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Effect of Un-Ionized Ammonia (NH_3) on *Oreochromis niloticus* Physiological Status with a Probiotic Treatment Trial

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Abstract: ***The possible effects of un-ionized ammonia (UIA-N) on the physiological status of Oreochromis niloticus reprersented by blood indices, total erythrocyte count (RBC), hemoglobin (Hb) concentration, white blood cells count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), packed cell volume (PCV), glucose and cortisol as well as survival rate (SR) were examined. Moreover, liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as well as antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD) in addition to creatinine, hepatosomatic index (HSI), spleenosomatic index (SSI) were tested. Furthermore, the possibility of using a probiotic (micropan) 5mg/L for remediating the expected ammonia stress was investigated. One hundred and fifty fish were divided into 5 groups; 30 fish each with three replicates. The first was considered as a control group and the remaining groups were subjected to 0.1 mg/L^{-1} UIA-N, 0.1 mg/L^{-1} UIA-N plus 5 mg/L probiotics, 0.5 mg/L^{-1} UIA-N and 0.5 mg/L^{-1} UIA-N plus 5 mg/L probiotics, respectively. The results showed that all the examined blood indices (except MCV and MCH), HSI and SR had significant reversible relationship (P≤ 0.05) with UIA-N concentration. Controversially, liver enzymes (ALT, AST), antioxidants (GPX and SOD), SSI, glucose, cortisol and creatinine had significant proportional relationship (P≤ 0.05). Meanwhile, probiotic treatments resulted in an obvious relieve of the fish physiological status. It could be concluded that the tested probiotics concentration helped to relieve the harmful effects of UIA-N especially with low UIA-N concentrations, hence prolonging the suitability of the aquacultures water***

Keywords: un-ionized ammonia; *Oreochromis niloticus*; physiological status; probiotics

1. Introduction

Tilapia is one of the most important Egyptian fish (Philippart and Ruwet, 1982). On which very large number of the population depend on as a cheap source of protein. The rapidly growing population increases its demand. Egypt is the world's second largest producer of farmed Tilapia after China (Mur, 2014). However, the expansion of its farming became an urgent request to be on a larger scale. Increased production of fish through intense culture practices often leads to not only stress and disease problems but also alteration in water quality. The organic wastes and bad water quality influence the growth of pathogenic microorganisms. One of the most important problems facing its farming is the high cost of renovation of the farming water due to its deterioration by ammonia **(**El-sherif and El-feky, 2008). Likewise, elevated ammonia concentration is one of the important factors in fish farming (Randall and Tsui, 2002). Therefore, lowering the farming water ammonia levels minimizes its adverse harmful effects on the fish as well as the other aquatic organisms, resulting in prolonged viability of the farming water which is a national demand. Therefore, using probiotics is considered a promising applicable way to circumvent these problems (Sunitha and Padmavathi, 2013).

Ammonia has several forms of deterioration (Randall and Tsui, 2002; Felipo and Butterworth 2002; McKenzie et al., 1993). It blocks oxygen transfer from the gills to the blood (Arana, 1997). Furthermore, intermediate and long term damage, destruction and reduction of intestinal external and internal layers (Thangam, 2014). The occurrence of ammonia (collectively Total Ammonia Nitrogen TAN) in aquatic environments is mainly in two major forms ionized and unionized. Under normal conditions (TAN) exists mainly in ionic form, however, increased temperature or pH, shifts towards formation of unionized ammonia (NH3) (Wood, 1993). The unionized ammonia (NH3) molecule is highly toxic, rapidly permeating gill and tissue membranes and impeding central nervous system functions (Evans et al., 2006; Eddy, 2005; Fairchild et al., 2005; Randall and Tsui 2002). Hematological indices represent a good monitoring parameter in both laboratory and field studies responding to low doses of pollutants (Seriani et al., 2010). *O. niloticus* hematological indices were negatively impacted by ammonia exposure in the form of elevated cortisol and glucose levels (Hanna et al., 2013; Metwally and Mohamed, 2014). Similarly, an elevated level of liver enzymes in common carp exposed to ammonia was recorded paralleled with reduction of Hb and PCV (Abbas, 2006).

