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Efficacy of Dietary Nucleotides (Nucleoforce™)on growth, haemato-immunological response and disease resistance in *Pangasianodon hypophthalmus* fish (Sauvage, 1878) in Egypt

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ABSTRACT

A feeding trial was performed to evaluate the efficacy of Dietary Nucleotides (Nucleoforce™) on Growth performance, haematological, biochemical, immunological indices and disease resistance of *Pangasianodon hypophthalmus* fish to *Pseudomonas aeruginosa*. Dietary nucleotides were incorporated in diet at different concentrations; group I control, group II 250 g/ ton diet and group III 500 g/ ton diet for 8 weeks. At the end of feeding trial, weight gain and specific growth rate (SGR) were recorded. Results showed that there was a significant increase in fish groups received diet supplemented with nucleotides in comparison with the control group. Haematogram indices showed a significant elevation in RBCs count, HB value, WBCs and leukocytic count. Biochemically, High super oxide dismutase (SOD) activity, total protein and globulin levels were recorded in fish fed on 500g/t nucleotide (NT). Immunologically, Lymphocytic proliferation activity, Nitric oxide concentration and Serum lysozyme activity were continuously increased in 250g/t and the 500g/t NT treatment groups along feeding duration. At the end of 8th week, the experimentally reared fish were challenged intraperitoneally with virulent strain of *Pseudomonas aeruginosa* (0.2 ml of 3× 10^{7} CFU) and the cumulative mortalities were recorded to be lower in nucleotide supplemented groups compared to the control group. In conclusion, the supplementation of fish diet with nucleotides in concentration of 500g/t nucleotide can improve the general health status of *P. hypophthalmus* via increasing the disease resistance and minimizing stressful conditions.

INTRODUCTION

Pangasianodon hypophthalmus is one of the major freshwater fish species originally inhabit south and South-east Asia, Pangasius have been classified in the genus Pangasianodon, and they are good candidates for intensive culturing at high stocking densities, with disease resistance and relatively rapid growth rate even in adverse environmental conditions **(Hill and Hill, 1994)**.

Several literature heightened attention on the usage of nucleotides as Growth promotor feed additives that are needed particularly for enhancing fish body metabolism and for developing of immune and intestinal tissues. **(Peng *et al.*, 2013 and Ridwanudin *et al.*, 2019).** Nucleotides are low molecular weight biological compounds that play major roles in most biological processes which Can occur as subunit nucleobases or as polymeric nucleic acids **(low *et al*., 2003).**

The endogenous synthesis of these nucleotides are not sufficient to satisfy the body needs so an additional exogenous supply is needed to ensure their availability to the body especially in case of high physiological demand and different stressful conditions as injury or infection **(Whitehead *et al*., 2006)**. The insufficiency in dietary nucleotides caused various diseases in mammals as immunodeficiency and decreased in hepatic or intestinal protein synthesis**. (Maldonado *et al*., 2001; Sanchez-Pozo and Gil, 2002).**

Dietary nucleotides (Nucleoforce™) has been reported to have beneficial role in growth, increasing immunity and disease resistance in different fish species as rainbow trout (*Oncorhynchus mykiss)* ,Channel catfish (*Ictalurus punctatus),* grouper (*Epinephelus malabaricus) and* red sea bream (*Pagrus major).* **(Lin *et al*., 2009; Yousefi *et al*., 2016 and Hossain *et al*., 2016),** making it the best choice to replace the usage of antibiotics in different aquaculture sectors via increasing stress tolerance **(Huu *et al*., 2012).**

This immunomodulatory and stress tolerance potential of nucleotides make fish more resistant to different bacterial diseases as with *Pseudomonas aeruginosa* bacteria which was recorded to cause high mortalities and severe economic losses in both marine and freshwater fish species **(El-Nagar, 2010 and Tahmasebi-Kohyani *et al*., 2011)**.

So, the current study was conducted to estimate the effect of different concentrations of nucleotides (250g/ton and 500g/ton) on growth and immune responses of *Pangasianodon hypophthalmus* with emphasis to resistant capability of dietary supplemented groups against *Pseudomonas aeruginosa* infection.

MATERIALS AND METHODS

Experimental Design

One hundred and thirty-five apparently healthy striped catfish (*Pangasianodon hypophthalmus*) with body weight (75 ± 0.9 g) were obtained from private fish farms in European countryside. The fish were acclimated to laboratory conditions in the Department of Fish Diseases and Management, Desert Research Center.

