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Effect Of Glutamine on Growth Performance, Biochemical Parameters and Diseases Resistance in Cultured Nile Tilapia Fish

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ABSTRACT

The current investigation was done to examine the impact of some amino acids as glutamine with different concentrations on growth performance, biochemical parameters and resistance to bacterial infection *as Aeromonas hydrophila* in Nile tilapia. Total number used in our study were two-hundred and eighty O. *niloticus* fish (100 diseased fish for *Aeromonas hydrophila* isolation and 180 apparently healthy fish for experimental design). 180 fish were divided into four groups, 45 fish / group. The control group was feeding a baseline diet without additives and the treated groups were feeding a diet containing glutamine of 1, 1.5, or 2% for groups 2, 3 and 4 respectively. Fish from the treated and control groups received intraperitoneal (IP) injections of 0.2 ml suspension of *A. hydrophila* after the feeding trial has concluded. Results of our investigation showed a total of 14 *A. hydrophila* strains were recovered from 100 samples of diseased fish with a percentage of 14%. Through the use of, cultural, morphological, biochemical characteristics and PCR technique, the isolates were identified. According to the results of the PCR examination all 7 examined isolates were positive for the *Aeromonas hydrophila* specific gene (*16S rRNA*) and act gene and 5 isolates were positive for aerolysin gene but the 7 examined strains were negative for ast gene. Also our study showed a significant differences with control and higher growth performance for groups fed on diet containing glutamine 1.5% and 2% concentrations and also showed that diet containing 2% Glutamine augmented Hb%, RBCs, and MCV% and increased serum SOD, CAT, GPx, and bactericidal activities in addition to elevation in the levels of total protein than the other Glutamine inclusion levels (1% or 1.5%). Also the mortality rate and re-isolation rate decreased in groups fed on diet containing glutamine 1.5% and 2% concentrations than the other groups. In conclusion, dietary supplementation with glutamine at a concentration of 2% enhanced the growth performance, improve biochemical parameters and the resistance to *Aeromonas hydrophila* infection.

1. INTRODUCTION

Fish supply inexpensive and healthful source of protein for people particularly in coast countries, such as Egypt. So, it is not surprising to observe a quick rise in the aquaculture production industry in Egypt over the past two decades. But, this huge aquaculture resulted in elevation of incidence, pathogenicity, and drug resistance of some types of bacteria such as *Aeromonas hydrophila*.(Al-Maleky *et al*., 2011).
The second most farmed fish in the world is tilapia, and its production has increased in the previous ten years as a result its adaptability for aquaculture, best marketing and constant costs (Wang and Lu, 2016). Aquaculture is disrupted by a broad variety of microorganisms that resulting in infections (Van Hai, 2015). *Aeromonas hydrophila* is a pathogenic bacteria which lead to major economic losses resulting in a disease defined as motile septicemia or hemorrhagic septicemia. The usual signs of this disease include tail rot, hemorrhage, ulcer,

exophthalmia and desquamation of scale. (Citarasu *et al*., 2011).

A. hydrophila is a widespread rod-shaped gram-negative bacterium that is frequently cut off from ponds of freshwater and it is an ordinary gastrointestinal tract habitant of the fish. This bacterium mostly causes disease in freshwater fish including catfish, various bass species and other ornamental or tropical fish species. (Yardimci and Aydin, 2011)

A. hydrophila is regarded by the majority of researchers as an opportunistic pathogen. In most cases, opportunistic pathogens not causing illness unless there are further conditions. This implies that *A. hydrophila* can usually cause disease if it gets the opportunity like that fish stressed because of improper manipulation or low quality of water. While tilapia was firstly recognized to become strong and resistant to disease, however, under unsuitable conditions, it may result in death because of secondary infection (Laith and Najiah, 2013).

Because of many virulence factors of aeromonads, they can penetrate the defenses system of the host, proliferate, and causing killing of the host. From these factors, there are different enzymes and toxins including Lipase (Lip), Serine protease (Ser), Aerolysin (Aer), Cytotoxic enterotoxin (ACT) and temperature-sensitive protease, Epr (CAI).

The using of chemicals and antibiotics to treat and prevent bacterial infections caused by *A. hydrophila* can poison fish and persistently harm the aquatic environment and result in resistance of bacteria to the antibiotics, so that using antibiotics becomes no longer beneficial (Irawan *et al*., 2003).

With the aquaculture progress which is rapid, the need for fish meals has flown. Over the past years, the whole of fish meal supply has declined, while the cost has elevated. So the essential aspects to decrease feed costs is to increase the rate of utilization of protein and optimize feed composition and reducing the present conditions of shortage of fish meal. Glutamine (Gln), a plentiful functional amino acid present intracellularly in muscle, is an important source for inosine monophosphate (IMP) and protein synthesis, which both of them significantly affect the growth of fish and the flavor of meat. (Yoo et.al ., 2020). The level of glutamine

in living organisms is maintained by a series of enzymes in a dynamic equilibrium. Fish tissues use glutamine and glutamate which are functional amino acids, as their primary source of energy for metabolism. (Jia *et al*., 2017). Due to the animals' physiological requirements, glutamate and glutamine are frequently integrated into diets as additives for feed even though they can be produced by the body. (Li and Wu, 2020). They enhance development of intestine, acquired and innate immune responses, development of skeletal muscle and the fillet's quality, elimination of ammonia, and endocrine health as feed additives. (Xu *et al*., 2014 and Jiang *et al*., 2016). Additionally, (Hu *et al*., 2015) demonstrated that in grass carp, exogenous dipeptides of glutamine improved the intestinal GS mRNA expression. Thus, it was postulated that the addition of glutamine and glutamate enhances fish growth performance due to their impact on the activity of glutamine synthetase.

