THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Efficacy of *Bacillus* probiotic mixture on the immunological responses and histopathological changes of Nile tilapia (*Oreochromis niloticus*, L) challenged with *Streptococcus iniae*

Citation for published version:

Moustafa, EM, Farrag, FA, Dawood, MAO, Shahin, K, Hamza, A, Decamp, O, Mohamed, R, Elsabagh, M, Eltholth, M & Omar, AA 2020, 'Efficacy of *Bacillus* probiotic mixture on the immunological responses and histopathological changes of Nile tilapia (*Oreochromis niloticus*, L) challenged with *Streptococcus iniae*', *Aquaculture research*. <https://doi.org/10.1111/are.15073>

Digital Object Identifier (DOI):

[10.1111/are.15073](https://doi.org/10.1111/are.15073)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Aquaculture research

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Efficacy of *Bacillus* probiotic mixture on the immunological responses and histopathological changes of Nile tilapia (*Oreochromis niloticus*, L) challenged with *Streptococcus iniae*

Eman M. Moustafa^{1,*}, Foad A. Farrag², Mahmoud A.O. Dawood^{3,*}, Khalid Shahin^{4,5}, Ahmad Hamza⁶, Olivier Decamp⁷, Radi Mohamed⁸, Mabrouk Elsabagh^{9,10}, Mahmoud Eltholth^{11,12&13}, Amira A. Omar¹

¹Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt

²Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt

³Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University, Kafrelsheikh, Egypt

⁴Aquatic Animal Health Laboratory, Department of Medicine and Epidemiology, University of California Davis, 95616, CA, USA

⁵Aquatic Animal Diseases Laboratory, Aquaculture Division, National Institute of Oceanography and Fisheries, PO Box 43511, Suez, Egypt

⁶AQUAVET for Fish Nutrition and Health Solutions, Alfarouk Towers, Zohdy Square, Kafrelsheikh, Egypt

⁷INVE Asia Services, 471 Bond St., Tambon Bangpood, Amphur Pakkred, Nonthaburi 11120, Thailand

⁸Department of Aquaculture (Fish Welfare), Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafrelsheikh, Egypt

⁹Department of Animal Production and Technology, Faculty of Agricultural Sciences and Technologies, Niğde Ömer Halisdemir University, Niğde, 51240 Turkey

¹⁰Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, 33516 Egypt

¹¹Department of Hygiene and Preventive Medicine, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt

¹²Institute of Aquaculture, University of Stirling, Stirling, United Kingdom, FK9 4LA.

¹³Global Academy of Agriculture and Food Security, The Royal (Dick) School of Veterinary
Studies and The Roslin Institute, The University of Edinburgh, Edinburgh, UK

***Corresponding authors:**

Moustafa, Eman Moustafa; emantarek2002@yahoo.com ; <https://orcid.org/0000-0002-7788-8533>

Mahmoud A.O. Dawood; Mahmoud.dawood@agr.kfs.edu.eg ; <https://orcid.org/0000-0002-4887-8838>

Short Title: Efficacy of *Bacillus* probiotics on *Streptococcus iniae* infected tilapia

Abstract

Isolates of *S. iniae* were recovered from the diseased fish during summer season, identified and characterized using different laboratory techniques. Three hundred and sixty Nile tilapia (100 ± 5.0 g average weight) were divided into two subgroups in triplicates with 20 fish per replicate and received either sterile PBS (B0, B1 and B2, respectively) or *S. iniae* (B0+ *S. iniae*, B1+ *S. iniae* and B2 *S. iniae*, respectively). At 15 days post-challenge, results showed low mortality rates in probiotic-fed groups. The mortalities ranged from 6.67% in B2 to 81.67% in B0 following *S. iniae* challenge. The hematological parameters showed a significant increase ($p<0.05$) in hemoglobin, red blood cells and white blood cells in B1, B1+*S. iniae*, B2 and B2+*S. iniae* groups compared to B0+ *S. iniae* group. The alanine aminotransferase (ALT) level was elevated ($p<0.05$) in tilapia challenged with *S. iniae* compared to the control groups. Glucose and creatinine levels were elevated ($p<0.05$) in all groups compared with B0. Both albumin and globulin levels were significantly low ($p<0.05$) in B0+ *S. iniae* compared to other groups. The total protein level, phagocytosis and phagocytic index were significantly lower ($p<0.05$) in B0+ *S. iniae* than other groups. Histological analysis showed that the hepatopancreas of B0, B1 and B2 groups represents normal hepatocyte architecture, while the infected tilapia showed severe diffused necrosis, mononuclear cell infiltration and loss of the normal architecture. Spleen of PBS control groups did not show any pathological changes, while that of infected tilapia revealed several necrotic areas in addition to marked depletion of lymphoid cells. A loss of skin layers was observed in infected tilapia with marked interstitial edema and leukocytic cell infiltration which was not observed in the probiotic-fed fish. The current study highlights the immunomodulatory effect of *Bacillus* probiotic mixture against *S. iniae* infection in tilapia that can be further applied to control the disease in farms.

67 **Keywords:** Probiotics; Aquaculture; Disease Resistance; Mortality; Nile tilapia; *Streptococcus*
68 *iniae*
69
70
71
72
73
74
75
76

Introduction

Nile tilapia (*Oreochromis niloticus*, L), remains one of the most commonly farmed fish species worldwide, due to their tolerance to different environmental conditions and diseases, fast growth, and high market demand (Hai, 2015, Dawood et al., 2019a,b). Global tilapia production has developed rapidly in recent decades, reaching approximately 5.4 Million MT in 2016 (FAO, 2018). High mortality rates in farmed tilapia could be due to many reasons including high stocking densities, intensive feeding, environmental and management factors and diseases (Dawood et al., 2020; Fathi et al., 2017). Bacterial pathogens including *Streptococcus* spp., *Edwardsiella* spp., *Francisella orientalis* and *Vibrio* spp., can induce septicemia, meningoencephalitis, skin lesions, exophthalmia, nervous manifestations leading to high economic losses (Lee et al., 2019).

Antibiotics are used to control infectious diseases in tilapia farms; however, they may affect beneficial bacteria, enhance the development of antibiotic resistance, and potential food safety hazards (Cerezuela et al., 2012; Cha et al., 2013). The detrimental effects of antibiotics have drawn global attention to look for alternative products to be applied in fish farms (Elsabagh et al., 2018; Ming et al., 2013).