Two weeks prior to the experiment fish were stocked in nine glass aquaria (80x50x30cm) and divided into three groups(15 fish/ group in replicates);Group I act as control group fed on basal diet, Group II fed on diet supplemented with 250g/ton dietary nucleotides and Group III fed on diet supplemented with 500g/ton dietary nucleotides. Fish were fed about 3% of their body weight twice daily for 8 weeks; the amount of feed was readjusted

every two weeks according to the increase in fish body weight. Water temperature (30 ± 2.0 °C) and dissolved oxygen (6 mg L−1) were adjusted and the water was exchanged every two days to maintain constant water quality parameters

Experimental Diets

The formulated diets were supplemented with 0% (basal that contained 30.17% crude protein and 9.12% crude lipid).The diets formulation adjusted according to the nutrient requirements of *Pangasianodon hypophthalmus* species **(FAO, 2011).** Nucleoforce™ Fish (Bioiberica, Spain) was supplemented in extruded it contains 34% of free nucleotides from inactivated yeast extract). These nucleotides comprised 80% pyrimidine and 20% purine. *Pangasianodon* fish fed on diets at the rate of 250 g and 500 g /ton feed in Aller Aqua, Egypt.**Table 1.** Growth was monitored every 2 weeks by random weighing of each group of fish and the weight gain %, SGR and feed conversion ratio (FCR)were determined using the following equations:

Weight gain (%) = [final weight-initial weight/initialweight]×100

SGR (%)= [Loge final weight-Loge initial weight/number of days]×100

FCR=Feed given(dry weight)/Body weight gain (wet weight).

Blood sampling

Blood samples were randomly collected at the 4^{th}, 8^{th} week and post experimental infection. Blood samples were collected by puncturing the caudal vessels in two sets. One set of the blood sample was collected using a 1 mL heparinized syringe (three samples/replicate; n=9 fish/group) for evaluation of white blood cell count (WBC; 103/mL) and differential leukocyte counts (lymphocytes and granulocytes). The second part of blood sample was collected without anticoagulant and centrifuged at 3000 rpm for 15 min for serum separation for the evaluation of serum total protein (g/dL), albumin (g/dL) , total globulin (subtracting albumin from total protein), and The non-specific immune parameters and biochemical parameters analysis.

Bacterial strain

A previously isolated virulent strain of *Pseudomonas aeruginosa* (accession number MT006361) used in the experimental infection of *p. hypophthalamus* was supplied from The Desert Research Center, Department of Animal Hygiene, Mathf elmataria Egypt.

Analysis of Haematological and Biochemical indices

Hemoglobin concentrations (Hb) were estimated by cyanmethemoglobin method. Red and White cell countwere estimated according to **Schaperclaus *et al.*, (1991)**.Field stain was used for the staining of blood films for the differential leukocytic count according to **Thrall, (2004)**. Biochemically**,** Serum Aspartate transaminases (AST) and Alanine Aminotransferase (ALT) activity were estimated using Commercial Stanbio-laboratory

diagnostic kits, USA according to the protocol supplied **Reitchman and Frankel, (1957)**.Serum urea and creatinine levels were determined using the Diamond Diagnostics commercial kits, Egypt according to **Henry *et al*., (1974)**. Total proteins was determined as method described by **Weichselbaum, (1946)**, Serum Albumin level were estimated calorimetrically using ELITech clinical System SAS, USA, at wave length 550 nm according to **Dumas and Biggs, (1972)**, Globulin was calculated by mathematical subtraction of albumin value from total protein value.

Table 1. Physical and chemical compositions of basal *Pangasianodon* fish diets.