It has been reported that dietary glutamine supplementation promotes growth (Pereira *et al.*, 2017), improve efficiency of feed (Han *et al*., 2014) and retention of protein (Caballero-Solares *et al.*, 2015), and improve development of fish intestine (Cheng *et al*., 2011). Supplementing with glutamine and glutamate has recently been thought to be beneficial for reasons other than disease resistance and growth performance. (Zhang *et al*., 2017).

2. MATERIALS AND METHODS

2.1. Samples of fish:

2.1.1. Diseased fish: for *A. hydrophila* isolation

One hundred *Oreochromis niloticus* fish which appeared healthy and infected naturally were obtained from several fish farms in governorate of Kafr El-Sheikh. The samples of fish were put in containers full of the identical pond water after that taken to the Laboratory.

2.1.2. Experimental Fish:

One hundred and eighty Nile tilapia with a 20 ±5 g average body weight and appeared healthy were acquired from Kafre EL-Sheikh Governorate's private fish farm and transferred a live to Kafr EL-sheikh's Animal Health Research Institute laboratory and they put in well-maintained aquariums that measure (90x45x45 cm). The tap water used in these glass aquariums is devoid of chlorine, according to **(Innes, 1966)** and kept at 27±20C while being continuously aerated by an electric pump. Every day, fifty percent of the water was replaced. Fish were adapted for 14 days and throughout the adaptation period, fish is feeding only on the baseline diet

2.2. Isolation and identification of *A. hydrophila* from samples of fish:

With a sterile loop, swabs from the liver, kidney, gills and spleen were obtained. The samples were then placed in trypticase soy broth and incubated for 24 hours at 37 °C. A loopful of the resulting broth was streaked over Tryptic soya agar and *Aeromonas* Medium Base (Oxoid, Ltd.) after adding ampicillin as a supplement, then incubated for 24 hours at 37 °C.

Morphological characteristics of the pure colonies were assessed using shape, motility testing and Gram staining. To confirm the identification of *A. hydrophila*, biochemical tests including oxidase, catalase, methyl red, Voges Proskauer, indole, citrate, urea, and gelatinase tests were performed (Markey *et al*., 2013).

2.3. Using polymerase chain reaction (PCR) for molecular identification and detection of some virulence genes of *Aeromonas hydrophila*:

DNA extraction. The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used for extracting DNA from samples, with some modifications occured according to the manufacturer's guidelines. In summary, 200 µl of the suspension of sample was incubated for 10 minutes at 56^{O}C with 10 µl of proteinase K and 200 µl of lysis buffer. 200 µl of 100% ethanol was added to the lysate post incubation. Then the sample was centrifuged and washed according to the manufacturer's guidelines. 100 µl of elution buffer provided in the kit was used to elute the nucleic acid.

Primer for Oligonucleotides. The primers chosen, that are included in table (1), were supplied by Metabion (Germany).

Amplification by PCR. In a 25 µl reaction with 12.5 µl of EmeraldAmp Max PCR Master Mix, primers were used. **(Takara, Japan)**, 1 µl of each primer at a concentration of 20 pmol, 5 µl of DNA template and 5.5 µl of water. Applied Biosystems 2720 thermal cycler was employed to complete the reaction.

Examination of the PCR Products.

Using 5V/cm gradients, the products of PCR were split off by electrophoresis on agarose gel 1.5% (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature. 20 µl of the PCR products were placed in every gel slot used for gel analysis. The sizes of fragment were measured with the use of Generuler ladder with 100 bp (Fermentas, Thermofisher, Germany). The gel was photographed by a system of gel documentation (Alpha Innotech, Biometra) and computer software was used to analyze the data.

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions for conventional PCR.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Aeromonas hydrophila 16S rRNA	GAAAGGTTGATGC CTAATACGTA CGTGCTGGCAACA AAGGACAG	685 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Gordon *et al*., 2007**
Act	AGAAGGTGACCAC CACCAAGAACA AACTGACATCGGC CTTGAACTC	232 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Nawaz *et al*., 2010**
Ast	TCTCCATGCTTCCC TTCCACT GTGTAGGGATTGA AGAAGCCG	331 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
Aerolysin (Aero)	CACAGCCAATATG TCGGTGAAG GTCACCTTCTCGCT CAGGC	326 bp	94˚C 5 min.	94˚C 30 sec.	52˚C 30 sec.	72˚C 40 sec.	72˚C 10 min.	**Singh *et al.*, 2008**