Probiotics are one of the alternatives that have been widely used in the aquaculture industry (Ali et al., 2008; Dawood et al., 2015a,b; Elsabagh et al., 2018). Probiotics are live microbial feed additives which beneficially affect the host by improving its intestinal microbial balance (Fuller, 1989). Previous studies highlighted the use of probiotics in aquaculture to enhance growth performance, digestive enzymes, feed conversion ratio (FCR), immune response and resistance to diseases (Adel et al., 2017; Gobi et al., 2018; Guardiola et al., 2016; Yilmaz et al., 2019; Zaineldin et al., 2018). *Bacillus subtilis* is intensively used in aquaculture and has been reported to promote growth, feed utilization, high antioxidant responses, improve water quality and increase disease resistance (Chiu et al., 2010; Giri et al., 2014; Kumar et al., 2008; Newaj-

Fyzul et al., 2007; Yilmaz et al., 2020). In our previous trial, *B. subtilis* was added to Nile tilapia feed and showed improved growth, FCR, health condition and immune response (Elsabagh et al., 2018). In addition, *Bacillus* species enhanced fish health against variable bacterial agents (e.g. *Streptococcus iniae*, *Aeromonas hydrophila* and *Vibrio parahaemolyticus*) (Cha et al., 2013; Ramesh et al., 2018; Yi et al., 2018).

Streptococcus iniae is a major fish pathogen affecting aquaculture (Acar et al., 2015). It possesses many virulence genes that are responsible for entrance, propagation, and evasion of immune defenses of fish and other aquatic species (Vazirzadeh et al., 2019). *Interleukin-8* protease (*IL-8*) is one of the important *S. iniae* virulence genes which is a cell envelope protease that can degrade the chemokine *IL-8* resulting in increased neutrophil resistance and contribute to the pathogenesis of invasive streptococcal infection (Zinkernagel et al., 2008). M-like protein (*simA*) is a surface protein and a major virulence factor of *S. iniae* that protects the bacterium from the phagocytic activity and contributes to adherence to fish epithelial cells. C5a peptidase (*scpl*) is a surface protein that hydrolyses the neutrophil chemoattractant complement factor C5a and impairs the ability of the infected fish to fight *S. iniae* infection. Streptolysin S "SLS" (*sagA*) is a major virulence factor in the pathogenesis of *S. iniae* that hemolyses erythrocytes and damages host cell membranes. *S. iniae* cytolysin affects erythrocytes, neutrophils, lymphocytes and promotes cerebrovascular trauma. Capsular polysaccharide (*cpsD*) is one of the most effective *S. iniae* genes that facilitates effective binding to the host epithelial cells and to overcome phagocytosis (Baiano and Barnes, 2009).

The main objectives of this study were to identify and characterize *S. iniae* isolates collected from infected Nile tilapia fish farms during summer outbreaks and assess the impact of using the probiotic "*Bacillus* spp. mixture" on the immune response and resistance of Nile tilapia to *S. iniae*.

Materials and methods

Ethical approval

The protocol for this study was approved by the ethical review committee, Faculty of Veterinary Medicine, Kafrelshikh University, Egypt.

Isolation and identification of *S. iniae* field strain

One hundred and fifty Nile tilapia, *Oreochromis niloticus*, were randomly sampled from different tilapia farms during disease outbreak in Kafr El-Sheikh governorate in summer 2016. These farms had a history of high mortality, and the collected fish showed abnormal swimming behavior, exophthalmia and hemorrhagic patches on pectoral, dorsal and caudal fins. Swab and tissue samples were taken aseptically from eyes, hepatopancreas, posterior kidney, and brain of the collected tilapia. The swabs were plated on tryptic soy agar (TSA, Oxoid, USA) supplemented with 5% sheep blood (Sigma-Aldrich, USA) and incubated at 37°C for 24 h. Well-differentiated single bacterial colonies were sub-cultured on the same medium to obtain pure colonies which were further identified by Gram staining, standard phenotypic and biochemical tests (Table 1). The bacterial isolates were inoculated into brain heart infusion broth (BHI, Sigma-Aldrich, USA), incubated at 28°C at 150 rpm for 24h then stored in 20% glycerol at -80°C till used.

Molecular Identification

For DNA extraction, 1 mL of a 24h-old bacterial culture grown in BHI was pelleted by centrifugation at 4,000 rpm for 30 s. The bacterial pellets were re-suspended in 200 µL phosphate buffer saline (PBS) then centrifuged at 10,000 rpm for 30 s twice for washing the bacterial pellets. The pellets were used for DNA extraction using QIA amp DNA Mini Kit (Qiagen inc., USA) according to the manufacturer's instructions. The extracted DNA was amplified by *S. iniae* specific PCR following the protocol published by Shah et al. (2009). Selected *S. iniae* virulence genes including *sagA*, *pdi*, *cpsD*, *pgmA*, *scpl* and *simA* were

amplified by multiplex PCR using a thermal cycler (Master cycler, Eppendorf, Hamburg, Germany). Sequences of primers used for *S. iniae* virulence genes detection are listed in Table 2. The 50 μ L PCR reaction mixture included 1.5 mM $MgCl_2$, 0.2 mM each of the four deoxynucleotide triphosphates, 2.0 U TaqDNA polymerase, 116 nM of *simA* primers, 58 nM of *scpI*, *pgm* and *cpsD* primers, 93 nM of *pdi* primers, 116 nM of *sagA* primers, 5 μ L of template DNA and RNase free water to the volume. The amplification conditions included an initial denaturation step at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, annealing for 1 min at 58°C, elongation at 72°C for 1 min and final extension for 2 min at 72°C. The amplified DNA fragments were analyzed on a 1% agarose gel after electrophoresis at 100v for 45 min and visualized on a MyECL Imager (Thermo Scientific, USA) (ThermoScientific, USA). The *IL-8* gene was amplified using singleplex PCR using a primer concentration of 5 nmol for both forward and reverse primers. The amplification conditions included initial denaturation at 95 °C for 10 s followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s extension at 72 °C for 60 s and final extension at 72 °C for 5 min. PCR products were visualized on a 2% agarose gel after electrophoresis at 80 V for 90 min.

