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The Immune Responses and Histopathology of Asian Seabass (*Lates calcarifer*) Fed Diets Supplemented with Various Single Pure Nucleotides

Sri Dwi Hastuti[1, 2*], James L. Munro[1], Stephen B. Pyecroft[1], Mary D. Barton[3], Maurizio Costabile[3, 4], Ben C. Jones[1]

[1]School of Animal and Veterinary Sciences, The University of Adelaide, Australia
[2]Department of Aquaculture, University of Muhammadiyah Malang, Indonesia
[3]School of Pharmacy and Medical Sciences, University of South Australia, Australia
[4]Centre for Cancer Biology, University of South Australia and SA Pathology, Frome Road, Adelaide, SA 5000, Australia

[*]**Corresponding Author: hastuti@umm.ac.id**

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ABSTRACT

This study investigated the immune responses and histopathology of the Asian seabass (*Lates calcarifer*) fed diets supplemented with 0.5% of different single pure nucleotides. Juvenile seabass (initial average weight: 16.43 ± 0.88 g) were stocked in 250L plastic tanks (10 fish/tank) and fed laboratory-prepared diets containing adenosine monophosphate (AMP), inosine monophosphate (IMP), uridine monophosphate (UMP), guanosine monophosphate (GMP), or cytidine monophosphate (CMP) for 56 days. A control group received a basal diet without nucleotide supplementation. At the end of the trial, growth performance, immune parameters, and organ histology were assessed. Fish fed IMP- and GMP-supplemented diets exhibited significantly higher weight gain (WG) and specific growth rate (SGR) (P<0.05). Serum interleukin-1β (IL-1β) levels were significantly elevated in the AMP group (P<0.05), though other immune responses showed no significant differences. However, a trend suggested that nucleotide-supplemented diets generally enhanced immune responses compared to the control. Histological examination revealed tissue alterations in most samples, with more severe changes observed in the basal diet group. These findings indicate that dietary supplementation with IMP, GMP, and AMP may improve growth performance for the Asian seabass and could positively influence immune function.

INTRODUCTION

Supplementation of fish diets with particular nutrients such as immunomodulators or immunostimulants has shown positive effects for maintenance of health status when fish are under stress conditions **(Uribe *et al.*, 2011; Pohlenz *et al.*, 2014)**. This suggests that there is a correlation between nutrient availability and immune responses of teleost fish **(Kiron 2012; Olivia-Teles, 2012; Pohlenz *et al.*, 2014)**. Among the wide range of immunomodulators, nucleotides have achieved greater prominence within the aquaculture

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industry over the last decade. This interest stems from a number of reports from studies showing nucleotide supplementation improves health and growth of aquatic organisms **(Lin *et al.*, 2009; Cheng *et al.*, 2011; Kenari *et al.*, 2013; Hossain *et al.*, 2016a; Asaduzzaman *et al.*, 2017)**. The benefits of dietary nucleotide supplementation in improving health status of fish may help reduce the dependence on antibiotics and other chemotherapeutic agents for the treatment of disease in aquaculture species and may also improve aquaculture production and generally reduce disease occurrence on farms due to improved immune competency of cultured species **(Kiron, 2012; Olivia-Teles, 2012; Pohlenz *et al.*, 2014)**.

Previously, research on the potential benefits of dietary nucleotides for increasing growth and health status of aquatic organisms has been predominated by the use of commercially produced nucleotide supplement products. Whether the benefits from these products are from nucleotides alone or from other components of the supplements such as other components from yeasts, has led to more work being undertaken investigating the benefits of pure nucleotide supplements **(Lin *et al.*, 2009)**. The first experiment which used pure nucleotide in fish diets was undertaken using Red drum (*Sciaenops ocellatus)* **(Li *et al.*, 2007)**, in which five single pure nucleotides (AMP, IMP, UMP, GMP and CMP) were mixed at equal amount and incorporated into fish diet. The supplementation produced significantly increases in the growth rates of treated fish. **Lin *et al.* (2009)** studied the same mixture of pure nucleotides within a grouper (*Epinephelus malabaricus*) diet with supplementation leading to higher weight gains (WG) and concentration of head kidney superoxide anions in the fish fed nucleotides diet compared to the control group. A recent study on red sea bream (*Pagrus major*) fed a purified mixture of nucleotides in the diet at different levels, showed that supplementation of 1.0–1.5g kg^{-1} mixed nucleotides into the fish diet promotes growth, immune responses and stress resistance of juvenile red sea bream **(Hossain *et al.*, 2016a)**. On the other hand in our previous study the inclusion of the same nucleotide mixture into the Asian seabass diet at 0.5% resulted in a significant increase on fish WG and serum total protein and globulins **(Hastuti *et al.*, 2021)**.

To date there are few studies of single purified nucleotide supplementation effects on aquatic animal diets. For example, AMP or IMP or UMP or GMP or CMP have been supplemented individually in red sea bream diets **(Lin *et al.* 2009; Hossain *et al.*, 2016b, 2016c, 2016d, 2017b, 2017c)** and Amber jack (*Seriola dumerili*) **(Hossain *et al.*, 2017a)**. However, there is still no information about Asian seabass (*Lates calcarifer*) fed a single pure nucleotide diet.

The Asian seabass (*Lates calcarifer*), is an economically important species for aquaculture throughout Southeast Asia, Australia, Western Pacific and India **(Ngoh, 2015; Siddik *et al.*, 2018)**. Asian seabass is an ideal aquaculture candidate species due to its excellent attributes such as high consumer demand, delicately-flavored white meat, rapid growth, the ability to be cultured in a wide range of environments and the ability to

adapt to artificial feed **(Anil *et al.*, 2010; Philipose *et al.*, 2010; Ngoh, 2015)**. In order to support the development of Asian seabass aquaculture, optimum nutrition (i.e., diets that provide the fish with all components for health and guarantee high growth rates) as well as possibly optimizing resistance in the fish against a wide range of stresses and potential infections, is required. Supplementation with immunonutrients such as nucleotides in diets could be expected to increase production in Asian seabass aquaculture. Therefore, the current study aimed to evaluate the effects of the supplementation of single pure nucleotides into diets of the Asian seabass on immune responses and the histopathological changes in its organs.

MATERIALS AND METHODS

Fish and ethics statement

All fish used in this study were covered by the University of Adelaide Animal Ethics Committee (approval number S-2017-044). A total of 180 juvenile Asian seabass with an average weight of 16.43 ± 0.88g (mean ± standard deviation) from a local supplier were used in this study. Upon arrival, fish were acclimated to laboratory condition for four weeks prior to the commencement of the feeding trial, and water temperature was maintained at 22-25°C during the acclimation period.

Diet preparation

In this experiment, we used diets containing different types of single pure nucleotide namely: adenosine monophosphate (diet AMP), uridine monophosphate (diet UMP), cytidine monophosphate (diet CMP), inosine monophosphate (diet IMP), guanosine monophosphate (diet GMP) and the diet without supplementation of nucleotide (diet C). All the nucleotides were purchased from Sigma Aldrich Germany and supplemented into a commercial diet base (Ridley, Australia). The diets were prepared by mixing fish oil, wheat flour and single pure nucleotide and water with the commercial grower mash to form a dough. The mixture was then put into a New Flora domestic meat grinder mincer (Flora Livings, Australia) to form pellets. Tables (1, 2) showed the formulation and approximate composition of the diet.

Table 1. Experimental diet formulation

Ingredient (%)	Basal (C)	AMP	IMP	UMP	GMP	CMP
Commercial grower mash	75	75	75	75	75	75
Fish oil	10	10	10	10	10	10
Wheat flour	15	14.5	14.5	14.5	14.5	14.5
AMP nucleotide	-	0.5	-	-	-	-
IMP nucleotide	-	-	0.5	-	-	-
UMP nucleotide	-	-	-	0.5	-	-
GMP nucleotide	-	-	-	-	0.5	-

CMP nucleotide	-	-	-	-	-	0.5
Approximate composition of commercial mash (%)						
Moisture						8.4
Ash						8.2
Crude protein						53.8
Crude lipid						6.4

Note: C (control/no supplemented nucleotides), AMP (adenosine monophosphate), IMP (inosine monophosphate), UMP (uridine monophosphate), GMP (guanosine monophosphate), CMP (cytidine monophosphate).

Table 2. Proximate analysis of the experimental diets

Parameter	Diet groups					
(% in diet)	C	AMP	IMP	UMP	GMP	CMP
Dry matter	91.22	89.77	88.82	90.16	90	91.07
Ash	9.07	9.2	9.31	8.93	9.39	9.07
Crude protein	49.37	48.13	50.55	49.61	48.76	48.4
Crude lipid	8.66	7.82	8.37	7.90	7.97	8.16
Fibre	3.3	2.58	3.09	2.41	1.24	3.3

Note: C (control/no supplemented nucleotides), AMP (adenosine monophosphate), IMP (inosine monophosphate), UMP (uridine monophosphate), GMP (guanosine monophosphate), CMP (cytidine monophosphate).

Feeding trial and blood sampling

Ten fish were placed into a 250L square plastic tank equipped with a filter aeration for 56 days. Water was changed at 30% every day to maintain water quality. Fish were fed once a day to near satiation. At the end of the feeding trial, three fish were euthanised by using overdose of AQUI-S® (AQUI-S New Zealand Ltd), and fish were weighed to calculate WG using the following formulae: WG = final body weight (g) – initial body weight (g) and specific growth rate (SGR) using this formulae: SGR = 100 × [ln final body weight (g) − ln initial body weight (g)/duration of feeding (day)]. After being weighed, blood was taken from the caudal vein of the fish by needle and syringe. The blood sample was centrifuged at 3000g for 15 minutes and serum was collected and pooled from the five fish then stored at –80°C until analyzed. The serum was used to assay lysozyme and complement activity, total protein, albumin, and globulins, and also interleukin(IL)-1β, while the respiratory burst assay was conducted by placing fish blood into a heparinised tube and processed within five hours of collection.