2.4. Feed preparation and experimental design

Fish were feeding a baseline diet consisting of commercial ingrdients twice a day until apparent satiation. After thoroughly mixing all of the commercial ingredients with oil until they formed a stiff, doughy consistency, the mixture was chopped into strands. The latter were dried, crushed, sieved into pellets with a diameter of 1.5 mm, and kept in plastic bags at 4 °C until used. According to NRC (2011), this basal diet was formulated to satisfy *O. niloticus's* nutritional needs. Chemical analysis of the experimental diets was done according to (AOAC, 1995) (Table 2). Glutamine was thoroughly mixed with the commercial ingredients during the pellet preparation. 180 apparently healthy *O. niloticus* were collected from a special farm in Kafrelsheish governorate, Egypt. Fish were transferred to lab of Animal Health Research Institute in Kafrelsheish governorate. The fish were adapted for 14 days and were given a baseline diet *ad libitum*. Water parameters were checked regularly and revealed that pH, dissolved oxygen and temperature recorded, 6.7±0.1, 6.5±1.1 mg/l and 26±1 °C respectively. Debris was removed and every day, one-third of the water was replenished. After adaptation period, fish were randomly distributed into 4 groups (n = 45/group); The control group received a baseline diet with additional ingredients, the treated groups

were given a diet supplied with glutamine at 1, 1.5 and 2% of feed for groups 2, 3 and 4 respectively. The fish received two daily hand feeds at 9:00 and 14:00 hours. Great attention took place to make sure that all feed was eaten. Each fish within the aquarium was subjected to weight twice a week for eight consecutive weeks beginning on the first week (W0). All the growth parameters were determined accordance with Abu-Zahra *et al*. (2024)

$$\text{weight gain (g)} = \text{final weight} - \text{intial weight}$$

$$\text{gain \%} = \frac{\text{weight gain}}{\text{intial weight}} \times 100$$

$$\text{Feed effeciency ratio\%} = \frac{\text{weight gain}}{\text{feed intake}}$$

$$\text{Feed conversion ratio} = \frac{\text{total feed intake}}{\text{total weight gain}}$$

Table 2. Ingredients proximate analysis of the experimental different dietary treatments:

Proximate analysis					**Chemical analysis**	
Ingredients	**Basal diet**	**1% Glutamine**	**1.5% Glutamine**	**2% Glutamine**	**Items**	**%**
Soybean (44%)	39	39	39	39	Moisture%	6.1
Yellow corn (8.5%)	32.145	31.145	30.645	30.145	Dry matter%	93.9
Fish meal (55%)	10	10	10	10	Crude protein%	32.29
Wheat bran (13.8%)	2	2	2	2	Ether extract%	2.8
Corn gluten (60%)	11	11	11	11	Ash	5.9
Soy oil	5	5	5	5	Crude fiber	3.9
Vit and mineral mix	0.3	0.3	0.3	0.3	NFE	49.01
Salt	0.2	0.2	0.2	0.2	Calcium	0.75
Carboxymethylcellulose	0.2	0.2	0.2	0.2	Total phosphorus	0.81
DI. Meth	0.155	0.155	0.155	0.155	Lysine	1.72
Glutamine	---	1	1.5	2	Methionine	0.73
					Digestible energy (DE)	3242.6

2.5. Experimental challenge

The virulent strains *A. hydrophila* which was already obtained from *O. niloticus* was grown overnight at 37 °C on tryptone soy broth and centrifuged at 3000 ×g for 10 minutes at 4 °C.; by using a McFarland standard tube, the pelleted cells were adjusted to 1×10^7 CFU/ml after being rinsed twice and re-suspended in sterile physiological saline. according to Ezzat *et al*. (2018). In the ending of the feeding trial (8 weeks), fish of the treated and control groups received intraperitoneal (IP) injections of 0.2 ml suspension of *A. hydrophila*. After the challenge, fish were monitored for 10 days, during which time clinical symptoms and deaths were recorded. Fish that were dead or moribund were examined bacteriologically and clinically. On the fifth day following the challenge, samples from gills, liver, kidneys, brain and spleen of 10 fish (dead and moribund) from each group were collected in aseptic conditions for re-isolation of bacteria, then inoculated in trypticase soya broth and incubated for 24 hours at 37 ^{0}C. After that a loopful of the inoculated broth was cultured on nutrient agar and *Aeromonas* Medium Base with its supplement (Ampicillin) then incubated at 37ºC for 24 to 48 hours.

2.6. Erythrocytic and leukocytic counts determination:

Leukocytes and erythrocytes were determined using the procedure identified by Stoskopf (1993**)** use the Natt-Herrik solution and hemocytometer.

2.7. Determination of hemoglobin concentration:

The cyanomethemoglobin method Drabkin's solution was used to measure the hemoglobin concentration in accordance with (Stoskopf, 1993). By using ferricyanide and cyanide ions, the cyanomethemoglobin technique changes all hemoglobin derivatives into methemoglobin. The methemoglobin is a red substance which is stable and could be determined colormetrically.

2.8. Determination of packed cell volume:

The PCV% was calculated by using the method of micro hematocrit accordance with **(Dacie and Lewis, 1991)**.

2.9. Differential leukocytic count (DLC) determination

A thin blood film was formed, let to air dry, fixed for three to five minutes with methanol, then stained for eight to ten minutes with Gimsa stain. After that it was washed with distilled water and allowed to dry. Among the 100 blood smears, the white blood cells were estimated according to **(**Stoskopf, 1993). The calculation of the absolute DLC was done accordance

with (Thrall, 2004) according to the equation that follows:

Absolute DLC = no. of each white cell x no. of total leukocytic count/100

2.1.0. Biochemical analysis of Blood serum

Total proteins in serum (Spectrum, Egyptian Company for Biotechnology, Egypt) were measured colorimetrically at a wave length of 546 nm according to **(**Cannon *et al*., 1974). Albumins (Biodiagnostic Co. Egypt) were measured colorimetrically at a wavelength of 630 nm according to (Doumas *et al.*, 1981). The content of globulins was estimated mathematically. Aspartate aminotransferase (AST) activities (Biodiagnostic co. Egypt.) were estimated colorimetrically at 505 nm wavelength according to (Reitman, and Frankel, 1957). Alanine aminotransferase (ALT) Biodiagnostic co. Egypt. were estimated colorimetrically at 505 nm wavelength according to (Reitman and Frankel, 1957). Alkaline phosphatase (Biodiagnostic co. Egypt.) were measured colorimetrically at 510 nm wavelength according to (Belfield and Goldberg, 1971). Creatinine (Biodiagnostic co. Egypt.) were colorimetrically measured according to (Bartles *et al.*, 1972). Urea (Biodiagnostic co. Egypt) were colorimetrically measured according to (Fawcett and Scott, 1960).