Total bacterial count

6 x 6 drop plate method was used for estimating the concentration of *S. iniae* strain and the dose for the experimental studies (CFU/mL) following protocol of Chen et al. (2003) after incubation at 37 °C for 24 h on TSA.

Fish and experimental diet

Before the challenge, tilapia were fed a commercial diet (30% crude protein, ALEKHW[®] feed factory, Kafrelsheikh, Egypt) top-coated with sunflower oil (20 ml/kg diet), mixed with a probiotic blend of lyophilized *Bacillus* strains (*Bacillus subtilis* 3.25×10^9 CFU/g, *Bacillus licheniformis* 3.50×10^9 CFU/g and *Bacillus pumilus* 3.25×10^9 CFU/g; Sanolife PRO-F, INVE Aquaculture, Belgium with a total concentration of 1.0×10^{10} CFU/g) at 0 g (B0:

control), 0.1 g (B1) and 0.2 g (B2)/kg diet for ten weeks, respectively (Elsabagh et al 2018). The final feed probiotic concentration for (B1) and (B2) diet, was 1×10^6 and 2×10^6 CFU/g of feed, respectively.

By the end of the feeding trial, 360 Nile tilapia (100 ± 5.0 g average weight), were divided into six groups; 60 fish/group (three replicates of 20 fish each). Fish were randomly allocated in glass aquariums ($50 \times 60 \times 100$ cm); (20 fish/ aquarium) that was equipped with a continuous aeration system to maintain enough dissolved oxygen. Tilapia were fed on the same diet as above for 15 days. Three groups (B0, B1, B2) were identified as a control and were injected intraperitoneal with 0.2 ml of PBS. The other three groups (B0 + *S. iniae*, B1+ *S. iniae* and B2+ *S. iniae*) were injected intraperitoneal with *S. iniae* (0.1 mL/fish) at a dose of 3.5×10^7 CFU that matches a predefined LD₅₀ dose (Data not shown). Fish were observed for 15 days post-challenge (dpc), mortalities were recorded daily, according to Cha et al. (2013) and dead fish were moved for further analyses. The relative percentage of mortality determined according to the following equation: Mortality rate = $100 - (\text{test survival}/\text{control survival}) * 100$.

Blood sampling and analysis

At 14 dpc, blood samples were collected (Feldman et al. 2000) from 3 fish/replicate. Each sample was divided into two parts; one part in a 5 mL test tube on EDTA for haematological analyses and the second part in a 1.5 mL Eppendorf tube for serum separation. Blood samples were left to clot at 4°C for 60 min then centrifuged at 3000 rpm using an Eppendorf centrifuge for 10 min and serum was collected. Serum samples were stored at -20 °C till analysis.

Red blood cells (RBCs) and white blood cells (WBCs) were counted immediately with a hemocytometer after dilution using Hayme's, Natt and Herrick's solutions, respectively (Houston, 1990). Packed cell volume (PCV) was determined according to Karimi et al. (2013). Hemoglobin (Hb) was evaluated using the method of Blaxhall and Daisley (1973). Blood smears were prepared for the determination of differential leukocyte counts (Anderson and

Siwicki 1995; Scalm et al., 1975). Serum total proteins and albumin were determined according to Doymas et al. (1981) and Dumas and Biggs (1972), respectively. Serum globulins was calculated by subtracting albumin values from total protein. Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined according to Reitman and Frankel (1957). The serum alkaline phosphatase (ALP) was determined colorimetrically according to the method described by Kind and King (1954).

Phagocytic activity and phagocytic index

The number of phagocytized cells was counted to calculate the phagocytic index according to Kawahara et al. (1991). Briefly, 50 mg of *Candida albicans* culture was added to 1 ml of blood sample and shaken in a water bath at 25 °C for 5 h. The blood smears were then stained with Gimsa stain solution. Phagocytosis was estimated by determining the proportion of macrophages that were contained intracellularly in yeast cells in a random count of 300 macrophages and expressed as a percentage of phagocytic activity according to the following equations:

Phagocytic activity = macrophages containing yeast/total number of macrophages ×100

Phagocytic index = number of cells phagocytized/number of phagocytic cells.

Histopathological examination

Nine randomly selected fish from each group were euthanized using overdose of MS-222. Tissue samples from the liver, spleen, skin, and eye were collected and fixed in 10% neutral buffer formalin (NBF, Sigma-Aldrich, USA). Tissue samples were dehydrated in ascending concentration of ethanol (Sigma, USA) followed by clearing in xylene (PubChem, USA) and embedding in paraffin wax. Paraffin blocks were sectioned at 5 µm thickness using a rotary microtome (Leica 2025, USA), stained with Hematoxilin and Eosin (H&E) and examined under a light microscope (Bancroft and Gamble 2007).

Statistical analysis

Survival analyses were performed on GraphPad prism version 8 (Graphpad software Inc., San Diego, CA, USA) using Kaplan-Meier method with Mantel-Cox log-rank test (Kaplan and Meier, 1958). Bonferroni correction test was applied for validation of multiple comparisons data set and the alpha value (α) for each comparison was corrected using the current equation $\alpha \frac{1}{4} 0.05/n$, where “n” is the total number of comparisons. Variance analysis (one-way ANOVA) and Tukey's multiple comparison test were used to determine the significant variation ($p < 0.05$) using GraphPad Prism 5 (Graph-Pad Software, San Diego, CA, USA). Results were expressed in mean $\pm$ SE.

Results

Isolates characterization and identification of *S. iniae*

The presumptive identification of the bacteria in the present study was carried out from colony morphology grown on TSA enriched with 5% sheep blood. Colonies of *S. iniae* appeared as transparent white colonies, punctiform in shape and producing complete hemolytic zones (β -haemolysis). *S. iniae* was isolated from 77 fish (51.3%) of the sampled tilapia. The biochemical profile of the isolated bacteria is shown in Table 2. The selected *S. iniae* isolate was positive for 5/7 of virulence genes (*sagA*, *cpsD*, *scpl*, *simA* and *IL-8*) using the multiplex PCR (Figures 1 and 2).

