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Characterization of probiotic *Bacillus subtilis* isolated from Nile tilapia (*Oreochromis niloticus*) digestive tract and evaluation its positive impact on health and nonspecific immunity of Nile tilapia

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Abstract

In the present study, a probiotic, *Bacillus subtilis* was isolated and identified from the digestive tract of freshwater Nile tilapia collected from production ponds of the Central Laboratory of Aquaculture Research, Egypt. The isolated strain of *Bacillus subtilis* was biochemically identified using API 20E and 16S rRNA. The biosafety assay confirmed that the isolate was not pathogenic to Nile tilapia fish. Isolated strain was able to survive at increasing concentrations of bile. The isolated strain was sensitive to most used antibiotics. The Isolated strain showed antagonistic activity against bacterial fish pathogens, including *Aeromonas hydrophila* and *Pseudomonas aeruginosa*. In addition, the isolated strain did not show any hemolytic activity against human blood in the blood agar medium. To evaluate the positive impact of the supplementation of an isolated strain of *Bacillus subtilis* on hematological, biochemical and immunological parameter of Nile tilapia. A total of 120 fish (20 g) were randomly divided into 2 groups (control and treatment with 3 replicates). The control groups were fed the basal diet. Treatment groups were supplemented with *B. subtilis* $1X10^9$ cfu/g. Results showed that Nile tilapia fed with diets containing *B.subtilis* for 7 weeks had a significant increase in Hb%, RBCs count, HCT values and WBCs count. Also, there is a significant improvement in liver function test (ALT, AST, Total protein and Albumin). Also, there is a significant increase in immunological parameter (NBT, Lysozyme). Moreover, fish fed with *B.subtilis* supplemented diets showed improved survival rates after *A. hydrophila* infection.

Keyword: *Bacillus subtilis* , Nile tilapia , API 20E, Positive impact.

INTRODUCTION

The rapid development of aquaculture from last years has increased feed ingredients demand and its prices, for this reason feeding represent 40-60% of the total production. Probiotics are live microorganisms able to establish, multiply and colonize the intestine of the host in order to promote a beneficial balance of microorganisms. These benefits are explain due to these microorganisms improve the digestibility, absorption of nutrients and inhibit the proliferation of harmful agents in the intestinal mucosa, **(Nayak, 2010),** promote the synthesis of vitamins **(Lee *et al*., 2013)**, and improve the growth performance of animals **(Mohapatra, *et al*., 2013)** by increasing the survival percentage **(Wu *et al*., 2014)** and water quality **(Chi, *et al*., 2014).** Bacteria of the *Bacillus genus* are among the most widespread microorganisms in nature, they can be found in soil, water and air. Bacillus constitutes a diverse group of rod-shaped, Gram-positive bacteria, characterized by their ability to produce a robust spore **(Sonnenschein, *et al*., 1993).** The intestinal microbe is an important component of mucosal barrier has resulted in the promotion of the use of beneficial probiotic **(Gomes, 2008).** Bacteria from genus *Bacillus* are one of the main probiotics used in aquaculture. These bacteria are ease to cultivate and form spores, which facilitates its conservation (**Nayak, 2010; Han, *et al*., 2015**). In addition, several bacteria of this genus have the capacity to secrete antimicrobial compounds and different exoenzymes that aid digestion (**Ziaei-Nejad, *et al*., 2006)**. Probiotic bacteria could produce digestive enzymes and essential growth nutrients such as amino acids and vitamins, which are benefit for enhancing the best growth, also they could benefit to their invertebrate host by competitive exclusion against pathogens **(Austin, *et al*., 1995; Gomez-Gil, et al., 2000)** or by increasing the host immunity and resistance **(Uma, *et al*., 1999)** which are benefit to achieve the higher survival rate and healthier animals. The use of probiotics in the aquaculture is increasing with demand for more environment friendly aquaculture practices (**Gatesoupe, 1999**). The aim of this study was to isolate probiotic Bacillus subtilis from the Nile tilapia digestive tract, identification of the isolated strain, Biosafety. assay, probiotic prosperities and evaluation the positive impact of isolated stain on Nile tilapia health and immunity.

MATERIAL AND METHODS

Fish sample

Healthy fresh water Nile tilapia samples with an average weight of 100 g were collected from the production pounds of Central laboratory for Aquaculture Research (CLAR) and transported alive to the laboratory of Fish Health and Management Department ,Central Laboratory for Aquaculture Research.

Isolation of gut bacteria

Under complete aseptic conditions, the fish digestive tract was dissected out and homogenized with 5 ml of normal saline. To remove fungal contaminants, the homogenate was kept in a boiling water bath for 20 min at 80 °C. The homogenate was serially diluted and pour plated onto Bacillus agar medium. Plates were incubated for 24 h at 28 °C under aerobic conditions. Individual colonies were picked up and purified by streaking method in a fresh Bacillus agar medium. Bacterial isolates were identified and stored at −80 °C in nutrient broth supplemented with 40% glycerol according **(Priest, *et al*., 1987).**

Biochemical characterization

The isolated bacteria was characterized by standard bacteriological methods according to (**Boone , *et al*., 2005).**

API-20E microbiological identification

Further identification of the isolates was performed by API-20E microbial identification kit (BioMerieux). The sample was prepared by suspending a single colony from a fresh culture into 5 ml of sterile saline solution. Then the suspension was carefully inoculated in all the tube sections according to the manufacturer's instructions. All incubations were done at 28 ◦C.