2.1.1. Antioxidant's determination:

Super oxide dismutase (SOD) (Biodiagnostic co. Egypt.) were estimated colorimetrically at 560 nm wavelength according to (Nishikimi et al., 1972). Catalase (Biodiagnostic co. Egypt) were estimated colorimetrically at 510 nm wavelength according to (Aebi, 1984). Glutathione peroxidase (Biodiagnostic co. Egypt) were estimated colorimetrically at 340 nm wavelength according to (Paglia and Valentine, 1967).

2.1.2. The serum lysozyme activity:

Was examined using the micro-well technique of ELISA by using ELISA kit for fish lysozyme (Sunlong Biotech co. China.) at the wavelength of 450 nm by using the micro plate ELISA reader in accordance with the manufacturer's guidelines.

2.1.3. Serum bactericidal activity

Bacterial strain *Aeromonas hydrophila* which obtained from *O. niloticus* used for study the serum bactericidal activity. Serum was extracted from each sample's hemocytes before diluting. To determine bactericidal activity, samples were diluted three, four, and five times with a Tris buffer (pH 7.5) according to Iida *et al*. (1989). A bacterial suspension (0.001 g ml−1, *Aeromonas hydrophila*) was mixed with the dilutions and incubated for 24 hours at 25 °C. Fifty microliter of the reaction solutions were incubated on TSA for 24 hours at 25 °C. The plate counting method was used to count the colony forming units (CFUs). The results were documented using the survival index (SI) (Wardlaw and Unkles., 1978). Value determined as the following: SI = (CFU at end / CFU at start) x 100.

2.14. Whole blood respiratory burst activity:

Nitro-blue-tetrazolium (NBT) test was used to quantify whole blood respiratory burst activity in accordance with (Anderson and Siwicki, 1995). A volume of 0.2% NBT (Sigma, St. Louis, MO, USA) was put to a microtiter plate well containing a sample of blood (0.1 mL) that had been treated with anticoagulant. Then at room temperature, the NBT-blood cell suspension was incubated for 30 minutes. An NBT-blood cell suspension sample (0.05 mL) was removed and put to a glass tube contain 1.0 mL of N,N-dimethylformamide solution. The mixture was then centrifuged at 3000 rpm for 5 minutes. A spectrophotometer was used to measure the absorption of the supernatant at 540 nm after it was transferred into a glass cuvette.

2.1.5. Statistical analysis:

The variance Analysis (ANOVA) one-way analysis of variance was used to do statistical analysis in order to examine the impact of various groups of treatment on the various examined variables which include (growth performance parameters, hematological and biochemical) variables using (SAS, 2004).

3. RESULT AND DISCUSSION

A. hydrophila bacteria that harm tilapia, as well as other types of freshwater fish causes Motile Aeromonas Septicemia disease. The antibotics and chemicals used to prevent and treat that disease can cause damage to the aquatic environments in addition to increasing antibiotic-resistant bacterium, which renders antibiotic usage is unsuccessful. (**Lestari and Astriana, 2021).**

3.1. Clinical and postmortem examination of Oreochromis niloticus infected naturally:

The examination of *Oreochromis niloticus* infected naturally in our study revealed skin discolouration, internal organs congestion, and hemorrhages on the external surface and surrounding the anus. These lesions are identical to those documented by (Abu-Elala *et al*., 2015 and Carvalho-Castro *et al.*, 2010). The gross pictures found in recently dead fish included enlarged spleen and kidney as well as pale and swollen liver. These findings concurred with the results by Noor El-Deen *et al.* (2014), Kaleeswaran *et al.* (2012) and Islam *et al.* (2008).

3.2. Incidence of *A. hydrophila* isolated from diseased *Oreochromis niloticus*:

The obtained data showed that 14 (14%) of the 100 Nile tilapia samples that were examined were positive for *Aeromonas hydrophila* based on biochemical identification and the colonial properties on Aeromonas Agar media. The obtained results nearly similar to that reported by Abd El-Malek, 2017 who reported that *A. hydrophila* could be detected in cultured and wild Nile Tilapia samples with the percentage of 12 % and 16 %, respectively. But disagreed with Nhinh *et al*., 2021 who stated that 236 isolates of *A. hydrophila* were identified and confirmed from 506 diseased fish by using phenotypic testing, PCR assays, and *rpoB, gyrB* sequencing studies, which corresponded to a 46.4% infection rate. Also, our result disagreed with Noor El-Deen *et al.* (2014) who cited that10 isolates of *Aeromonas hydrophila* were identified from 40 cultured O. niloticus which *randomly* collected from fish farms in Kafr El Sheikh Governorate with an incidence of 25%.