Feed ingredient	Inclusion rate (%)
Yellow corn, ground[a]	28.00
Soya bean meal 46 % CP[b]	32.00
Corn gluten meal 60 % CP[c]	5.00
Fish meal 65 % CPd	12.00
Wheat bran[e]	5.00
Rice bran[f]	16.20
Common salt[g]	0.20
DL-Methionine[h]	0.10
L –Lysine HCL[i]	0.10
Ground limestone[j]	1.00
Fish Premix[k]	0.30
Toxin binder[l]	0.10
Nucleoforce™ Fish[m]	0.00
Calculated Chemical analysis of fish diets	
CP %	30.17
ME Kcal/Kg	2603
EE %	5.02
CF %	4.46
Lysine %	2.36
Methionine %	0.81
Calcium %	1.01
Available phosphorus %	0.50

[a] Corn (Argantine Co).[b]Soya bean meal (Argantine Co).[c]Corn gluten meal (U.S).[d]Fish meal 65 % CP (Morocco).[e]Wheat bran (local Egypt.)[f]Rice bran (Local Egypt).[g]Common salt (Local Egypt).[h]DL-Methionine (Evonike co).[I] L.lysine (ADM Company, U.S).[j]Ground limestone (Egypt).[k]Fish premix (DSM Company).[l]Toxin binder(Kemin company , Belgharia).[m]Nucleoforce™ Fish (Bioiberica company, Spain).Chemical analysis of fish diets **(AOAC, 2006)**.

Measurement of Antioxidant parameter

Liver tissue samples were get after surgical anatomy of the fish then homogenates with cold saline and analyzed for superoxide dismutase (SOD) activities according to **Kakkar *et al*., (1984).**

Determination of Immunological Assays

Nitric oxide concentration was measured at 570nm wave length by spectrophotometer according to the method described by **Rajaraman, (1998).** Lymphocytes proliferation activity estimated calorimetrically at 470nm wave length using MTT reduction assay according to **Rai-Elbalhaa *et al*., (1985)**.Lysozyme activity was measured using *Micrococcus lysodeikticus* lyophilized Gram positive bacteria. **(Sigma®) (Ellis, 1990)**.

Experimental infection

At the end of feeding trial,15 fish from each NT fed groups were separately restocked in another glass aquaria and challenged intraperitoneallywith0.2 ml of a previously isolated virulent strain of *Pseudomonas aeruginosa*(accession number MT006361)at a dose of 3×10^7 CFU /ml.Control negative group of 15 fish were I.P. injected with sterile physiological saline **(Hanna *et al*., 2014)**. *Pseudomonas aeruginosa* was incubated in a tryptic soya agar for 24 h, at 28^{o}C. Then, it was collected and suspended in a 0.85% sterile saline. The bacteria CFU /mL^{-1}was determined by matching with McFarland standard. The clinical signs and mortalities were monitored 7 days post challenge **Sarker and Faruk, (2016).** Also biochemical and immunological parameter of challenged fish were recorded.

Statistical analysis:

Data were expressed as mean ± SE (standard error of mean). Statistical comparison between the mean of the different groups was made by two-way ANOVA (Univariate ANOVA) and multiple comparisons between groups (post hoc) LSD using SPSS version (24) **(SPSS Inc., Chicago, IL).** A probability (P value) of ≤ 0.05 was assumed for statistical significance.

RESULTS

1. **Growth performance**

The growth rate, weight gain and (SGR) significantly increased in group III supplemented with 500g/ton NT than group II supplemented with 250g/ton NT and control group respectively Furthermore, Feed conversion ratio (FCR) recorded a significant decrease in group III throughout the duration of feeding trial. **Table 2.**

2. **Haematological and biochemical indices**

Hematogram indices revealed a significant increase in hemoglobin level, RBCs count and PCV in group III in comparison with control group before and after challenge. The WBCs count was elevated in group III after challenge and there was remarkable difference in Lymphocytes between groups. Biochemically, Total protein and Globulin levels elevated in group III before and after challenge. Urea, Creatinine levels; ALT and

AST activity showed notable decrease in group III after challenge in comparison with the control group. **Table 3.**

3. Determination of Antioxidant parameter

The antioxidant status of *p. hypophthalmus* that were fed on different dietary yeast nucleotide doses is showed in **Table 4**. A marked elevation in SOD activity was recorded in nucleotides supplemented groups before and after bacterial challenge than in control.

Table 2. Growth rate, weight gain, specific growth rate and feed conversion ratio of *Pangasius* fish in Nucleotides supplemented groups.

