly lowest feed utilization performances (Feed Efficiency ratio, FER and protein efficiency ratio, PER) were also observed in FM0 diet group. While the other replaced diet groups shoed no significant difference with FM based control diet group (FM100). Significantly higher feed intake observed in fish fed diet group FM50 and not differed significantly with diet group FM75. Diet group FM25 showed intermediate value and not differed significantly with control diet group (FM100). Alike other growth performance parameter lowest feed intake reported in diet group FM0.

3.2. Hematological parameters

Table 6.2.3 represents the blood parameters of juvenile red sea bream after 56 days of feeding trial. Although wide variations were observed on some of the blood chemical parameters, no

Table 6.2.2 : Growth performance and feed utilization parameters of red sea bream fed test diets for 56 days*

Parameters	Diet groups				
	FM100	FM75	FM50	FM25	FM0
IBW	4.86±0.00	4.87±0.01	4.87±0.01	4.86±0.01	4.87±0.01
FBW	19.04±0.40^{b}	20.66±0.3b^{c}	22.57±0.5^{c}	18.46±0.98^{b}	11.07±0.12^{a}
WG%	291.5±8.12^{b}	324.8±6.3^{bc}	363.7±9.5^{c}	279.6±20.07^{b}	127.5±2.54^{a}
SGR	2.43±0.04^{b}	2.58±0.02^{bc}	2.74±0.04^{c}	2.38±0.1^{b}	1.47±0.02^{a}
FI	12.53±0.11^{b}	15.03±0.77^{c}	16.26±0.53^{c}	12.64±0.23^{b}	9.05±0.35^{a}
FCE	1.13±0.02^{b}	1.06±0.06^{b}	1.09±0.07^{b}	1.08±0.08^{b}	0.69±0.04^{a}
PER	2.49±0.05^{b}	2.30±0.14^{b}	2.42±0.14^{b}	2.40±0.19^{b}	1.54±0.08^{a}
Sur	91.67±3.33	91.67±4.41	91.67±4.41	93.33±1.67	85.00±2.89

[a] IBW: initial body weight (g); [b] FBW: final body weight (g); [c] WG: percent weight gain (%); [d] SGR: specific growth rate (% day^{-1}); [e] FI: feed intake (g fish^{-1} 50 days^{-1}); [f] FCR: feed conversion ratio; [g] PER: protein efficiency ratio; [h] Sur: survival (%).

* Values are means of triplicate groups±S.E.M. Within a row, means with the same letters are not significantly different (P>0.05).

significant alteration was identified among treatments except those of total cholesterol, BUN and TG. Total cholesterol was significantly higher in diet group FM75 and not differed significantly with control diet group and FM50 and FM25 diet groups. BUN was significantly lower in diet group FM100 and FM75, where as diet group FM50 and FM25 showed intermediate value. Significantly highest BUN value was observed in diet group FM0. Total bilirubin GPT were numerically higher in FM0 diet group compared to control and other replaced diet groups. TG level was significantly increased with the increasing replacement level and it was significantly highest in FM0 diet group where as FM based control group (FM100) showed significantly lower TG value. Numerically higher ($P>0.05$) hematocrit content was observed in diet group FM25 while, the other FM replaced diet groups with IMP supplementation showed increased hematocrit content in comparison to control diet group.

3.3. Oxidative stress parameters

Dietary treatments had no significant effects of oxidative stress parameters measured in the preset study (no data illustrated here). However oxidative stress responses were evaluated by comparing the combined effects of both stress intensity and tolerance ability of fish (d-ROM and BAP) fed diet for 56 days which is expressed in Fig. 6.2.1. From Fig 1 it indicates that fish fed diet groups FM100 was located in zone A which was categorized as lower intensity of oxidative stress and higher tolerance ability against oxidative stress. Simultaneously, diet groups FM75 and FM50, FM25 were located in zone B and zone C, respectively. Complete FM replaced diet group (FM0) located in zone D indicated higher intensity of oxidative stress and lower tolerance ability against oxidative stress.

Table 6.2.3: Blood parameters of juvenile red sea bream fed test diets for 56days.[*]

Parameters	Diet groups				
	FM100	FM75	FM50	FM25	FM0
Hematocrit (%)	31.0±1.1	33.8±1.2	33.3±2.7	34.8±1.0	31.7±0.7
Glucose (mg/dl)	47.7±3.2	47.0±2.7	55.7±4.4	50.3±2.7	51.7±2.6
T-Cho (mg/dl)[a]	243.7±11.2[b]	248.7±4.4[b]	220.0±8.0[b]	230.7±8.7[b]	176.0±5.6[a]
BUN (mg/dl)[b]	6.33±0.7[a]	6.0±0.6[a]	8.0±0.6[ab]	8.3±1.9[ab]	12.3±0.3[b]
T-Bill (mg/dl)[c]	0.23±0.03	0.20±0.00	0.20±0.00	0.23±0.03	0.27±0.03
GOT (IU/l)[d]	18.0±4.6	16.7±2.4	21.0±8.6	25.3±10.4	22.0±6.1
GPT(IU/L)[e]	<10	<10	12.0±2.0	12.7±2.7	20.67±9.6
TG (mg/dl)[f]	133.3±13.0[a]	234.0±13.4[ab]	273.7±49.2[abc]	361.3±61.5[bc]	459.0±41.0[c]

[a] T-Cho: total cholesterol.