3.3. Using polymerase chain reaction (PCR) for molecular identification and detection of some virulence genes of *Aeromonas hydrophila*:

PCR is a highly quick and precise technique for diagnosing bacteria isolated from cultured fish. (El-Adawy *et al*., 2018). As demonstrated in Fig (1) a,b,c and d, our investigation showed that the 16S rRNA gene of *A. hydrophila* were identified in 100% of the fish samples and this was in contrast to the findings published by Lee *et al.* (2002). Who discovered that that14% of the examined fish samples were infected with *A. hydrophilia* by using the 16S rRNA gene-based PCR. However, Onuk *et al*. (2013) discovered a lower prevalence of 23.33% as *A. hydrophila* through using PCR-RFLP pattern analysis. and also reported by Hussain *et al*., (2014) who using presence of the 16S rRNA gene and discovered that Aeromonas spp. were present in 56 % of the fish samples. Virulence genes play a multifunctional and multifactorial role in determining the potential pathogenicity of microorganisms and they can also be utilized for virulence typing of isolates of *A. hydrophila*. (Li *et a*l., 2011 and Oliveira *et al*., 2012). This bacteria's aerolysin (aer) revealing increased RBCs and cellular lysis (Niamah, 2012).

Aerolysin which is present in pathogenic *A. hydrophila* may help in diagnosis, treatment and control of the disease. And it may also help to decrease mortality and the spread of disease in aquaculture. (Al-Maleky *et al*., 2011). Our findings demonstrated that the aero gene recorded five out of seven isolates, representing 71.4% and providing product of 326 bp. The results achieved were in line with the findings of Mansour *et al*. (2019), who discovered that 86% (18/21) of the isolates of *Aeromonas hydrophila* harbored the aerolysin gene. However, greater than other studies obtained by Tahoun *et al*., 2016, Yogananth *et al*.2009 and Ahmed *et al*., 2018. The *act* gene is responsible for *Aeromonas* enterotoxigenicity, In our investigation, Act was the most common virulence gene in which 100% of our isolates harbored this gene. This was inconsistent with lower detection rate of the gene in some studies reported by Ahmed *et al*., 2018. Furthermore, our findings closely matched with those published by Simon *et al*. (2016) who cited that 94 % of the *A. hydrophila* isolates harbored *act* gene. The *ast* has the capacity to increase the gut's vascular permeability and cause detachment of the intestinal mucosa (Harikrishnan and Balasundaram, 2005). Our results did not identify the ast gene in the isolates and this disagreed with Mansour, *et al*., 2019 and Ye *et al*., 2013 who identified it with a percentage of 57% and 75% respectively.

Table 3. Effect of different treatments on growth performance of *Oreochromis niloticus* (0-8weeks):

parameters	Control	Glutamine 1%	Glutamine 1.5%	Glutamine 2%
W1 (g/fish)	25.56± 0.61 [a]	25.54± 0.60 [a]	25.53± 0.64 [a]	25.55± 0.66 [a]
W2 (g/fish)	44.56± 0.90 [a]	46.89± 0.89 [a]	48.42± 1.27 [a]	50.44± 1.18 [b]
WG (g/fish)	19.00± 0.28 [a]	21.40± 0.28 [b]	22.90± 0.63 [c]	24.89± 0.51 [d]
G%	74.36± 0.65 [a]	83.83± 0.84 [b]	89.69± 0.23 [c]	97.44± 0.49 [d]
TFI	38.77± 0.91 [a]	40.89± 1.16 [a]	39.89± 1.90 [a]	40.56± 1.67 [a]
FCR	2.04± 0.01 [a]	1.91± 0.02 [a]	1.74± 0.03 [b]	1.62± 0.04 [c]
FE	0.49± 0.01 [a]	0.52± 0.01 [a]	0.57± 0.01 [b]	0.61± 0.01 [c]

The values are presented as the means ± SE. Significant differences at $P<0.05$ are indicated by different letters in the same row. W1: initial body weight; W2: final body weight; WG: weight gain; G%: Gain%; TFI: total feed intake; FCR: feed conversion rate; FE: feed efficiency.

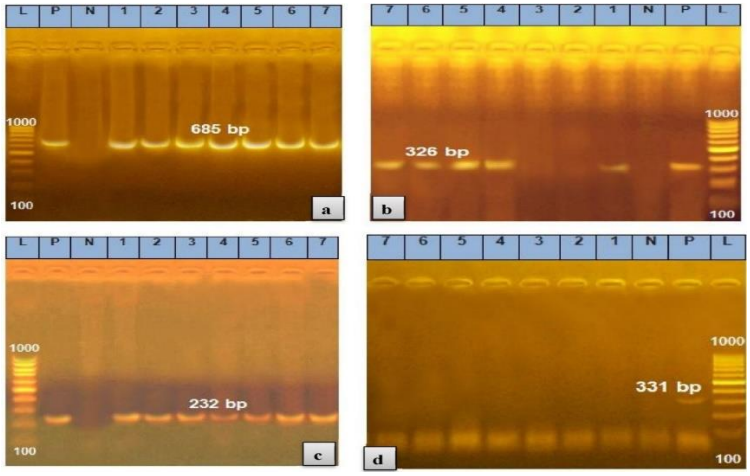

Fig. 1. Agarose gel electrophoresis of PCR amplification products for characterization of *A. hydrophila* a) *16S rRNA* gene, Lane1-7: Positive for *A. hydrophila* strains at amplicon of 685 bp; b) aerolysin gene, Lane1,4,5,6 and 7 Positive for *A. hydrophila* strains at amplicon of 326 bp; c) act gene, Lane1-7: Positive for *A. hydrophila* strains at amplicon of 232 bp; d) ast gene, Lane1-7: negative for *A. hydrophila* strains at amplicon of 331 bp. Lane L: Molecular size marker (100-1000 bp), Lane P. and N.: Positive and negative controls.