Molecular identification

DNA extraction. DNA extraction from sample was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer. Primer used was supplied from **Metabion (Germany)** are listed in table (1).

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		
B. subtilis Group 16S rRNA	AAGTCGAGCGG ACAGATGG	595 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Wattiau *et al.*, 2001**
	CCAGTTTCCAAT GACCCTCCCC							
	GGTGATCGATC ACCACCAGC							

PCR amplification. Primer was utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix **(Takara, Japan)**, 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler

Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. Gelpilot 100 bp ladder (Qiagen, Gmbh, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Bio-safety assay

Sixty apparently healthy Nile tilapia of average weight 20 g were obtained from production ponds of CLAR , were divided into two groups with 3 replicates . fish were acclimatized under laboratory condition for 7 days. Experimental fish were injected intraperitoneally with 100 µl of bacterial suspension (approximately 10^9 CFU/ml), whereas the control groups were injected with sterile saline **(Mukherjee & Ghosh, 2016).** All groups were fed at 5% of the body weight per day for 7 days divided into two time daily. All injected fish were subjected for close observation for any abnormal alteration for 7 days post injection. Also fish were sacrificed and examined the disease symptoms (**Sharifuzzaman & Austin, 2009)**

Screening for probiotic properties:-

Bile tolerance

From gall bladder of apparently heath Nile tilapia crude bile was collected, using a syringe and stored at –20 °C until use. To assess the bile tolerance, the 500 µl of the fresh bacterial suspension (10^8 CFU ml^{-1}) of the test culture was added in 10 ml medium supplemented with 2.5, 5.0, 7.5 and 10% (v/v) of bile salts. All bacterial were incubated at 28 ^oC. for 3 and 6 h and survivability of the isolates was monitored by culturing 0.1 ml on the Bacillus agar plate . Growth medium with 0% bile served as control and survivability of the isolate was represented in percentage. **(Nikoskelainen** , *et* ***al*.,** **2001)**.

Haemolytic activity

The isolated strain of *B.subtilis* was subjected to a hemolytic activity, according to **(Argyri, *et al*., 2013).** Bacillus subtilis was streaked on blood agar plates containing 5% (w/v) human blood and incubated for 48 h at 28°C.

Antibiotic sensitivity:-

The sensitivity of isolated probiotic *Bacillus subtilis* to different antibiotics was determined according to . **(Bauer, *et al*, 1966).**

Antimicrobial activity

The antagonistic property of *Bacillus subtilis against* pathogenic strains of *Aeromonas hydrophila* and *pseudomonas aeruginosa* was performed according to **(Ahire, *et al*., 2011)**.

Evaluation of positive impact of the isolated strain of *Bacillus subtilis* on Nile tilapia health and immunity:-

Diet preparation

Bacillus subtilis culture was grown for 48 h at 28°C in Tryptic soya broth. The culture was centrifuged at 4000 g for 10 min at 4°C, was washed three times in 0.9% (w/v) saline, and was prepared a suspension in 0.9% (w/v) saline **(Newaj, *et al*., 2007).** Commercial feed (Aler aqua) was used as the basal diet for the supplementation of probiotic strains. Bacillus subtilis strain was sprayed into the feed to give a final concentration of $1X10^9$ CFU/g according to **(Kuan., *et al* 2010)**Then the feed was oven-dried at 35ºC for 1 - 2 h. 200 g portions of the feed were packed in sealed plastic bags and stored at 4ºC for 2 weeks until used.

Experimental design

The total numbers of 120 apparently healthy Nile tilapia with average body weight of (20±1 g) were obtained from production ponds of

CLAR. Fish were acclimated to the laboratory conditions for one week . In well equipped 6 glass aquaria (80 x 40 x 50cm) filled with dechlorinzed tap water at 25±1^{O}C and continuous aeration using air pumps in the wet laboratory fish were divided into two groups (Control and treatment groups) with 3 replicates. Fish were fed at a rate of 5% from the biomass and about half of the water was changed with chlorine free water and fecal matters were siphoned out once daily during the experiment. Fish were fed twice daily on standard commercially prepared pellets 30% protein (Aler aqua) . Control groups were fed on basal diet and treatment groups were supplemented with *B.subtilis* 1X10^{9} cfu/g for 7 weeks .

Blood sampling

Collection of blood was carried out according to (**Lied , *et al.*, 1975**). For serum separation, peripheral blood was clotted at 4 °C for 4 h. Blood clots were centrifuged at 10,000×g for 10 min and stored at−20 °C for use in determination of biochemical parameter and lysozyme activity. Remaining blood was placed in 0.6 ml plasma separation tubes containing heparin-lithium for use in the determination of hematological parameter and Nitroblue tetrazolium.