Lysozyme activity

The level of lysozyme activity in each blood sample was measured using a method described by **Ellis (1990)**, with a slight modification. A total of 10µL of Asian seabass serum was placed into Corning 96 well plate flat bottom (Adelab Scientific, Australia) and mixed with 10µL of 0.05 M sodium phosphate buffer (Na_2HPO_4

dodecahydrate "12 H_2O") (Sigma Aldrich, Australia) at pH 6.2. As much as 130µL of lyophilized *Micrococcus lysodeikticus* (Sigma Aldrich, Australia) suspension at a concentration of 0.6mg mL^{-1} in phosphate buffer, pH = 6.2 was added to the wells. Absorbance was monitored at 450nm at 0 and 10min using a Benchmark Plus microplate spectrophotometer, Bio-Rad version 5.2.1. One unit of lysozyme activity was defined as the quantity of serum which caused a 0.001min^{-1} decrease in absorbance. The serum lysozyme activity was expressed in unit mL^{-1} serum.

Complement activity

Alternative complement activity was measured following the methods described by **Hastuti *et al.* (2020)**. Rabbit red blood cells (RRBC) in Alsever's solution (Applied Biological Products Management (Adelaide, Australia) were washed twice with equal amount of EGTA-GVB (ethylene glycol-bis tetracetic acid-gelatin veronal buffer) (Sigma Aldrich, Australia) by centrifugation at 1000g for 5 minutes at 22°C and resuspended in the same buffer at 4×10^7 cells mL^{-1} and used for the assay. Briefly, Asian seabass serum was serially diluted in Mg-EDTA-GVB, pH 7.5 at 1:8 dilution. The dilution was added with 100 µL of RRBC suspension, and then incubated at 25°C for 30 minutes. Following incubation, the sample was centrifuged at 2000g for 5 minutes at 22°C. A total of 100µL of supernatant was removed, transferred to a 96-well flat bottom plate (Sarstedt, Germany) and the optical density was measured at 540nm using an Ascent Multiskan microplate reader.

Respiratory burst activity by dihydrorhodamine (DHR)-123 flow cytometry

Respiratory burst activity was performed using whole blood DHR-123 flow cytometry followed method by **Hastuti *et al.* (2019)**. Briefly, 25µL of blood sample was placed in 12 x 75mm round bottom polystyrene tubes. Amount of 225µL phosphate buffered saline (PBS) supplemented with DHR-123 (Sigma-Aldrich) was added to the blood and mixed carefully, then incubated at 37°C for 15 minutes in a water bath. Phorbol 12-myristate 13- acetate (PMA) was added to the tubes at final concentration of 100nM, and incubation was continued for 15 minutes. A control tube without PMA stimulation was included with every sample tested. Subsequently, one mL of ammonium chloride lysis solution (0.15 M NH_2Cl, 10 mM $NaHCO_3$, 1 mM disodium EDTA, pH 7.4) was added to all tubes. The tubes were vortexed and incubated at room temperature for 10 minutes followed by centrifugation at 500 x *g* for 3 minutes. The supernatant was discarded, the cells were washed and resuspended in 100 µL of PBS then analysed by a BD FACSCanto I flow cytometer, using BD FACSDiva v8.0 software. Respiratory burst activity was determined by the increasing of Rhodamine-123 fluorescence (RHO) in activated cells against unstimulated cells.

Total protein, albumin, and globulins

Total protein, albumin, and globulins in serum were measured by autoanalyzer AU480 (Beckman-Coulter) using standard protocols provided in machine user guide. A total of 200µl of pooled fish serum from each treatment group were measured collectively.

IL-1β assay

The concentration of fish IL-1β in serum was determined by specific Fish IL-1β ELISA Kit (My Bio Source, Product code: MBS026888, San Diego, USA) according to manufacturer's instructions. 50µL serum was placed in 96-well plate, then 100µL of HRP-conjugate reagent was added and the plate incubated for 60 minutes at 37°C. Following the incubation, the plate was washed four times with washing solution and dried. After that, 50µL of Chromogen Solution A and Chromogen Solution B were added to each well, and the plate was incubated again for 15 minutes at 37°C. Finally, 50µL of stop solution was added to each well, and the optical density was read at 450nm using a Benchmark Plus microplate spectrophotometer, Bio-Rad version 5.2.1.

Histology examinations

Briefly, three fish from each group of feeding trial were sampled, humanely killed and rapidly fixed in 10% neutral buffered formalin solution and then stored until processed for histopathological examination. Necropsies were performed, and tissue specimens were collected from gills, liver, spleen, kidney, and intestine. Samples were trimmed and put into the cassettes, processed routinely through graded alcohols, embedded into metal moulds, sectioned at 4 µm thickness and stained with Mayer Bennet hematoxylin and eosin (HE), then examined by using an Olympus BX 41 microscope to visualise any pathological changes present. The type of alterations that was assessed in the histoloical analysis included congestion, hyperplasia, oedema, vacuolation, infiltrations of inflammatory cells, curling/clubbing/fusion of gill lamellae, parasites in the gills, haemorrhage, necrosis/degeneration/pyknosis, melano macrophages center present, lymphoid depletion or ellipsoids of spleen, and loss of nuclei of the hepatic cells. Any alterations and abnormalities found in each organ were identified and scored subjectively as follows: score 0 = no alteration, score 1 = mild/moderate alterations, and score 2 = marked alterations. If the evaluated organ has more than one abnormalities feature then the score of each feature is summed to give a histopathology score for the organ. Histopathology score of the organ may therefore be greater than 2.

Statistical analysis

Each diet was fed to three replicates groups of fish according to a completely randomized design. Results were analyzed by one-way analysis of variance (ANOVA) using IBM SPSS version 22.0 statistical software, followed with Duncan's test to

compare the means between individual treatments. Significance was set at $P< 0.05$. Descriptive and semi quantitative analysis were used for histopathological analysis.

RESULTS

Growth rate

Growth performance of the fish during 56 days of the feeding trial are shown in Table (3). The results of the present study suggest that supplementation of different types of pure nucleotides significantly affect growth performance of the Asian seabass under study. It was found that the WG of the fish fed diet containing 0.5% of GMP and IMP was significantly higher compared to other diet groups, with the value of 49.96 ± 8.23g and 48.24 ± 2.41, respectively, while the SGR of diet containing IMP and GMP improved significantly, with the value of 2.53 and ± 0.02 and 2.51 ± 0.20, respectively.

Lysozyme activity

The serum lysozyme activity of each diet group is presented in Table (4). Different types of single pure nucleotides added to the diet did not significantly affect the lysozyme activity ($P= 0.246$). However, the highest lysozyme activity was found in fish fed diet supplemented with IMP followed by UMP with the values of 436.18 ± 202.15 and 374.06 ± 90.17 Unit mL^{-1} (mean ± standard deviation "SD, respectively).

Complement activity

There were no differences in the serum alternative complement activity of the Asian seabass fed diets containing different types of single purified nucleotides for 56 days, as presented in Table (4). However, nucleotide supplemented diet groups tended to have greater lysis activity compared to the control group. The highest lysis activity of serum complements occurred in the diet supplemented with GMP with the lysis activity of 136.5 ± 5.79 (mean ± SD)

Table 3. Growth performance of Asian seabass during 56 days of feeding trial

Diet Group	Initial Weight (g)	Final Weight (g)	Weight Gain (WG, g)	Specific Growth Rate (SGR, % BW day^{-1})
C	15.75 ± 0.41	53.74 ± 5.44	36.35 ± 3.27^{a}	2.14 ± 0.14^{a}
AMP	16.78 ± 1.07	56.90 ± 4.23	40.12 ± 3.90^{ab}	2.18 ± 0.13^{ab}
IMP	15.43 ± 0.67	63.67 ± 3.06	48.24 ± 2.41^{b}	2.53 ± 0.02^{b}
UMP	16.57 ± 0.68	58.07 ± 5.79	41.50 ± 6.41^{ab}	2.24 ± 0.25^{ab}
GMP	16.21 ± 1.43	66.17 ± 8.89	49.96 ± 8.23^{b}	2.51 ± 0.20^{b}
CMP	17.20 ± 0.13	56.20 ± 8.91	39.01 ± 8.73^{ab}	2.11 ± 0.26^{a}

Note: Data presented as means of triplicates ± SD. Different superscripts in the column indicate significant ($P<0.05$) difference between different diet groups. C (control/no supplemented nucleotides), AMP (supplemented with adenosine monophosphate), IMP (supplemented with inosine monophosphate), UMP (supplemented with uridine monophosphate), GMP (supplemented with guanosine monophosphate), CMP (supplemented with cytidine monophosphate).

Respiratory burst activity

The respiratory burst activities were measured by DHR-123 flow cytometry using PMA for stimulating cells activity. It was found that supplementation of diets with different types of pure nucleotide did not affect the respiratory burst activity, evidenced by the percentage of RHO positive cells, as shown in Table (4). However, it was observed that, diet supplemented with 0.5% AMP gave the highest respiratory burst activity with the percentage of RHO positive cells of 32.16 ± 12.23 (mean ± SD).

Table 4. Serum biochemical assay of Asian seabass after 56 days of feeding on diet containing single pure nucleotide

Diet Group	Lysozyme (unit/ml)	Complement (% lysis)	Respiratory burst (% RHO-positive cell)	IL-1β (pg/ml)
C	342.66 ± 15.51	107.37 ± 41.99	31.03 ± 11.62	20.59 ± 18.63[a]
AMP	341.98 ± 39.51	117.54 ± 9.49	32.16 ± 12.23	110.25 ± 73.80[b]
IMP	436.18 ± 202.15	123.86 ± 13.33	27.16 ± 7.67	42.38 ± 16.52[ab]
UMP	374.06 ± 90.17	126.67 ± 7.39	28.90 ± 7.63	51.02 ± 2.67[ab]
GMP	271.67 ± 63.84	136.49 ± 5.80	31.23 ± 11.86	54.76 ± 42.56[ab]
CMP	317.75 ± 68.06	123.68 ± 18.53	17.29 ± 2.72	63.05 ± 4.61[ab]

Note: Data presented as means of triplicates ± SD. Different superscripts in the column indicate significant (P<0.05) difference between different diet groups. C (control/no supplemented nucleotides), AMP (supplemented with adenosine monophosphate), IMP (supplemented with inosine monophosphate), UMP (supplemented with uridine monophosphate), GMP (supplemented with guanosine monophosphate), CMP (supplemented with cytidine monophosphate).

IL-1β

The activity of the Asian seabass cytokines IL-1β was measured with a MyBioSource IL-1β Kit, developed specifically for fish. Diet enriched with dietary nucleotides resulted in higher serum IL-1 β levels, as shown in Table (4). It was found that the level of IL-1β in fish fed diet containing AMP only was significantly higher, compared to other diet groups, with the value of 110.3 ± 73.80 pg mL^{-1}.