[b] BUN: blood urea nitrogen.

[c]T- Bill: Total bilirubin

[d]GOT: glutamyl oxaloacetic transaminase.

[e]GPT: glutamic pyruvate transaminase.

[f]TG: triglyceride.

[*]Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

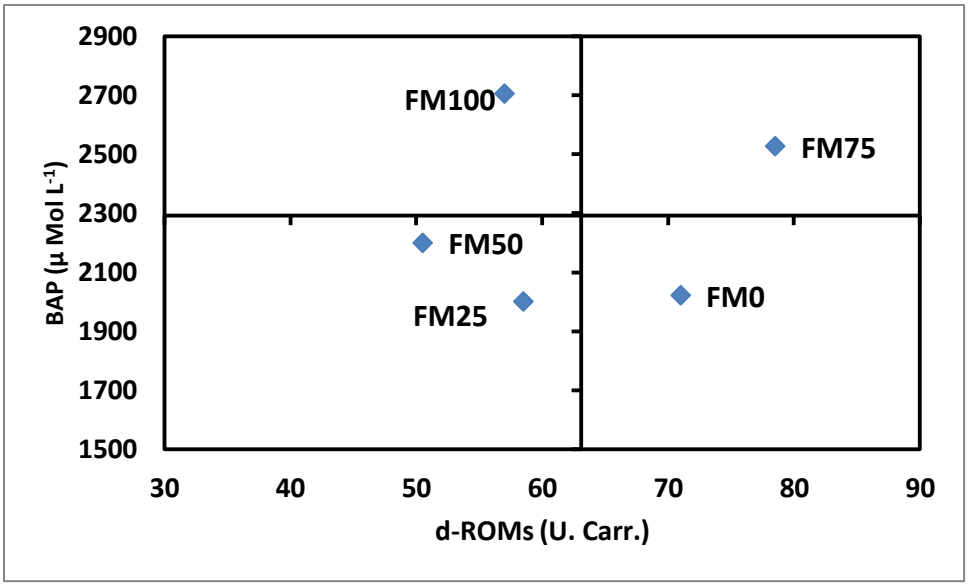


Fig. 6.2.1. Oxidative stress parameters in red sea bream fed test diets for 56 days. (Values are means of triplicate groups. The abbreviations of experimental treatments are illustrated in the text. Central axis based on mean values of d-ROM and BAP from each treatment.) Zone A: High antioxidant potential and low reactive oxygen metabolites (good condition). Zone B: High antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone C: Low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone D: Low antioxidant potential and high reactive oxygen metabolites (stressed condition).

3.4. Low salinity stress test

The results of lethal stress test against fresh water shock on LT_{50} obtained by regression analysis are shown in Fig. 6.2.2. Supplementation of IMP increase the LT50 values of red sea and significantly highest value (102.84 min.) obtained in FM50 diet group. Fish fed up to 75% FM replaced diet groups were not significnaly differed with FM based control diet. Significantly lower LT50 was found in complete FM replaced diet group (FM0).

3.5. Innate immune responses

Table 6.2.4 showed the innate immune responses of red sea bream fed experimental diets for 56 days. Among the different measured non specific immune parameters PA and NBT were significantly influenced by dietary treatments. Overall, increased non specific immune response observed in the entire IMP supplemented FM replaced diet groups in comparison to control diet. Significantly higher PA was observed in fish fed diet group FM25 and not differed significantly among diet groups FM50 and FM75.FM0 showed intermediate value.NBT also significantly higher in diet group FM25 and the IMP supplemented FM replaced diet groups shoed intermediate values. PA and NBT were significantly lower in FM based control diet group. In comparison to control lysozyme activity and total serum protein also showed increased value in IMP supplemented FM replaced diet groups. In contrast, numerically reduced CAT activity observed in IMP supplemented FM replaced diet groups compared to FM based control diet (FM100).

4. Discussion

Effective utilization of terrestrial plant ingredients as FM replacer in aquafeed has been distinguished as a key measure of enhancing aquaculture sustainability. Soy-derived ingredients are the most important terrestrial plant ingredients has been widely used as the

