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THE EFFECT OF *BACILLUS SUBTILIS* ON GROWTH RATE AND IMMUNE RESPONSE IN CATFISH

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ABSTRACT

The study assessed the impact of dietary *Bacillus subtilis* supplementation on the growth and immune response of catfish. A 2-month feeding trial included control groups (Gr1&Gr2) and *Bacillus*-treated groups (Gr3&Gr4) with $1X10^{10}$CFU/kg *Bacillus subtilis*. *Bacillus*-treated groups (Gr3 and Gr4) exhibited significant improvements in the last weight, gaining weight, SGR%, and condition factor to the Gr1 and Gr2 groups of controls (Gr1 and Gr2). Additionally, the *Bacillus*-treated groups exhibited considerably reduced levels of ammonia (NH3) and nitrite (NO2) at the end of the trial. Afterward, (Gr2&Gr4) were infected with *Aeromonas hydrophila*, and hematological, blood serum parameters, and *Aeromonas hydrophila* count were assessed. The highest erythrocyte, hemoglobin, and PCV values were observed in the *Bacillus*-treated group (Gr3). However, RBCs, Hb, and PCV decreased significantly after the pathogen challenge in Gr2 compared to the unchallenged group. A leucogram revealed slight changes in (Gr2, Gr3, and Gr4) compared to (Gr1). The phagocytic activity showed significant enhancement in (Gr3) and significant reduction in (Gr2) compared to other groups. The infected group (Gr2) had increased AST, ALT, urea, creatinine, and TNF-α, along with decreased catalase enzyme, total protein level, albumin, globulin, and lysozyme activity relative to the control group (Gr1). However, Gr4 exhibited significant improvements in all these parameters compared to Gr2. Bacterial load was higher in group (Gr2) but lower in group (Gr4). In summary, this study suggests that adding *Bacillus subtilis* to the diet may improve the health and growth characteristics of catfish.

Key words: *Bacillus subtilis*, fish, growth, lysozyme, AST.

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INTRODUCTION

These days, one of the sectors with the quickest rate of growth is aquaculture for the production of meat which is the main source of protein for the general public (Kamran *et al*., 2020). In order to meet the growing demand for proteins, aquaculturists must increase their aquaculture practices due to the dwindling global fish populations (Ahmad *et al*., 2020).

The primary source of lipids and proteins in aquafeed formulations is fish products. Therefore, it is crucial to have a balanced supply of the essential fatty and amino acids. It is necessary for the optimal growth, development, and reproduction of aquacultured animals (Olmos *et al*., 2022). Catfish is thought to be appetizing and has a high palatability. Catfish flesh can be considered a functional food, since it contains omega-3 fatty acids and has an adequate amount of essential amino acids (Romanova *et al*., 2020).

Antibiotic use in aquatic environments has increased significantly in the last few decades as a means of battling disease. Drug resistance and bioaccumulation in aquatic life forms are just two of the environmental issues that have arisen from this scenario (Yaqub *et al*., 2022). Probiotic use has recently become well-known as a trustworthy substitute that could reduce the overuse of antibiotics in aquaculture (Olmos *et al*., 2020; Seethalakshmi, 2021).

Potential probiotics, *such as Bacillus spp*. are said to have an impact on the immune system and growth of *Tilapia spp* (Sookchaiyaporn *et al*., 2020; Tachibana *et al*., 2021). Probiotics, including *Bacillus subtilis*, have been employed as a viable microbial food supplement to increase the performances of growth, immunological responses, the balance of gut microbes and the activity of digestive enzymes. This has been an alternative, environmentally friendly approach to developing dependable aquaculture (Lee *et al*., 2017).

One of the most popular probiotic strains in aquaculture is *Bacillus* (Doan *et al*., 2016). Because they are spore-based, stable, and able to settle in the intestines, they are special because they can create a variety of digestive enzymes, including lipase, amylase, and protease. Additionally, it is useful for unique industrial usage (Delwin Abarike *et al*., 2018).

The Food and Drug Administration has classified *B. subtilis* as generally recognized as safe (GRAS) for ingestion by people and animals (Chen *et al*. 2017). Additionally, through pond bioremediation, *B. subtilis* enzymes could improve water quality and prevent disease (Olmos *et al*., 2011; Zorriehzahra *et al*., 2016).

Motile *Aeromonas* Septicemia (MAS) in fish caused by *Aeromonas hydrophila* (Shoemaker *et al*. 2018), causes the death rate to rise (Li *et al*. 2019). Therefore, in order to maintain sustainability and address the issues associated with aquaculture intensification, an environmentally friendly strategy is required. The blood biochemistry and hematological parameters are thought to be useful markers for tracking the health of fish when they are fed probiotic-enriched diets and subjected to various stresses in fish farming. (Ahmadifar *et al*., 2019).

Therefore, the current study was carried out to examine the effect of *B. subtilis* on growth parameters, water quality, resistance to *Aeromonas hydrophila*, and some haematological, immunological and serum biochemical parameters of catfish.

MATERIALS AND METHODS

Ethical approval

The Animal Health Research Institute Ethical Committee Approval Number is ARC/AHRI/23 /41, and this study was approved by the local committee of the

ARC-IACUC committee. The Animal Health Research Institute recommendations and the OIE criteria for the use of animals in research and education were followed in all methodological aspects.