Total protein, albumin and globulins

Total protein, albumin, and globulins in the serum of fish fed different types of single pure nucleotides did not differ significantly. However, it was marked that the diet supplemented with GMP resulted in higher total protein, albumin and globulins levels with the values of 37.47 ± 2.78; 10.03 ± 1.90 and 26.33 ± 1.53 (mean ± SD) g L^{-1}, respectively. Table (5) presents the data for total protein, globulins and albumin of the Asian seabass serum.

Table 5. Total protein, albumin, and globulins of Asian seabass serum associated with diets containing different types of pure nucleotides

Treatment	Total Protein ($g\ L^{-1}$)	Albumin ($g\ L^{-1}$)	Globulins ($g\ L^{-1}$)	A : G
C	36 ± 2.86	9.43 ± 1.30	26.33 ± 1.53	0.36 ± 0.02
AMP	34.9 ± 1.71	9.43 ± 0.49	25.67 ± 1.53	0.37 ± 0.01
IMP	35.97 ± 2.78	9.77 ± 0.93	26.33 ± 2.08	0.37 ± 0.03
UMP	36.7 ± 5.0	9.63 ± 2.28	27.0 ± 2.65	0.35 ± 0.05
GMP	37.47 ± 2.78	10.03 ± 1.90	27.67 ± 1.53	0.36 ± 0.06
CMP	36.05 ± 2.47	9.8 ± 1.31	26.0 ±1.41	0.37 ± 0.02

Note: C (control/no supplemented nucleotides), AMP (supplemented with adenosine monophosphate), IMP (supplemented with inosine monophosphate), UMP (supplemented with uridine monophosphate), GMP (supplemented with guanosine monophosphate), CMP (supplemented with cytidine monophosphate).

Histopathology of fish organs

Table (6) shows the degree of abnormalities in fish organs. The most affected organs with observable changes found were gills, kidney, and spleen.

Table 6. Semi-quantitative analysis of tissue abnormalities at the different groups of fish fed on diets supplemented with nucleotides

Treatment	Gill	Kidney	Spleen	Liver	Intestine
C	4.33 ± 1.00^{b}	4.00 ± 1.32^{b}	3.00 ± 1.32^{ab}	3.33 ± 0.87^{b}	0.33 ± 0.71
AMP	2.66 ± 1.22^{a}	3.66 ± 1.73^{b}	2.66 ± 1.41^{ab}	1.33 ± 1.12^{a}	0.33 ± 0.50
IMP	2.00 ± 1.12^{a}	3.00 ± 0.71^{ab}	2.66 ± 0.87^{ab}	0.66 ± 0.71^{a}	0.00 ± 0.00
UMP	2.66 ± 1.87^{a}	3.66 ± 1.22^{b}	2.00 ± 1.12^{a}	1.33 ± 0.87^{a}	0.00 ± 0.00
GMP	2.00 ± 1.41^{a}	2.00 ± 0.87^{a}	2.00 ± 1.58^{a}	0.66 ± 0.87^{a}	0.00 ± 0.00
CMP	2.33 ± 1.32^{a}	3.33 ± 2.12^{ab}	3.66 ± 1.73^{b}	0.66 ± 0.71^{a}	0.00 ± 0.00

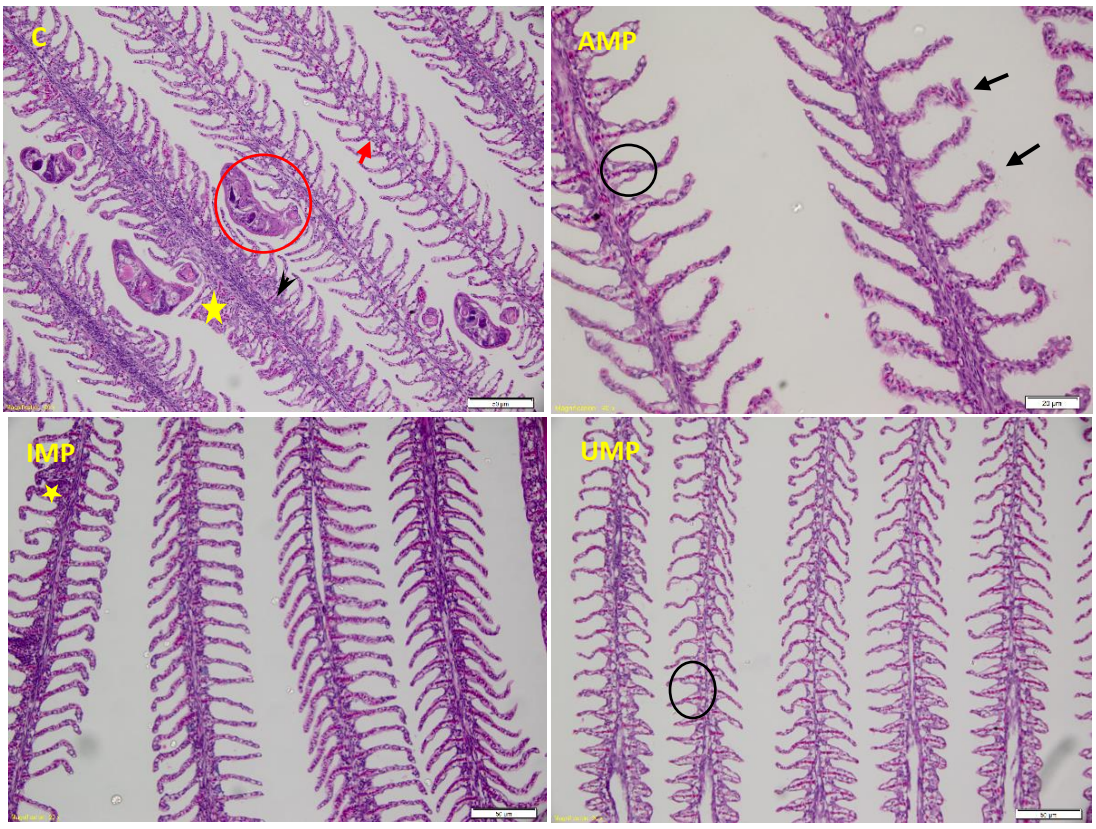
Note: Data presented as means of triplicates ± SD. Different superscripts in the column indicate significant ($P<0.05$) difference between different diet groups. C (control/no supplemented nucleotides), AMP (supplemented with adenosine monophosphate), IMP (supplemented with inosine monophosphate), UMP (supplemented with uridine monophosphate), GMP (supplemented with guanosine monophosphate), CMP (supplemented with cytidine monophosphate).

Tissues of the fish demonstrated abnormalities in most samples. Common histological alterations in the gills, found in our present study, included congestion of blood vessels, curling at the secondary gill lamellae, lifting of the respiratory epithelium (clubbing), vacuolation, secondary gill lamellae fusion, oedema, hyperplasia of the lamellar epithelium, the presence of melano-macrophage (melanin pigment), haemorrhage and rupture/degeneration of gill lamellae, as shown in Fig. (1). In addition, the gills of some fish were infected with monogenean parasites, in which hyperplasia and congestion, with an accumulation of inflammatory cells near the site of infection. The most severe infected gills with parasites were found in control group. The infection of the monogeneans led to the necrosis and damaging tissues around the infection site. In contrast, fish fed nucleotide diet experienced fewer alterations than those fed the control

diet. However, some samples from GMP and IMP diet treatment showed normal gill structure.

Histology examination of the posterior kidney of the Asian seabass showed moderate to marked vascular congestion and melano-macrophage centres (MMC), with hyperactivation of the MMC mostly in control (free nucleotide diet) group of fish. Other alterations including interstitial inflammation, necrosis or degeneration of the kidney tubules, vacuolation, narrowing of tubular lumen, oedema, pyknosis and haemorrhage were also found in some samples. Both the kidney samples in control and treated group seemed to experience alterations, however the degree of severity was greater in the control group, in which the inflammation and tissue damage with the sign of structural disruption and disorganization of tubular architecture were shown. Fig. (2) displays the changes in the posterior kidney of fish.

The spleen tissues of control and some treated groups showed red pulp degeneration with signs of lymphoid depletion, resulting in the disruption of spleen structure and unclear boundaries between red pulp and white pulp regions. Blood vessel congestion, focal haemorrhages and mild to moderate leucocytes infiltration were also detected in the tissue samples. Ellipsoids were frequently found at the red pulp area. In addition, hyperactivation of melano-macrophage center and necrosis were also observed in spleen tissue, as shown in Fig. (3).



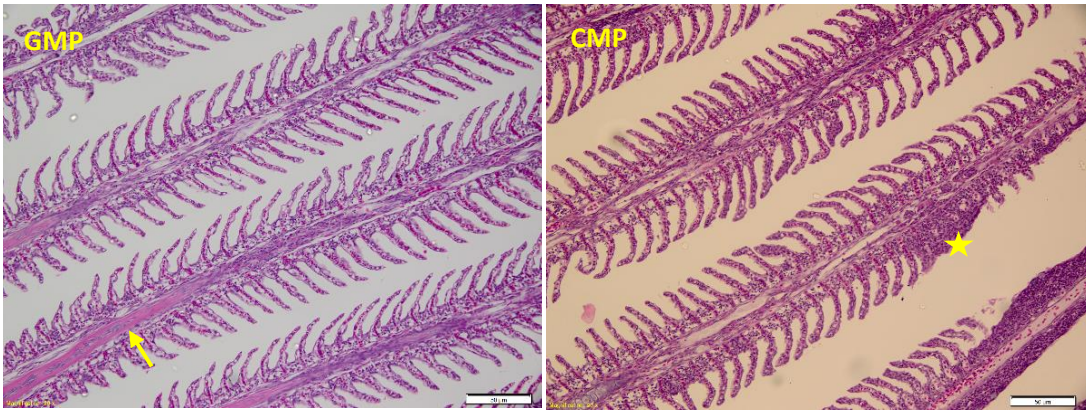


Fig. 1. Histology of the gill of Asian seabass fed dietary nucleotides. C (control/no supplemented nucleotides) (H&E, 200X), AMP (adenosine monophosphate) (H&E, 400X), IMP (inosine monophosphate) (H&E, 200X), UMP (uridine monophosphate) (H&E, 200X), GMP (guanosine monophosphate) (H&E, 200X), CMP (cytidine monophosphate) (H&E, 200X). The abnormalities found on the gill were infestation of monogeneans parasite (red circle), hyperplasia of the goblet cells (red arrow), leucocyte infiltration (arrow head), curling at the tip of secondary gill lamellae (arrow), hyperplasia of the secondary gill lamellae resulting of gill fusion (yellow star), lifting of the epithelial cells (circle), congestion (yellow arrow)