Experimental challenge

In the present study, the median lethal dose fifty (LD_{50}) experiments revealed that the concentration of 10^6 CFU caused 50% mortalities within (24-48 h). The *S. iniae* challenged tilapia revealed lethargy, abnormal swimming behavior, exophthalmia, ulceration on the abdomen, erosion of tail and anal fin, hemorrhagic patches randomly distributed over

operculum and isthmus area, eye cloudiness and complete loss of scales with tail rot. However, the groups injected with PBS did not show any clinical signs (Figure 3).

Different mortality rates were observed with the different concentration of probiotics 15 dpc. The lowest mortality was recorded in group B2 (6.67%), while the highest was in B0+*S. iniae* group (81.67%) (Figure 4).

Haematological and biochemical parameters

Results showed that hemoglobin content and RBCs count were significantly higher in B0, B1, B1+*S. iniae*, B2 and B2+ *S. iniae* compared to B0+ *S. iniae*. Moreover, the RBCs count of B1 was significantly higher than that in B2+ *S. iniae*. The PCV% of B0+ *S. iniae* was lower ($p<0.05$) than the other groups. On the other hand, there was no significant variation in PCV% between B1 and B2 groups, but a significant decrease was noticed between B1+ *S. iniae* and B2+ *S. iniae* compared to B1 and B2. The WBCs count was higher ($p<0.05$) in B1+*S. iniae* than B0 but there was no significant elevation in the other groups compared to B0.

There was a significant ($p<0.05$) increase in lymphocyte % in all groups compared to B0. Moreover, lymphocyte % of the B2+ *S. iniae* was significantly increased compared to other groups. Neutrophil% was elevated ($p<0.05$) in B2 and B2+ *S. iniae* compared to other groups while there was no significant difference between B0, B1 and B1+ *S. iniae*. Eosinophil% was elevated in all groups compared to B0+ *S. iniae*. The highest degree of elevation was present in B2+ *S. iniae* followed by B1+ *S. iniae*, B1, B0 and B2. Basophil% was significantly increased in all groups compared to B0. There was no significant difference in monocyte% among all groups. The hematological parameters are shown in Figure 5.

There were no significant difference in AST level among all groups (Figure 6). The ALT level was significantly higher ($p<0.05$) in B0+ *S. iniae*, B1+ *S. iniae* and B2+ *S. iniae* compared to B0, B1 and B2. The alkaline phosphatase and creatinine levels were significantly higher ($p<0.05$) in all groups compared to B0. The creatinine level was significantly higher ($p<0.05$)

276 in B1, B1+ *S. iniae*, B2 and B2+ *S. iniae* compared to B0+ *S. iniae*. The glucose level was
277 significantly higher ($p<0.05$) in all groups compared with B0 but it was higher in B1+ *S. iniae*,
278 B2 and B2+ *S. iniae* compared to B0+ *S. iniae* and B1. Both albumin and globulin levels were
279 significantly reduced ($p<0.05$) in B0+ *S. iniae* compared to other groups. The total protein level
280 was lower ($p<0.05$) in B0+ *S. iniae* than in other groups. Moreover, there was a marked increase
281 ($p<0.05$) in the total protein level in B0.

282 The phagocytic index of B0+ *S. iniae* was lower compared to other groups (Figure 7).
283 Concerning the phagocytosis parameter, there was a significant increase ($p<0.05$) among all
284 groups compared to B0+ *S. iniae*, while there was no significant variation between B0, B1 and
285 B2 (Figure 7).

286 **Histopathological findings**

287 The results of the histopathological examination are shown in Figures (8-11). The
288 hepatopancreas of B0, B1 and B2 showed normal hepatocyte architecture. The pancreatic
289 venous tracts showed normal architecture within the liver parenchyma (Figures 8A-8C). The
290 liver of *S. iniae* infected fish revealed severe diffuse necrosis, mononuclear cell infiltration in
291 addition to loss of the hepatic and pancreatic architecture (Figures 8D). The liver of B1+ *S.*
292 *iniae* and B2+ *S. iniae* showed mild to moderate fatty changes within hepatocytes (Figures 8E
293 & 8F).

294 The spleen of B0, B1 and B2 did not show any pathological changes. The white pulp was
295 formed from irregular areas of lymphoid cells that surround the blood vessels and
296 melanomacrophage centers (Figures 9A-C). The spleen of *S. iniae* infected fish revealed the
297 presence of several necrotic areas in addition to marked depletion of lymphoid cells (Figures
298 9D). The spleen of B1+ *S. iniae* fish showed a mild degree of necrosis and depletion of
299 lymphoid cells (Figures 9E) while that of B2+ *S. iniae* did not show any evidence of necrosis
300 or lymphoid cells depletion (Figures 9F).

The skin of B0, B1 and B2 groups was composed of intact epidermis and dermis connected with the underlying muscular layers which are characterized by elongated, acidophilic and multinucleated muscle fibers (Figures 10A-C). The *S. iniae* infected fish showed loss of skin layers due to skin ulcer in addition to diffuse degeneration and necrosis of underlying musculature with marked interstitial edema and leukocytic cell infiltration (Figures 10D). The skin of B1+ *S. iniae* fish showed a moderate degree of cutaneous necrosis in addition to interstitial edema and a moderate degree of leukocytic infiltration in the muscle layers (Figures 10E). The degree of necrosis and interstitial edema was decreased in B2+ *S. iniae* (Figures 10F).

The eyeball of B0, B1 and B2 revealed normal architecture of cornea; corneal epithelium, corneal stroma, and corneal endothelium (Figures 11A-C). The eye of the *S. iniae* infected fish showed loss of corneal epithelium and endothelium in addition to interstitial edema in the corneal stroma (Figures 11D). The corneal epithelium appeared vacuolated with interstitial edema in the corneal stroma in B1+ *S. iniae* while the cornea of B2+ *S. iniae* revealed a marked decrease in the vacuolation and interstitial edema (Figures 11E and 11F, respectively).