Cellular Oxidative processes are essential for the organismal life and death, Oxidative stresses cause damages to the cellular macromolecules such as nucleic acids, proteins, and lipids (Noor, 2012). The levels of oxidative stress biomarkers and the activities of the enzymes were significantly increased in liver and white muscle of fish exposed to both low and high total ammonia levels, due to ammonia induce reactive oxygen species (ROS) generation in liver and white muscle causes oxidative stress in Nile tilapia (Mona et al., 2010).therefore, Oxidative stress in liver and white muscle is proportional with the total ammonia levels (Ching et al., 2009). The Antioxidant system either

enzymatic or non-enzymatic scavengers protect cells against oxidative damage (Winzer et al., 2002; Noor, 2012). Antioxidant enzymes such as SOD, catalase (CAT) and GPx provide the first line of cellular defense against toxic free radicals which cause oxidative stress; increased enzymatic antioxidant activities enhance the fish tolerance to ammonia (Mona et al., 2010; Halliwell and Gutteridge, 1999; Hermes and Tania, 2002; livingstoone, 2001; Hayes and Pulford, 1995; Noor, 2012). According to stress intensity and duration, the antioxidant activities may be increased or inhibited **(**Nahed, 2011).

From the etymological point of view, probiotic term is a Greek word. It means for life (Gismondo et al., 1999). Also, it defined as a live microbial food supplement that confers health benefits or disease resistance to the host (Lara-Flores and Aguirre-Guzman, 2009). Besides, it can be administered through the food or the rearing aquaculture water (Merrifield et al., 2010). Probiotics has several advantages, it enhances the nature of processes such as degradation and decomposition of organic matter, it also reduce nitrogen and phosphorus levels, restrain and controlling ammonia , decreased nitrite and the incidence of diseases, promoting both algal growth and consequently greater dissolved oxygen availability (Boyd and Gross, 1998). Zokaeifar et al. (2014) confirmed Boyd and Gross (1998) suggestions pinpointing that probiotics Bacillus subtilis strains enhances the shrimp rearing water quality by reducing ammonia, nitrite and nitrate ions. The majority of probiotics micro-organisms proposed for aquiculture belong to the lactic-acid bacteria (LAB). *Lactobacillus* and *Lactococcus* genera are mostly used, they are considered as GRAS (Generally recognized as safe), a warranty that the implementation of isolated probiotics will not cause collateral damage to the cultivated organisms or to the final consumers (Holzapfel et al., 1998). Use of probiotics as LAB in the avian gastrointestinal tract helps establishment of an intestinal microbial equilibrium, as well as the improvement of some immune responses. (Netherwood et al., 1999).The current study aims to investigate the impact of UIA-N on *O. niloticus* physiological status and the possible role of probiotics (micropan) in relieving such stress.

2. Materials and Methods

Experimental design

A total number of on hundred fifty same weight 30.17±1.5 gram healthy *O. niloticus* were collected from a private fish farm at El-Hamol Kafr El-Sheikh Governorate**.** The fish were acclimated in fiberglass tanks at Kafr El-Sheikh provincial lab, animal health research institute for 15 days to laboratory conditions. Fish were randomly distributed in glass aquariums (50 x 40 x 40 cm). Each aquarium was filled with about 60 liters of dechlorinated tap water; its temperature was adjusted at 25±1.5 ^{o}C, with continuous oxygen supply by air pump. The fish were fed pelleted ration with daily percentage 3% of body weight six day per week. Water conditions were adjusted according to Boyed and Tucker. (1992).

The fish were divided into 5 treatments the first group was the control, the second group was 0.1mg/L^{-1} UIA-N, the third group was 0.1mg/L^{-1} UIA-N plus 5mg/L^{-1} probiotics, the fourth group was 0.5mg/L^{-1} UIA-N and the fifth group with 0.5mg/L^{-1} UIA-N plus 5mg/l probiotics Respectively (each treatment had 3replicates).

The experimentation period lasted for 28 days, divided into two main equal sub-periods. In the first 14 days, the second and the fourth group was exposed to UIA-N only (0.1mg/L^{-1} and 0.5mg/L^{-1}) respectively. The third and the fifth group was subjected to (0.1mg/L^{-1} plus 5mg/L^{-1} probiotics and 0.5mg/L^{-1} plus 5mg/L^{-1} probiotics) respectively. In the second 14 days, exposure to UIA-N has been ceased.