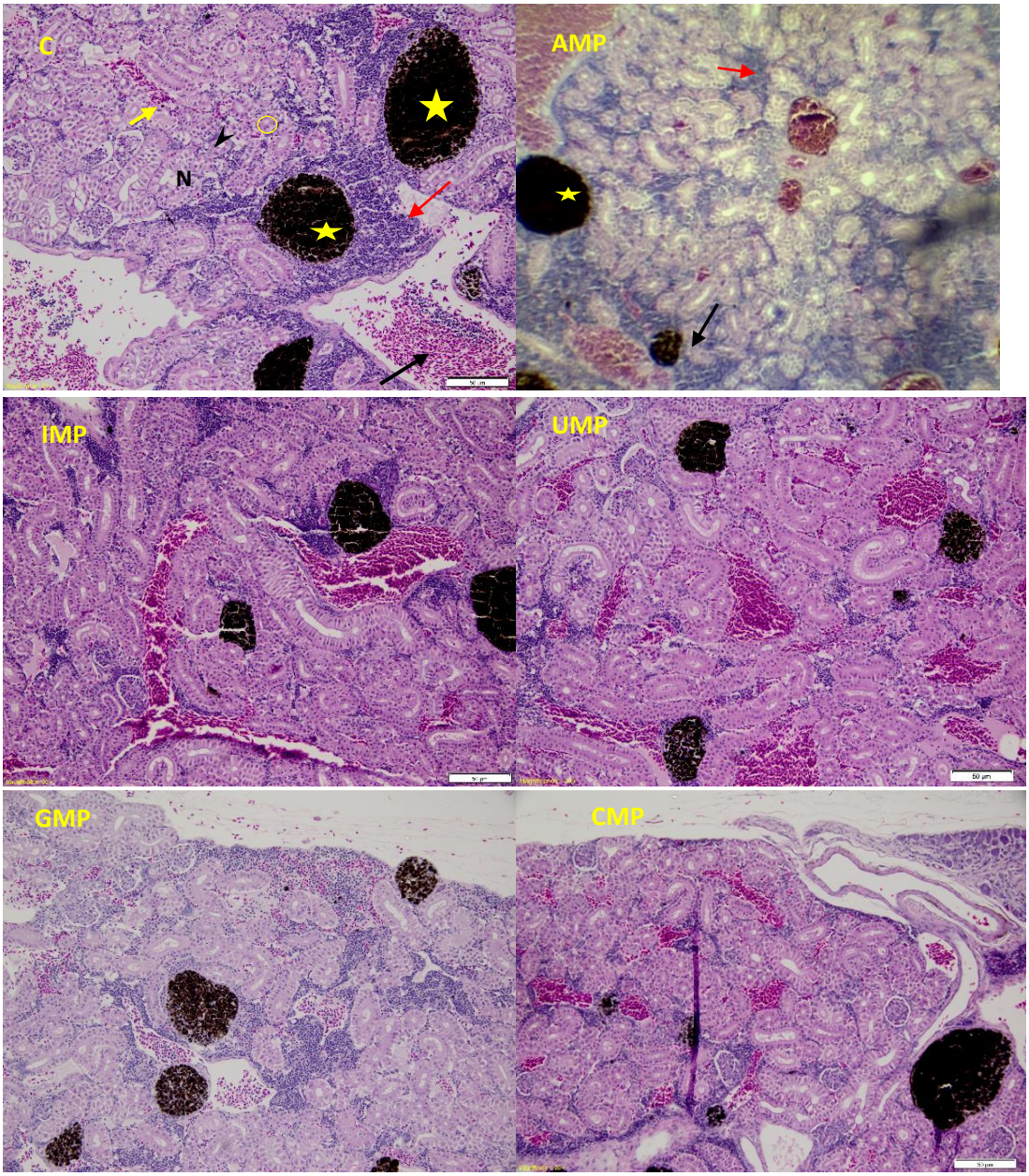


Fig. 2. Histopathology alteration of the Asian seabass kidney. C (control/no supplemented nucleotides), AMP (adenosine monophosphate), IMP (inosine monophosphate), UMP (uridine monophosphate), GMP (guanosine monophosphate), CMP (cytidine monophosphate). Abnormalities found in the kidney were hyperactivation of melanomacrophages (MMC) (yellow star), congestion of blood vessels (arrow), leucocyte infiltration (red arrow), vacuolation (arrow head), haemorrhage (yellow arrow), pyknosis (yellow circle) and necrosis (N) (H&E, 200×)

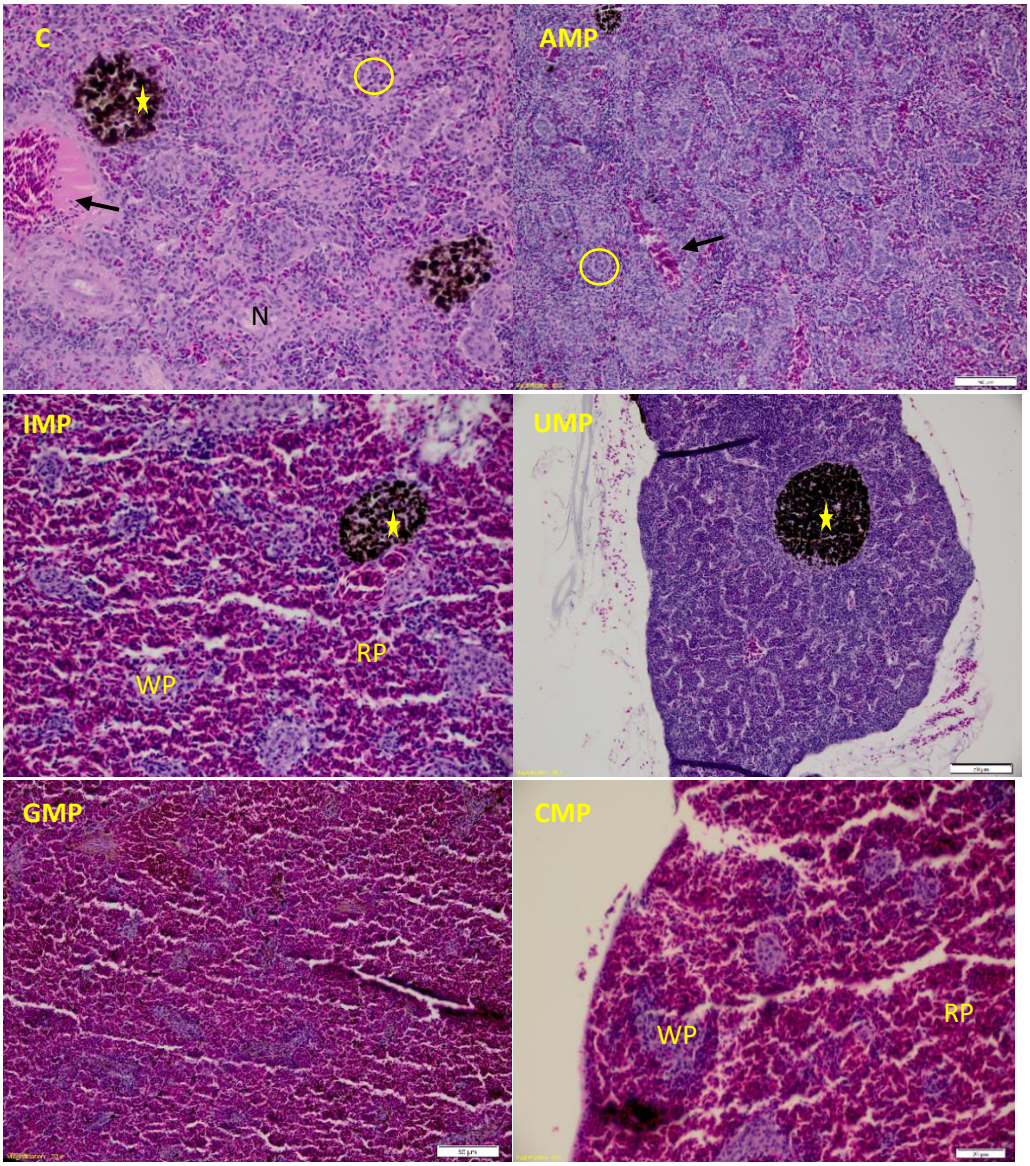


Fig. 3. Histopathology alteration of the Asian seabass spleen. C (control/no supplemented nucleotides) (H&E, 200X), AMP (adenosine monophosphate) (H&E, 200X), IMP (inosine monophosphate) (H&E, 400X), UMP (uridine monophosphate) (H&E, 100X), GMP (guanosine monophosphate) (H&E, 200X), CMP (cytidine monophosphate) (H&E, 400X). Abnormalities found in the spleen were red pulp degeneration and lymphoid depletion (C and AMP), melano-macrophage centre (MMC) (yellow star), ellipsoids (yellow circle),, congestion (arrow) and N (necrosis). RP (red pulp), WP (white pulp)

Liver tissue from control fish exhibited mild to marked congestion of blood vessels, accompanied by infiltration with inflammatory cells, oedema and marked vacuolation, suggesting fatty degeneration or toxic stress. Loss of nuclei in hepatocytes, hypertrophy of hepatocytes, pyknosis, degeneration and hepatocytic necrosis were also found in the organ sample of the control fish. Some samples showed congestion and mild activation of MMC, which might be an indication of oxidative stress. Less alterations

were found in tissue sample from fish fed nucleotide diet. Fig. (4) shows the histopathological changes in the liver.