Probiotic supplementation

Bacillus subtilis was previously isolated from the intestine of *Clarias gariepinus* and identified by 16 S rRNA gene sequencing and submitted to the Gene Bank database, with accession numbers KX015881 (through project no. 5589, supported by a grant from the Science and Technology Development Fund in Egypt (STDF) to the Aquatic Animal Medicine Department, Faculty of Veterinary Medicine, Zagazig University, Egypt). One ml of the culture (24 hours) of *Bacillus subtilis* was centrifuged at 3000 rpm for 30 minutes at 4ºC. After being cleaned with sterile saline, the pellets were centrifuged for five minutes at 3000 rpm. Using a McFarland standard tube, the probiotic isolate's final concentration in saline was adjusted to 10^{10} CFU/ml. The components of the fish feed were combined with the isolate's bacterial suspension. A meat mincer with a 3 mm diameter was used to mechanically combine the components before they were pelletized. Pellets were stored at 4ºC after being allowed to air dry for 24 hours at ambient temperature (27 ◦C). (Reda *et al*., 2018).

The tested organism: *Aeromonas hydrophila* was isolated from naturally infected fish

Experimental design:

A private farm in Sharkia Governorate provided a total of 120 catfish (*Clarias garipeinus*), ranging in length and weight from 25–30 cm and 155–156 g respectively. Four equal groups were formed out of them (15 for each with two replicates) and allowed to acclimatize to dechlorinated tap water for two weeks in a well-aerated glass tank. Group 1 and Group 2 (Gr1&Gr2) were healthy normal fish fed on ration without any supplement (control), Group3 and Group4 (Gr3 & Gr4) fish were fed on ration supplemented with *Bacillus subtilis* ($1X10^{10}$CFU/kg) for 2 months. After that (Gr2 & Gr4) were inoculated intra-peritoneally with 0.5 ml of *Aeromonas hydrophila* from 24 h of previously prepared *Aeromonas hydrophila* inoculum. The injected fish were transferred to the aquaria and observed daily for any abnormal clinical appearances.

Growth performance

At the beginning and end of the experiment, catfish were captured in each group to estimate the following: The weight gain (WG) is equal to the difference between the starting and final weights (g). The specific growth rate (SGR) (%) is calculated as [(ln (final weight) – ln (initial weight))/60 days] × 100, where ln is the Napierian logarithm and the condition factor (K) is equal to (weight (g)/(length (cm))3) × 100. (Mohammadi *et al*., 2020).

Water quality

Water samples were collected from each tank at the beginning and end of the experiment for the detection of inorganic nitrogenous compounds. Ammonia (NH3) and nitrite (NO2) were measured at the beginning and end of the experiment, according to APHA (1985).

Collection of fish samples

A total of 40 catfish were collected from different localities in El-Sharkia governorate. Fish were transported alive to the Animal Health Research Institute, Zagazig branch. Fish were examined clinically in glass aquaria, supplied with aerated chlorine-free tap water. Fish were examined clinically for any abnormal lesions and bacteriologically, according to (Austin and Austin, 2007). After that, the samples were placed in a sterile plastic bag, shipped to the laboratory for bacterial isolation and identification, and maintained in an aseptic isolated box that was cooled.

Preparation of fish samples

Fresh samples of gills, kidneys, livers, and intestines were aseptically collected from naturally infected fish. The time between collection of samples and the beginning of the analysis did not exceed 2 hours, being compliant with the recommendations of ISO (2013). Subsequently, 45 milliliters of aseptic 1% peptone water were placed in a sterile homogenizer tube along with five grams of each sample. In accordance with APHA (1992), the contents were homogenized at 14000 rpm for 2.5 minutes, before being left to stand for 5 minutes.

Bacterial isolation and biochemical identification of *A. hydrophila*

After the homogenate was ready, to serve as an enrichment broth, 1 ml and 9 ml of brain heart infusion broth (BHI) were placed into a sterile test tube. The test tube was then incubated at 28°C for a whole day. According to (Handfield *et al*., 1996), an *Aeromonas* Agar medium was streaked with a loopful of the enrichment broth and incubated aerobically at 37°c for 18 to 24 hours. By carrying out the additional identification, suspected colonies (translucent, dark green opaque colonies with a darker center and 0.5–3.0 mm diameter) should be verified as presumed *Aeromonas* species. To identify *Aeromonas* species, tests for oxidase, catalase, H2s production, citrate, indole production, Voges-Proskauer, motility, and methyl red were performed. Biochemical identification was based on standard techniques (Fawole and Oso, 2004). All the media and reagents for biochemical tests were prepared, according to the instructions of the manufacturers.

Detection of *Aeromonas hydrophila* and its virulence gene by PCR

Following the manufacturer's instructions, DNA was extracted from five probable isolates in order to use the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) to detect the *16S rRNA* of *Aeromonas hydrophila* and the *aerolysin* virulence gene. 200 µl of the sample suspension, 200 µl of lysis buffer, and 10 µl of proteinase K were incubated for ten minutes at 56°C. Following that, 200 µl of 100% ethanol was added to the lysate. The manufacturer's recommendations were followed when rinsing and centrifuging the sample. Elution buffer (100 µl) included in the kit was used to elute the nucleic acid. The primer sets were purchased from Metabion in Germany. The cycling parameters are shown in Table 1. The results of the PCR were separated by electrophoresis using gradients of 5V/cm on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature. Twenty microliters of the PCR products were put into each gel slot for the gel inspection. The Gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was utilized to calculate the fragment sizes. A gel documentation system (Alpha Innotech, Biometra) was used to take the gel photo. Computer software was used to analyze the data.

Table 1: Primer sequences, target genes, and amplicon sizes of *Aeromonas hydrophila*.