Probiotic (micropan) composition and dosage

The used probiotic was a mixture of two bacterial genera namely, Lactobacillus and Bifedobacterium. Lactobacillus was represented by two species acidophilus & planetarium. Bifedobacterium was represented by two species longhum & thermophilum. Their concentration was 90,000,000,000 CFU/KG. It has been prepared to be constantly as 5 mg/L^{-1}. The two UIA-N concentrations (0.1 mg/L^{-1} and 0.5 mg/L^{-1}) were prepared by dissolving ammonium chloride NH_4CL 4.7 and 23.5 gram in 60 litter water respectively (Xu et al., 2005).

Ammonia level was monitored using Hach kits to maintain constant level and the percentage of UIA-N ammonia added in water was calculated using the equation:-
NH_3_N= (total ammonia × percentage of ammonia in the pH, temperature, ammonia Relationship tables) / 100 (Emerson et al., 1975).

Temperature, dissolved oxygen, pH and Total dissolved solid were estimated using Hana's instrument (Boyed and Tucker, 1992).

Clinical and post mortem examination

The collected fish were clinically examined according to (Amlacher, 1970); they were examined for any abnormalities including exophthalmia, skin, erosion, ulcers, hemorrhages and detachment of scales. The collected fish were opened according to (Amlachar, 1970), internal organs were exposed by making three cuts. The first from infront of Anus through abdominal cavity toward the head. The second perpendicular to the first behind the bronchial cavity. The third cut ran from anus to head parallel to the lateral line then the abdominal wall was removed and internal organs were exposed.

Blood analysis

Blood samples were collected according to (sayed and Moneeb , 2015). Blood analyses in schedule time 24hr, 48hr, 72hr, 14days and 28 days. Red blood cell (RBC) and White blood cell (WBC) counts were counted by haemocytometer according to Stoskopf **(**1993). Blood hemoglobin (Hb) was assessed by cyanometahemoglobin method Drubkin (1964). Packed Cell Volume (PCV) was assessed by centrifuge the blood. In addition, Mean Corpuscular Volume (MCV), Mean Corpuscular hemoglobin (MCH) and Mean Corpuscular hemoglobin concentration (MCHC) were calculated according to the formula mentioned by Dacie and lewis (1975). MCHC (g/dl)

= (HB / PCV) *100, MCH (pg) = (HB / RBCs) * 10, MCV (μm^3) = (PCV / RBCs) * 10

Biochemical analysis

Glucose was determined calorimetrically according to Trinder (1969). Cortisol was estimated using radio immunoassay technique according to the method of (Pickering and Potinger, 1983; Wedemyer, 1970).The activity of the liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined according to Reitman and Frankel (1957). The activities of the serum creatinine were determined according to Henry (1974).

The survival rate percent

The survival rate percent were calculated on the basis of the following equation

Survival rate % = (Number of live fish in specific period / Total population during that period) * 100

Somatic indexs, hepatosomatic index and Spleenosomatic index

At the end of experimental period, 5 fish from each group were dissected and the viscera were exposed. The liver and spleen were taken and weighed after which the indices were calculated according to these equations:

Hepatosomatic index (HSI) = weight of the liver/fish body weight. (Htun-hun, 1978).

Spleenosomatic index (SSI) = weight of the spleen/fish body weight.

Antioxidant studies GPx, glutathione peroxidase activity was

Assayed by the method of (Mohandas et al., 1984) and SOD superoxide dismutase was assayed by the method of (Misra, 1972).

Statistical analysis

Duncan's Multiple Range (Duncan, 1955) was used to determine differences among means at significance level of 0.05. All statistics were run on the computer using the SPSS program (SPSS, 2004).

3. Results

The reported UIA-N impacts varied between darkened skin, rapid breathing, gasping for air, lethargy and loss of appetite, depending on the used UIA-N concentration. While, these signs were not clear in case of the controlled and probiotics treatments.

Physicochemical water parameters

Table 1 represents the measured water parameters including temperature, dissolved oxygen (DO), PH and total dissolved salts (TDS) were in the range which is suitable for fish culture (Boyed and Tucker, 1992).