Discussion

Infection with *S. iniae* is one of the challenges of farmed tilapia intensification in Egypt, where *S. iniae* induces severe mortality in farms and results in a substantial economic loss (Neamat-Allah et al., 2019; Zahran et al., 2019). The irrational use of antimicrobials in livestock production and aquaculture resulted in antimicrobial resistance against infectious bacteria (Garlock et al., 2020). Alternatively, a wide range of functional supplements with immunomodulation potentials is recommended to substitute the antibiotics for sustainable aquaculture (Dawood, 2020). Probiotics are proven to potentiate the immune response of aquatic animals and are recommended as an alternative strategy for enhancing fish immunity (Elsabagh et al., 2018). More specifically, beneficial bacterial cells exert bacteriocins that

enhance the phagocytosis and fish's innate immunity and increase the resistance against the infections (Dawood and Koshio, 2016; Dawood et al., 2018).

In the present study, presumptive identification of *S. iniae* was carried out from colony morphology on blood agar (Rahmatullah et al., 2017; Saleh et al., 2017). Phenotypic characters of the isolates were similar to those reported by Oretega et al. (2018) and Rahmatullah et al. (2017). The biochemical results were similar to those reported by previous studies (Tison et al., 1982; Austin et al., 1997). Seventy-seven isolates were positive for *S. iniae* among 150 fish samples that were collected during summer outbreaks. The high incidence of Streptococosis in summer may be attributed to the increase of water temperature (Saleh et al., 2017). High water temperature during summer is considered a stress factor, promoting the expression of various virulent factors, facilitating the invasion of *Streptococcus* to their hosts (Kayansamruaj et al., 2014; Rodkhum et al., 2011). Interestingly, the five virulence genes (*sagA*, *cpsD*, *scpl*, *simA* and *IL-8*) identified in the present study were specific to pathogenic *S. iniae* (Locke et al., 2007; Locke et al., 2008; Baiano and Barnes 2009).

The median lethal dose fifty (LD₅₀) observed in the current study (3.5×10^6) CFU is similar to that used by Oretega et al. (2018) and Rahmatullah et al. (2017). However, Dong et al. (2015) obtained LD₅₀ at a concentration of 10^2 CFU, while Baums et al. (2013) reported that 10^8 CFU per mL of *S. iniae* could cause mortality in Nile Tilapia. The differences in LD₅₀ doses may be attributed to the number of virulent genes in *S. iniae* strain, pathogen properties, as well as size, the weight of fish species and variations in water temperature at time of the challenge or natural disease outbreak (Moustafa et al., 2016).

Observed signs in the experimentally infected fish may be due to variations of the virulent genes of the selected isolates. Exophthalmia might be attributed to the pressure of accumulated fluid in the abdomen on the eyeballs and/or due to the cytotoxic effect of *S. iniae* on the fish cells (Locke et al., 2007). Ulcerative areas on the abdomen, erosion of the fins, loss of scales

and detachment might be attributed to streptolysin S (SLS) virulence factor which has a direct cytotoxic effect against the fish cells (Locke et al., 2007). Haemorrhagic patches over the operculum, mouth and pectoral fins may also attributed to the virulence factors (SLS) produced by *S. iniae* causing blood cells death leading to hemorrhage in different parts of the body (Locke et al., 2007). Eye cloudiness may attributed to *S. iniae simA* virulence factor which facilitates invasion of streptococcus to the fish eyes leading to cloudiness due to bacterial growth. Also streptococcus itself can penetrate the epithelial and endothelial barriers (Locke et al., 2008). Little mortalities in other infected groups may be attributed to the immunostimulant effect of the used probiotics. Similar results were observed in Nile tilapia fed with *Bacillus* probiotic (Addo et al., 2017; Iwashita et al., 2015).

Hemato-biochemical variables are simple and applicable diagnostic tools to reveal the immune response and the resistance against infections in many aquatic animals (Fazio et al., 2019). The results showed that tilapia infected with *S. iniae* have impaired RBCs, WBCs, haemoglobin, total protein, globulin, and albumin values; however, incorporating *Bacillus* probiotics into their feed enhanced these values. These results indicate that *Bacillus* could potentiate the immunity and metabolic functions in Nile tilapia and raise its resistance to *S. iniae* (Panigrahi et al., 2010). Our results are supported by previous studies that illustrated that probiotics could enhance the hemato-biochemical indices in fish (Dawood et al., 2019a,b; Elsabagh et al., 2018; Firouzbakhsh et al., 2011; Reda and Selim, 2015; Irianto and Austin, 2002; Firouzbakhsh et al., 2011). A high count of RBCs in tilapia treated with *Bacillus* shows non-anemic features and availability of high Ferrin with the possible capability to scavenge the free radicals (e.g., ROS) which causes oxidative stress during the infection with *S. iniae*. In addition, the increased count of WBCs indicates the enhanced immune responses (e.g., phagocytosis) to counteract the infection in tilapia treated with *Bacillus*. The improved blood protein profile (total protein, albumin, and globulin) indicates enhancement of humeral immunity associated with enhanced

376 systemic immunity during the infection (Alexander et al., 2011; Ebrahimi et al., 2012).
377 Probiotics exert several functional substances in the intestine of fish (e.g., peroxide,
378 bacteriocin, siderophore, and lysozyme enzymes), enhancing the local intestinal immunity and
379 competing with the intestinal pathogenic bacteria. Once the intestinal immunity is triggered by
380 probiotic cells, the general immune system would be activated and tolerate the infection (Fuller,
381 1989).

382 Infection with pathogenic *S. iniae* caused impaired hepatic-renal function in tilapia under the
383 current trail conditions, but the addition of *Bacillus* probiotics relieved the harmful impact on
384 the hepato-renal tissues. Under severe infection conditions, harmful bacteria secretes toxins that
385 damage the hepatocytes in the liver and encourage the liver to secret enzymes (e.g., ALT, ALP,
386 and AST) to cope with the negative impacts of toxins (Cagauan et al., 2004). More specifically,
387 the increased values of ALP and ALT in Nile tilapia infected with *S. iniae* refer to the impaired
388 liver condition, but mediated ALT and ALP in tilapia fed *Bacillus* confirming the healthy
389 status. The impaired renal function, as shown by the level of creatinine, due to infection with
390 *S. iniae*, may result from the injured kidney tubules. The infection with *S. iniae* induces protein
391 leakage in the fish tissues and dysfunction the capacity of the kidney to filtrate the over
392 nitrogen-based molecules in the blood.