Bacteria	Gene	Sequence	Amplified product	Reference
Aeromonas hydrophila	*16S rRNA*	GAAAGGTTGATGCCTAATACGTA CGTGCTGGCAACAAAGGACAG	685 bp	**Gordon *et al.* (2007)**
	Aerolysin	CACAGCCAATATGTCGGTGAAG GTCACCTTCTCGCTCAGGC	326 bp	**Singh *et al.* (2008)**

Preparation of *Aeromonas hydrophila* inoculum (bacterial suspension):
After being freshly grown on *aeromonas* agar medium at 37°c for 18-24 hours, *A. hydrophila* colonies were combined with sterile physiological saline and adjusted to (6 x 10^6 CFU/ml) using McFarland's standards (McFarland, 1907). In the experiment, 0.5 ml of the prepared inoculum was administered intraperitoneally to the fish (Emeish *et al*., 2018). Care was used when administering injections to prevent internal organ punctures. All of the injected fish were subsequently moved to aquariums.

Blood Samples
In the first week following infection, three aseptic samples were taken from the caudal vein of each group. The initial blood sample for hematological analysis was drawn on EDTA (1 ml). A sterile tube containing heparin was used to collect the second blood sample (2 ml). (50 IU/ml) for phagocytic activity analysis. The third blood sample (3 ml) was drawn into a clean, dry centrifuge tube without anticoagulant. It was then allowed to clot at room temperature and rotated for 10 minutes at 3000 rpm. For biochemical analysis, serum was gathered, tagged, put in dry, clean tubes with caps, and frozen at -20°C.

Hematological studies:
Red blood corpuscles (RBCs), concentration of hemoglobin (Hb) and total leukocytic counts were determined according to the hematological procedures routine described by (Feldman *et al*., 2000).

Phagocytic activity and phagocytic index:

A. Peripheral blood mononuclear cells separation:
The method described by (Goddeeris *et al*., 1986) was used to isolate peripheral blood mononuclear cells (PBMC).

B. Phagocytic Assay:
To test cell phagocytic activity, we placed 0.25 ml of heat-inactivated *C. albicans* in plastic tubes, followed by 0.25 ml of adjusted viable leukocyte solution on top. For 30 minutes, the tubes were incubated in a humidified CO2 incubator at 37°C. After 5 minutes at 2500 rpm, using a Pasteur pipette, the supernatant was taken out of the tubes, leaving a drop in which the sediment was re-suspended. The deposit was spread out, allowed to dry in the air, and then stained with Leishman's stain.

C. Evaluation of phagocytic activity:
A light microscope with an oil immersion lens was used to count hundreds of phagocytic cells at random across ten microscopic areas. The quantity of yeast cells eaten by each phagocyte was counted in order to ascertain the phagocytic cell activity in each of the tested groups. By using a microscope field, the percentage of phagocytic cells is used to compute the phagocytic activity. The average amount of *Candida albicans* eaten by a single phagocytic cell is known as the phagocytic index.

Biochemical studies:
Each biochemical parameter was measured using commercial kits, and the manufacturer's instructions were followed for each parameter's technique. The activity of the liver transferases, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), was calculated using (Murray, 1984). According to Kaplan (1984), serum urea was measured, and serum creatinine was approximated using Henry (1974). The total protein in the serum was measured in accordance with (Tietz, 1995). The serum albumin level was determined using (Domas, 1971). Serum globulin was estimated by subtracting albumin level from the total protein level described by (Doumas and Biggs, 1972). Catalase (CAT) activity is measured in accordance with (Aebi, 1984). Serum lysozyme activity was determined according to (Demers and Bayne 1997). Tumor necrosis factor alpha (TNF-α) was estimated according to (Wallach, 2001).

Aeromonas count

Aseptic dissection of the liver, kidney, and intestine was performed during the first week after infection, and the samples were then placed into individual, sterile plastic Petri dishes. To create a stock solution, the samples were weighed and homogenized, before being suspended in sterile physiological saline (1 part sample: 9 parts PS). From the stock solution, three successive decimal dilutions were prepared. *Aeromonas* agar plates were used for the cultivation of various dilutions. For 48 hours, all plates were incubated at 25 °C. The number of expanding colonies was determined and utilized to evaluate the impact of the probiotic therapy (ISO, 2004).

Statistical analysis:

The statistical analysis employed the analysis of variance (ANOVA). At a significant threshold of 0.05, Duncan's Multiple Range was employed to identify changes in the treatment groups. The SPSS application was used on a PC to run all statistics (SPSS, 2004).

RESULTS

Growth performance

Table 2 shows the impact of a 60-day *B. subtilis* addition in the diet on the growth performances of catfish. The *Bacillus* groups (Gr3 & Gr4) showed a significant increase in final weight, weight gain, SGR % and condition factor compared with control groups (Gr1 &Gr2).

Table 2: The effect of *Bacillus subtilis* on growth performance of catfish before infection (mean ± SE).

Parameters	Groups			
	Gr1	Gr2	Gr3	Gr4
Initial weight (g)	155.80±1.90	156.00±1.87	155.82±1.90	158.60±2.11
Final weight (g)	287±8.88^{b}	298±3.74^{b}	384±9.27^{a}	386±9.27a
Weight gain (g)	131.20±8.48^{b}	141.00±3.67^{b}	228.20±8.20^{a}	227.40±8.61^{a}
SGR %	1.01±0.04^{b}	1.06±0.02^{b}	1.49±0.03^{a}	1.47±0.03^{a}
Condition factor	1.67±0.10^{b}	1.74±0.08^{ab}	1.99±0.08^{a}	2.01±0.10^{a}

There was a significant difference at $p<0.05$ when different letters appeared in the same rows. n=10

Water parameters

The effects *of B. subtilis* on the concentrations of ammonia (NH3) and nitrite (NO2) in fish water were represented in Table 3. Both NH3 and NO2 showed non-significant changes between groups at the beginning of the experiment. At the end of the experiment, after 60 days, both NH3 and NO2 were significantly lower in *bacillus* groups compared with control groups.