Growth performance	Group I (Control)	Group II (250g/t NT)	Group III (500g/t NT)
Initial weight (g)	75.44 ± 1.676^{A}	75.44 ± 1.741^{A}	76.00 ± 2.461^{A}
2-week weight (g)	87.44 ± 1.215^{A}	93.56 ± 0.988^{B}	95.00 ± 0.898^{B}
4-week weight (g)	112.56 ± 2.028^{A}	119.89 ± 1.637^{B}	124.44 ± 2.102^{B}
6-week weight (g)	123.78 ± 1.605^{A}	136.89 ± 1.532^{B}	141.33 ± 1.700^{B}
final weight (g)	148.67 ± 2.415^{A}	185.78 ± 1.245^{B}	194.89 ± 1.274^{C}
Weight gain (g)	73.22 ± 2.847^{A}	110.33 ± 1.929^{B}	118.89 ± 2.664^{C}
Specific growth rate (SGR) (g/day)	1.22 ± 0.048^{A}	1.84 ± 0.032^{B}	1.98 ± 0.045^{C}
Feed conversion ratio (FCR)	1.71 ± 0.071^{A}	1.18 ± 0.031^{B}	1.13 ± 0.036^{B}

- Data represented as Mean ± SE
- SE: Standard error of mean
- Means in the same row followed by the different capital letter/s are significantly different according to ANOVA (LSD, $p \leq 0.05$).

Table 3. Hematogram (Hb, RBCs, Total WBCs, PCV, Lymphocytes, Monocytes and Heterophils) and biochemical indices (Total protein, ALT, AST, urea and creatinine) of Nucleotides supplemented groups before and after challenge with *Ps. aeruginosa*

Parameters	Before challenge			After challenge		
	Group I	**Group II**	**Group III**	**Group I**	**Group II**	**Group III**
Hb (g/dl)	10.15 ± 0.127^{A}	12.45 ± 0.157^{B}	12.86 ± 0.112^{B}	8.99 ± 0.168^{D}	10.93 ± 0.073^{E}	10.95 ± 0.114^{E}
RBCs (x 10^6 /ml)	2.65 ± 0.099^{A}	3.59 ± 0.092^{B}	3.63 ± 0.123^{B}	2.27 ± 0.043^{C}	3.57 ± 0.048^{B}	3.62 ± 0.037^{B}
WBCs (x 10^3 /ml)	8.14 ± 0.22^{A}	8.22 ± 0.034^{A}	8.36 ± 0.085^{A}	9.09 ± 0.172^{B}	9.86 ± 0.035^{C}	9.98 ± 0.003^{C}
PCV (%)	31.13 ± 0.128^{A}	33.05 ± 0.162^{B}	32.93 ± 0.164^{B}	29.93 ± 0.107^{C}	32.30 ± 0.193^{D}	31.95 ± 0.232^{D}
Lymphocyte (%)	70.3 ± 0.056^{A}	72.3 ± 0.057^{B}	72.7 ± 0.068^{C}	71.6 ± 0.025^{D}	78.7 ± 0.059^{E}	80.3 ± 0.049^{F}

Monocyte (%)	1.77 ± 0.033^{A}	3.04 ± 0.171^{B}	3.39 ± 0.155^{C}	1.81 ± 0.026^{A}	3.31 ± 0.114^{B}	4.37 ± 0.140^{D}
Heterophils (%)	9.52 ± 0.076^{A}	9.79 ± 0.063^{C}	9.90 ± 0.053^{BC}	9.98 ± 0.079^{BC}	10.04 ± 0.088^{B}	9.96 ± 0.054^{B}
Total protein (g/dl)	3.20 ± 0.207^{A}	4.30 ± 0.134^{B}	4.65 ± 0.151^{BC}	3.59 ± 0.157^{A}	5.02 ± 0.061^{C}	5.55 ± 0.148^{D}
Albumin (g/dl)	0.60 ± 0.078^{A}	0.59 ± 0.050^{A}	0.58 ± 0.051^{A}	0.78 ± 0.068^{B}	$0.69 \pm 0.057A^{B}$	0.74 ± 0.037^{AB}
Globulin (g/dl)	2.67 ± 0.249^{A}	3.71 ± 0.150^{B}	4.08 ± 0.171^{B}	2.81 ± 0.185^{A}	3.91 ± 0.317^{B}	4.74 ± 0.154^{C}
Urea (mg/dl)	4.53 ± 0.123^{A}	4.45 ± 0.133^{A}	4.26 ± 0.137^{A}	7.06 ± 0.114^{B}	5.61 ± 0.168^{C}	5.32 ± 0.110^{C}
Creatinine (mg/dl)	0.19 ± 0.020^{A}	0.17 ± 0.018^{A}	0.16 ± 0.018^{A}	0.35 ± 0.018^{Ac}	0.22 ± 0.028^{B}	0.20 ± 0.023^{B}
ALT (U/L)	14.69 ± 0.196^{A}	14.56 ± 0.143^{A}	14.42 ± 0.125^{A}	17.50 ± 0.289^{B}	15.89 ± 0.326^{C}	15.49 ± 0.147^{C}
AST (U/L)	45.12 ± 0.630^{A}	43.92 ± 0.817^{A}	43.64 ± 0.825^{A}	53.66 ± 1.109^{B}	43.76 ± 0.879^{A}	43.32 ± 0.753^{A}