Hematological parameters

Hemoglobin concentration was determined using the cyanomet-hemoglobin method according to **(Stoskopf, 1993).** Packed cell volume was estimated by the micro-haematocrite method described by **(Decie & Lewis, 1991)** A manual method for counting RBCs using a hemocytometer counting chamber and Natt-Herrik solution was carried out according to **(Stoskopf, 1993).** WBC count were estimated according to **(Thrall , 2004).**

Biochemical parameters

Alanine aminotransferase activity (ALT) and Aspartate aminotransferase activity (AST) were performed according to (**Reitman & Frankel, 1957).** Assay of total proteins and albumin were carried by a test kit according to biuret method described by (**Weichselbaum , 1946).**

Immunological parameters

Lysozyme assay

Lysozyme activity was measured using spectrophotometer with attachment for turbidity measurement. A series of dilution was prepared by diluting the standard lysozyme from hen egg- white and mixed with

Micrococcus lysodeikticus (ATCC No.4698) suspension for establishing the calibration curve. 50 µl of standard or serum was added in cuvtts to 1.0 ml of *Micrococcus lysodeikticus* suspension . The change in the extinction was measured at 620 nm, by measuring the extinction immediately after adding the solution which contained the Lysozyme (start of the reaction) and after 20 minute incubation of the preparation under investigation at 40 ^{o}C. (end of reaction). The Lysozyme content is determined based on the calibration curve and extinction measured according to **(Schäperclaus, *et al,* 1992).**

Nitroblue tetrazolium (NBT)

After fish bleeding, 100 µL of heparinised blood was added to 100 µL of 0.2% nitroblue tetrazolium solution (NBT, Sigma,) and the final solution was homogenised and incubated for 30 minutes at 25 °C. The NBT solution was prepared in phosphate buffered saline (pH 7.4). After incubation and a second homogenisation, 50 µL from the solution were added to 1 ml of N, N-dimethyl formamide (DMF, Sigma,) in a glass tube. This new solution was homogenised and centrifuged at 3000 g for 5 minutes. The optical density (OD) of supernatant was determined on spectrophotometer at 540 nm. The blank consisted of the same components and steps except blood that was exchanged with distilled water. The values of the extinction here were transposed according to a standard curve into mg of NBT/ 1 ml of blood. Extinction reading X 4 = mg NBT formazan/ 1 ml of blood according to **(Siwicki, 1989).**

Challenge test and estimation of relative percent of survival:-

The fish pathogen, *Aeromonas hydrophila* was previously isolated and biochemically identified from clinically diseased Nile tilapia, in fish health and management department , CLAR. After 7 weeks of feeding , 20 fish/replicate were intraperitoneal injected with 0.1 ml pathogenic *A. hydrophila* (containing 1.5×10^{8} cells/ml). Clinical signs, postmortem lesions and daily mortalities were monitored for 14 days . The reisolation of *A. hydrophila* from the kidney, and intestine of morbid fish confirmed the infection. The average mortality among all replicates was used to calculate the relative percent survival (RPS) (**Amend, 1981**).

Statistics

All data were analyzed using Independent Sample T Test. The results are displayed as mean values with their standard errors (mean ± SE) using the statistical package SPSS 19.0 (IBM SPSS statistics).

Results

Identification of the bacteria isolated from the Nile tilapia gut:-

After 24 h of incubation under aerobic conditions, colonies appeared on the Bacillus agar medium. The biochemical characteristics of isolated probiotic *Bacillus subtilis* were illustrated in table (2), Fig (1,2) in which the isolated bacteria was, gram positive, bacilli, positive for Catalase, Oxidation, Citrate utilization, Gelatin hydrolysis, VP, Nitrate reduction, Gelatinase, and Glucose ,Sucrose, Sorbitol, Rhamnose, Inositol and manitol fermentation. The strain was negative for Arginine dihydrolase, ,Indole production, Tryptophan, Lysine decarboxylase, Ornithine decarboxylase, Urea hydrolysis , Melibiose, Arabinose , Amygdalin and Bata galactosidase .The PCR technique was successfully detected the 16s rRNA *Bacillus subtilis* at gene locus 595 bp(Fig.,3). .

Bio-safety assay:-

In the present study, in vivo biosafety assay, all control and the experimental groups did not show any pathological alteration or mortalities as recorded after 7 days (after I/P injections of probiotic) of observation.

Screening for probiotic properties :-

Bile tolerance

The *B.subtilis* isolate was able to survive at increasing concentration of bile , showed higher survivability of 71, 68, 65 and 55% at 2.5, 5.0, 7.5, and 10% of bile salt, respectively after 3 h of incubation. However, after 6 h incubation the survivability was reduced to 50.0, 47.0, 45.0 and 40%, respectively at increasing concentration of bile salt. As shown in table (3).

Haemolytic activity

The isolated *Bacillus subtilis* strain had no clear transparent or greenish zone surrounding their colonies on the blood agar plates. The B.subtilis had no haemolytic activity against human blood in the blood agar medium as shown in Fig (4).

Antibiotic sensitivity

The isolated study the isolated *Bacillus subtilis* was sensitive to Norfloxacin, Levofloxacin, Streptomycin, Gentamycin, Trimethoprime /sulphamethoxazole, Amoxicillin and Ampicillin /sulbactam on the other hand, it was resistant to Cefuroxime and Flucloxacillin . Table (4) Fig (5).