Hematological studies and (glucose &cortisol)

Table 2 shows a clear negative impact of UIA-N on RBCs, WBCs, HB, PCV, and MCHC represented by their reduction (especially with the higher UIA-N concentration – 0.5 mg/L^{-1}). Probiotic had enhancement role reducing the gap between the treated and the control groups, (especially in the case of lower UIA-N concentration 0.1 mg/L^{-1}). Similarly, the temporal variation of these parameters has also taken a consistent pattern. The levels of these parameters decreased significantly (P=0.0000) with its maximum reduction in the third period (72 h). This pattern has deviated in case of the control and 0.1 mg/L^{-1} UIA-N plus probiotic treatments where this decline was not significantly different from the beginning to the end of the treatment. However, the greatest reduction occurred in the 0.5 mg/L^{-1} UIA-N then 0.1 mg/L^{-1} UIA-N treatment. 0.5 mg/L^{-1} UIA-N plus probiotic treatment showed a moderate decreasing between the mere UIA-N (0.1 and 0.5 mg/L^{-1}) and both control and 0.1 mg/L^{-1} UIA-N plus probiotic. The afterwards cessation of UIA-N and probiotics applications resulted in the recovery of the previously mentioned parameters. The recorded elevations were not the same (although the same shape). In case of 0.1 and 0.5 mg/L^{-1} treatments (mere UIA-N) the fish relief was not enough to reach the control values as in the case of 0.1mg/L^{-1} plus probiotic treatment. However, the fish relief in case of 0.5 mg/L^{-1} UIA-N plus probiotic treatment still below the control level but better than its case in both 0.1 and 0.5 mg/L^{-1} UIA-N. This pattern applies for the mentioned parameters except RBCs and WBCs. The remaining parameters (Table 3) MCV, MCH, glucose and cortisol showed the same negative result with reversed image.

Survival rate (SR), liver enzymes, creatinine, and somatic indices

Table 4 shows Survival rate (SR) as well as hepatosomatic index (HSI) .They had the same pattern, more reduction in the higher UIA-N concentration and vice versa. Moreover, probiotic exerts a remediation effect reflected on the relatively elevated levels of (SR) and (HIS) especially with the low UIA-N concentration. Controversially, splenosomatic index (SSI), creatinine, ALT and AST were high in UIA-N exposed fish only, and low (especially with the low UIA-N concentration with probiotic) in the other fish.

Blood antioxidants (SOD and GPX)

Table 5 also reveals blood antioxidants (SOD and GPX), it was noted that, SOD had different behavior than GPX in terms of probiotic impact on their recovery. Both SOD and GPX had elevated levels in 0.1 and 0.5 mg/L^{-1} UIA-N. Probiotic enhanced SOD levels especially in 0.1 mg/L^{-1} UIA-N but not as the control treatment. Meanwhile, probiotic significantly lowered GPX in case of 0.1 mg/L^{-1} to the control level but not in case of 0.5 mg/L^{-1} despite of improving its level.

The temporal variation of SOD and GPX showed a significant difference between the first and final period (*P=0.0001* and *0.0003* respectively). However, GPX showed relative improvement than SOD in the final period. Temporal variation of GPX within each treatment showed the following pattern. No significant difference between the first and the final period in case of the control treatment. Presence of probiotic resulted in no significant difference between the first and the final period. The absence of probiotic resulted in a significant difference between the first (high level) and the final (low level) period. However, SOD showed a different model. No significant difference

between the first and the final periods in case of control, 0.1 mg/L^{-1} UIA-N and 0.1 mg/L^{-1} UIA-N plus probiotic treatments. However, significant difference existed between the first (high level) and the second (low level) period in case of the other treatments.

4. Discussion

The clinical signs of the treated fish are in an agreement with the results of (Miyazaki et al., 1984; Ortega et al., 2005; Evans et al., 2006; EL-Shebly and Nahed, 2011; Sherif et al. 2014) who summarized them in two main categories. The first category was pre-mortem symptoms appeared in loss of appetite which was represented by the presence of uneaten pellets, tail rot and blackening of skin and the second category was the post-mortem symptoms as congested gills, hemorrhage in liver, distended gall bladder and splenomegaly.