- Data represented as Mean ± SE
- SE: Standard error of mean
- Means in the same row followed by the different capital letter/s are significantly different according to ANOVA (LSD, $p \leq 0.05$).

4. Determination of Immunological Assays

4.1. lysozyme activity:

The highest lysozyme activity was recorded in group III supplemented with 500g/ton NT group followed by group II supplemented with 250 g/ton NT; The lowest lysozyme activity was recorded in the control group (0% NT).The percentage of serum lysozyme activity increased significantly with time , after challenge and dose of dietary yeast nucleotide administration. **Table 5.**

4.2. Nitric oxide:

Group III after the feeding trials demonstrated significantly higher serum nitric oxide than the control group before and after challenge. **Table 5.**

4.3. lymphocytic activity:

lymphocytic proliferation activity were notably enhanced in group III throughout feeding and also after challenge experiment .**Table 5.**

5. Experimental infection:

No apparent clinical signs were observed on Nucleotides supplemented fish groups challenged with pathogenic *Ps .aeruginosa* versus the control group that exhibited hemorrhages and Ulceration on the skin, gill cover and tail rot **Fig. 1.** The observed Mortalities within 7 days are recorded in **Table 6.**

Table 4. Superoxide dismutase (SOD) activities of Nucleotides supplemented groups on 4th and 8th week before challenge and after challenge with *Ps. aeruginosa*

Groups	SOD (U/mg)		
	Before challenge		After challenge
	4th week	8th week	
Control group	71.32 ± 0.218^{Aa}	71.95 ± 0.293^{Aa}	73.10 ± 0.293^{Aa}
250	72.87 ± 0.105^{ABa}	73.38 ± 0.229^{ABa}	77.58 ± 0.504^{Bb}
500	74.02 ± 0.271^{Ba}	74.98 ± 0.298^{Ba}	85.18 ± 2.148^{Cb}

- Data represented as Mean ± SE
- SE: Standard error of mean
- Means in the same column followed by the different capital letter/s are significantly different, while means in the same row followed by the different small letter/s are significantly different according to ANOVA (LSD, $p \leq 0.05$).
- Univariate test (Two-way ANOVA test): There was a statistically significant Two-way interaction between time and group on the dependent variable (SOD), as p-value = 0.000.

Fig. 1 . Skin, Gill cover ulcers and tail rot observed in control group after challenge with pathogenic *Pseudomonas aeruginosa*.

Table 5. Lysozyme activity, Nitric oxide concentration and Lymphocyte proliferation activity of Nucleotides supplemented groups on 4th and 8th week before challenge and after challenge with *Ps. aeruginosa.*

Immunological Parameters		Before challenge		After challenge
		4th week	8th week	
Lysozyme activity (Ug/ml)	**Group I**	171.05 ± 0.374^{Aa}	171.48 ± 0.244^{Aa}	171.93 ± 0.331^{Aa}
	Group II	181.35 ± 0.543^{Ba}	191.03 ± 0.398^{Bb}	197.60 ± 0.221^{Bc}
	Group III	184.15 ± 0.768^{Ca}	197.92 ± 0.436^{Cb}	203.85 ± 1.743^{Cc}
Nitric oxid (U mol/L)	**Group I**	26.05 ± 0.257^{Aa}	26.62 ± 0.218^{Aa}	29.25 ± 0.169^{Ab}
	Group II	28.12 ± 0.095^{Ba}	32.27 ± 0.351^{Bb}	34.83 ± 0.333^{Bc}
	Group III	30.38 ± 0.320^{Ca}	35.80 ± 0.389^{Cb}	42.38 ± 0.649^{Cc}
Lymphocyte proliferation (µg/ml)	**Group I**	1.14 ± 0.229^{Aa}	1.89 ± 0.029^{Ab}	1.87 ± 0.036^{Ab}
	Group II	2.05 ± 0.027^{Ba}	2.89 ± 0.036^{Bb}	2.91 ± 0.134^{Bb}
	Group III	2.77 ± 0.133^{Ca}	3.48 ± 0.154^{Cb}	4.48 ± 0.155^{Cc}