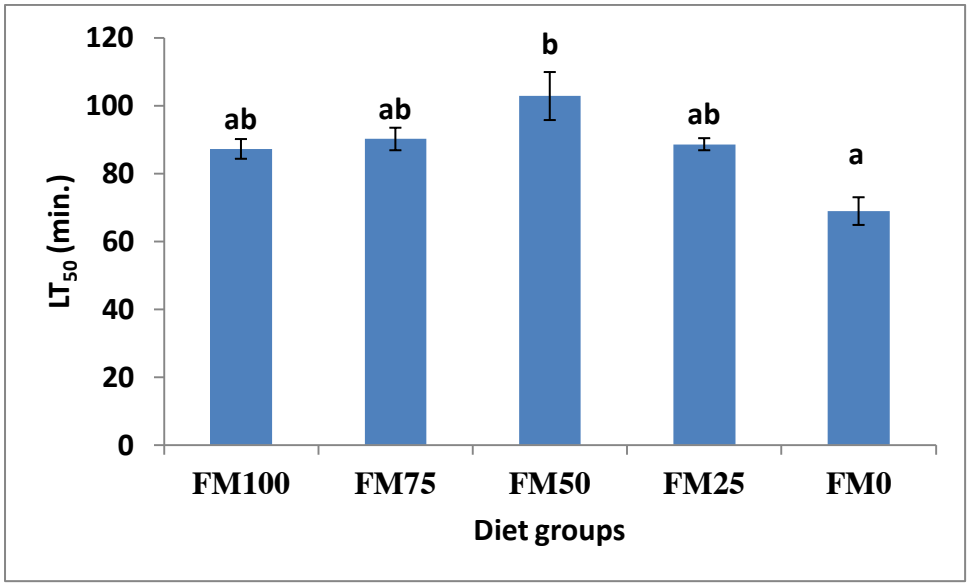


Fig.6.2.2. LT_{50} (min) calculated from the lethal time of juvenile red sea bream exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from triplicate groups. Values with different letters are significantly different (P<0.05).

Table 6.2.4: Non-specific immune response of juvenile red sea bream fed test diet for 50 days.*

Parameters	Diet groups				
	FM100	FM75	FM50	FM25	FM0
TSP (g/dl)[a]	2.50±0.20	2.55±0.05	2.70±0.10	2.60±0.10	2.50±0.10
LA (unit/mL)[b]	41.7±8.33	75.0±25.0	112.5±12.5	83.3±20.1	62.5± 12.5
PA[c]	1.85±0.08[a]	2.12±0.06[b]	2.12±0.06[b]	2.16±0.03[b]	1.94± 0.04[ab]
NBT[d]	0.51±0.02[a]	0.57±0.02[ab]	0.57±0.02[ab]	0.58±0.01[b]	0.56± 0.01[ab]
CAT[e]	30.16±21.4	18.86±1.22	25.93±8.4	17.62±3.2	21.78± 6.5
B-Agglu[f]	0.80±0.1	1.00±0.1	1.10±0.1	0.80±0.1	0.70± 0.1

[a]TSP: Total serum protein

[b]LA: lysozyme activity

[c]PA: Peroxidase activity (measured at OD of 450nm).

[d]NBT: Nitro-blue-tetrazolium activity

[e]CAT: Catalase activity

[f]B-Agglu: Bacterial agglutination antibody titer

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different (P>0.05).

most cost effective alternative for high quality fish meal in diets of many aquaculture species. SPC is a soya derived ingredient has been evaluated as a FM substitute in wide range of fish species due to its high utilization efficiency (Walker et al. 2010; Colburn et al.2012; Storebakken et al., 2000; Carter & Sajjadi, 2011; Freitas et al., 2011; Deng et al., 2006; Mambrini et al. 1999 and Wu et al., 2015). However, in most cases high inclusion of SPC as well as soya derived protein caused reduced growth and health performance of aquatic species. Supplementations of functional nutrients in alternative protein based diet could overcome the negative effects of alternative protein. In our recent study dietary nucleotide, IMP has been reported as a most efficient functional material in casein based semi purified red sea bream diets to improve growth and health performance. However, almost no information was available on the supplemental effect of IMP in FM protein replaced with SPC protein based diet on growth and immunity of red sea bream fish. Under these circumstances, it is important to investigate the supplementation effects of IMP as feeding stimulant as well as functional materials in low FM based aquafeed.

Results from the present study clearly demonstrated that, up to 75% of fish meal protein replaced by SPC as sole protein with IMP supplementation did not negatively affect growth performance, nutrient utilization and health performance of juvenile red sea bream. In some previous studies on red sea bream it was found that red sea bream is not able to utilize higher levels of SPC in their diet and it was less than 60% according to Kader et al. (2010). Takagi et al., 1999) also found that only 50% FM protein could be replaced by SPC protein in diets for juvenile red sea bream. That group also tried to replace higher levels of FM in red sea bream diet with supplementation of lysine and methionine, but failed to obtain similar performance of fish as that in the control group (Takagi et al., 2001).Kissil et al. (2000) and Deng et al. (2006) reported that growth was significantly depressed even at 25–30% substitution of SPC in the diets of gilthead seabream and Japanese flounder, respectively.

Day and González (2000) suggested that 25% FM protein could be substituted with SPC in diets for turbot. In contrast, higher or complete replacements of FM with SPC were reported in Senegalese sole, *Solea senegalensis* (Aragão et al., 2003), cobia (Salze et al., 2010) and rainbow trout (Kaushik et al., 1995) which were more or less consistent with the present study. The increasing acceptance of FM replaced SPC based diet by juvenile red sea bream in the present study was due to supplementation of functional nutrients IMP, which has both growth and health promoting capacity as reported in our previous study (Hossain et al. 2016a, Song et al., 2012). Supplementation of methonine and lysine which are the most limiting amino acids in soya protein based diet, are also beneficial in recovering essential amino acid balances in FM replaced with SPC protein based diet in the present study. Significantly lower growth performance was observed in complete FM replaced diet group (FM0) was associated with the reduced feed intake which also consistent with the findings of Bureau et al., 1998; Chen et al., 2011). Where they also stated that suppression of feed intake could be the main reason for reducing growth performance when FM was replaced by SBM.