Table 3: The effect of *Bacillus subtilis* on the concentration of NH3 and NO2 in fish water (mean ± SE).

Parameters	At the beginning of the experiment			At the end of the experiment		
	Control groups	*Bacillus* groups	Sig.	Control groups	*Bacillus* groups	Sig.
NH3 (mg/l)	0.96±0.031	0.98±0.006	0.512	1.70±0.08	0.80±0.06	0.001
NO2(mg/l)	0.087±0.008	0.086±0.008	0.979	0.89±0.009	0.05±0.003	0.017

A statistically significant difference was defined as a probability value (P) of less than 0.05.

Isolation and identification of *Aeromonas hydrophila*

Bacteriological examination of the collected samples, based on their colony morphology, and biochemical characterization, revealed recovery of 20 *A. hydrophila* out of 40 examined catfish, with a percentage of 50%. Each isolate was from a different fish, regardless of the number of examined organs, and the recovered isolates had grown on Aeromonas agar media, producing green colonies with dark centers. Motile gram-negative bacilli which were positive for oxidase, catalase, H2s production, citrate, indole production, Voges-Proskauer and methyl red negative were considered *Aeromonas hydrophila.*

PCR Detection of *Aeromonas hydrophila* and aerolysin virulence factor

PCR was applied to five randomly selected *A. hydrophila* isolates for the detection of the ***16S***rRNA gene and results showed that this gene was detected in 3 of 5 examined isolates and gave a characteristic band at 685 bp, as shown in Fig (I). Based on the presence of the *aerolysin* virulence gene in *Aeromonas hydrophila* strains, all three isolates under investigation carried the *aerolysin* gene (*aer*A) at the expected product size of 326 bp.

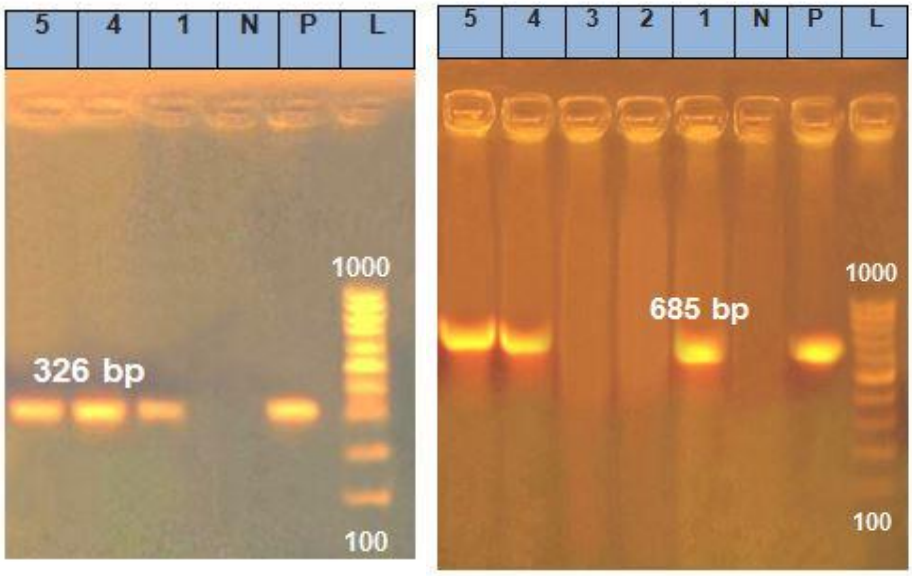

Fig. (I): PCR was applied to five randomly selected *A. hydrophila* isolates for the detection of *the 16S*rRNA gene and results showed its detection in 3 of 5 examined isolates and gave a characteristic band at 685 bp, while the aerolysin gene was detected in the 3 examined isolates at the expected product size 326 bp with a percentage of 100%.

Hematological and immune response

Table 4 showed that the highest erythrocyte, hemoglobin and PCV values were obtained from addition *bacillus* (Gr3). However, RBCs, Hb, and PCV were significantly reduced in (Gr2) after the pathogen challenge compared to the unchallenged group. A leucogram revealed that (Gr2), (Gr3) and (Gr4) catfish had mild leucocytosis, neutrophilia, lymphocytopenia, and monocytosis, when compared to (Gr1) catfish. The immune parameters of catfish showed a significant improvement in Phagocytic% and Phagocytic index. The highest value was found in catfish treated with Bacillus (Gr3). The previously reported immunological parameters were considerably reduced in (Gr2) after the *A. hydrophila* challenge compared to other groups.

Table 4: The effect of *Bacillus subtilis* on Erythrogram, leukogram and phagocytic activity of clinically healthy and infected catfish with *Aeromonas hydrophila* (mean ± SE).