Cruz et al. (2012) pin pointed that probiotics especially the gram-positive bacteria are more effective in transforming organic matter to Co_2 improving water quality by minimizing its accumulation. This finding supports the results of the current study, where the main constituents of probiotics used were Lactobacillus acidophilus, Bifedobateruim longhum, Bifedobacterium thermophilum and Lactobacillus plantarium which belongs to the gram positive bacteria. Moreover, Lalloo et al. (2007) highlighted the importance of gram positive bacteria in improving the water quality of ornamental fish aquaria by inhibiting the growth of the pathogenic bacteria as well as over 70% reduction in ammonia, nitrate and phosphate. Haroun et al. (2006) and Sivakumar et al. (2009) confirmed that addition of probiotic as supplementary food or direct to the culturing water greatly enhanced its quality. This was clearly shown in the current study especially in case of 0.1 mg/L^{-1} UIA-N with probiotic which had no significant differences with the control treatment. While in case of 0.5 mg/L^{-1} UIA-N, probiotic enhanced the fish physiological state but not as good as the control treatment. Probably because more time (more than 4 weeks) is needed for the used probiotic concentration to exert its action with the higher UIA-N concentration or the used probiotic concentration (5 mg/L^{-1}) was not strong enough to enhance the water quality in case of UIA-N concentrations greater than 0.1 mg/L^{-1}.

Mere UIA-N exposure resulted in a significant reduction in all blood parameters. This result is in an agreement with) Pickering 1984; Ahmed et al., 1992; Atle et al., 2004; El-sherif and El-feky, 2008; Seriani et al., 2011) in terms of reduction of WBCs, RBCs numbers, PCV%, and suppression of leukopoietic centers respectively. However, Hrubinko et al. (1996) had a contradicted result, which is an increase in the Hb concentration with 0.1 mg l^{-1} UIA-N which may be due to the short period of exposure.

The elevated serum glucose and cortisol in the current study reflected the stress condition. This result agreed with the findings of)Bonga, 1997; Davis and Mc Entire, 2006; Sherif et al., 2014) who observed that glucose and cortisol increased significantly thereafter recovered to the normal condition after cessation of ammonia exposure. Despite the similarity of the starting and the ending of the experimentation in the current study, in terms of the hematological parameters, glucose and cortisol concentrations, there were significant temporal differences among the different treatments. This could highlight the important role played by probiotics in relieving the fish physiological state. Where probiotic existence with 0.1 mg/L^{-1} UIA-N suppressed the harmful UIA-N effect. On the other hand, its existence with 0.5 mg/L^{-1} did relieve the fish physiological state but not as good as in case of 0.1 mg/L^{-1} or control. This could be explained by the fact that more time is needed for higher UIA-N concentrations to deviate its harmful effects. Or more probiotic concentrations may be needed to overcome the high UIA-N concentration. However, fish recovery even in the case of mere UIA-N treatments at the end of the experiment returns to the stress cessation.

The parallel trends of both cortisol and glucose is in an agreement with Porchas et al. (2009) and could be attributed to that cortisol mobilize and elevate glucose production in fish through glucogenesis and glycogenolysis pathways Iwama et al.(1999) to cope with the energy demand produced by the stressor. Similar results obtained by EL-Shebly and Nahed (2011) who observed accumulative mortalities of O. niloticus 23.7% and 43.3% occurred within 0.4 and 0.6 mg.l^{-1} NH_3 respectively. Observed mortalities could be explained by tissue damage which resulted in high cortisol levels leading to corruption of the homeostatic mechanisms as reported by Stein-Behrens and Sapolsky (1992). Moreover, Smart (1978) argued that ammonia toxification can cause a wide harmful effects including the impairment of the cerebral energy metabolism, as well as gill, liver, kidney and spleen damage in the different aquatic organisms including fish, crustaceans and mollusks. The results of Sherif et al. (2014) coincide with the current study in terms of the highest creatinine concentration were a result of fish exposure to 0.5 mg/l^{-1} UIA-N. Salah El-Deen (1999) argued that the reason of the elevated creatinine levels is due to dysfunction of kidneys and leakage of these enzymes from injured tissue into blood stream. General system failure prior to death stands behind the physiological and biochemical fluctuations (Abass, 2006).

El-Shehawi et al. (2007) pinpointed that inhibition or induction of liver enzymes is a good parameter for measuring the potential impacts of pollutants supporting the current recorded fluctuated levels of ALT, AST, creatinine, SSI and HSI of *O. niloticus* stressed by ammonia. Furthermore, ALT and AST high levels at the end of the experiment coincide with the results of Abbas (2006). However, Niels et al. (1998) stated that high liver enzymes could be due to tissue necrosis.