Antimicrobial activity

The observed results of the antimicrobial effect of the probiotic *B. subtilis* against selected fish pathogen showed that the probiotic *B. subtilis* was efficient in inhibiting the growth of fish pathogen *Aeromonas hydrophila and Pseudomonas aeruginosa.* Fig (6,7).

Evaluation of positive impact of the isolated strain of B.subtilis on Nile tilapia health and immunity:-

Hematological parameter:-

Results of hemoglobin concentration, RBCs count , HCT values, WBCs count were summarized in table (5). It shows that fish fed diet supplemented with B. *subtilis* at concentration of $1X10^9$ cfu/g significantly increased of the examined blood parameters. compared with the control group.

Biochemical parameters.

The results of liver enzymes (AST, AST), total protein and albumin showed significant improvement in fish fed diet containing probiotic

($1X10^9$ CFU/g feed) compared with the control groups. (table ,6).

Immunological parameter

Lysozyme activity:-

As shown in table (7), serum lysozyme of the fish fed diet containing probiotics ($1X10^9$ CFU/g feed) had significantly higher activity than the control groups after 7 weeks of feeding (3.20 + 0.034 μg /mL)

Nitroblue tetrazolium (NBT)

As shown in table (7), NBT activity of the fish fed diet containing probiotics ($1X10^9$ CFU/g feed) had significantly higher activity than the control groups after 7 weeks of feeding (0.644+ 0.06 mg /mL).

Challenge test

After 14 days post-infection of *A. hydrophila*, the obtained results revealed that the mortality rate was 90 % and 60 % in control groups and fish fed diet containing probiotics ($1X10^9$ CFU/g feed) groups respectively and the relative percent of survival was 33.3% (Table ,.8).

Discussion

In the current investigation, the probiotic *Bacillus subtilis* was isolated and characterized from digestive tract of apparently healthy Nile tilapia collected from production ponds of Central Laboratory of

Aquaculture Research, Egypt. *Bacillus subtilis*, a saprophytic Gram-positive, spore forming, rode shape bacteria, is not a animal or human pathogen, nor is it toxigenic like the other members of this genus (**U.S. EPA, 1997**). In addition, this species is known to be non- or low virulent and requires very high bacterial number for causing disease in human **(Mathialagan Kavitha, *et al.*, 2018)**. The isolated strain of *Bacillus subtilis* was gram positive, Catalase positive that showed the ability to ferment Esculin, glucose, dextrose, sucrose, glycerol, salicin and malonate. The present results have been found to be similar to the findings of earlier investigations **(Rajashekhar, et al., 2017 and Lee, *et al.*, 2017).** As such, an extensive research on Bacillus was initiated only 15 years ago (**Hong, *et al*., 2005; Mazza, 1994; Sanders *et al*., 2003)** and consequently a number of Bacillus spp. were evaluated for their efficiency in various livestock production sectors like cattle, poultry, and fishery. The sequence of 16S rRNA was frequently used for bacterial classification at species level (**Alam. *et al* ., 2011)** The genes encoding DNA gyrase beta subunit (gyrB) and RNA polymerase beta subunit (rpoB) were previously used as phylogenetic markers in identification and delineation of the genus Bacillus **(Guo, *et al*. 2012)**. Thus in this study, the bacteria isolated from Nile tilapia digestive tract was exactly confirmed as *B. subtilis* species by phenotypic characterization using API 20E and detection of *Bacillus subtilis* 16S rRNA. Safety evaluation of the selected candidate should be non-pathogenic to the host, which is an important precondition towards consideration of it as probiotic **(Verschuere et al., 2000).** In the present study, biosafety experiment demonstrated that isolated strain of *B. subtilis* isolate was safe to Nile tilapia. (**Banerjee, *et al.*, 2017**) reported that the *B. subtilis* are safe to be as probiotics for aquaculture application.The bile salt tolerance is essential for bacterial colonization and metabolic activity in the small intestine during the host (**Havenaar, et al., 1992**). In the present study *Bacillus subtilis* isolate was able to survive in wide range of bile concentration of 2,5 , 5.0 , 7% and even higher up to 10%, as reported earlier by (**Lee *et al*. 2017).** Hemolytic activity is one of the pathogenic properties of bacteria, as it facilitates infection by microbial entry in to the small lesions in the mucous and skin (**Madigan et al., 1984**). In the present study the isolated strain of Bacillus subtilis did not show any haemolytic activity on blood agar. Similarly, **(Ramesh ,*et al.* 2015)** have confirmed that Bacillus spp., have showed the non-haemolytic activity. **(Mathialagan Kavitha, *et al* ., 2018**) reported that *Bacillus subtilis* strains did not show any haemolytic activity against human blood in the blood agar medium**.**The required properties for a bacterium to be