Parameters	Gr1	Gr2	Gr3	Gr4
RBCsx10^6 /µl	2.85±0.15^b	2.12±0.05^c	3.20±0.0.7^a	2.70±0.10^b
Hb gm/dl	10.06±0.20 ^{b}	7.50±0.23 ^{d}	11.10±0.27 ^{a}	8.90±0.25^c
PCV %	38.80±0.92^b	32.00±0.68^c	41.00±0.70^a	37.30±0.66^b
TLCx10^3 /µl	26.90±1.07^b	30.22±1.15^a	26.75±1.22^b	28.50±1.08^a
Neutrophilsx10^3/µl	5.35±0.26^c	8.73±0.25^a	5.25±0.45^c	7.20±0.36^b
Lymphocytesx10^3/µl	19.20±0.25^a	17.80±00.35^b	19.00±0.40^a	17.75±0.62^b
Monocytesx10^3 /µl	1.60±0.14^c	3.00±0.15^a	1.82±0.09^b	2.90±0.08^a
Eosinophilsx10^3 /µl	0.65±0.01	0.69±0.02	0.68±0.02	0.65±0.03
Phagocytic%	80.62±1.15^b	75.60±2.40^c	83.65±1.60^a	81.00±1.10^b
Phagocytic index	1.69±0.04^b	1.55±0.03^c	1.93±0.05^a	1.75±0.09^b

There was a significant difference at $p<0.05$ when different letters appeared in the same rows. n=10

Biochemical parameters

The biochemical parameters of catfish that are both infected with *Aeromonas hydrophila* and clinically healthy are summarized in Table (5). The infected group (Gr2) revealed notable elevation of AST, ALT, urea, creatinine and TNF-α, in addition to a significant decrease in catalase enzyme, total protein, albumin, globulin and lysozyme activity compared with the control group (Gr1). While Gr4 (*B. subtilis* supplemented then infected) showed significant improvement in all mentioned parameters compared with Gr2. Also, Gr3 (*B. subtilis* supplemented non infected) showed a significant decrease in AST and creatinine, as well as a significant increase in catalase enzyme, total protein, albumin, globulin and lysozyme activity compared with the control group (Gr1).

Table 5: The effect of *Bacillus subtilis* on some biochemical parameters of clinically healthy and infected catfish with *Aeromonas hydrophila* (mean ± SE).

Parameters	Gr1	Gr2	Gr3	Gr4
AST(IU/L)	99.91±0.47^c	124.48±0.96^a	90.97±1.15^d	102.90±0.86^b
ALT(IU/L)	14.06±0.49^c	25.38±0.63^a	13.59±0.52^c	16.61±0.28^b
Urea(mg/dl)	1.28±0.03^{bc}	1.84±0.08^a	1.19±0.02^c	1.37±0.02^b
Creatinine(mg/dl)	0.24±0.004^c	0.51±0.018^a	0.18±0.004^d	0.31±0.012^b
Catalase(ng/ml)	4.65±0.16^b	2.67±0.16^d	5.45±0.44^a	3.82±0.10^c
Total protein(g/dL)	2.86±0.16^b	1.96±0.06^c	3.81±0.11^a	3.08±0.015^b
Albumin(g/dL)	1.51±0.03^b	1.27±0.04^c	1.76±0.09^a	1.48±0.03^b
globulin(g/dL)	1.34±0.17^b	0.68±0.03^c	2.06±0.03^a	1.59±0.13^b
Lysozyme(ng/ml)	4.40±0.17^b	2.58±0.09^c	6.45±0.53^a	4.38±0.21^b
TNF-α (mmol L^{-1})	38.00±0.31^c	54.00±0.45^a	37.00±0.40^c	45.00±0.35^b

There was a significant difference at $p<0.05$ when different letters appeared in the same rows. n=10

Clinical and Post-mortem Examination of Experimentally Infected Fish

Extensively distributed haemorrhagic skin ulcers and severe hyperaemic patches all over the fish body, especially at the base of

fins, and tail and skin ulceration were observed in Gr2, also abdominal distention, liver paleness, enlargement in some fishes and congestion of spleen with hemorrhagic enteritis were observed with high mortality. while the effect of *B. subtilis* supplementation in Gr4 was obvious without neither observed lesions nor mortalities similar to the control group.

***Aeromonas hydrophila* count:**

Aeromonas hydrophila enumeration in the first week after experimental infection revealed that Gr1 (control negative) showed a count ($1x10^2$ CFU/ml) while Gr2 (infected without *Bacillus* supplementation) showed a very high count, ranging from $4x10^7$ to $4x10^9$ CFU/ml. regarding Gr3 (non-infected with *Bacillus* supplementation) had no detectable *A. hydrophila* count, while Gr4 (infected with Bacillus supplementation) showed a low count ranging from $2x10^3$ to $2x10^5$ CFU/ml.

DISCUSSION

Recently, *Bacillus species* have gained a lot of interest because of their ability to enhance the health and growth of aquacultured animals. can also alter the host microbiome, enhancing their state of health in the process (Olmos *et al*., 2022). The supplementation of *Bacillus subtilis* in the diet can improve the final weight, weight gain, specific growth rate and condition factor of catfish. A similar result was obtained by (Cao *et al*., 2022) for *Penaeus vannamei* supplemented with 0.5% *Bacillus subtilis* in diet. Mohammadi *et al*. (2020) revealed that Nile tilapia fish fed on probiotics (Bacillus subtilis) had noticeably improved growth performances over the control group. Bacillus probiotics have been shown to have similar positive benefits on tilapia growth performance (Elsabagh *et al*., 2018). The improved growth performance of fish supplemented with probiotic diets might be due to improved histology of the intestines and enzyme activity (Won *et al*., 2020). Liu *et al*. (2017) revealed that supplementing *Nile tilapia* with *B. subtilis* could increase their activity levels of digestive enzymes and hence boost their growth performance (Wang, 2007).