393 The high count of WBCs is also associated with the enhanced phagocytosis in tilapia fed
394 *Bacillus* and infected with *S. iniae*. Phagocytosis is a systemic immune response activated
395 during the infection to decrease bacterial infection spread in the body of fish (Harikrishnan et
396 al., 2011). Probiotics are known for their action against the infection by enhancing
397 phagocytosis and antibacterial activities (Zhang et al., 2008). This was in agreement with
398 previous studies where tilapia-fed probiotics showed enhanced phagocytic activity (Ren et al.,
399 2013; El-Boshy et al., 2010; Pirarat et al., 2011).

Bacterial infection can be diagnosed by histopathological examinations, which present a clear image of the possible inflammatory features induced by the infection (Chen et al., 2006; Turner, 2006). The intestinal function is disturbed by the pathogenic bacteria due to impaired barriers immunity and mucosal integrity, followed by the overexpression of inflammation-related cytokines. Once the inflammation occurred in the intestine, the remaining tissues in the body are also damaged and lose their regular function. During the infection, disturbance of the intestinal barrier's function is often associated with inflammation of the intestine and other tissues (Liu et al., 2014). In the present study, the tissue damage was detected in the skin, liver, spleen, and cornea of Nile tilapia infected with *S. iniae* and fed with or without *Bacillus*. The results showed severe inflammation in these tissues, which were attributed to the impaired function induced by toxins produced by *S. iniae*. These results also associated with the dysfunction in the hepatic tissue (high ALT and ALP levels). Notably, incorporating *Bacillus* in tilapia diets revealed a protective effect against the impact of *S. iniae* on liver, spleen, skin, and cornea tissues.

As the skin is the first line of defence (Guardiola et al., 2014), it is crucial to determine the impact of *S. iniae* and the expected defensive role of *Bacillus* in tilapia. The results showed normal features in the tilapia skin fed on *Bacillus* and infected with *S. iniae*, but inflammation and tissue damage were observed in infected tilapia that did not receive any probiotic supplement in their feed. These results clearly showed the possible anti-inflammatory role of *Bacillus* against *S. iniae* infection (Kong et al., 2017). The *S. iniae* infected tilapia showed loss of skin layers due to skin ulcer in addition to diffuse degeneration and necrosis of underlying musculature with marked interstitial edema and leukocytic cell infiltration (Das et al., 2013). However, further studies are required to investigate the role of anti-inflammatory genes in tilapia fed on *Bacillus* probiotics.

Conclusion

In conclusion, the infection with *S. iniae* induced severe impairment in the hepato-renal functions, immune response, and anti-inflammatory capacity of Nile tilapia. However, incorporating the *Bacillus* probiotic mixture relieved the inflammation induced by *S. iniae* through the modulation of haemato-biochemical indices, immune response, and histopathological features. In this sense, the *Bacillus* mixture is recommended as a feasible probiotic in tilapia diets for better performance and resistance against infectious diseases.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

Author Contributions

Authors contributed to the current study equally.

Conflict of interest

No conflict of interest.

Acknowledgement

This study was funded by the Research Funding Unit, Kafrelsheikh University, Egypt (Grant No: KFURF-13).

References

- Adel M, Yeganeh S, Dawood MAO, Safari R, and Radhakrishnan S (2017) Effects of *Pediococcus pentosaceus* supplementation on growth performance, intestinal microflora and disease resistance of white shrimp, *Litopenaeus vannamei*. Aquacult Nutr 23(6):1401-9.
- Acar, Ü., Kesbiç, O.S., Yılmaz, S., Gültepe, N. and Türker, A., 2015. Evaluation of the effects of essential oil extracted from sweet orange peel (*Citrus sinensis*) on growth rate of tilapia

450 (*Oreochromis mossambicus*) and possible disease resistance against *Streptococcus iniae*.
 451 Aquaculture, 437, pp.282-286.

452 Addo, S., Carrias, A.A., Williams, M.A., Liles, M.R., Terhune, J.S. and Davis, D.A., 2017.
 453 Effects of *Bacillus subtilis* strains on growth, immune parameters, and *Streptococcus iniae*
 454 susceptibility in Nile tilapia, *Oreochromis niloticus*. Journal of the World Aquaculture Society,
 455 48(2), pp.257-267.

456 Iwashita, M.K.P., Nakandakare, I.B., Terhune, J.S., Wood, T. and Ranzani-Paiva, M.J.T.,
 457 2015. Dietary supplementation with *Bacillus subtilis*, *Saccharomyces cerevisiae* and
 458 *Aspergillus oryzae* enhance immunity and disease resistance against *Aeromonas hydrophila*
 459 and *Streptococcus iniae* infection in juvenile tilapia *Oreochromis niloticus*. Fish & shellfish
 460 immunology, 43(1), pp.60-66.

461 Alexander, C., Sahu, N.P., Pal, A.K. and Akhtar, M.S., 2011. Haemato-immunological and
 462 stress responses of *Labeo rohita* (Hamilton) fingerlings: effect of rearing temperature and
 463 dietary gelatinized carbohydrate. Journal of animal physiology and animal nutrition, 95(5),
 464 pp.653-663.

465 Ali, S.M.; Abdel-Galil A. Y.; Abdel-Aziz Gh. A. and Mohamed, M.F. (2008): Studies on
 466 *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune
 467 response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish
 468 Shellfish Immunol. 25: 128-136.

469 Anderson, D. P., & Siwicki, A. K (1995). Basic haematology and serology for fish health
 470 programs. In M. Shariff, J. R. Arthur, & J. P. Subasinghe (Eds.), Diseases in Asian aquaculture
 471 II (pp.185-202). Manila, Philipines: Fish Health Section. Asian Fisheries Society.

472 Austin B, Alsina M, Austin DS, Blanch AR, Grimont F and Grimont PAD (1997). A
 473 comparison of methods for the typing of fishpathogenic *Vibrio spp.* Systematic and Applied
 474 Microbiology: 20(1): 89-101.

475 Baiano, J.C.F. and Barnes, A.C. (2009): Towards Control of *Streptococcus iniae*. Emerging
 476 Infectious Diseases. 15 (12): 1891-1896.