In the present study, significantly higher feed intake observed in fish fed diet group FM50 and not differed significantly with diet group FM75. Diet group FM25 showed intermediate value and not differed significantly with control diet group (FM100). Feed intake was significantly lower in complete FM replaced diet group. Addition of palatability enhancers is an effective approach when developing diets containing high plant protein in order to maintain feed attractiveness and induce adequate feed consumption rate by fish (Kissil et al., 2000; Papatryphon and Soares, 2000). Chemo-attractive and feeding stimulatory effect is well documented for certain nucleotide and nucleoside (Li and Gatlin 2006). Mackie (1973) first analyzed the low-molecular weight fraction of squid and hypothesized nucleotide (AMP) and nucleoside (inosine) components as the main chemo-attractants for aquatic animals such as the lobster. inosine and inosine-5-monophosphate have been reported as

specific feeding stimulants for turbot fry, *Scohthalmus maximus* (Mackie and Adron, 1978), Dover sole, *Solea solea* (with betaine and glycine; Metailler et al., 1983), and yellowtail, *Seriola quinqueradiata* (Takeda et al., 1984). In our recent studies it was also found that supplementations of IMP improved the palatability of the diets of sea bream (Hossain et al., 2016a). Improved FI with supplementing functional supplement IMP in the present study also helped to improve palatability of diets with high SPC (upto 75%) which completely supports the previous statements. However, at complete replacement level of FM with SPC supplementation of IMP couldn't improve the palatability of diets like as FM based control diets. Similar lower palatability was also reported in high SPC based diet (100%) by Salze et al., (2010) where mannan oligosaccharide supplementation couldn't improve the palatability of experimental diets of cobia.

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as welfare of fish (Hossain et al., 2016 a,b). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to those of the previous findings (Hossain et al., 2016 a,b; kader et al., 2010). Cholesterol content is significantly higher in FM based diet and it was not differed up to 75% FM replaced diets with SPC. Cholesterol is an important component of cell membranes (Cheng and Hardy, 2004) and the substrate for many substances, including bile acid, steroid hormones and vitamin D (Hernández et al., 2004; Holme et al., 2006). The concentration of cholesterol in plant protein was lower than that in FM (Cheng and Hardy, 2004). In some previous research it was found that feeding high plant protein based diet had the depressed growth which was generally accompanied by a hypocholesterolemic effect in many fish species (Afuang et al., 2003; Kaushik et al., 2004; Sagstad et al., 2008; Wang, 2007; Yagi et al., 2006), which indicated that the low concentration of cholesterol in diets could be one of the reasons leading to the poor growth

performance of fish when FM was replaced by plant protein. In the present study at 100 % replace level cholesterol level significantly reduced which completely supports the above findings. However, FM replaced with SPC based diet (≤75%) did not significantly differed with control. This is might be due to the supplementation effects of IMP in alternative protein based diet. TG content showed opposite trend of total cholesterol content and significnaly highest TG level was reported in FM0 diet group. Similarly, increasing TG level in SPC based diet also reported by Kader et al., (2010) for juvenile red sea bream. BUN content was significantly lower in diet groups FM100 and FM75, whereas FM50 and FM25 showed intermediate value indicated that supplemntion of IMP helps to maintain lower BUN level up to 75% replacement level of FM by SPC. Similarly, capabilities of reducing BUN levels through nucleotide supplementation was also reported previously by (Hossain et al., 2016a) . Result of the present study also revealed that plasma bilirubin, and GPT level showed lower values ($P>0.05$) in IMP supplemented FM replaced diet group FM75,FM50 and FM25 which are also similar with complete FM based diet group. Plasma bilirubin and GPT (or alanine aminotransferase, ALT) are often used for the evaluation of liver function as they are released into the blood during injury or damage to the liver cells (Lemaire et al., 1991). Lower values of these parameters in diet groups FM75, FM50, FM25 and control diet groups indicated that, Complete FM based diet and IMP supplementation upto 75% FM replacement level with SPC induced an optimal physiological condition as well as better liver health condition of fish.

Fish meal contained abundant nucleic acids and nucleotides, while little was found in soybean meal (Mateo et al., 2004). Along with reduced growth performances, higher replacement levels of soya protein caused morphological changes of distal intestinal epithelium and stimulated immune responses because of inflammation in distal intestine (Baeverfjord and Krogdahl, 1996; Burrells et al., 1999; Chen et al., 2011; Krogdahl et al.,

2003). In such circumstances, supplementation of nucleotides as a potential functional nutrient as well as immunomodulators in soya protein based aquafeeds will be helpful to positively influence the immune response as well as overall health condition of fish. Non specific immune responses measured in the present study influenced by dietary IMP supplementations. PA and NBT activity in the present study significantly increased by dietary IMP supplementation in FM replaced diet groups. Significantly higher PA was observed in diet groups FM75, FM50, FM25. Meanwhile diet group FM25 showed highest ($P<0.05$) NBT activity. In a 60 days growth trial Peng et al. (2014) also found significantly higher NBT activity in 50 % FM replaced with soyabean protein based diet with nucleotide supplementation for juvenile turbot, *Scophthalmus maximus* L. Similar increased PA and NBT was also observed by Song et al. (2012) in Japanese olive flounder fed diet supplemented with IMP. Our previous studies on red sea bream fed diets with supplemented nucleotides (inosine, IMP, mixed nucleotides) also showed increased PA which are in agreement with the present study.