The addition of *Bacillus licheniformis* to the diet can improve the health and growth performance of tilapia (Yaqub *et al.*, 2022). *B. subtilis* enhanced the growth performance, health, and gut microbiota of *Totoaba Macdonaldi* (Olmos *et al*., 2022). Enhancing the activity of digestive enzymes with probiotic treatment may facilitate better food digestion and absorption, which in turn may enhance growth performance and feed utilization rate (Jjx *et al*., 2019). Probiotics in the diet can alter the gut flora of fish and imitate digestive processes that are advantageous for effectively utilizing feed macromolecules (Amir *et al.*, 2019). Based on reliable data, probiotics are being used as nutritional supplements in aquaculture as a sustainable and environmentally friendly way to improve fish health and growth. A few Bacillus species show promise in aquaculture applications. (Monica and Jayaraj, 2021). The physiologically active compounds produced by probiotic bacteria boost the defense mechanism, improve feed conversion, and accelerate the fish growth rate. (Gatesoupe, 2010).

Increased loads of organic debris and the buildup of nitrogenous compounds and organic wastes like nitrite and ammonia are linked to aquaculture. These wastes can accumulate and become poisonous to farmed fish, causing stress and possibly death (Loh, 2017). Probiotic *Bacillus* is a recent development in aquaculture operations that promotes water quality. (Kuebutornye *et al*., 2019; Soltani *et al*., 2019). The NH3 and NO2 were significantly lower in the Bacillus groups. Similar results were obtained by Elsabagh *et al*. (2018) and Mohammadi *et al*., (2020), who investigated the impact of feeding commercial probiotics derived from *Bacillus* to *Oreochromis niloticus*. The impact of various *Bacillus* strains obtained from *Cyprinus carpio* on enhancing water quality in ornamental fish production was

investigated by Lalloo *et al.* (2007). According to their findings, three of the nine isolates caused the concentrations of phosphate, nitrate, and ammonia to drop at rates of 74%, 76%, and 72%, respectively. According to Martínez-Cruzova *et al.* (2015), probiotics and other microorganisms use various types of nitrogen, such as total ammonia nitrogen (TAN), N-NO3, N-NO2, and total Kjeldahl nitrogen (TKN), for their metabolism. This helps remove nitrogen from the water cycle. Therefore, different types of nitrogen in aquaculture wastewater can be eliminated by *Bacillus species* (Hlordzi, 2020).

According to (Rashad *et al*., 2017), *A. hydrophilla* is one of the bacterial species that is frequently detected in cultured organisms. Fish breeders may suffer significant losses and numerous damages due to the opportunistic bacterium *A. hydrophila* (Moori Bakhtiari *et al*., 2017). Fish farming ponds and various organs may sustain damage from it. Therefore, fish farmers should use suitable health management procedures to prevent fish disease. According to (Praveen *et al*., 2016), diagnosis has the power to stop threats and manage *Aeromonas* disease outbreaks. The prevalence rates of *A. hydrophila* in catfish were previously reported as 55% by Emeish *et al*., (2018). According to El-ghareeb *et al*., (2019), out of 75 *Mugil cephalus* samples, 38 isolates of *Aeromonas* strains were found, accounting for 50.67% of the total. Their findings were supported by the results that were obtained. Whereas 50 samples of *Mugil cephalus* were obtained from different fish markets within the governorate of Kafr El-sheikh, the results of this analysis were not as compelling as those of (Ebeed *et al.*, 2017). The researchers discovered that 62% of the samples carried *Aeromonas* species. According to (Hafez *et al*., 2018), the different species, sample location and time, geographic range, and post-capture contamination can all be factors in the changes in *Aeromonas* species incidence. This result contradicted those reported by (Rahayu Kusdarwati *et al*., 2017), who stated that the percentage of catfish infected with *Aeromonas hydrophila* was 95%. The isolation and identification results illustrated that *Aeromonas hydrophila* percent was high, this result agreed with some authors (Daood, 2012).

A. hydrophila isolates were found to have similar results in several publications. despite some differences in their biochemical properties, because they were isolated from various organs of freshwater fish (Sahu *et al*., 2013). They were also found to be positive for Voges Proskauer and ornithine decarboxylase but negative for the DNase test (Jayavignesh *et al*., 2011).

According to Venkataiah *et al*., (2013), *Aeromonas hydrophila* can release a range of virulence factors linked to enterotoxic, cytotoxic, and hemolytic activities that cause adhesion and colonization of mucosa. These events, when followed by fluid accumulation or epithelial change, are likely to result in human disease. The isolation of 16S rRNA can confirm the presence of *Aeromonas hydrophila* (Daskalov, 2006). Three of the five isolates under examination had 16S rRNA found by PCR, representing a 60% detection rate. These results imply that *Aeromonas hydrophila* can be identified by using 16S rRNA as a specific target.

Hematological indices are strong indications of fish health, and an increase in RBC, Hb, and Hct speeds up tissue oxygenation and carbon dioxide removal. (Abdel-Tawwab *et al*., 2006). Our studies revealed that the erythrocytic count, Hb concentration, and packed cell volume of infected non-treated fish had significantly decreased. This could be attributed to bacterial toxins that obstruct normal erythrocyte development (Sutili *et al*., 2014). The current findings were in line with prior studies by Ahmed (2000) and Amer *et al.* (2009), which found that *Clarias lazera* infected with *A. hydrophila* had significantly lower haemoglobin concent-ration, packed cell volume, and erythrocytic

count. Our results showed that the addition of Bacillus produced the highest erythrocyte, hemoglobin, and PCV values, which were consistent with previous findings. Zhao *et al*., (2019) discovered that tilapia fed the probiotic B. subtilis LT3-1 had a higher hematocrit value than controls. Reda *et al*., (2018) when compared to the control group, all probiotic-supplemented groups had higher hemoglobin content, platelet counts, MHC, and MCHC. The immune-modulatory impact of *B. subtilis* on liver cells boosts the anabolic capacity of hepatocytes to create blood proteins, as demonstrated by the considerable improvement in liver function tests. The hepatic enzymes study results, which revealed a drop in *O. niloticus* fed on probiotics compared to the control group, further corroborated this and suggested that the inhabitant's maintenance was normal, positive, and beneficial. Numerous writers concurred with these conclusions (Safinaz, 2006).