The liver is the place of manifold oxidative reactions and maximal free radical generation (Avci et al., 2005). An antioxidant enzymes play an important role in defends and protects aquatic organisms from free radicals that cause oxidative stress. Responses or activities of these enzymes are related with increased ROS production leading to oxidative stress, when the ROS generation rate exceeds that of their elimination this resulted in occurrence oxidative stress (Martinez-Alvarez et al., 2005). Oxidative stress occurs as a result of exposure to ammonia (Walsh et al.,

2007). SOD antioxidant enzymes stimulate remove peroxides, and superoxide radicals by converts' superoxide anion radical to water and hydrogen peroxide(Hegazi et al., 2015; Tripathi et al., 2006), which detoxified by the CAT activity (Zhang et al. ,2007) Moreover, GPX play important role in detoxifies hydrogen peroxide in living cells (Scholz et al. , 1981; Tripathi et al., 2006). This reaction plays a very important role in defending the cells and maintaining it from damage which occurring by free radicals, which formed by peroxide decomposition. The GPx enzymes using glutathione to decrease peroxides into alcohols, therefore preventing the free radicals formation (Hegazi et al., 2015). The reduced activity of the antioxidants (GPX and SOD) in the current study was in an agreement with the results of Mona et al.(2010) who found that the *O. niloticus* fish exposed to sublethal total ammonia nitrogen (TAN) showing a negative effects on The activity of GPX and SOD. The level of these enzymes was increased with increase of (TAN) concentrations. Furthermore, Hegazi et al. (2015) reported that when *O. niloticus* exposed to some polluted substance including ammonia during four season's leads to increase in SOD and GPX activity.

5. Conclusion

0.1and 0.5 mg/L^{-1} UIA-N physiologically impacted *O. niloticus.* The fish restored its normal physiological status after two weeks' stress suspension. Usage of probiotics reduced the period of refreshment and enhanced the restoration to the normal physiological status.

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Table 1: water parameters of fish aquarium.

Parameters	Treatments				
	control	0.1UIA-N	0.1UIA-N+pro	0.5UIA-N	0.5UIA-N+ pro
Temp	26.5±2	26.3±2.1	27±1.5	26.5±1.5	27±1
DO	6.5±1	7±0.5	7.1±0.4	6.5±1.2	6.5±0.3
pH	7.2±0.2	7±0.3	7.3±0.1	7.2±0.2	7±0.4
TDS	340±12.5	345±10.4	347±11.1	345±9.8	348±12.5

Temp=Temperature, DO=Dissolved Oxygen, TDS=total dissolved salts, UIA-N= Unionized ammonia (used with 0.1mgl^{-1} & 0.5mgl^{-1}) and pro=probiotics 5mg/L

Table 2: Explain (RBC, WBC, Hb, PCV and MCHC) changes (mean value ± SE)