- Data represented as Mean ± SE
- SE: Standard error of mean
- Means in the same column followed by the different capital letter/s are significantly different, while means in the same row followed by the different small letter/s are significantly different according to ANOVA (LSD, $p \leq 0.05$).
- Univariate test (Two-way ANOVA test): There was a statistically significant Two-way interaction between time and group on the dependent variable (Lymphocyte transformation), as p-value = 0.000.

Table 6. Mortality percent of *Hypophthalmus* challenged with *Ps. aeruginosa* for 7 days

Fish group	No. of fish/group	No of dead fish	Mortality %
Control	15	14	90%
250g/ton NT	15	2	20%
500g/ton NT	15	1	10%

DISCUSSION

Sustainability of aquaculture industry and fish welfare against negative impacts of disease outbreaks remain the main concerns in last decades **(Subasinghe *et al*., 2009)** especially after the banned usage of antibiotic growth promoters (AGP) and chemotherapeutics to overcome those outbreaks in fish farms. So the recent approach was to study and evaluate natural immunostimulants products as probiotics, prebiotics, synbiotics, nucleotides and other functional dietary supplements (**Denev, 2008).** Nucleotides could act as prebiotics

favoring the modulation and proliferation of the beneficial intestinal microflora and inhibiting that of potential pathogens. **(Borda *et al*., 2003)**.
Recent researches proved the efficacy of dietary nucleotides in improvement of both innate and adaptive immunity, enhancing the plasma cortisol levels so that reduce the adverse effect of environment, increase stress tolerance and enhancing disease resistance **(Fuchs *et al*., 2017)**.

The continuous significant increase in growth rate, weight gain, specific growth rate and decrease in feed conversion ratio recorded in fish groups treated with nucleotides (250 and 500g/t)come in agreement with **Tahmasebi-Kohyani *et al*., (2012); Abedian *et al*., (2012); Abtahi *et al*., (2013) and Selim *et al*., (2019**) in rainbow trout, Caspian brown trout, *Salmo trutta caspius* and Nile Tilapia Fish, respectively fed on dietary nucleotides. This escalation in growth parameters may be due to the chemo-attractive effect of dietary nucleotides which by turn lead to an increase in fish voluntary feed intake **(Li and Gatlin, 2007)**, minimizing the high energy needed for nucleotide synthesis **(Guo *et al*., 2017)** and increasing enterocyte regeneration and the intestinal mucosal surface area in fish so, enhancing the intestinal morphology **(Cheng *et al*., 2011)**.In contrast with **Yaghobi *et al*., (2014)** and **Barros *et al*., (2015**) that Nucleotide mixture supplementation had no effect on weight gain nor feed conversion ratio in striped catfish (*Pangasianodon hypophthalmus*) and Nile tilapia. The variation in growth stimulatory effect of dietary nucleotides mainly depend on the fish species, feeding period, nucleotide mixture composition, incorporation level and fish life stage **(Li and Gatlin, 2007; Abedian *et al*., (2012)**.

Haematogram results indicated a significant increase in blood indices of fish groups treated with nucleotides in comparison with the control group. This come in accordance with **Abedian *et al*., (2012); Abtahi *et al*., (2013) and Yousefi *et al*., (2016)**,and may be related to the effect of nucleotides on the dietary bioavailability of iron and the increase in intestinal iron absorption that is required for haemoglobin and RBC synthesis. **(Grimble, 1996)** likewise, the stimulatory effect of Nucleotides on haemopoietic tissues such as head kidney **(Tahmasebi-Kohyani*et al*., 2012)**. In opposite, no recorded alteration in haematocrit values of Atlantic salmon and channel catfish, striped catfish and Nile tilapia with dietary nucleotide supplementation as reported by **(Burrells *et al*., 2001; Weiss and Wardrop, 2010; Welker *et al*.,2011; Yaghobi *et al*., 2014 and Barros *et al*.,2015).**

WBCs count was elevated only after bacterial infection as that documented by **Abedian *et al*., (2012)** in Beluga sturgeon *(Huso huso*) juveniles and Caspian brown trout fed with NT.This may be linked to the activation of haemopoietic tissues as a cellular immune response to infectious diseases **(Whyte, 2007)**.