Stress is one of the emerging factors in aquaculture activities, which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization (Hossain et al., 2016a,b). Dietary nucleotide increases the resistance of fish to a variety of stressors is well documented (Burrells et al., 2001; Leonardi et al., 2003 Tahmasebi-Kohyani et al., 2012, Hossain et al., 2016a,b). In our study, fish fed IMP supplemented diets showed increase in stress resistance of red sea bream after the exposure of low salinity stress test. Supplementation of IMP increase the LT50 values of red sea bream and up to 75% FM replaced groups were not significnaly differed with FM based control diet. Significaly lower LT50 was found in complete FM replaced diet group (FM0). Increased LT50 value up to 75 % FM replaced diet group indicates healthy status of red sea bream (Hossain et al., 2016a; Yokoyama et al., 2005). Oxidative stress is considered to involve in plenty of diseases and

pathological status in fish (Martinez-Alvarez , 2005), and is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants (Celi et al., 2010). Simultaneous measurements of d-ROMs with BAP can provide a suitable tool for measuring the oxidative stress in humans, pig, rabbit and dog (Oriani et al., 2001; Pasquini et al., 2008; Ballerini et al., 2003). Recently, d-ROMs and BAP were reported to be reliable parameters for determining the oxidative stress condition of fish (Hossain et al., 2016a,b). Fish with higher d-ROM values indicate that they are under more oxidative stress conditions. On the other hand, fish with higher BAP values indicate stronger tolerance against oxidation. As shown in Fig. 6.2.1, it is interesting to note that diet groups FM100 was located in zone A, which represents the best condition with low oxidative stress and high antioxidant levels. Diet groups FM75 and FM50, FM25 located in zone B and zone C, respectively and considered as acceptable. While the complete FM replaced diet group FM0 was located in zone D is indicated as high oxidative stress and low antioxidant levels as considered as stressed condition of fish. However, overall no significant difference of d-ROMs and BAP values were observed among treatments in the present study which is also comparable with the values obtained in earlier study by Kader at al., (2010) with juvenile red sea bream fed SPC based diet. However, to date, there remains a lack of explanation about how nucleotides work to affect these parameters, so further study is also needed.

Lysozyme, being an enzyme with antimicrobial activity, can split peptidoglycan in bacterial cell walls especially of the Gram positive species and can cause lysis of the cells (Chipman and Sharon, 1969). In the present study IMP supplemented FM replaced with SPC based diet groups showed increased LA but not at a significant level. Similarly, Peng et al. (2013) also reported that dietary nucleotide supplementation levels did not significantly affect the activity of lysozyme under 40% or 50% soybean protein level. However, numerically higher ($P>0.05$) LA in IMP supplemented diet groups might be due to increasing immune-

stimulating properties of IMP nucleotide. Increase LA with fish fed nucleoside and nucleotide supplemented diet were also reported previously in common carp (*Cyprinus carpio*) (Sakai et al., 2001), rainbow trout (Tahmasebi-Kohyani et al., 2011), Japanese flounder (Song et al., 2012) and red sea bream (Hossain et al., 2016a,b). Agglutination antibody titer against different bacteria is another mechanism of innate immune response which has high activity in fish (Oriol Sunyer and Tort, 1995). Fish fed IMP supplemented FM replaced (upto 75%) with SPC based diet showed higher agglutination antibody titer compared with control and complete FM replaced diet(FM0). Proteins are the most important compounds in the serum and are essential for sustaining a healthy immune system (Kumar et al., 2005). In the present study supplementation of IMP significantly increased TSP compared to the control and FM replaced (upto 75%) with SPC based diets. Increased TSP due to nucleotide supplementation were also reported in *Catla catla* (Jha et al., 2007), red sea bream (*P. major*) (Hossain et al., 2016a,b) and rainbow trout (Tahmasebi-Kohyani et al., 2012). These findings are in agreement with the present study. Increased CAT activity rates were attributed to elevated levels of exogenous hydrogen peroxide (H_2O_2) which is the main cellular precursor of the hydroxyl radical (HO^-), a highly reactive and toxic form of ROS (Hossain et al., 2016). In human increased serum CAT activity is used as a diagnostic tool in acute pancreatitis (Goth, 1982), hemolytic disease (Goth, 1983) and some liver diseases (Goth, 1987). Reduced CAT activity in IMP supplemented diet groups indicated an optimal physiological condition as well as better liver health condition of fish.Overall, all the non specific immune response parameters measured in the present study showed improved value in IMP supplemented FM replaced with SPC based diet groups confirming the benefits of IMP nucleotide for the non-specific innate immunity of red sea bream.