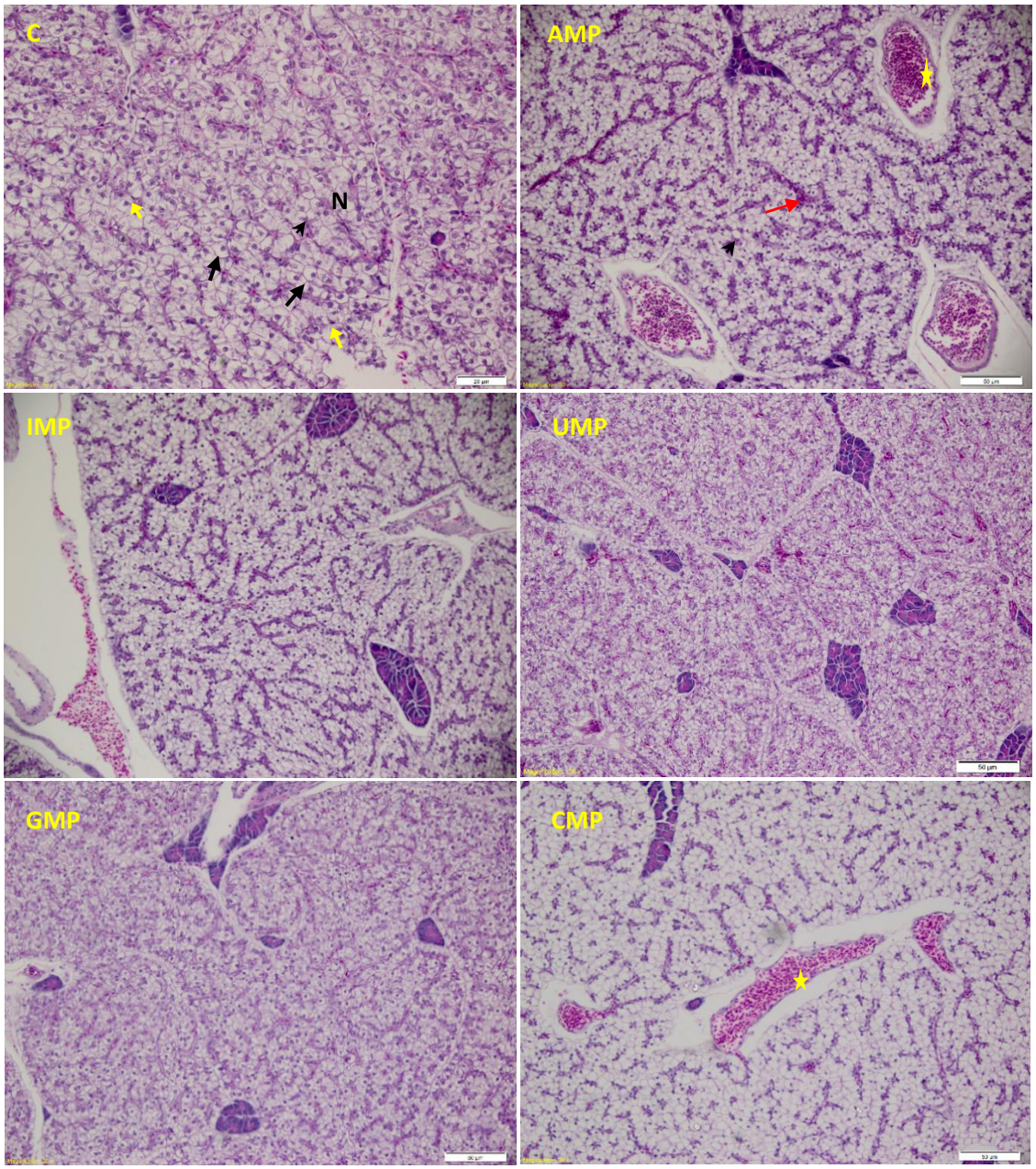


Fig. 4. Histopathology alteration of the Asian seabass liver. C (control/no supplemented nucleotides) (H&E, 400X), AMP (adenosine monophosphate) (H&E, 200X), IMP (inosine monophosphate) (H&E, 200X), UMP (uridine monophosphate) (H&E, 200X), GMP (guanosine monophosphate) (H&E, 200X), CMP (cytidine monophosphate) (H&E, 200X). Abnormalities found in the liver were vacuolation (arrow head), hypertrophied hepatocytes (arrow), pyknosis (yellow arrow), congestion (star), infiltration of inflammatory cells (red arrow)

There were not many histopathological changes found in gastrointestinal of the sample, in which most of the stomach and intestine appeared normal. Some samples experienceed mild congestion. Notably, samples from nucleotides diet group showed increased fold density and branch of the villi intestine compared to fish fed nucleotide free diet (control diet), as shown in Fig. (5). It is worthy to mention that, the increase of villi fold and height will increase the absorption area of the intestine for better nutrition absorption for fish growth and health.

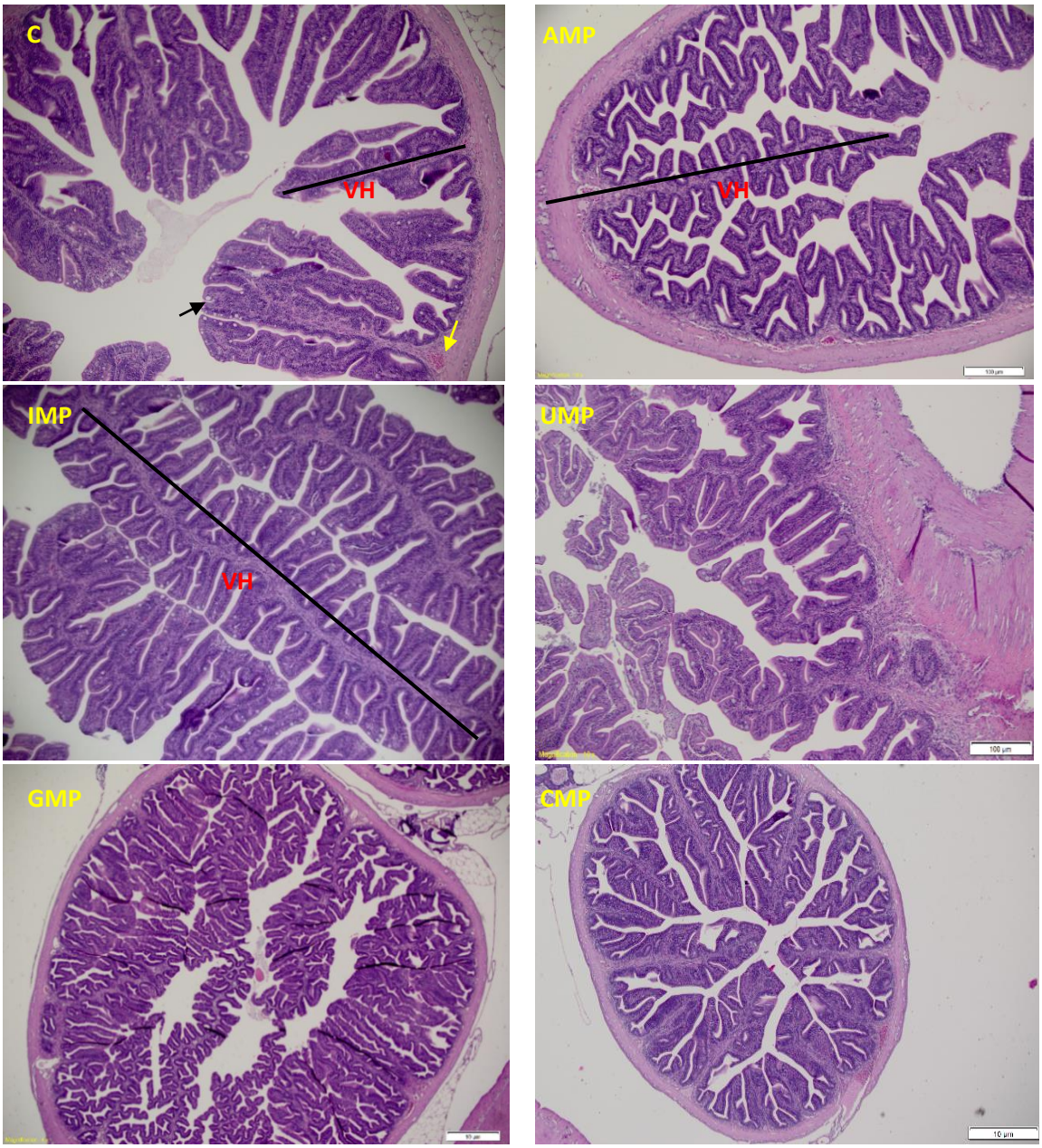


Fig. 5. Microphotographs of enteric section of Asian seabass. C (control/no supplemented nucleotides) (H&E, 200X), AMP (adenosine monophosphate) (H&E, 100X), IMP (inosine monophosphate) (H&E, 200X), UMP (uridine monophosphate) (H&E, 200X),

GMP (guanosine monophosphate) (H&E, 40X), CMP (cytidine monophosphate) (H&E, 40X). Congestion (yellow arrow), goblet cell (arrow), VH (villus height)

Water quality during the experiment

During the feeding trial, the water quality in each tank was evaluated. The average temperature range during the trial was 27.9-28.1ºC; pH was around 6.2-6.3; and ammonia level was recorded at 8mg/ L (uncontrolled and very high), while average nitrite range was at 1.6-2.3mg/ L, and nitrate was at 64.1-81.1mg/ L. Table (7) shows the average water quality during the feeding trial. It could be seen that ammonia level could not be controlled during the trial, though efforts has been conducted to reduce the ammonia level by using filter aeration in each tank and cahanging water at 30% daily.

Table 7. Water quality measurements during the feeding trial

Treatment	Temperature	pH	Nitrate	Nitrite	Ammonia
C	28.1	6.3	81.1	2.2	8
AMP	279	6.3	68.5	2.3	8
IMP	27.9	6.2	78.9	1.7	8
UMP	27.9	6.2	70.7	2	8
GMP	27.9	6.4	64.1	2.3	8
CMP	28	6.3	62.0	1.6	8

Note: C (control/no supplemented nucleotides), AMP (adenosine monophosphate), IMP (inosine monophosphate), UMP (uridine monophosphate), GMP (guanosine monophosphate), CMP (cytidine monophosphate).

DISCUSSION

Dietary nucleotides have been studied widely in the field of human and animal nutrition **(Boza *et al.*, 2002; Hess *et al.*, 2012; Weaver *et al.*, 2014; Huu, 2016)**. In aquaculture the first study in nucleotides-supplemented diet in 1994 was on tilapia with a promising result **(Ramadan *et al.*, 1991)**. From then study on the role of nucleotide-supplemented diets became a focus in the field of fish nutrition and health, and many studies have been done on different species of aquatic organism, using different sources and types of nucleotide supplements.