Lymphocytes significantly increased in 500 g/ton NT supplemented group than the control group. This comes in harmony with the study of **Sakata and Sakai, (2010)** and **Tahmasebi- Kohyani *et al*., (2012)** on Caspian brown trout fingerlings and rainbow trout fed on nucleotide diets. The difference in physiological body responses to dietary nucleotides varies according to fish species, water temperature, age, sex, pollution and the experimental period.

Total protein and globulin considered as the principle line of defense against extracellular bacteria providing immediate and broad spectrum protective immune responses against pathogens **(Nayak *et al*., 2004; Elkamel and Mosaad, 2012).**Our results declared a significant increase in total protein and globulin levels in group II and group III before and after challenge when compared to the control group correspondingly to results of **Sakata andSakai, (2010)** and **Hossain *et al*., (2016)** in Caspian brown trout fingerlings and Pagrus major fish. However, **Yousefi *et al*., (2016)** reported a decrease in protein levels by dietary nucleotide supplementation in rainbow trout (*Oncorhynchus mykiss*).

Concerning ALT and AST levels, they registered lower values in fish fed on 250 and 500g/ton NT than the control group after experimental infection. This indicates the positive beneficial effect of nucleotides on maintaining the hepatocytes integrity and liver function **(Shi *et al*., 2006)** even in the presence of pathogenic bacteria which may cause hepatic cellular damage and hepatic dysfunction. These results are in consistent with **Glencross and Rutherford, (2010); Abedian *et al*., (2012)** and **Tahmasebi- Kohyani *et al*., (2012**) in barramundi (*Lates calcarifer*) fish,Caspian brown trout fish and rainbow trout fish fed on nucleotides diet. In contrast, some studies showed marked increases in ALT and AST levels in *P. major* juveniles and Nile tilapia, *Oreochromis niloticus* ,fish fed on 2.5 g NT/kg diet **(Hossain *et al*., 2016 and selim *et al*., 2019)**.

Urea is a major excretory products in fish, usage of high levels of dietary amino acids as energetic compounds activates hepatic uricase enzyme which convert the uric acid by oxidation to allantion then to urea **(Oliva-Teles and Goncalves, 2001; Li and Gatlin, 2006)** which consequently excreted in urine in large amount lead to thedecrease in serum urea levels as recorded in our results.These were supported by listed results of **Fournier *et al*., (2003)** in turbot (*Psetta maxima*) and rainbow trout *(O. mykiss*) fish. Nonetheless, Nile tilapia, *Oreochromis niloticus,* fish recorded high serum urea levels in 2.5 g NT/kg diet supplementation **(Selim *et al*., 2019)** this may be related to the lack of liver uricase in some fish species **(Rumsey *et al*., 1992).**

The recorded creatinine results showed a significantly lower values in group II and group III after challenge with pathogenic *Ps. aeruginosa* as that mentioned by **Yousefi *et al*., (2016)**. This may be attributed to the alternative source of energy provided by dietary nucleotides that not only used for intrinsic (de novo) synthesis of nucleotides but also act

as a source of energy for cellular metabolism. Therefore, a decrease in processes of body protein catabolism including creatinine metabolism is noticed **(Low *et al*., 2003)**.

Oxidative stress condition resulted from alteration of ROS and free radicals production above the cellular intrinsic neutralizing capacity in response to several causes of tissue injuries **(Patra *et al*., 2011)**.Superoxide dismutase enzyme (SOD)is one of the antioxidant biomarkers which increase in case of many degenerative diseases. This enzyme work synergistically with other antioxidant enzymes to protect body against cellular damage **(Ahmed *et al*., 2019 and Mohamed *et al*., 2019)**.

The increase in SOD activity in group III post challenge were in parallel with **Xu *et al*., (2015)** and **Tie *et al*., (2019)**. This may be attributed to the ability of nucleotides to enhance the antioxidant defense-system and decrease the oxidative damage via the radical scavenging capacity **(Regoli, 2000 and Tang *et al*., 2016)**.On contrary with **Peng *et al*., (2013)** that SOD activity of *S. maximus* was not significantly affected after 60 days of feeding trial with nucleotides.