3.4. Growth performance:

Our study's findings demonstrated a substantial difference with control and higher growth performance for groups fed on diet containing glutamine 1.5% and 2% concentrations, Initial weight of the body, final weight of the body, total body weight gain, gain percent, total feed intake, ratio of feed conversion and feed efficiency of Nile tilapia taking feed containing various glutamine concentrations of 1%, 1.5% and 2% . The initial body weight and total feed intake in all groups did not differ significantly ($P \geq 0.05$) according to statistical analysis of the previously mentioned data. On the other hand, the group fed a diet containing 2% glutamine showed a substantial ($P \leq 0.05$) increase in other growth parameters, for example final body weight only. Total body weight gain and gain percent showed linear significant ($P \leq 0.05$) increase with all groups fed glutamine compared with control one. Finally ratio of feed conversion and feed efficiency showed a substantial ($P \leq 0.05$) differences in Nile tilapia fed on diet containing 1.5% and 2% concentrations of glutamine in relation to that fed on control diet and diet containing 1% glutamine.

The results of our study proved that glutamine is important for growth performance of Nile tilapia and indicated its capacity to utilize the glutamine crystalline. In the represented work, the growth performance increased linearly with increasing the percent of addition of glutamine to diet as reported by Graciano *et al*. (2014) and Li and Wu.(2020) who observed that the addition of glutamine with 2% to diet is necessary for maximal feed efficiency and growth. The result of our study agreed with Pereira *et al.(*2017) who proved that 1% to 2% addition of glutamine to diet of Nile tilapia increased growth performance and feed efficiency as it improve and increase antioxidant activity and efficiency of dietary protein utilization as observed in table (3). The feed intake in our study is not significantly affected with the increasing of percent of addition of glutamine from 1%, 1.5% to 2% as in table (3) and this agreed with Pereira *et al*., 2017 who reported lower feed intake in fish feed glutamine 1% and 2%. Also they agreed with our study in the weight gain. Glutamine is required to improve growth performance parameters may be due to it is considered as major substrate of energy for intestine and immune cell (Jia *et al*.2017). Jiang *et al*. 2016 repored that the addition of 1% to 2% glutamine improve the antioxidant capacity for fish gut, moreover Song *et al.* 2018 proved that glutamine is considered a major substrate for ATP production. So in our study we did not use glutamine with concentration more than 2% because other studies obtained by Pohlenz et al. (2012) proved that an increased supply of dietary glutamine may reduce the catabolism of other amino acids, at the enteric level, as it happens in rats (Salloum *et al.*, 1990). Another reason may because glutamine is metabolized to glutamate and ammonia, both of which have neurological effects (Garlick, 2001). Excess supply of glutamine could cause some neurotoxic effects, but compared to the control group, adverse effects were not observed in the present study when dietary glutamine supplemented to 2.0% so we not use glutamine more than 2%.

3.5. Mortality rate and bacterial re-isolation post-experimental challenge:

Tests for bacterial challenges are frequently employed as a final indicator of fish health **(Jin *et al*.**, 2013 and Zhao *et al*., 2015). In our study mortalities were firstly recorded in the 2nd day post challenge. After 10 days post challenge, we found that the rate of re-isolation and the rate of mortality reduced in the glutamine-fed groups than the control group especially in the fish group fed 2% glutamine as shown in Table (4). These findings concurred with the findings of Li *et al*. (2020), who found that the rate of fish survival following experimental *A. hydrophila* infection improved when dietary glutamine levels increasing. Examining fish clinically and after mortality showed severe signs of infection in control group such diffuse skin hemorrhage, exophthalmia, hemorrhagic liver, distended gall bladder, and congested gills (Figure 2). The severity of sings was greatly reduced in groups fed different grades of glutamine compared to the control group.

Table (4): The re-isolation and mortality rate after experimental challenge

Groups	No. of fish before challenge	No. of fish after challenge	Mortality rate		Re-isolation rate	
			No	%	No	%
Control	45	25	20	44.44	10/10	100
1%glutamine	45	29	16	35.5	5/10	50
1.5% glutamine	45	32	13	28.8	4/10	40
2% glutamine	45	38	7	15.5	2/10	20

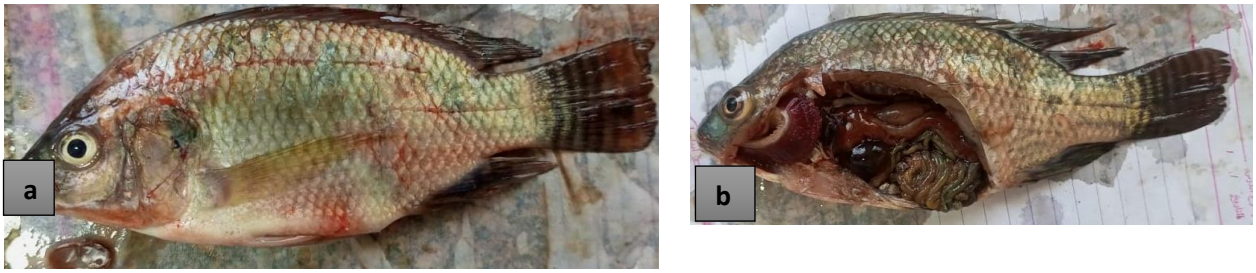

Fig. 2 clinical and postmortem examination of fish post challenge. a) fish from CTR group showing diffuse skin hemorrhage, b) fish from CTR group showing exophthalmia, hemorrhagic liver, distended gall bladder, and congested gills