One of the fish's non-specific defense mechanisms was leukocytes (Uribe *et al*., 2011). The leukocytes were one of the fish's non-specific defense systems (Tanbiyaskur *et al*., 2015). In untreated infected fish, there was leukocytosis, neutrophilia, lymphocyte-penia, and monocytosis. Following pathogen challenge, neutrophilia, monocytosis, and lymphopenia may indicate either a bacterial infection attacked and taken up by neutrophils and monocytes or a stress response to the infection (Mahmoud *et al*., 2007). In our study, *B. subtilis*-supplemented groups had significantly higher leukocyte counts after *A. hydrophila* infection. Our findings are consistent with those of Reda and Selim (2015), who discovered that supplementing Nile tilapia with *B. amyloliquefaciens* enhanced their leukocyte count. Using *L. plantarum* as a probiotic enhanced the number of leukocytes in *juvenile Siberian sturgeon*, according to a study by Pourgholam *et al.* (2017).

Phagocytes are frequently used in the evaluation of defense against specific pathogenic illnesses (Giri *et al.,* 2012). The first step of the cellular immune system following a pathogenic infection is phagocytosis, which is carried out by monocytes and granulocytes (Tamamdusturi *et al.,* 2016). Fish that receive probiotic supplements have stronger cellular immune systems and are more resistant to harmful illnesses (Djauhari *et al.,* 2016). After the challenge test, African catfish with *Bacillus* NP5 showed a sharp rise in phagocyte index. Our findings were similar to Doan *et al. (*2015) on *A. hydrophila*-infected *Pangasius* catfish and (Zhao *et al.,* 2019), who gave probiotic *B. pumilus* to gigantic freshwater prawns.

Serum creatinine, urea, and alanine aminotransferase (ALT) are thought to be vital parameters for assessing novel feed additives and unconventional feedstuffs at the proper time of addition (Al-Hisnawi and Beiwi, 2021). In the present study, the infected non-treated group showed a significant increase in transaminase activities, urea and creatinine. similar results obtained by Amer *et al*. (2009) and El Alem *et al*. (2017). The groups supplemented with *B. subtilis* revealed improvement in transaminase activities and kidney function. Likely, the addition of probiotics reduced the activity of transaminase in *Penaeus vannamei* (Cao et al., 2022). Ghaly *et al*. (2023) suggested that probiotics decrease AST, ALT, urea and creatinine. Also, Sayed *et al*. (2011) revealed that as compared to the control group, all treatment groups of (Lin Fingerlings) Nile tilapia exhibited a substantial lower creatinine, urea, AST and ALT. On the other hand, when *B. subtilis* was added to common carp feed for six weeks, there was no effect on blood urea and serum creatinine (kidney function tests) (Al-Hisnawi and Beiwi, 2021).

Significant improvement in total protein, albumin and globulin in *B. subtilis* supplemented groups. These results agree with Elsabagh *et al*. (2018) and Mohammadi *et al*. (2020). According to Asadi *et al.*

(2012), high blood protein levels, especially globulin, are associated with a successful fish immune response and are considered a critical indicator of fish health. The amount of total protein in the body increased greatly when probiotics were used (Chelladurai *et al*., 2013). Serum and mucus protein levels in *Catla catla* and *Labeo rohita* treated with various probiotic strains, including *B. subtilis* and B. *amyloliquifaciens*, significantly raised (Sutthi and Doan, 2020). The improvement noticed in biochemical parameters may be due to the antibacterial effect of *B. subtilis* (Krishnan, 2014) who discovered that Bacillus species produced a chemical resembling bacteriocin, which had a probiotic action in the laboratory against *A. hydrophila* and *V. harveyi*. Based on our findings, a *Bacillus subtilis*-supplemented diet improved the activity of the catalase enzyme. The function of defense enzymes against antioxidants catalase was employed as a useful oxidative stress indicator. Catalase is primarily involved in the breakdown of hydrogen peroxide, which is produced in fish cells as a result of oxidases action. It also acts as a barrier against hydrogen peroxide, which has the ability to damage cellular structures. *Bacillus subtilis* and *Bacillus licheniformis* supplemented diet can improve the catalase enzyme in blood serum of catfish (Romanova *et al*., 2020)

By interacting with different types of immune cells, probiotics can increase immunological function in fish (Gobi *et al.,* 2018). High levels of lysozyme activity in Tilapia were linked to dietary probiotics (Bacillus) and an enhanced immunological response (Yaqub *et al*., 2022). Some previous studies have also suggested an increase in lysozyme activity by the application of this probiotic in fish (Mohammadi *et al*., 2020 and Ghaly *et al*., 2023).

TNF-α, a pleiotropic cytokine, is associated with inflammation, apoptosis, cell proliferation, and strong pleiotropic stimulation of the immune system. It is a member of the TNFs superfamily. (Goetz *et al*., 2004). Pro-inflammatory cytokines TNF-α can increase the permeability of the intestinal epithelium, which could aggravate inflammation (Al-Sadi *et al*., 2008). After pathogen infection, TNF-*α* can interact with bacteria and parasites through a domain containing lectin-like ability for N, N'-diacetyl chitobiose (Wang *et al.,* 2012). Fiocchi (2006) discovered that probiotics can prevent the activation of NF-κB and the regulation of extracellular signaling kinase, which promotes the inflammatory pathway, and so lower the generation of TNF-α and IL-8 by blocking the release of a range of pro-inflammatory cytokines. Won *et al.* (2020) demonstrated that, in comparison to the control group in *Oreochromis niloticus*, the probiotic-supplemented groups showed improvements in pro-inflammatory cytokines.