Supplementation of nucleotides into fish diets aims to improve the immune responses of aquaculture organism against diseases, as well as enhancing the growth performance of cultured organism **(Li *et al.*, 2015; Huu, 2016)**. Commonly, studies on dietary nucleotides have focused only on the use of commercial nucleotide supplement. However recently, the potential value of adding pure nucleotide to the diet has become a focus of exploration in aquaculture nutrition studies **(Lin *et al.*, 2009; Huu *et al.*, 2013; Hossain *et al.*, 2016a, b, c, d, 2017b, c)**. It should be noted that commercial nucleotides usually contain trace element and polysaccharides in addition to the nucleotides, and

these have immunostimulant properties, and so possibly interfere with results recorded in any study of nucleotide-supplemented diets **(Gatlin *et al.*, 2007)**.

It has been well reported that dietary nucleotide increased growth performance and survival rate of some aquaculture species. On the contrary, few studies revealed that nucleotide inclusion in the diet did not significantly increase the growth performance and survival of the studied fish **(Li *et al.*, 2004; Welker *et al.*, 2011; Barros *et al.*, 2015; Fuchs *et al.*, 2015)**. In our present study, Asian seabass diet was supplemented with each of the following single pure nucleotide: AMP, IMP, UMP, GMP at 0.5% for 56 days and resulted in significant differences in the WG and SGR of the fish fed diet containing IMP and GMP. This result is in line with that of **Huu *et al.* (2013)**, who postulated that the supplementation of 0.5% GMP, GMP + AMP, GMP + IMP significantly increased shrimp growth rates compared to those fed basal diet. Study by **Hossain *et al.* (2017a)** found that the amberjack fed IMP-supplemented diet gave significantly higher weigh gain, and the authors suggested that the optimal level of the supplementation of IMP into fish diet is 0.54%, while in a prior study, the optimal level suggested for growth performance of the red seabream was determined to be 0.4% inclusion of IMP **(Hossain *et al.*, 2016b)**. In addition, **Asaduzzaman *et al.* (2017)** reported a significant improvement on the final body weight as well as muscle fiber frequency of the Nile tilapia fed diet supplemented with 0.2– 0.8% IMP compared to free-nucleotide diet. Therefore, it can be assumed that, IMP and GMP might have benefitial effects increasing the growth performance of the Asian seabass. The positive effect of IMP supplementation in the diet on growth performance was also reported for the olive flounder **(Song *et al.*, 2012)**. The optimal inclusion level for different species and size of fish warrant a further study.

Though advantageous, the way exogenous nucleotides work to improve fish growth is still not cristal clear. However, it has been known that IMP and GMP are flavor enhancers and usually added to fish diet as a chemoattractant to increase palatability of the fish toward the diet **(Ishida *et al.*, 1987; Hartati *et al.*, 1993)**. Thus, their effect in increasing growth performance of the fish presumably works through the increasing of the feed intake from the diet given **(Weaver *et al.*, 2014)**, while **Borda *et al.* (2003)** and **Roige (2017)** stated that nucleotide supply exogenously possibly enhance the high rate of cell replication during early stages of fish development. In addition, **Kader *et al.* (2018)** reported that supplementation of nucleotides diet changed the mRNA expression pattern of growth-related genetic factors gene which can improve the activation of hyperplastic and hypertrophic growth of the Nile tilapia, and this might explain the way through which nucleotide promotes the growth performance of the fish.

Remarkably, the innate immunity is the first line of defence mechanism in fish which contains cellular and humoral components including lysozyme, complement system, transferrins, antiproteases, lectins, pentraxins, interferon, C-reactive proteins and bactericidal peptides **(Whyte, 2007; Biller-Takahashi *et al.*, 2014)**. Parameter of innate

immune system can be used as an indicator for determining the health status of an animal and evaluating the effect of immunomodulatory substances in fish farming **(Biller-Takahashi *et al.*, 2012)**. It has been known that nucleotides are potential to optimize the mechanism of the immune system components **(Cosgrove, 1998)**. Several components of immune system, such as IgM level, lysozyme, respiratory burst and complement activity, increased as a result of feeding the fish with nucleotides supplementation diet **(Sakai *et al.*, 2001; Lin *et al.*, 2009; Tahmasebi-Kohyani *et al.*, 2011; Song *et al.*, 2012)**. Our present study investigated whether different sources of single pure nucleotide affect differently the immune responses in terms of lysozyme, complement and respiratory burst activity, total protein and globulins, also serum IgM and IL-1β level in the Asian seabass.

Lysozyme is a component of fish innate immune system which plays important role on preventing the invasion of pathogens via bacterial lysis process **(Magnadottir, 2006; Caruso *et al.*, 2011)**. Lysozyme activity could be affected by nutrition found in the diet viz, nucleotides supplementation **(Tahmasebi-Kohyani *et al.*, 2011; Song *et al.*, 2012; Hossain *et al.*, 2016a)** or other immunomodulator such as LPS, prebiotic and probiotic **(Ringo *et al.*, 2012)**. A study on the red drum **(Cheng *et al.*, 2011)** and the rainbow trout **(Tahmasebi-Kohyani *et al.*, 2011; Hunt *et al.*, 2016)** found that feeding the fish with nucleotide supplemented diet increased the fish lysozyme activity significantly. Similarly, feeding the Caspian brown trout with nucleotide supplementation diet increased lysozyme activity of the fish **(Kenari *et al.*, 2013)**; the same effect was noticed in a previous study on the turbot **(Peng *et al.*, 2013)**, the hybrid tilapia **(Shiau *et al.*, 2015)** and the Nile tilapia **(Kader *et al.*, 2018)**.

Based on the results of the current experiment, the supplementation of different single pure nucleotides was recorded with no significant effect on the lysozyme activity of the Asian seabass. Unfortunately, no study was found in literature addressing the effect of the single nucleotide diet on the Asian seabass serum lysozyme activity, except for those conducted on the red seabream investigating the effect of different levels of supplementation of each single nucleotide AMP, IMP, UMP, GMP and CMP in separate studies. All studies' results coincide with the present outcomes, revealing no significant difference in fish lysozyme activity obtained as an effect of different levels of single nucleotide implemented, although the authors found in all their studies that there was higher trend in the lysozyme activity of fish fed nucleotide diet compared to diet without supplementation of nucleotides **(Hossain *et al.*, 2016a, c, d, 2017b, c)**. Even though no significant differences were found in our study, it was noted that IMP supplementation tends to have higher serum lysozyme activity compared to basal diet and other single nucleotide diet. **Kumari *et al.* (2006)** hypothesized that IMP could stimulate immune system of the animal through increasing the number of phagocytic cells, which produce lysozyme, or increasing the quantity of lysozyme discharged in a cell.

Complement is one component of the immune system which plays an important role in innate defence mechanisms, destroying microorganisms via the inflammatory

reaction, direct microbial killing, opsonisation, phagocytosis, immune complex elimination, and modulation of adaptive immune responses **(Yano *et al*., 1988; Holland *et al*., 2002; Kirschfink *et al*., 2003)**. Analysis of complement activity based on the evaluation of lytic activity of complement system can be used as an immune indicator for determining the health status of fish **(Biller-Takahashi *et al*., 2012)**. It has been found that the activity of serum complement in fish is affected by particular nutrients such as α-tocopherol, n-3 HUFA (omega-3 highly unsaturated fatty acids), vitamins C and E **(Li *et al*., 1985; Tort *et al*., 1996; Montero *et al*., 1998; Bagni *et al*., 2000)**. It has been reported that dietary nucleotide significantly increased serum complement activity of the common carp compared to fish fed control diet **(Sakai *et al*., 2001)**. In the present study, no differences were detected among fish fed diet at the different types of single pure nucleotides. However, our study revealed that GMP- supplemented diet tends to give higher complement activity in the Asian seabass, followed by UMP diet.

Respiratory burst, usually called oxidative burst, is the rapid production of reactive oxygen species (ROS) from activated macrophages and neutrophils. The ROS components such as superoxide anion radical (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) have bactericidal activity property, giving the potential to phagocytize and destroy foreign particles including pathogenic microorganisms, thus they play a crucial role to remove microbes from the body **(Biller-Takahashi *et al*., 2013; Ringo *et al*., 2012)**. The respiratory burst is regarded to be the powerful and efficient defence mechanism in fish **(Haugland *et al*., 2012)**, and its activity can be used as an indicator of the status of macrophage and neutrophil activation **(Ringo *et al*., 2012)**. Respiratory burst activity can be affected by nutrition intake, for example feeding the rainbow trout diet supplemented with 1% of powdered ginger roots for three weeks experienced a significant higher of extracellular respiratory burst activity of blood leukocytes compared to control group **(Dügenci *et al*., 2003)**, while juvenile Turbot fed with a purified mix nucleotides experienced an increased respiratory burst activity **(Peng *et al*., 2013)**.

In this present study, respiratory burst activity was performed using whole blood DHR-123 flow cytometry which we developed previously **(Hastuti *et al*., 2019)**. Our result suggested that different types of single pure nucleotide did not significantly affect the respiratory burst activity in the Asian seabass. However, there is a trend that AMP supplementation diet gives higher respiratory burst activity followed by diet containing GMP. This result similar to study by **Lin *et al* (2009)** on grouper which showed that the oxygen production ratio was significantly higher in fish fed the diet contain AMP, while the red seabream fed different levels of AMP resulted in significantly higher nitro blue tetrazolium activity at level supplementation of 0.4 and 0.8% **(Hossain *et al*., 2016c)**. This suggests that AMP might have advantage to increase immune responses of fish, particularly on respiratory burst activity. However, it is possible that the level of AMP we implemented into the diet was not optimal to give significant differences for fish

respiratory burst activity. Therefore, further study on dietary AMP in the Asian seabass is required in order to obtain the optimal dose of administration. It can be assumed that nucleotides affect the fish respiratory burst activity through the stimulation of leukocytes production which are the important cells of the immune system, particularly in phagocytosis and respiratory burst activity **(Carver *et al.*, 1995; Carver, 1999)**.