In summary , the present experiment shows that supplementation of IMP increased the efficiency of utilizing SPC (≤75%) as a sole protein source for the diet of juvenile red sea

bream. In comparison with complete FM based diet fish growth, feed utilization performances and blood chemical parameters were not differed significantly up to 75% FM replace with SPC based diet. In case of oxidative stress condition IMP supplementation in up to 75 % SPC based diet showed relatively better condition. In terms of non specific immune response all replacement diet groups showed improved immune responses compared with protein based diet with 100 % fishmeal based diet. Considering over all performances finally it can be concluded that IMP supplementation in 75% FM replacement with SPC as sole protein source could be helpful to improve the growth, feed utilization, blood chemistry, immune responses and stress resistance of juvenile red sea bream.

CHAPTER VII

General discussion

7.1.General discussion

One of the major goal in all types of aquaculture is to maximize the efficiency of production to optimize profitability. Intensified aquaculture not only increased the production of culture organisms but also increased the susceptibilityof different types of bacterial, viral or parasite infections due to deterioration of water quality and elevation of stress. Antibiotics are commonly used in aquaculture to treat the bacterial infections. However, antibiotics and some chemicals/drugs used in aquaculture have been criticized for potential development of antibiotic resistant bacteria and destruction of environmental microbial flora as well as being cost intensive and in some cases with marginal effects only. Moreover development of drug resistance in human body through consumption of long term antibiotic treated fish has lead to ban of sub- therapeutic use of antibiotics in Europe and more stringent regulations on application of antibiotics in lots of other countries. Meanwhile, these situation drives aquaculture nutritionist to develop alternative strategies which are enabling a shift away from chemotherapeutic and antibiotic treatments of culture organisms. Development of function feed through supplementations of functional nutrient is one of the best alternative strategies in this regard. Because, functional nutrient as well as functional feeds has the capabilities to add benefits above and beyond the basic nutritional requirement and it is hoped these diets will improve both health status and growth of the fish. Recently among different functional supplements used in aquaculture, nucleotide has received both commercial and scientific interest as functional materials in aquafeeds.

The roles of nucleotides and metabolites in fish diets have been sparingly studied for over 25 years. The potential growth and health benefit of dietary nucleotide in aquaculture species instigated in early 2000s. Despite occasional inconsistency in physiological responses, dietary supplementation of nucleotides has shown rather consistent beneficial

influences on various fish species. However, currently there were numerous gaps in existing knowledge about exogenous nucleotide application to fish including functional effects of various individual nucleotide, doses and time of administration, various aspects of digestion, absorption, metabolism, oxidative stress resistance, and influences on various physiological responses especially expression of different genes etc. So, in the present study we tried to evaluate the major purine and pyrimidine nucleotides for better understanding of nucleotide nutrition in fish. Moreover, we supplemented individual nucleotide in low/ non fish meal based diet to develop eco-friendly low/ non fishmeal based functional feeds.

In the present study in chapter III nucleotide related products was evaluated initially in a feeding trial through supplementing a basal diet with NT related products such as nucleoside by- products (NBP) and inosine at 1, 3 and 0.03, 0.1%, respectively. After 60 day's results suggested that inosine and low concentration of NBP (1%) could be effectively used as dietary supplements for better growth and health performance. Alike our present results, the growth enhancing and feeding stimulating properties of nucleosides inosine have been reported earlier in some studies (Ishida and Hidaka, 1987; Kumazawa and Kurihara, 1990; Yamaguchi, 1991; Person-Le Ruyet et al. 1983). However, findings relating to improved health performance due to NBP and inosine supplementations were the first such reports on nucleotide / nucleoside nutrition in fish which instigated us to conduct more intensive research on this nucleoside.

In the chapter IV and V major purine and pyrimidine individual nucleotides were evaluated, including inosine nucleoside in six separate feeding trials with graded supplementation levels. After feeding trial (7-10 weeks) overall, all the individual nucleotides showed improved growth and health performance although all the parameters were not always significantly different compared to supplementation free control group. So far, there is no exact explanation how dietary nucleotide and nucleosides work to enhance growth.

However, it is assumed that the growth-enhancing effect of inosine and other nucleotide in the present study resulted from improved feed intake at the beginning of weaning, promoting more rapid food intake that reduced nutrient leaching into the water or possibly playing roles in metabolism (Metailler et al., 1983, Hossain et al., 2016a,b). It is also well known that growth response was strongly related to improve feed utilization. Our results also suggested that supplementations of nucleotide improve the feed efficiency ratio (FER) and protein efficiency ratio (PER) in most of the feeding trial. Recently, there are some researches on mixed nucleotides supplementation where the beneficial effects on health performance have been evaluated. Finding of our present study regarding improved health performance completely support the previous findings (Burrells et al., 2001a,b; Lin et al., Cheng et al., 2011; Song et al., 2012; Tahmasebi-Kohyani et al., 2011,2012). The supplementation level, which was optimized, based on the fish performance and in some cases regression analysis of those performance parameters were used. A wide range of optimum supplementation levels were observed depends on the variation of nucleotide and experimental fish species . For instance, in red sea bream, their optimal levels of IMP, inosine, UMP was 0.4%. For the GMP and CMP, their optimum supplementation ranged from 0.45 to 0.48% and from 0.44 to 0.50%, respectively. In contrast, relatively low and a wide range of optimum AMP supplementation level were observed through regression analysis (0.16% for growth and 0.41 % for immunity). While, in the case of amberjack supplementation of inosine at 0.6%, there was optimum performance of growth and health parameters. In case of mixed purified nucleotide optimum supplementation levels for red sea bream ranged between 1 to 1.5g kg^{-1} diets. Overall, at optimal supplementation level the growth, feed utilization, stress resistance, intestinal health and immune responses of both species were enhanced. On the contrary in excess supplementation level in most cased growth and health performance parameters showed reduced performance. Reduced growth performance with excessive NT