477 Bancroft JD and Gamble M (2007). Theory and Practice of Histological Techniques. 5th
 478 edition; Churchill Livingstone, London, UK., pp. 125-138.

479 Baums, C. G., K. Hermeyer, S. Leimbach, M. Adamek, C. P. Czerny, G. Hörstgen-Schwark,
 480 P. Valentin-Weigand, W. Baumgärtner, and D. Steinhagen. 2013. Establishment of a model of
 481 *Streptococcus iniae* meningoencephalitis in Nile Tilapia (*Oreochromis niloticus*). Journal
 482 Comparative Pathologists 149:94–102.

483 Blaxhall, P.C., Daisley, K.W. 1973. Routine hematological methods for use with fish blood. *J.*
 484 *Fish Biol.* 5(6):771-781.

485 Cagauan, G.G., R.H. Tayaban, J.R. Somga and R.M. Bartolome (2004). Effect of aflatoxin-
 486 contaminated feeds in Nile tilapia. Proceedings of 6th International Symposium on Tilapia in
 487 Aquaculture Philippine International Convention Center Roxas Boulevard, Manila, Philippines
 488 September, 12-16: 172-178.

489 Cerezuela, R.; Guardiola, F.A.; Meseguer, J. and Esteban, M. A. (2012): Increases in immune
 490 parameters by inulin and *Bacillus subtilis* dietary administration to gilthead seabream (*Sparus*
 491 *aurata* L.) did not correlate with disease resistance to *Photobacterium damsela*. Fish Shellfish
 492 Immunol. 32: 1032–1040.

493 Cha, J. H.; Rahimnejad S.; Yang S. Y.; Kim K.W. and Lee, K. J. (2013): Evaluations of *Bacillus*
 494 *spp.* as dietary additives on growth performance, innate immunity and disease resistance of
 495 olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives.
 496 Aquaculture; 402-403: 50-57.

497 Chen, M. L., Ge, Z., Fox, J. G. & Schauer, D. B. 2006. Disruption of tight junctions and
 498 induction of proinflammatory cytokine responses in colonic epithelial cells by *Campylobacter*
 499 *jejuni*. Infection & Immunity 74, 6581–6589.

500 Chen, C.Y. Nace, and G.W. Irwin, P.L. A 6 x 6 drop plate method for simultaneous colony
501 counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and
502 *Escherichia coli*, J. Microbiol. Methods 2 (2003) 475–479, [https://doi.org/10.1016/S0167-](https://doi.org/10.1016/S0167-7012(03)00194-5)
503 7012(03)00194-5.

504 Chiu CH, Cheng CH, Gua WR, GU YK, Cheng W (2010) Dietary administration of the
505 probiotic, *Saccharomyces cerevisiae* P13, enhanced the growth, innate immune responses, and
506 disease resistance of the grouper, *Epinephelus coioides*. Fish Shellfish Immunol 29: 1053-
507 1059.

508 Cruickshank, R., Duguid, J.P., Marmion, B.P., Swain, R.H. 1975. Medical Microbiology. The
509 practical of Microbiology. Chucohill Livingstone 12th ed. (11), Edinburgh, London and New
510 York.

511 Das, A.; Nakhro, K.; Chowdhury, S. and Kamilya, D. (2013): Effects of potential probiotic
512 *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and
513 disease resistance of catla (*Catla catla*). Fish Shellfish Immunol. 35: 1553–1547.

514 Dawood MAO, Magouz FI, Salem MFI, Abdel-Daim HA (2019a) Modulation of digestive
515 enzyme activity, blood health, oxidative responses and growth-related gene expression in GIFT
516 by heat-killed *Lactobacillus plantarum* (L-137). Aquaculture
517 <https://doi.org/10.1016/j.aquaculture.2019.02.053>

518 Dawood MAO, Eweedah NM, Moustafa EM, Shahin MG (2019b) Synbiotic Effects of
519 *Aspergillus oryzae* and β -Glucan on Growth and Oxidative and Immune Responses of Nile
520 Tilapia, *Oreochromis niloticus*. Probiotics and Antimicrobial Proteins 8:1-2.

521 Dawood MAO, Koshio S (2016) Recent advances in the role of probiotics and prebiotics in
522 carp aquaculture: a review. Aquaculture 454:243-251.

523 Dawood MAO, Koshio S, Esteban MÁ (2018) Beneficial roles of feed additives as
524 immunostimulants in aquaculture: a review. Rev Aquac 10(4):950-974.

525 Dawood MAO, Koshio S, Ishikawa M, Yokoyama S (2015a) Effects of heat killed
 526 *Lactobacillus plantarum* (LP20) supplemental diets on growth performance, stress resistance
 527 and immune response of red sea bream, *Pagrus major*. *Aquaculture* 442:29-36.

528 Dawood MAO, Koshio S, Ishikawa M, Yokoyama S (2015b) Interaction effects of dietary
 529 supplementation of heat-killed *Lactobacillus plantarum* and β -glucan on growth performance,
 530 digestibility and immune response of juvenile red sea bream, *Pagrus major*. *Fish Shellfish*
 531 *Immunol* 45(1):33-42.

532 Dawood, M.A., Metwally, A.E.S., El-Sharawy, M.E., Atta, A.M., Elbially, Z.I., Abdel-Latif,
 533 H.M. and Paray, B.A., 2020. The role of β -glucan in the growth, intestinal morphometry, and
 534 immune-related gene and heat shock protein expressions of Nile tilapia (*Oreochromis*
 535 *niloticus*) under different stocking densities. *Aquaculture*, p.735205.

536 Dawood, M.A., 2020. Nutritional immunity of fish intestines: Important insights for
 537 sustainable aquaculture. *Reviews in Aquaculture*. <https://doi.org/10.1111/raq.12492>

538 Dong, H. T.; Viet, V. N.; Le, H. D.; Sangsuriya, P.; Jitrakorn, S.; Saksmerprome, V.; Senapin,
 539 S. and Rodkhum, Ch. (2015): Naturally concurrent infections of bacterial and viral pathogens
 540 in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms. *Aquaculture*. 448:
 541 427–435.

542 Doymas, B.T., Bayso, D.D., Carter, R.J., Schaffer, R. 1981. Determination of total serum
 543 protein. *Clin. Chem.* 27:1642-1643.