The undetectable mortalities attained in the probiotic treatment are consistent with the findings of (Villamil *et al*., 2014), who observed that Nile tilapia performed better using *B. subtilis* probiotic with *A. hydrophila* challenge than in the control group.

It was demonstrated through experimental infection that *A. hydrophila* was extremely harmful to fish, which could result in a significant loss of commercial production (Hasan, 2007). Group (Gr2) had a high bacterial load, whereas Gr4 had a low count. Santos *et al*. (2018) and Olmos *et al*. (2020) found similar results, indicating that adding *Bacillus spp* as a probiotic supplement prevents pathogens like *Vibrio* and *Aeromonas hydrophila* in the aquaculture industry. The ability to reisolate the bacteria *A. hydrophila* from the kidney, liver, and intestine of the experimentally infected fish demonstrated how effectively the pathogen was able to propagate throughout the fish's organs. The findings published earlier by (Mona *et al*., 2015) corroborated this outcome. After isolating *A. hydrophila* from the impacted shing, Hasan (2007) discovered that the load ranged from $1.67X10^4$ to

6.46x10^8 CFU/g. *Aeromonas hydrophila* was isolated from *Thai pangus* (Alam, 2009), and the bacterial load was discovered to be between 4.8 and 7.2 × 10^7 CFU. *Aeromonas hydrophila* was isolated by (Mostofa *et al*., 2008) from *Heteropneustes fossilis*. The highest bacterial load was found in the liver, 2.4 × 10^7 CFU/g, and the lowest in the kidney, 2.1 × 10^2 CFU/g.

CONCLUSION

The diet supplemented with *Bacillus subtilis* for 2 months can improve growth rate and water quality in addition to improving the health status of fish through its impact on hematological and biochemical profile. Also, enhance the immune response of fish against pathogenic bacteria, such as *Aeromonas hydrophila*.

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تأثير البسيلس ستيلس علي معدل النمو والاستجابة المناعية في أسماك القرموط

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صممت هذه الدراسة لتقييم التأثير الغذائي للمكملات الغذائية لبكتيريا *Bacillus subtilis* على أداء النمو والاستجابة المناعية في أسماك القرموط (*Clarias gariepinus*). تم تنفيذ تجربة التغذية لمدة شهرين، حيث تم تغذية المجموعات الضابطه (الاولى والثانية) علي عليقة متوازنة بدون اضافات و مجموعتين من الأسماك تم تغذيتها على عليقة مكملة بـ *Bacillus subtilis* (المجموعه الثالثة والرابعة).

تم تقييم معاملات النمو ومعايير الماء (NO2 & NH3) و أظهرت مجموعات (المجموعه الثالثة والرابعة) زيادة معنوية في الوزن النهائي وزيادة الوزن ونسبة النمو وعامل الحالة SGR مقارنة بالمجموعتين الضابطين (الاولى والثانية). ومع ذلك، كان NH3 وNO2 أقل بشكل ملحوظ في مجموعات محفزات النمو مقارنة بمجموعات الضابطه في نهاية التجربة. بعد ذلك تم عمل عدوي للمجموعات (المجموعه الثانية والرابعة) تجريبيا ببكتيريا *Aeromonas hydrophila* و تم تقييم مؤشرات الدم ومصل الدم وعدد *Aeromonas hydrophila*.

تم الحصول على أعلى قيم لكريات الدم الحمراء والهيموجلوبين وPCV من إضافة *Bacillus* في المجموعه الثالثة. وكانت كرات الدم الحمراء والهيموجلوبين و PCV أقل بشكل ملحوظ، بعد العدوي بميكروب *Aeromonas hydrophila* في المجموعة الثانية مقارنة بالمجموعة الضابطة. أظهر العد الكلي والنوعي لكريات البيضاء زيادة في عدد الكريات البيضاء، الخلايا المتعادلة الصبغة والخلايا أحادية النواة بينما وجد نقص في الخلايا الليمفاوية في سمك القرموط (المجموعات الثانية، الثالثة والرابعة) مقارنة بتلك الموجودة في سمك القرموط (المجموعة الأولي). أظهرت نسبة الخلايا البلعمة ومؤشر البلعمة تحسنا معنويا في (المجموعه الثالثة) وانخفاضا ملحوظا في (المجموعه الثانية) مقارنة مع المجموعات الأخرى.

وأظهرت المجموعة المصابة (المجموعه الثانية) ارتفاعا معنويا في نشاط AST وALT واليوريا والكرياتينين بالإضافة إلى انخفاض معنوي في نشاط أنزيم الكاتلاز والبروتين الكلي والألبومين والجلوبيولين والليزوزيم مقارنة مع المجموعة الضابطة (المجموعه الأولي). في حين أظهرت (المجموعه الرابعة) تحسنا معنويا في جميع المعايير المذكورة مقارنة مع (المجموعه الثانية). فيما يتعلق بعدد الـ *A. hydrophila* بعد الإصابة التجريبية، وجد أن العد البكتيري مرتفع في المجموعة (المجموعه الثانية) بينما لوحظ انخفاض العدد في المجموعة الرابعة. أظهرت نتائج الدراسة الحالية دوراً محتملاً لمكملات *Bacillus subtilis* في النظام الغذائي و تعزيز أداء النمو، والحالة الصحية لسمك القرموط.