supplementation level was also observed in some previous studies for both fish and other terrestrial animal. For example, a commercial NT product, Ascogen (Chemoforma, Augst, Switzerland) at a high dietary concentration (5%) caused growth depression in rainbow trout, *O. mykiss* (Adamek et al., 1996); Similarly, a high dietary concentration of IMP (1.0%) resulted in depressed growth performance of olive flounder (*P. olivaceus*) compared to lower levels of dietary IMP (0.1 - 0.2%) in a 14 weeks growth study (Song et al., 2012). Adenine has been shown to be toxic to rats at concentrations in excess of 0.1% (Akintonwa et al., 1979) and to inhibit growth rate in chickens at dietary concentrations of 0.8% and above (Baker and Molitori, 1974).

Evidence from animal and human studies supports the theory that dietary nucleotides are important for optimal functioning of several components of the immune system (Cosgrove, 1998). In the present study in most cases supplementation increased the non specific immune response through increasing the lyoszyme activity, peroxidase activity, respiratory brust acitivty, bactericidal activity, total serum protein and agglutination antiboday tier. The findigs of the present study completely supports the theory for optimal functioning of immune system by nucleotides. Moreover, our present study also supports the findigs of some previosus research (Burrells et al., 2001a,b; Lin et al., Cheng et al., 2011; Song et al., 2012; Tahmasebi-Kohyani et al., 2011,2012)

In all the experiments we measured the oxidative stress response of fish through measuring d-ROMs and BAP. Oxidative stress is considered to involve in plenty of diseases and pathological status in fish (Martinez-Alvarez et al., 2005), and is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants (Celi et al., 2010). After measuring d-ROM and BAP we made a comparison of both of these parameters. It is interesting to note that, supplementation increased the oxidative stress resistance of fish and at the optimum supplementation level it

showed best condition with low oxidative stress and high antioxidant levels, while supplementation free control group showed oxidative stress condition. To date, there is a lack of explanation about how these additives work to affect these parameters, so more studies are needed.

The beneficial effects of dietary nucleotides on gastrointestinal tract (GIT) is well documented in human and other terrestrial animals (Uauy et al., 1990; Bueno et al., 1994; Carver, 1994) but very little is known about fish and other aquatic animals (Burrells et al., 2001b; Borda et al., 2003; Cheng et al., 2011). In the present study we determine the effects of nucleoside, inosine on intestinal health condition of amberjack. In another comparative study comparison of the effects of inosine and IMP on gut health condition were evaluated. In both species results showed that supplementation of inosine and IMP improved intestinal fold height, microvillus height and enterocyte height of anterior and posterior intestine. Recently Cheng et al. (2011) also reported that nucleotide supplementation significantly increased fold height of the proximal intestine, and enterocyte height of the pyloric caeca, proximal and distal enteric sections of red drum, which completely supports our findings. Moreover, in comparative study results also showed that, with the exception of anterior hF, the inosine supplemented diet groups showed improved gut morphology compared to the IMP supplemented diet groups at the same supplementation level. Although the mechanism by which dietary nucleoside inosine exerts its positive effects over the nucleotides IMP on the intestine morphology is not clear, most of the ingested nucleotides will be degraded in the intestine to nucleosides by alkaline phosphatase and nucleotidases, and may be further broken down by nucleosidases to produce purine and pyrimidine bases. On the other hand, some investigations on land animals suggest that nucleosides are the primary form absorbed (Uauy, 1989). Most of the absorbed nucleosides and bases are rapidly degraded within the enterocytes (Sonoda and Tatibana, 1978), but certain levels may be incorporated into the

tissue pools, primarily in the small intestine, liver and skeletal muscle (Burridge et al., 1976; Saviano and Clifford, 1978). Inosine as a nucleoside absorbed directly by intestine mostly and beneficially can influence intestinal health of fish. The beneficial effects on intestine in present study might be due to the positive effects of inosine on mucosa associated with lymphoid tissue (MALT) although the knowledge on MALT in fish is very limited (Li and Gatlin, 2006).

As an initial step of low/non fishmeal (FM) based functional feed development for amberjack and red sea bream, in the final step of my research, inosine nucleoside and IMP nucleotide were supplemented in alternative protein (dehhulled soyabean meal and soya protein concentrate, SPC) based diet. Results indicated that amberjack can effectively utilize at least half of the replaced FM protein with soyabean meal protein without any compromising growth performances. Moreover, inosine supplementation in 50 to 75% FM replacement groups could be helpful to improve the digestibility, immune responses, stress resistance and intestinal health condition of amberjack. In the case of red sea bream trial, results showed that supplementation of IMP increased the efficiency of utilizing SPC (≤75%) as a sole protein source for the diet of juvenile red sea bream. Fish growth, feed utilization performances and blood chemical parameters were not significantly different up to 75% FM replacement groups in comparison with complete FM based control diet. In terms of non specific immune response, all replacement diet groups showed improved immune responses when compared with control diet group. Fish meal contained abundant nucleic acids and nucleotides, while little was found in soybean meal (Mateo et al., 2004). So, high inclusion of soybean protein might caused the nucleotide deficiency in the diet and supplementations of nucleotide recover these deficiency which results improved utilization of soybean protein (≤75%) in the present study.