Cytokines are important regulators of the immune system **(Sahoo *et al.*, 2010)** and mediators of disease progression **(Prachar *et al.*, 2013)**. Among them, IL-1β is a pro-inflammatory cytokine and a key mediator which induces inflammation and host immune response, and subsequently activates the adaptive immune system **(Ringo *et al.*, 2012; Yildirim *et al.*, 2014)**. Hence, IL-1β could become an interesting marker for the detection of early stages of inflammation **(Prachar *et al.*, 2013)**. Cytokines including IL-1β are produced by activated phagocytes **(Pelegrin *et al.*, 2004; Prachar *et al.*, 2013)** and the function of IL-1β in fish is analogous to that found in mammals **(Zou *et al.*, 2016)**. The increase of IL-1β level in serum could be a response to infection **(Prachar *et al.*, 2013)**. Additionally, this increase can also be affected by the administration of immunomodulators **(Corripio-Miyar *et al.*, 2007)**. In addition, **Yildirim *et al.* (2014)** suggested that a variety of agents such as endotoxin could stimulate IL-1β production. In our present study there was no differences in serum IL-1β levels in Asian seabass fed different types of nucleotides. However, the highest level of IL-1β in serum was found in fish fed diet supplemented with AMP. There might be a correlation between the level of IL-1β with respiratory burst activity since both immune components are produced by activated phagocytes. In this study we found that, dietary AMP also gave better result in the burst activity of the Asian seabass serum similar to what we found in serum IL-1β. However, further study is needed to explain the mechanism of nucleotides modulation of the immune responses in terms of cytokines production and the correlation with other immune response parameters. According to **Low *et al.* (2003)** increasing of IL-1β production may enhance phagocytosis activity, by stimulating the activation of lymphocytes or induction of the release of other cytokines which are able to activate macrophages, NK cells and lymphocytes.

Proteins are the greatest significant compounds in the serum and are important in maintaining a healthy immune system of the animal **(Kumar *et al.*, 2005)**, among them albumin and globulins are the major protein compounds in fish serum since their level could inform the health status of the animal since it is associated with the fish innate immune response **(Syeed *et al.*, 2018)**. As a part of globulins serum, gamma globulins play an important role to maintain fish immune system. Higher globulins concentration and lower albumin:globulins ratio could indicate an increase of antibody response in fish **(Kumar *et al.*, 2005)**. Our present study showed there was no significant differences on the serum proteins level of the Asian seabass fed different types of nucleotides. However higher concentrations of total protein, albumin, and globulins in serum were found in fish fed diet containing GMP. While our results confirmed no significant effects of

supplementation nucleotide diet on total protein, albumin, and globulins of the Asian seabass serum; it has been reported that the Indian major carp fed dietary nucleotides experienced a significant increase in total protein and globulins in serum **(Jha *et al*., 2007)**. Likewise, dietary nucleotide increased total protein, albumin, and globulins of the rainbow trout **(Tahmasebi-Kohyani *et al*., 2012)** and the juvenile red seabream **(Hossain *et al*., 2016a)**.

Histopathological examination of tissues provides a rapid method to detect the effects of stressors including pathogens and irritants in various tissues and organs as well as providing an indicator of abnormal environmental conditions **(Silva & Martinez, 2007; Mohammadi *et al*., 2012)**. In the present study, we investigated histopathological alterations in organs of the Asian seabass fed diet supplemented with different types of single pure nucleotides. The results showed that fish from all groups experienced abnormalities in their organ targets from mild to severe levels. Overall, histopathological alterations found in the fish organs included congestion, haemorrhage, oedema, inflammation, degeneration and necrosis in the gills, kidney, spleen and liver, while mild alteration in the intestine was observed in a few number of fish.

There were moderate to severe abnormalities found in the gills of the Asian seabass including congestion of blood vessels, curling at the secondary gill lamellae, haemorrhage and lifting of the epithelium surface which is characterized by displacement of the lining epithelium of the secondary lamellae and oedema. **Santos *et al*. (2014)** stated that lifting of the epithelium is the first sign of gill damage and it can be triggered by chemical contaminants or infection with pathogens. These finding agrees with other studies reported histological changes in the gills of different species such as gill damage detected in the white seabass exposed to cadmium contamination **(Thophon *et al*., 2003)**, the common carp and the Nile tilapia exposed to ammonia contamination **(Benli *et al*., 2008; Chezian *et al*., 2012; Esam *et al*., 2022)** and the striped catfish exposed to water borne silver nanoparticles and silver nitrate **(Pournori *et al*., 2017)**. In addition, **Modu *et al*. (2012)** reported contamination from pathogens such as monogenean parasites and the poor water quality that caused gill damage in fresh water catfish. Hyperplasia accompanied with infiltration with inflammatory cells is also commonly observed in the gill lamellae. In addition it was found that, secondary gill lamellae were completely ruptured in addition to infestation with monogenean parasites *Dactylogyrus* sp. as can be seen in the histology of the control group. This parasite is primarily a gill parasite of freshwater fish that causes histopathological changes such as gill filament fusion and secondary filament hyperplasia. The infestations of the parasite reduces the inter-lamellar spaces resulting in focal or multifocal fusion of lamellae **(Modu *et al*., 2012; Mohammadi *et al*., 2012)**.

Our present study suggested that high level of ammonia in experimental tank could be the main cause of histopathological alterations in the gills of Asian seabass. The concentration of ammonia in the tanks during the trial was 8mg/ L, which is beyond the

acceptable level of 0.5-2.00mg/ L **(Benli *et al*., 2008; Naeem *et al*., 2023)**. Although each tank was equipped with a filter and aeration system, the ammonia level could not be adequately controlled during the experiment. To reduce the adverse effects of the high ammonia level, water changes were conducted daily. The main source of ammonia in the tanks was from fish excretion. It was reported that fish excretion is the primary source of ammonia in aquaculture system and the excretion is directly related to protein levels in the diet **(Naeem *et al*., 2023)**. Since Asian seabass were fed high protein diet during the study, their excretion would increase the ammonia level of water. **Edwards *et al*. (2015)** and **Zeitoun *et al*. (2016)** reported that 75-90% ammonia nitrogenous waste products are discharged to the environment through the gill and kidney excretion. Elevated ammonia level could inhibit oxygen uptake from the gills into the bloodstream causing gill damage. Due to its toxicity, fish must excrete ammonia into the surrounding water **(Nochalabadi *et al*., 2023)**.

Gills are the organ in which gas exchange and waste excretion take place. Research indicates that structural changes in the gills is a fish strategy to survive against environmental stress caused by ammonia **(Guo *et al*., 2023)**. Since the gills are an important organ in respiration, osmoregulation, and nitrogenous waste excretion, they are more susceptible to the effects of ammonia toxicity which causes gill damage **(Esam *et al*., 2022)**. The abnormalities found in histology, such as congestion of blood vessels, curling of the secondary gill lamellae and haemorrhage, could be an indicator of gill damage which leads to the reduction of the gill surface area and disruption of gas exchange **(Chezian *et al*., 2012; Modu *et al*., 2012; Esam *et al*., 2022)**.

The current study showed that the most severe gill changes was experienced in the control fish group, receiving no supplementation of nucleotide in the diet. It could be seen that the score resulted from semi quantitative analysis was 4.33, which was significantly higher than those of the groups of fish treated with diet containing nucleotides. In contrast, less gill alterations were observed in the group of fish treated with nucleotide diets, especially that containing GMP and IMP. These results suggest that nucleotides may help mitigate the effects of ammonia-induced gill damage. Nucleotides are known to enhance the immune response by promoting the proliferation of lymphocytes and the production of antibodies, which may support the ability of the gill to repair damage caused by ammonia exposure since the nucleotides play a crucial role in cellular metabolism, repair, and immune function **(Hossain *et al*., 2017a)**.

Histopathological examination of kidney revealed severe congestion of blood vessels, hyperactivation of MMC, and haemorrhage. The MMCs are macrophage aggregates containing pigments such as melanin, lipofuscin and hemosiderin **(Agius & Roberts, 2003)**. The number and morphology of MMCs could alter in response to a range conditions such as starvation, bacterial or viral infections, parasite infestations and poor environmental conditions **(Agius & Roberts, 2003; Pronina *et al*., 2014)**. Our present study recorded a hyper activation of MMC in some kidney samples in which the

number or size of MMCs were increased. Activation occurs as a response to a stressor such as chemical contamination or the presence of pathogens **(Benli *et al*., 2008; Pronina *et al*., 2014; Triana *et al*., 2023)**. In this present study, the concentration of ammonia in fish tank during the experiment was very high, and it could be the reason of MMC hyperactivation. **Nochalabadi *et al*. (2023)** reported that MMCs aggregation can be used as a bio–indicator for environmental state.

In this present study, congestion, inflammation, infiltration of inflammatory cells at the edges of blood vessels, vacuolation and degeneration of epithelial cells in the kidneys were also found. The alteration detected in this study is consistent with those of **Benli *et al*. (2008)**, **Banihashemi *et al*. (2016)** and **Triana *et al*. (2023)**, who studied the effect of high ammonia levels on the kidney of different species fish. In addition, **Yanchev *et al*. (2015)** suggested that the exposure of fish to contaminated water resulted in histopathological changes in the kidney such as tubule degeneration, dilation of capillaries in the glomerulus, reduction of Bowman's space, hyaline degeneration, and shrinkage of the glomerulus. Ammonia and other toxic contaminants causing alterations in the kidney leads to the disruption of the normal function of the kidney; as an excretory organ, it filters waste materials from the blood, and plays a crucial role in producing blood cells, and is responsible for the immune defence mechanisms. Since kidney is an important organ for detoxification and osmoregulation, the structural changes will disturb its function and lead to the inability of fish to detox and manage body fluid **(Rombout *et al*., 2005; Dastan *et al*., 2017)**. This present study found that kidney from control group showed more severe alterations with the highest value (4), based on the semi quantitative analysis. Interestingly, feeding fish with dietary nucleotide, especially with the inclusion of GMP into the diet, showed mild alterations in fish kidney, suggesting that nucleotide supplementation of GMP provides a protection through the improvement of immune responses, reducing the inflammation and damage of the kidney, and supporting cells regeneration mechanism under ammonia stress. This finding matches that of **Hess and Greenberg (2012)**, who stated that exogenous nucleotide is essential during stress condition, such as in the period of rapid growth, development and injury recovery. Furthermore, nucleotides may have contribution to the prevention of DNA damage. Additionally, **Burrels *et al*. (2001)**, **Low *et al*. (2003)** and **Li and Gatlin (2006)** elucidated that, nucleotide diet can help reduce the negative impacts of aquaculture stressors such as poor water quality caused by ammonia stress through supporting the fish immune response, maintaining cells integrity, and promoting cellular repair which could mitigate the systemic impacts of elevated ammonia levels.