considered as a potential aquatic probiotic are that it must not only be safe for fish, but also do not harbor acquired and transferable antibiotic resistance **(Vizoso Pinto, *et al.*, 2006).** In addition, antibiotic susceptibility assay showed that the isolate was sensitive to Norfloxacin, Levofloxacin, Streptomycin, Gentamycin, Trimethoprime /sulphamethoxazole, Amoxicillin and Ampicillin /sulbactam. Resistant to Cefuroxime and Flucloxacillin . **Xia Guo *et al* 2016)** founded that isolated stains of B.subtilis were sensitive to vancomycin, gentamicin, erythromycin, kanamycin, tetracycline, and chloramphenicol, but resistant to clindamycin and streptomycin . **(Mathialagan Kavitha, *et al,* 2018)** found that *B. subtilis* found to be susceptible to the antibiotics Viz; kanamycin , ampicillin, erythromycin , amoxicillin, gentamycin , streptomycin, penicillin- G, chloramphenicol, , rifampicin and tetracycline. Antagonism between microorganisms is widespread in nature. Bacillus species was found to be effective to inhibit the growth of Pseudomonas fluorescens, Aspergillus niger, Saccharomyces sp, Fusarium moniliforme, Rhizoctonia sp. and Escherichia coli. **(Xia Guo, *et al.*, 2016)** The inhibitory activities against some tested pathogens demonstrated that B. subtilis could produce antibacterial substances (such as bacteriocin, fengycin, .bacilysin, surfactins, iturin, subtilin, and/or sublancin) as previous studies have shown **(Ansari, *et al.*, 2012) .** The antimicrobial activities of *B. subtilis* might be due to its competition for nutrients with other bacteria **(Moriarty, 1998).** In the present study, antimicrobial activity of the isolated strain *Bacillus subtilis* against bacterial fish pathogen showed that the probiotic *B. subtilis* was efficient in inhibiting the growth of *Aeromonas hydrophila and Pseudomonas aeruginosa***.** Probiotic bacteria have the ability to release chemical substances with bacteriostatic or bactericidal effect on pathogenic bacteria that are in host intestine, thus representing a barrier against the proliferation of opportunistic pathogens. In general, the antibacterial effect is due to one or more of the following factors: antibiotic production, bacteriocins, siderophores, enzymes (lysozymes, proteases) and/or hydrogen peroxide, as well as alteration of the intestinal pH due to the generation of organic acids **(Verschuere, *et al*., 2000).** The parallel use of biological products namely the probiotic is recently the goal of the disease biocontrol strategy in aquaculture as they modify the fish associated microbial community and improve the fish health **(Gibson and Roberfroid, 1995).** Probiotics have a beneficial effect on the digestive processes of aquatic animals because probiotic strains synthesize extracellular enzymes such as proteases, amylases, and lipases as well as provide growth factors such as vitamins, fatty acids, and

aminoacids **(Balc´azar, *et al* 2006) .** The hematological parameters are important assessments for fish nutrition and health. **(Das, et al., 2013).** In the present study , the isolated *B. subtilis* supplementation in Nile tilapia fed displayed an enhancement in hematological parameters at the end of feeding trials than those of the control. These results confirmed the safe harmless beneficial use of B. subtilis as probiotic on Nile tilapia diet. **(Rasha, et al., 2018)** found that all probiotic supplemented groups had higher hemoglobin content, platelet counts, MHC and MCHC compared with the control group. The results indicated a significant improvement of liver function tests which could be attributed to the immune- modulatory effect of *B. subtilis* on the liver cells which activate the anabolic capacity of the hepatocytes to produce blood proteins and this was also supported by the results of hepatic enzymes analysis which decreased in *O. niloticus* fed on probiotics in comparison to control group indicating a normal, positive and beneficial effect on maintenance of the integrity of hepatocytes . These results were supported by several authers **(Jessus , *et al.* 2002); (Nayak, *et al.* 2004) and (Safinaz , 2006).** In the present work, serum lysozyme activity, Nitroblue tetrazolium activity were enhanced in groups supplemented with isolated Bacillus subtilis after 7 weeks compare with Control groups as shown in Table (7)**.** The serum lysozyme is used as an indicator of innate immune response in fish **(Tort, *et al.*, 2003).** The lysozyme is a fish defence element, which causes hydrolysis of the N – acetylmuramic acid and N-acetylglucosamine which are constituent of the pepidoglycan layer of bacterial cell wall **(Ellis, 1999).** In agreement with the present results, **(Addo, *et al* 2016)** found that upon dietary supplementation of tilapia with strain NZ86 for 21 days, plasma lysozyme activity also was significantly greater**.** Plasma lysozyme content was also increased after 51 days of supplementation with both B. subtilis NZ86 and O14VRQ ($p < 0.05$). The result from this time-period indicates the ability of both candidates for long-term effects in immunity, which is similar to the findings of Sun et al. after feeding grouper (Epinephelus coioides) with two different bacilli for 60 days **(Sun ,. *et al* 2010) .** The nitroblue tetrazolium (NBT) assay is mostly used to measure the oxidative radical production by leukocytes in the defence against pathogens **(Cook, *et al*., 2003 and Sahoo,*et al*., 2005).** The NBT reduction is a simple assay widely used to demonstrate the production of superoxide anion. The NBT accepts electrons from a variety of donor substances, such as superoxide, and thus is converted into a reduced form which precipitates as a blue-black insoluble material (formazan) on the cytoplasm of phagocytes. **(Sheng Zhoua, *et al*., 2019)** found that dietary containing *B. subtilis* 7k administration at 10^6, 10^8