Therefore, based on the overall findings of the present study suggests that nucleotides acts as a potential functional supplements in the red sea beam and amberjack diets. Optimum supplementations of nucleotides in alternative protein based diet reduce the negative effects of alternative proteins concurrently increase the utilization efficiency of alternative protein (soybean protein), which will help to develop low/non fish meal functional feed for these species. Moreover, supplementation of functional supplements, nucleotides is more environmental friendly approach compared to the use of antibiotics and chemicals in fish culture.

CHAPTER VIII

Summary and conclusion

8.1.Summary and conclusion

The present research was conducted to evaluate functional effects of purine and pyrimidine nucleotides and development of functional feed through supplementation of nucleotide like functional supplements for marine species like red sea bram and amberjack. To summarize the resuts of different experiments, it was found that-

1. Nucleotide realted products like nucleoside by- products (NBP) and inosine could be effectively used as relatively low price of nucleotde soucrce for marine speices like red sea bream. Meanwhile, inosine and low concentration of NBP (1%) could be effectively used as dietary supplements for better growth and health performance of *P. major*.
2. Supplemnetaion of inosine increased the growth, survival and health performace of juvenile amberjack. The optimum optimal levels of dietary inosine were 0.54 and 0.67 %, respectively, for juvenile amberjack, which is also inline with the most of the growth performance and health parameters of fish under present experimental condition.
3. Inosine and inosine monophosphate can have positive influences on growth performance, survival, feed utilization, immune response, hematological parameters, and intestinal morphology of red sea bream. Even though some oxidative stress parameters, non-specific immunity and intestinal morphology improved at 0.2% supplementation level, most of the performance parameters showed relatively better condition at 0.4 % supplementation level. Considering overall performance, it is recommended that dietary inosine and IMP supplementations at 0.4% are beneficial to promote growth, immune responses, oxidative stress and intestinal health condition of juvenile red sea bream.

4. In red sea bream the optimal suppllementaiton levels of UMP was 0.4%. For the GMP and CMP, the optimum supplementation ranged from 0.45 to 0.48% and from 0.44 to 0.50%, respectively. In contrast, relatively low and a wide range of optimum AMP supplementation level were observed through regression analysis (0.16% for growth and 0.41 % for immunity). Overall, the growth, feed utilization, stress resistance, intestinal health and immune responses of red sea beam were enhanced by the dietary nucleotide supplementations and at the optimum level of supplementation all performace parameters showed best condition.

5. Inosine nucleoside and IMP nucleotide supplementation in alternative protein (dehhulled soy bean meal and soy protein concentrate, SPC) based diet for amberjack and red sea bream showed that supplementation increae the effeciecny of utilizing alternative protein through reducing the negative effects of alternative protein for both species. Amberjack can effectively utilize at least half of the replaced FM protein with soybean meal protein without any compromising growth performances. Moreover, inosine supplementation in 50 to 75% FM replacement groups could be helpful to improve the digestibility, immune responses, stress resistance and intestinal health condition of amberjack. Similarly, supplementaion of IMP increased the efficiency of utilizing SPC (≤75%) as a sole protein source in the diet of red sea bream. Red sea bream growth, feed utilization performances and blood chemical parameters were not significantly different up to 75% FM replacement groups in comparison with complete FM based control diet. In terms of non specific immune response, all replacement diet groups showed improved immune responses when compared with control diet group

Finally, we concluded that nucleotides could be effectively used as functional supplements for the development of low/non fish meal based funcitonl feed for marine species like, red sea bream and amberjack. Moreover, supplementation of functional supplements, nucleotides is more environmental friendly approach compared to the use of antibiotics and chemicals in fish culture.

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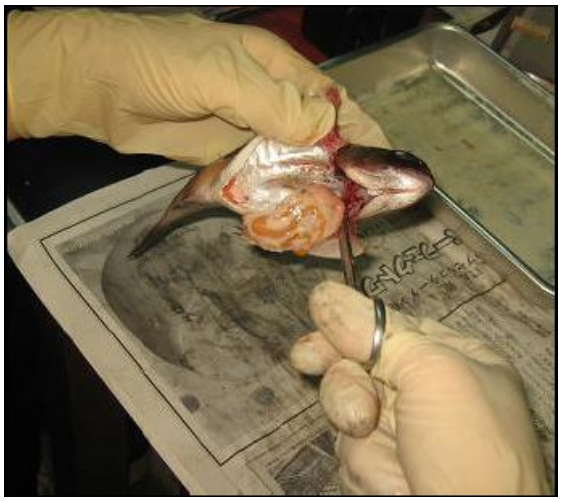
Appendix

Fig. 2.3: Experimental system and tank

Fig. 2.5: Test for freshwater stress resistance

(a)



(b)

Fig. 2.4a,b: Sample collection (blood, organs)

Fig. 2.2: Experimental diet preparation