	Treatments					
Period	control	0.1 UIA-N	0.1 UIA-N + pro	0.5 UIA-N	0.5 UIA-N + pro	
	mean +SD	mean +SD	mean +SD	mean +SD	mean +SD	
	Total erythrocyte count RBC (million/mm³)					
1	3.26±0.15	2.66±0.18	3.16±0.2	2.68±0.03	3.3±0.1	A
2	3.2±0.1	2.26±0.05	3±0.15	2.5±0.1	2.9±0.17	B
3	3.1±0.15	2.1±0.1	3±0.24	2±0.02	2.47±0.05	C
4	3.1±0.15	2.61±0.05	3±0.24	2.6±0.18	2.75±0.05	B
5	3.2±0.2	2.8±0.02	3.4±0.17	2.88±0.1	3.1±0.05	A
	a	c	a	c	b	
	white blood cells count WBC (Thousands/mm³)					
1	81.7±0.57	76.7±0.4	80.3±0.57	73.2±1.1	79.6±0.6	B
2	83±1	75.3±0.8	81.3±0.76	72.2±2.2	76.9±0.3	B
3	81.7±2	73±1.3	80.4±0.5	69.5±1.1	77.7±0.96	C
4	82.2±1.6	77.3±0.75	80.7±1.4	72.4±0.8	80.3±0.6	B
5	81.2±2	79.1±0.95	81.2±2.2	76.5±1.6	81.2±1.4	A
	a	d	b	e	c	
	Hemoglobin Hb (g/dl)					
1	9.5±0.3	9.2±0.3	9.2±0.05	9.2±0.15	9.3±0.26	A
2	9.3±0.46	8.5±0.17	8.9±0.1	8.1±0.1	8.6±0.3	B
3	9.1±0.2	7.1±0.1	8.9±0.2	6.9±0.05	8.3±0.15	D
4	9.2±0.1	7.8±0.2	9±0.1	7.3±0.05	8.7±0.2	C
5	9.3±0.15	8.7±0.43	9.1±0.1	7.9±0.05	8.9±0.05	B
	a	d	b	e	c	
	Packed Cell Volume PCV (%)					
1	29.95±0.99	29.2±0.99	29.2±0.17	29.25±0.47	29.4±0.8	A
2	29.3±1.4	26.95±0.5	28.2±0.31	25.8±0.35	27.2±0.99	B
3	28.8±0.62	22.6±0.31	28.2±0.62	22.1±0.17	26.23±0.47	D
4	29.1±0.3	24.7±0.64	28.5±0.31	23.13±0.17	27.5±0.64	C
5	29.3±0.47	27.6±1.3	28.8±0.31	25.1±0.31	28.3±0.17	B
	a	d	b	e	c	
	Mean corpuscular hemoglobin concentration MCHC (%)					
1	31.6±0.02	31.59±0.02	31.59±0.054	31.59±0.01	31.6±0.01	A
2	31.59±0.03	31.53±0.01	31.57±0.007	31.5±0.01	31.54±0.02	BC
3	31.59±0.01	31.47±0.01	31.57±0.01	31.4±0.007	31.5±0.01	C
4	31.59±0.007	31.55±0.02	31.57±0.007	31.4±0.006	31.55±0.01	BC
5	31.59±0.2	31.55±0.03	31.58±0.007	31.49±0.009	31.57±0.004	AB
	ab	bc	a	c	a	

Different letters indicate there is a significant difference at $p \leq 0.05$. 1=after 24 hour, 2=after 48 hour, 3=after 72 hour, 4=after 14 days, 5=after 28 days, UIA-N= Unionized ammonia (used with 0.1mgl^{-1} & 0.5mgl^{-1}) and Pro=probiotics 5mg/L

Table 3refer to (MCH, MCV, glucose and cortisol) changes (mean value ± SE)

	Treatments					
Period	control	0.1 UIA-N	0.1 UIA-N + pro	0.5 UIA-N	0.5 UIA-N + pro	
	mean +SD	mean +SD	mean +SD	mean +SD	mean +SD	
	Mean corpuscular hemoglobin MCH (Pg)					
1	28.99±0.65	34.9±3.7	29.5±0.6	34.4±0.6	27.9±1.4	A
2	28.97±1.6	37.5±0.8	28.2±1.5	32.6±1.4	29.6±2.7	A
3	29.1±1.1	33.87±1.8	29.4±0.8	34.4±1	33.5±1.3	A
4	29.4±1.2	29.7±0.18	29.98±2.1	27.7±1.7	31.5±1.3	B
5	29±1.5	30.9±1.7	26.8±1.1	27.4±0.9	29.1±0.48	B
	cd	a	d	B	bc	
	Mean corpuscular volume MCV (µm³)					
1	91.7±2.1	110.4±11.7	93.3±2	109±2	88.4±4.5	B
2	91.7±5.2	118.9±2.7	89.2±4.7	103.4±4.4	94±8.7	B
3	92±3.5	107.8±5.9	93±2.6	109.63±3	106.4±4.1	A
4	93.2±4.1	94.5±0.5	94.9±6.7	88.3±5.6	99.9±4.1	C
5	91.8±4.8	97.9±5.5	84.9±3.6	87.1±3	92.3±1.5	C

	c	a	c	B	b	
	Glucose (mg/dl)					
1	34±3.4	61.3±1.5	33.7±3.2	91±3.6	53±2.6	A
2	36±4	64.3±1.5	35.7±1.5	103±3.6	51.8±2	A
3	35.67±2	40±1	35.7±2	53±2.6	41.7±1.5	B
4	34±2	37±1	36±4	43.3±1.5	35.7±2.5	C
5	36.7±1.5	35±1	35.3±3	38.3±0.5	35±2.6	C
	a	b	d	A	c	
	Cortisol (mg/dl)					
1	0.826±0.06	1.54±0.12	0.85±0.06	1.86±0.05	1.16±0.1	A
2	0.823±0.01	1.72±0.02	0.8±0.03	1.76±0.05	1.42±0.1	A
3	0.83±0.04	1.026±0.06	0.81±0.01	1.1±0.1	0.84±0.03	B
4	0.79±0.01	0.836±0.005	0.81±0.02	0.85±0.05	0.79±0.005	C
5	0.826±0.01	1.143±0.6	0.82±0.02	0.8±0.01	0.80±0.01	BC
	c	a	c	A	b	