The innate immune system of fish is the first and primitive line of defense against invading pathogens where lymphocytes, macrophages, monocytes, granulocytes, and humoral elements, such as lysozymes, immunoglobulins and the complement system are the major components of the immune system **(Magnadóttir, 2006)**.

Immunological responses; lysozyme activity, nitric oxide concentration and lymphocytic proliferation activity; were significantly ($p \leq 0.05$) higher in Group III with 500g/ton NTalong the4^{th} and 8^{th} week of dietary nucleotide supplementation before the experimental challenge and also after challenge, indicting the positive relationship between the nucleotides supplementation level and the stimulation of immune cell response.

The elevation in Lysozyme activity pre and post challenge is indicator to the innate immunostimulatory effect of nucleotides via catalyzing the peptidoglycans hydrolysis of bacterial cell walls as mentioned by **Sakai *et al*., (2001) ; Jha *et al*., (2007) ; Saurabhand Sahoo, (2008)**;**Tahmasebi-Kohyani *et al*., (2011); Abedian *et al*., (2012); Shiau *et al*., (2015)** and **Hossain *et al*., (2016).**They assumed that lysozyme activity increased in common carp, *Cyprinus carpio*, catla juveniles ; hybrid Tilapia and red sea bream (*Pagrus major*) after feeding with dietary nucleotides. Moreover the recorded increase in lysozyme activity is directly proportional with the leukocytic count in consistent with **(Magnadóttir, 2006)**.Other studies reported non-significant effects of four and eight weeks nucleotide supplementation on lysozyme activity in juvenile Nile Tilapia. **(Anguiano, 2012)**. This may be correlated to a variation in time and dose of dietary nucleotide administration.

The increase in Nitric oxide (NO) level post challenge reflect the activation of fish immune system by stimulation of macrophage–inducible NO synthase **(Yeh and Klesius, 2012)** and since NO is one of killing byproducts of activated macrophages therefore, the increase in nitric oxide radicals production has high killing capability, as mentioned by **Reda *et al*., (2018)** after 15 and 30 days of dietary yeast nucleotide administration.

In the present study significantly high lymphocytic proliferation was recorded in group II and group III. These results were supported by **Burrells *et al*., (2001); Leonardi *et al*., (2003); Li and Gatlin,(2004) and Anguiano (2011)** in rainbow trout and hybrid striped bass fed on nucleotides, and attributed to the important regulatory role of Nucleotides on the generation and development of T-Lymphocytes additionally there is a direct relationship between nucleotides level and lymphocytes and macrophages activity **(Gil, 2002)**.

Dietary nucleotides have been reported to increase resistance against various pathogenic bacteria in different fish species including salmonids **(Burrells *et al*., 2001)**, common carp **(Sakai *et al*., 2001)** and hybrid striped bass **(Li *et al*., 2004)**. Pathogenic strain of previously isolated *Pseudomonas aeruginosa* was identified using 16SrDNA gene, virulent toxA gene and virulent OprL gene was used to challenge reared *Pangasius* fish intraperitoneally at dose of 2ml of 3×10^{7}CFU /ml. (**Somerville *et al*., 1999 ;Nafee, 2012; Nowroozi *et al*., 2012 and Markey *et al*., 2013)**

The Experimentally infected fish that was previously supplemented with 500g/ton nucleotides showed the lowest cumulative mortalities than control. This endorse the main role of nucleotides in enhancing innate, specific immune response, improving the production of antibodies and blocking the bacterial translocation by preventing endotoxin-induced mucosal damage **(Leonardi *et al*., 2003; Li and Gatlin, 2006 and Anguiano, 2012)**. On the contrary, **Welker *et al*., (2011)** reported that the highest survivability against *Edwardsiella ictaluri*in channel catfishwas recorded in the low nucleotide fish diet and it declined in the groups fed on high nucleotide diets, as increasing in nucleotides diet may be associated with increase in the nitrogen levels, which affect the metabolism and decrease the disease resistance **(Barros *et al*., 2015)**.

CONCLUSION

We can safely conclude that Dietary Nucleotides (Nucleoforce™) supplementation at 500g/ton have positive effects on *Pangasius* fish welfare and resulted in better improvement in FCR, growth rate, haematological and immunological body responses by enhancing lysozyme activity and nitric oxide levels that consequently increase disease resistance against *Ps.aeruginosa* challenge.

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