3.6. Hematological parameters of *O. niloticus* fed on diet with various glutamine concentrations:

Hematological parameters are essential items for identifying nutritional levels, detecting diseases, and evaluating fish health and hygienic conditions. Erythrocytes' primary roles in respiration are the transportation of O2 and the mediation of CO2 production (Cimen, 2008). The oxygen carrier protein in erythrocytes is hemoglobin (Hb) which can bind oxygen to form oxygenated hemoglobin and then releases oxygen into animal tissue. (Johnson *et al*., 2005). In the current investigation, dietary Gln demonstrates the changes in hematological parameters following the feeding O. niloticus with. diets with various glutamine concentrations as shown in Table (5). The additing of 2% Glutamine to the feed substantially ($P<0.05$) increased Hb%, RBCs, and MCV%, but the other Glutamine inclusion levels (1% or 1.5%) had no significant effect on the hematological parameters when comparing with the control group. Furthermore, there was no discernible effect of any dietary inclusion levels of glutamine on WBCs, PCV, MCH, MCHC, and differential leukocyte count comparing with that in the control group.

One explanation for the rise in Hb could be because Gln helps in fish erythrocytes development. These findings supported the idea that dietary Gln can improve the role of oxygen transportation in fish blood. Autoxidation of the oxygenated hemoglobin can yield O2 and Met-Hb, which is incapable of binding or transporting O2 in erythrocytes. (Johnson *et al*., 2005). Corresponding to our findings, Tomato paste by-product extract has been shown substantially to increase carp's RBC, Hb, and Ht values (Kesbiç *et al.*, 2022). According to another study, Ht, Hb, and RBC values of goldfish fed diet with 1‰ Ferula elaeochytris root powder (FRP) elevated (Inanan *et al.*, 2021). Our results unsimilar to Öz *et al*. (2024) who mentioned that the highest RBC, Ht, and Hb values were determined in 1.5% of Gln group.

Table(5): Hematological parameters of *O. niloticus* fed diets containing different concenterations of Glutamine

parameters	CTR	Glutamine 1%	Glutamine 1.5%	Glutamine 2%
Hb (g/100 ml)	10.34± 0.29 [a]	11.90± 0.28 [a]	11.99± 0.40 [a]	13.46± 0.45 [b]
RBCs (×10^6/mm^3)	3.34± 0.08 [a]	3.72± 0.12 [a]	3.82± 0.07 [a]	4.66± 0.23[b]
WBCs (x10^3/mm^3)	10.38± 0.29 [a]	10.37± 0.48 [a]	12.20± 1.28 [a]	14.35± 2.49 [a]
PCV%	33.33± 1.20 [a]	36.00± 1.52 [a]	36.67± 0.88 [a]	40.33± 0.88 [a]
MCV	93.42± 1.06 [a]	96.84± 1.21 [a]	96.91± 0.42 [a]	97.42± 0.29[b]
MCH	30.03± 0.48 [a]	29.68± 1.12 [a]	29.56± 0.53 [a]	30.01± 0.72 [a]
MCHC	31.04± 0.28 [a]	30.74± 0.85 [a]	30.51± 0.68 [a]	31.88± 0.51 [a]
Neutrophil %	18.00± 1.15 [a]	14.33± 2.84 [a]	10.33± 0.88 [a]	12.33± 1.85 [a]
Basophil %	0.66± 0.33 [a]	1.33± 0.33 [a]	1.00± 0.33 [a]	1.33± 0.88 [a]
Esinophil %	0.66 ± 0.33 [a]	0.66 ± 0.33 [a]	0.33 ± 0.33[a]	1.00 ± 0.00 [a]
Monocyte %	7.33± 0.33	7.66± 0.88	8.33± 0.33	7.66± 1.20
Lymphocyte %	73.33± 1.76 [a]	76.00± 2.88 [a]	80.00± 1.15 [a]	77.67± 0.88 [a]

The values are the means ± SE (n=6/group). The values (mean ± SE) bearing asterisks are significantly different (P< 0.05). Hb: hemoglobin; RBCs: red blood cells; WBCs: white blood cells; PCV: packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