and 10^{10} cfu g−1 for 8 weeks significantly increased the respiratory burst activity in Hulong grouper. The present study showed high mortality rate in *A. hydrophila* challenged Nile tilapia that were fed on basal diet (90 %) compared with that were supplemented with Bacillus subtilis (60%) **.**This evidence suggests that the increase of fish resistance to bacterial pathogens is related to the enhancement of antibacterial activity and immunity derived from the bacterial probiotics administered by the fish. Similar results were reported by **(Saputra *, et al.,* 2016)** This may be due to the ability of Bacillus spore to resist gastrointestinal conditions, survive and transit cross gastrointestinal tract, germinate and vegetate with heterologous antigen expression before being excreted (**Duc. *et al.,* 2003).**

Table (2) Biochemical characteristics of isolated *bacillus subtilis* using API 20E.

REACTION	***Bacillus subtilis***
Gram stain& shape	**+ Rod**
Catalase	**+**
O/F test	**Oxidation**
β-galactosidase	**-**
Arginine dihydrolase	**-**
Lysine decarboxylase	**-**
Ornithine decarboxylase	**-**
Citrate utilisation	**+**
H_2S production	**-**
Urea hydrolysis	**-**
VP	**+**
Tryptophan deamination	**-**
Indole production	**-**
Gelatin hydrolysis	**+**
Glucose fermentation	**+**
Mannitol	**+**
Inositol	**+**
Sorbitol	**+**
Rhamnose	**+**
Sucrose	**+**
Melibiose	**-**
Amygdalin	**-**
Arabinose	**-**
Nitrate reduction	**+**

Table (3) Bile tolerance test, showing that the *B.subtilis* isolate was able to survive at increasing concentration of bile.

Antibiotic	Symbol	Conc. (mcg)	Reaction
Levofloxacin	Lev	5	S
Norfloxacin	Nor	10	S
Trimethoprim/sulphamethazole	SXT	25	S
Flucloxacillin	Cx	5	R
Amoxicillin	AX	25	S
Cefuroxime	Ctx	30	R
Ampicillin/sulbactam	SAM	20	S
Gentamycin	CN	10	S
Streptomycin	S	10	S

Table (4) Antibiotic sensitivity of isolated *Bacillus subtilis*

Bile concentration	*B. subtilis* Survivability %	
	3hr	6 hr
2.5%	71	50
5%	68	47
7.5%	65	45
10%	55	40

S:- Sensitive

R:-Resistant

Table (5) Effects of the experimental treatment on the hematological parameters of Nile tilapia after 7 weeks of feeding means + - SE.

Hematological parameter	Control groups	*B. subtilis* treatment groups
Hb%	10.46 ± 0.14	11.26 ± 0.17
RBCs 1X 10^6 /mm3	1.6 ± 0.014	2.0 ± 0.053
HCT %	21.1 ± 0.12	22.3± 0.24
WBCs 1X 10^3 /mm3	50.0 ±0.10	57.2 ± 0.17

Table (6) Effects of the experimental treatments on the biochemical parameters of Nile tilapia after 7 weeks of feeding

Biochemical parameter	Control groups	B. *subtilis* treatment groups
ALT IU/ml	33 ± 2.08	25.0 ±1.0
AST IU/ml	59.3± 1.20	53.3 ± 1.45
Total protein gm/dl	2.47 ± 0.021	2.86 ± 0.026
Albumin gm/dl	1.37 ± 0.011	1.61 ± 0.0008

Table (7) Effects of the experimental treatment on the Immunological parameters of Nile tilapia after 7 weeks of feeding

Immunological parameter	Control groups	B. *subtilis* treatment Groups
Lysozyme (µg /mL)	2.49 ± 0.06	3.20 ± 0.034
NBT (mg/ml)	0.396 ± 0.06	0.644± 0.06

Table (8) challenge test and RPS after 14 days post-infection of *A. hydrophila*.

A.hydrophila	Rout Of injection	% of mortality		RPS %
		Control	Treatment	
0.1 ml /fish (1.5×10^8 cells/ml)	I/P	90%	60%	33.3