Different letters indicate there is a significant difference at $p \leq 0.05$.
1=after 24 hour, 2=after 48 hour, 3=after 72 hour, 4=after 14 days, 5=after 28 days, UIA-N= Unionized ammonia (used with $0.1mgl^{-1}$ & $0.5mgl^{-1}$) and Pro=probiotics 5mg/L

Table 4: describe liver (enzymes & antioxidants), SR, creatinine and somatic indices changes (mean value ± SE)

	TREATMENT					
period	parameter	control	0.1UIA_N	0.1 UIA_N +pro	0.5+ UIA_N	0.5 UIA_N +pro
2	GPX activity (µmol/mg prot./min.)	20.83±1.2c	25.3±0.9ab	20.3±0.5c	26.6±0.5a	23.97±0.55b
	SOD activity (Unit/mg protein)	157.7±2.5d	173.2±2.3b	164.8±0.8c	180.8±1a	170.3±1.5b
4	GPX activity (µmol/mg prot./min.)	20.8±1.7c	20.3±1.4c	19.8±0.2c	23.8±0.76b	24.4±0.8.5b
	SOD activity (Unit/mg protein)	157±3.6d	171±1b	162±1c	169.7±5.5b	163.7±1.5c
	SR %	96.7±5.7a	80±10b	86.7±5.7ab	66.7±5.7c	83.3±5.7b
	ALT (u/l)	7.97±0.5c	9.3±0.17b	8.73±0.3b	10.4±0.5a	8.8±0.4b
	AST (u/l)	25.5±0.5d	28.6±0.7b	26.57±0.4cd	31.17±0.7a	27.17±0.7c
	CREATININE(mg/dl)	0.77±0.028d	0.9±0.01b	0.84±0.04c	0.97±0.025a	0.87±0.05bc
	SSI %	0.33±0.02d	0.47±0.05ab	0.38±0.028cd	0.51±0.028a	0.43±0.028bc
	HIS %	1.65±0.05a	1.43±0.057c	1.53±0.057b	1.35±0.05c	1.46±0.05c

Different letters indicate there is a significant difference at $p \leq 0.05$
2=second week, 4=fourth week, GPX=glutathione peroxidase, SOD= superoxide dismutase, SR=survival rate, ALT=alanine aminotransferase, AST=aspartate aminotransferase, SSI= spleenosomatic index**,** HIS= hepatosomatic index, UIA-N= Unionized ammonia (used with $0.1mgl^{-1}$ & $0.5mgl^{-1}$) and Pro=probiotics 5mg/L.

Table 5: Growth performance of different fish groups

	Treatments					
Period	**control**	control	0.1 UIA-N	0.1 UIA_N +pro	0.5 UIA-N	0.5 UIA_N +pro
4	**IBW**	30.33±0.17a	30.17±0.4a	30.27±0.4a	29.6±0.38a	30.47±0.3a
	FBW	47.3±1.2a	37.87±0.6bc	47.7±0.9a	34.5±0.64c	40±1.9b
	WG	17±1.1a	7.7±0.7cd	11.4±0.6b	4.9±0.3d	9.5±1.8bc
	DWG	0.57±0.04a	0.26±0.02cd	0.38±0.02b	0.16±0.01d	0.32±0.06bc
	FCR	1.83±0.08c	3.41±0.28b	2.46±0.1bc	4.92±0.17a	3.06±0.6b
	FI	31.03±0.7a	25.9±0.35bc	27.9±0.55b	23.95±0.4c	27.1±1.1b
	SGR	56.16±3.24a	25.57±2.5cd	37.6±1.75b	16.5±0.7d	31.15±5.8bc

4= after 28 days, IBW=Initial body Weight, FBW=Final body Weight, WG=Weight Gain, FCR=Feed Conversion Ratio, DWG=Daily Weight Gain, FT=Feed Intake and SGR=Specific growth rate.