3.7. Immune responses and antioxidant activity of *O. niloticus* fed on diet with various glutamine concentrations:

In erythrocytes, the main enzymatic antioxidants are SOD, CAT, and GPx. (Cimen, 2008 and Fan *et al*., 2015). SOD guarantees that O2 is broken down enzymatically into H2O2. (Kochhann *et al*., 2009). CAT converts H2O2 to water and O2. (Pflugmacher, 2004). GSH serves as the substrate for GPx, which degrades H2O2 and lipid hydroperoxides. (Lin and Shiau, 2009). Our findings cited that fish were feeding diets with 2% of Glutamine substantially (P<0.05) elevated serum SOD, CAT, GPx, and bactericidal activities as indicated by table (6). These findings concurred with Zhang *et al*. (2017); Liu *et al*., 2015 and Carvalho *et al*., 2018 who reported that supplementing Gln to the culture media leading to a considerable (P < 0.05) elevation in anion superoxide generation and bactericidal capability. While there was no discernible effect of glutamine inclusion levels (1% or 1.5%) on the immunological response and antioxidant activities comparing with those of the control group. Additionally, there was no discernible effect of any dietary inclusion levels of glutamine on LYZ and NBT comparing with that in control group as shown in Table (6) and these results in line with Zhang *et al*., 2017. These findings suggested that the increased GSH content (rather than CAT and GPx) may be the primary mechanism by which glutamine improves OH-scavenging ability. Qu *et al*., 2019 concluded that the concentrations of malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GSH-PX), and total antioxidant capacity (T-AOC) could be improved by the 12 g/kg dietary Gln level. The studying on hybrid sturgeon by Wang et al. (2011) and hucho taimen by Xu et al. (2009) demonstrated that dietary glutamine might increase antioxidant capabilities by increasing SOD enzyme activities. Recent in vitro research suggests that glutamine may shield fish enterocytes from oxidative damage by reversing H2O2-induced suppression of SOD and GPX enzyme activity. (Hu *et al*., 2014; Li *et al*., 2013; Yan and Zhou, 2006).

Table (6): Immune responses and antioxidant activity of *O. niloticus* fed diets containing different concenterations of Glutamine:

parameters	CTR	Glutamine 1%	Glutamine 1.5%	Glutamine 2%
SOD	8.45± 0.29 [a]	9.51± 0.28 [a]	9.90± 0.50 [a]	11.95± 0.25[b]
CAT	9.21± 0.41 [a]	9.19± 0.27 [a]	9.53± 0.77 [a]	11.54± 0.75 [a]
GPx	6.88± 1.22 [a]	7.42± 0.32 [a]	9.51± 0.30 [a]	10.16± 0.27 [b]
BA	44.80± 2.60 [a]	44.14± 2.62 [a]	44.99± 2.59 [a]	60.80± 4.71 [b]
LYZ	5.71± 1.11 [a]	7.81± 1.01 [a]	8.8.32± 1.58 [a]	9.65± 0.33 [a]
NBT	0.17± 0.02 [a]	0.20± 0.03 [a]	0.17± 0.01 [a]	0.26± 0.03 [a]

The values are presented as the means ± SE. Significant differences at P<0.05 are indicated by different letters in the same row. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; BA: bactericidal activity; LYZ: lysosomal activity; NBT: nitro blue tetrazolium.

3.8. Biochemical parameters of O. niloticus fed diet with various glutamine concentrations:

In our results it was shown that feeding feed supplemented with 2% glutamine causing the levels of total protein to significantly increase within the experimental groups as in Table (7) . Moreover, the findings of this study are in line with those of Inanan *et al*. (2024), and all dietary inclusion levels of glutamine did not significantly affect albumin, globulin, ALT, AST, or ALP., creatinine, and urea

comparing with the control group. These results disagreed with Subandiyono and Hastuti (2022) and Ngaddi *et al* 2019 who mentioned that a drop in the activity of AST and ALT with the dietary glutamate supplementation was found in the carp (*Cyprinus carpio*) and catfish (*Clarias gariepinus*) respectively. Our results disagreed to Inanan *et al*. (2024) who mentioned that GOT, GPT, and CRE values decreased, whereas ALP, BUN, GLU, ALB, CHO, TPR, GLO, and URA values increased with increasing dietary Gln.

Table(7): Biochemical parameters of *O. niloticus* fed diets containing different concenterations of Glutamine:

parameters	CTR	Glutamine 1%	Glutamine 1.5%	Glutamine 2%
ALT	41.56± 2.99 [a]	37.45± 4.65 [a]	32.22± 2.60 [a]	30.89± 0.87 [a]
AST	22.27± 1.57 [a]	23.23± 3.45 [a]	19.82± 1.55 [a]	21.25± 0.91 [a]
Total protein	3.95± 0.09 [a]	3.62± 0.11 [a]	4.51± 0.30 [a]	4.85± 0.07 [b]
Albumin	1.49± 0.02 [a]	1.50± 0.05 [a]	1.51± 0.02 [a]	1.54± 0.02 [a]
globulin	2.46 ± 0.12 [a]	2.12± 0.10 [a]	3.00± 0.33 [a]	3.31± 0.07 [a]
ALP	40.81± 5.81 [a]	36.31± 7.64 [a]	34.43± 3.07 [a]	25.56± 3.49 [a]
Creatinine	0.39± 0.03 [a]	0.46± 0.01 [a]	0.41± 0.02 [a]	0.38± 0.01 [a]
Urea	1.40± 0.09 [a]	1.50± 0.13 [a]	1.45± 0.30 [a]	1.34± 0.17 [a]

The values are presented as the means ± SE. Significant differences at $P<0.05$ are indicated by different letters in the same row. ALT: alanine transaminase; AST: aspartate aminotransferase; ALP: alkaline phosphatase.

4. Conclusion

It can be concluded that dietary supplementation with glutamine particularly at a concentration of 2% enhanced the growth performance, augmented Hb%, RBCs, and MCV% and elevated serum SOD, CAT, GPx, and bactericidal activities in addition to elevation in the levels of total protein and increase the resistance to *Aeromonas hydrophila* infection.

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