RPS= Relative percent of survival

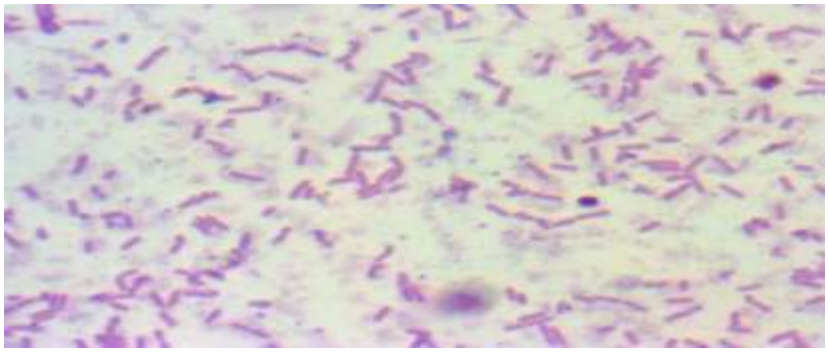
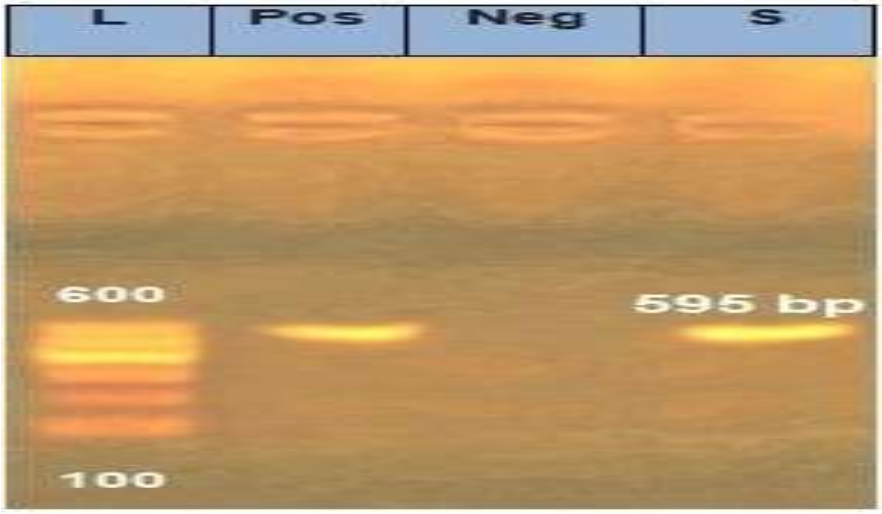


Fig (1) Gram positive rods of *Bacillus subtilis* , under light microscope (X 1000).

Fig (2) Phenotypic characterization of the isolated strain *Bacillus subtilis* using API20E



.

S=Sample, Neg = control negative , Pos = Control positive, L:- = Lan marker

Fig (3) The PCR result showing that the primer used successfully detected the 16s rRNA for isolated strain of *Bacillus subtilis* .The size of amplicon for the gene of interest was 595 bp.

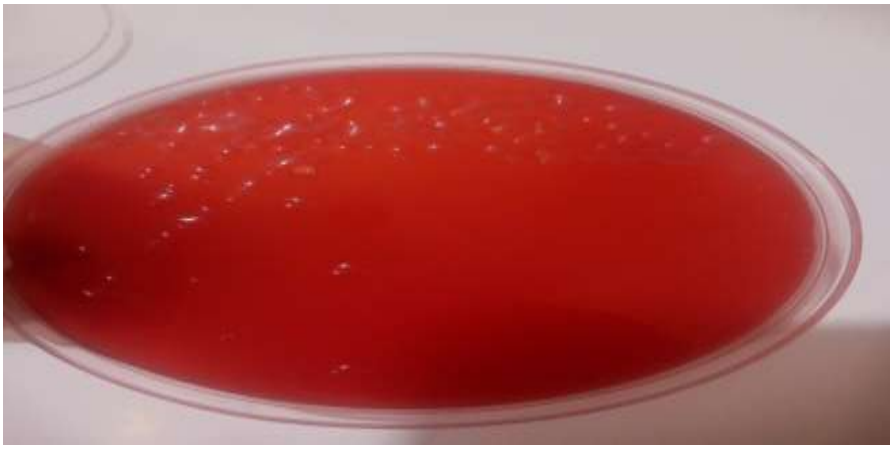


Fig (4) The haemolytic activity of the isolated *Bacillus subtilis* strain did not show any haemolytic activity against human blood in the blood agar medium.

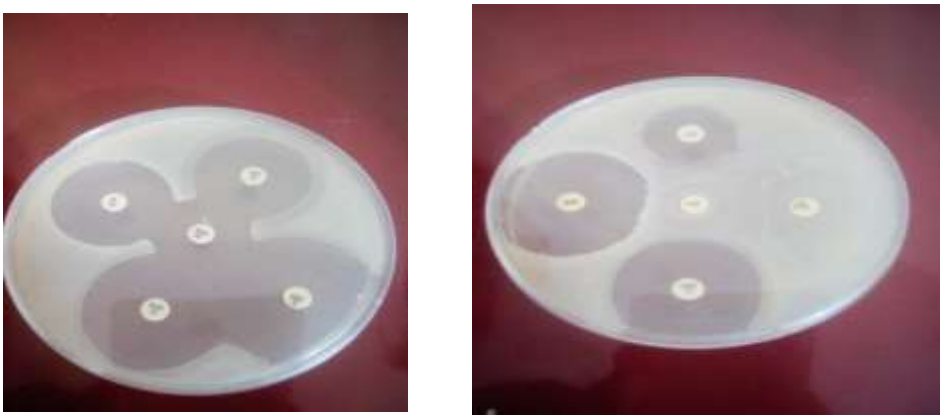


Fig (5) Antibiotic sensitivity test for the isolated *Bacillus subtilis* was sensitive to Norfloxacin, Levofloxacin, Streptomycin, Gentamycin, Trimethoprime /sulphamethoxazole, Amoxicillin and Ampicillin /sulbactam and resistant to Cefuroxime and Flucloxacillin.