Histopathological changes found in the spleen of the Asian seabass includ lymphoid depletion, congestion, haemorrhage, leucocyte infiltration and the presence of MMC and ellipsoids. This finding coincides with that of previous studies on carp **(David & Kartheek, 2015)**, the darkbarbel catfish **(Chuan-Jie *et al*., 2017)**, and the Nile tilapia **(Getnet *et al*., 2024)**. The lymphoid depletion observed in the control group, along with

white pulp proliferation and the presence of hyperactivated MMCs suggested that ammonia exposure may stimulate a systemic immune response. According to **David and Kartheek (2015)**, the depletion of lymphocyte might be caused by exposure to contaminants from the environment which leads to the suppression of the immune system and subsequently a decrease in the number of mature lymphocyte. **Dotta *et al.* (2018)** verified that the number of lymphocytes in the white pulp area increased when the fish immune system was activated, for instance, because of the presence of ammonia.

The increasing MMCs number and size found in the spleen were triggered by stressful condition experienced by fish due to the poor water quality **(Montero *et al.*, 1999; Getnet *et al.*, 2024)**. It was suggested that MMC could assist as a biomarker for toxic effects of the contaminants as well as the environmental pollution **(Agius & Robert, 2003; Sayed & Younes, 2016; Getnet *et al.*, 2024)**. This finding is in agreement with our present study in which the level of ammonia in the tank were recorded at 8mg/ L during the experiment causing the activation of MMC and other abnormalities in the Asian seabass spleen. Similarly, **Guo *et al.* (2022)** reported that spleen tissues of the Wuchang bream exposed to 5-30mg/ L ammonia nitrogen experienced an MMC activation. Whereas, **Kwon and Chang (1996)** found that the exposure of the black seabream to ammonia at 4.0-10.4mg/ L induced severe hemosiderin deposition and increased the melanin-macrophages. Additionaly, exposing *Pelteobagrus vachellii* to 1 and 5mg/ L total ammonia nitrogen resulted in the hyperactivation of melano-macrophage center in its spleen **(Chuan-jie *et al.*, 2017)**.

Since spleen is an organ which plays an important role in immune function, the degeneration of its tissue under ammonia stress can impair the fish's ability to support their immune response. In this study, it was observed that fish fed nucleotide supplemented diet exhibited fewer MMCs and ellipsoids with less lymphoid depletion and congestion, as shown in the histology of fish fed GMP supplemented diet. This indicates that nucleotides may help maintain immune function under stressful conditions, possibly by promoting the production and function of immune cells as well as reducing tissue damage. According to **Li *et al.* (2004)**, exogenous nucleotides play a role in the function of both antibody-mediated and cell-mediated immune responses, and they are important for the health and maintenance of lymphoid tissue such as spleen since spleen cannot produce enough nucleotides. In addition, the supplementation of nucleotide in fish diet provides immune-modulatory effects on lymphocyte maturation, proliferation, immunoglobulin responses and phagocytosis **(Sakai *et al.*, 2001)**.

The histopathological changes in the liver in the present study included mild to marked congestion of blood vessels, infiltration of inflammatory cells, haemorrhage, oedema, vacuolation, loss of nuclei in hepatocytes, hypertrophy of hepatocytes, nucleus pyknosis and hepatocytic necrosis, mostly found in fish fed control diet. Liver samples in some fish also showed mild activation of MMC. Our results are similar to those observed in several studies on different species, where abnormalities in liver were usually found,

for example, the study of **Magouz *et al*. (2021)** on European seabass; that of **Getnet *et al*. (2024)** on the Nile tilapia, and that of **Pu *et al*. (2024)** on the largemouth bass. Furthermore, abnormalities in liver affected by contamination of water with chemical toxic substances or pathogens have been widly recorded. According to **Tophon *et al*. (2003)** and **Younis *et al*. (2015)**, pollutants such as cadmium can cause a range of damaging effects on fish liver cells. These include fatty changes and the formation of vacuoles (vacuolization) within the cells. Moreover, the presence of leucocyte infiltration indicates an inflammation of the liver tissue while hepatocytes pyknosis could be a signal of necrosis caused by environmental toxins like pesticides **(Hasan *et al*., 2022; Shah & Parveen, 2022)**. In this context, **Esam *et al*. (2022)** reported the hepatic vascular congestion and visible necrotic changes with nuclear pyknosis shown in the liver of the Nile tilapia exposed to ammonia. In the present study, the abnormalities found in the Asian seabass liver might be affected by elevated ammonia as a result of fish excretion. The effect of ammonia toxicity on liver histopathology were also reported in the studies of **Benli *et al*. (2008)**, **Li *et al*. (2016)**, **Zeitoun *et al*. (2016)**, **Chuan-Jie *et al*. (2017)** and **Esam *et al*. (2022)**.

The liver is the primary site of ammonia detoxification, for that reason it represents the main organ which is more susceptible to ammonia toxicity and the first organ showing a signal of the toxic chemical presence, including ammonia, which can cause structural damage to the liver **(Pu *et al*., 2024)**. Similarly, **Zou *et al*. (2023)** reported that fish exposed to high levels of ammonia experienced alterations in their liver structure and nutrient metabolism impairment. Accordingly, the activity of metabolism, the number of hepatocytes and the hepatocytes nuclear area could be used as the indicator to determine health status of fish. Our recent study demonstrated that most fish samples exhibited mild to severe abnormalities in their liver, which suggests that ammonia toxicity affected the liver structure. Fish fed with nucleotide-enriched diets showed significantly different alterations compared to those subjected to treatments without nucleotide supplementation. Notably, fish treated with nucleotide-enriched diets exhibited fewer alterations compared to fish in the control group. These findings indicate that nucleotide supplementation has a significant impact in mitigating the risk of structural alterations in the liver caused by elevated ammonia levels.

Although the ammonia level was very high during the experiment, the majority of fish in all groups survived. Their survival might be attributed to the Asian seabass possessing a strategy to cope with high ammonia levels via activating the urea cycle in the liver and converting ammonia into urea **(Jahanbani *et al*., 2023)**. This adaptation helps them tolerate the impact of ammonia stress. Additionally, increasing detoxification activity in the liver and other organs are the other routes in combating oxidative stress caused by ammonia **(Li *et al*., 2020)**.

There were negligible histopathological abnormalities found in the gastrointestinal of fish, whereas stomach and intestinal tissues samples from few fish

showed mild congestion. This finding aligns with the outcome of **Raskovic *et al.* (2011)** who denoted no significant alterations in histological structure of fish intestine fed diet containing heavy metals. Although there were not much changes in the intestine of the Asian seabass, the histology examination revealed the increasing of fold density in enteric sections of fish fed nucleotide diet, assuming that nucleotide enriched diet might contribute to intestinal development and growth. It should be noted that, an increase in fold density in the intestine will increase the total gut surface area which subsequently increases the absorptive activity of the intestine. It was reported that dietary nucleotides enhanced lateral branching of the intestinal folds in the Atlantic salmon **(Burrells *et al.*, 2001b)** and the juvenile turbot **(Peng *et al.*, 2013)**. Moreover, it increased fold, microvillus formation and enterocyte height in the red drum intestine **(Cheng *et al.*, 2011)**, increased fold density in intestinal tissue sections of the striped catfish **(Yaghobi *et al.*, 2015)**, and enhanced intestinal structure of the red drum **(Meng *et al.*, 2017)**. Intestine is an important organ with a crucial role in the nutrient absorption as well as in immune defence. In addition, it functions as the first barrier against pathogens in aquatic environment **(Guo *et al.*, 2019)**. **You *et al.* (2023)** stated that nucleotides inclusion in fish diet exhibited an improvement in the structure and function of the aging intestine which includes an increasing number of goblet cells and intestinal protective factor. Thus, exogenous nucleotides may prevent fish intestine from inflammation by decreasing the levels of inflammatory factors and the number of lymphocytes which, in turn, stimulates immune system of the intestine.

Semi-quantitative analysis of the results of histopathological examination of organs showed a significant difference in the gills and liver of fish fed with a nucleotide-supplemented diets and those fed on a nucleotide-free diet. Meanwhile, the kidneys, spleen, and intestines of fish fed with a nucleotide-supplemented diet showed no significant differences compared to those fed a nucleotide-free diet. It can be deduced from histology result that fish fed nucleotide diet and control diet experienced histopathological changes in their gills, kidney, spleen and liver, although the control diet showed more severe changes compared to the nucleotide diet fish. Considering that during the experiment, the level of ammonia was high; nevertheless, all fish samples affected by ammonia toxicity showed different responses depending on their immune status. This finding is in congruent with the previous study of **Pournori *et al.* (2017)**, who found no differences in histopathological changes in the striped catfish fed dietary nucleotides and fish fed nucleotides-free diet when the fish were exposed to water-borne silver nanoparticles or silver nitrate, suggesting that nucleotide supplemented diet did not significantly increase the resistance of the fish to water contamination but could reduce the impact of ammonia toxicity. Interestingly, fish fed nucleotide diet, especially that supplemented with GMP and IMP, performed a better structure in their gastro intestinal, suggesting the benefit of nucleotides on the intestinal development and immunity.

CONCLUSION

Despite the variability of changes in the immune parameters, as a result of different single nucleotide supplementation of diet in Asian seabass, we found that fish fed dietary nucleotide enriched diets tend to have a better result compared to the control diet. Thus, it can be deduced that, dietary pure nucleotide supplementation of diet might benefit the Asian seabass growth, performance and health, particularly when the diet contains GMP, IMP or AMP; since GMP and IMP gave a significant result in terms of fish growth, while AMP gave a significant result in the level of IL-1B. However, the optimal doses of these nucleotides on fish still need to be explored. Although both fish fed nucleotide diet and nucleotide-free diet experienced histopathological alterations in their gills, kidneys, spleen, and liver; the nucleotide diet groups showed fewer changes and better intestinal structures. High ammonia concentration as a result of fish excretion may affect this present study result. It is necessary to conduct further research on the Asian seabass addressing the role of individual pure nucleotide supplemented diets enriched at different levels and using better recirculation aquaculture system to minimize the ammonia level. Given that GMP, IMP and AMP function as umami which can produce a more delicious taste when fish are consumed, further study on the effect of dietary nucleotide on the better taste of fish products should be conducted.

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Conflict of interest.

The authors declare no conflict of interest.

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