Fig (6) T he antimicrobial activity of the probiotic *B. subtilis* against *Aeromonas hydrophila*..

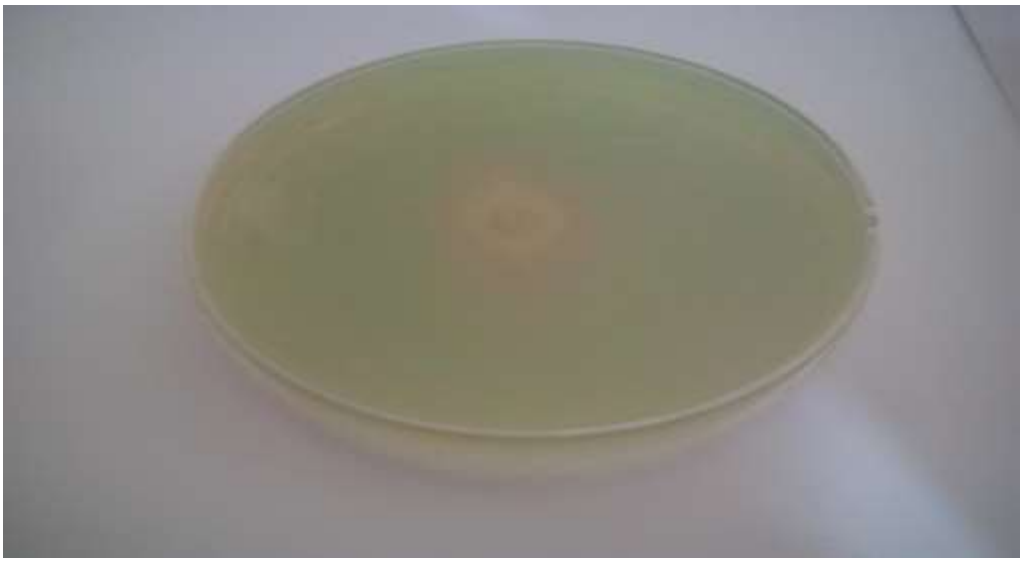


Fig (7) The antimicrobial activity of the probiotic *B. subtilis* against *Pseudomonas aeruginosa*..

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خصائص البروبيوتك بسلس ستلس المعزولة من القناة الهضمية لأسماك البلطي النيلي وتقييم أثارها الايجابية على الصحة والمناعة الغير نوعية في اسماك البلطي النيلي

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الملخص العربي

في الدراسة الحالية تم عزل البروبيوتك بسلس ستلس من القناة الهضمية لأسماك سليمة من البلطي النيلي المجمعة من المزرعة الإنتاجية بالمعمل المركزي لبحوث الثروة السمكية - مصر. تم تصنيف البروبيوتك بسلس ستلس بناء على الخصائص البيوكميائية باستخدام API 20E والتوصيف الجزيئي باستخدام 16s rRNA.

تبين من الدراسة أن البكتريا المعزولة غير ممرضة لأسماك البلطي النيلي؛ قادرة على النمو عند تركيزات مختلفة من العصارة المرارية؛ حساسة لمعظم المضادات الحيوية المستخدمة في الدراسة؛ كما أظهرت نشاط مضاد على البكتريا الممرضة للأسماك مثل الايرمونس هيدروفيلا والسودوموناس ارجونوزا

لدراسة الآثار الايجابية لبروبيوتك بسلس ستلس على صحة ومناعة اسماك البلطي النيلي تم تجميع عدد ١٢٠ سمكة بلطي نيلي متوسط وزن ٢٠ جرام وتم تقسيمهم إلى مجموعتين (كل مجموعة ثلاث مكررات) المجموعة الأولى تم تغذيتها على علف طافي ٣٠٪ بروتين والمجوعة الثانية تم تغذيتها على علف طافي ٣٠٪ بروتين مع إضافة البروبيوتك بسلس ستلس بتركيز $١٠^{٩} \times ١$ لكل جرام علف لمدة ٧ أسابيع. تبين من الدراسة أن إضافة البروبيوتك بسلس ستلس المعزولة من القناة الهضمية لأسماك البلطي النيلي أدى زيادة معنوية في نسبة الهيموجلوبين و الهيماتوكريت وعدد كرات الدم الحمراء وكرات الدم البيضاء كما أدى إلى تحسن في وظائف الكبد شاملة إنزيمات الكبد وبروتينات الدم (الالبيومين والبروتين) كما أن إضافة البروبيوتك بسلس ستلس أدى إلى زيادة معنوية في القياسات المناعية (الليزوزيم والنتروبلوتترازوليم).

تم إجراء العدوى الصناعية في نهاية التجربة بميكروب الايروموناسى هيدروفيلا وكانت نسبة النفوق في المجموعة الأولى (الكنترول) ٩٠٪ بينما في المجموعة الثانية (المغذاة على بسلس ستلس) كانت ٦٠٪ بنسبة أعاشه ٣٣,٣٪.