544 Dumas, B.T., Biggs, H.G. 1972. *Standard Methods of Clinical Chemistry*. ed., Academic Press,
 545 New York.

546 Ebrahimi, G., H. Ouraji, M. Khalesi, M. Sudagar, A. Barari, M. ZareiDangesaraki, and K. Jani
 547 Khalili. 2012. Effects of a prebiotic, Immunogen, on feed utilization, body composition,
 548 immunity, and resistance to *Aeromonas hydrophila* infection in the Common Carp (*Cyprinus*
 549 *carpio*, Linnaeus) fingerlings. *Journal of Animal Physiology and Animal Nutrition* 96:591–599.

550 El-Boshy, M.E., Ahmed, M., AbdelHamid, F.M. and Gadalla, H.A., 2010. Immunomodulatory
551 effect of dietary *Saccharomyces cerevisiae*, β -glucan and laminaran in mercuric chloride
552 treated Nile tilapia (*Oreochromis niloticus*) and experimentally infected with *Aeromonas*
553 *hydrophila*. Fish & Shellfish Immunology, 28(5-6), pp.802-808.

554 Elsabagh M, Mohamed R, Moustafa EM, Hamza A, Farrag F, Decamp O, Dawood MAO,
555 Eltholth M (2018) Assessing the impact of *Bacillus* strains mixture probiotic on water quality,
556 growth performance, blood profile and intestinal morphology of Nile tilapia, *Oreochromis*
557 *niloticus*. Aquac Nutr 24(6):1613-1622.

558 Fathi, M., Dickson, C., Dickson, M., Leschen, W., Baily, J., Muir, F., Ulrich, K. and
559 Weidmann, M., 2017. Identification of Tilapia Lake Virus in Egypt in Nile tilapia affected by
560 'summer mortality'syndrome. Aquaculture, 473, pp.430-432.

561 FAO, 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the Sustainable
562 Development Goals. Rome. in: Organizsation, F.a.A. (Ed.), Rome, Italy.

563 Fazio, F., 2019. Fish hematology analysis as an important tool of aquaculture: A review.
564 Aquaculture. 500, 237-242.

565 Feldman, B.F., Zink, J.G., Jain, N.C. 2000. Schalm's Veterinary Hematology. 5th ed.,
566 Lippincott Williams and Wilkins, New York.

567 Firouzbakhsh, F., Noori, F., Khalesi, M. K., & Jani-Khalili, K., 2011. Effects of a probiotic,
568 protexin, on the growth performance and hematological parameters in the Oscar (*Astronotus*
569 *ocellatus*) fingerlings. Fish Physiology and Biochemistry. 37, 833–842.

570 Fuller, R., 1989. Probiotics in man and animals. Journal of Applied Bacteriology, 66,365-378.

571 Giri SS, Sukumaran V, Sen SS, Jena PK (2014) Effects of dietary supplementation of potential
572 probiotic *Bacillus subtilis* VSG1 singularly or in combination with *Lactobacillus plantarum*
573 VSG3 or/and *Pseudomonas aeruginosa* VSG2 on the growth, immunity and disease resistance
574 of *Labeo rohita*. Aquacult Nutr 20(2):163-171.

575 Garlock, T., Asche, F., Anderson, J., Bjørndal, T., Kumar, G., Lorenzen, K., Ropicki, A.,
 576 Smith, M.D. and Tveterås, R., 2020. A global blue revolution: aquaculture growth across
 577 regions, species, and countries. *Reviews in Fisheries Science & Aquaculture*, 28(1), pp.107-
 578 116.

579 Gobi N, Vaseeharan B, Chen JC, Rekha R, Vijayakumar S, Anjugam M, Iswarya A (2018)
 580 Dietary supplementation of probiotic *Bacillus licheniformis* Dahb1 improves growth
 581 performance, mucus and serum immune parameters, antioxidant enzyme activity as well as
 582 resistance against *Aeromonas hydrophila* in tilapia *Oreochromis mossambicus*. *Fish Shellfish*
 583 *Immunol* 74: 501-508.

584 Guardiola FA, Porcino C, Cerezuela R, Cuesta A, Faggio C, Esteban MA (2016) Impact of
 585 date palm fruits extracts and probiotic enriched diet on antioxidant status, innate immune
 586 response and immune-related gene expression of European seabass (*Dicentrarchus labrax*).
 587 *Fish Shellfish Immunol*. 52: 298-308.

588 Guardiola, F.A., Cuesta, A., Arizcun, M., Meseguer, J., Esteban, M.A., 2014. Comparative skin
 589 mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus*
 590 *aurata*). *Fish Shellfish Immunol*. 36, 545-551.

591 Hai NV. Research findings from the use of probiotics in tilapia aquaculture: a review. *Fish*
 592 *Shellfish Immunol* 2015; 45: 592-597.

593 Harikrishnan R, Kim JS, Kim MC, Balasundaram C, Heo MS (2011) *Prunella vulgaris*
 594 enhances the non-specific immune response and disease resistance of *Paralichthys olivaceus*
 595 against *Uronema marinum*. *Aquaculture* 318:61-66.

596 Houston, A. 1990. Blood and circulation', *Methods for fish biology* 1990: 273-334.

597 Irianto A, Austin B (2002) Use of probiotic to control furunculosis in rainbow trout. *J Fish Dis*
 598 25:333-342.

599 Kaplan, E.L. and Meier, P., 1958. Nonparametric estimation from incomplete observations.
600 Journal of the American statistical association, 53(282), pp.457-481.

601 Karimi, S., Kochinian, P., Salati, A.P. 2013. The effect of sexuality on some haematological
602 parameters of the yellowfin seabream, *Acanthopagrus latus* in Persian Gulf. *Iran. J. Vet. Res.*
603 14(1):65-68.

604 Kawahara, E.; Ueda, T. and Nomura, S. (1991): In vitro phagocytic activity of white spotted
605 shark cells after injection with *Aeromonas salmonicida* extracellular products. *Gyobyo Kenkyu*,
606 Japan, 26(4): 213-214.

607 Kayansamruaj, P., Pirarat, N., Hirono, I., Rodkhum, C., 2014. Increasing of temperature
608 induces pathogenicity of *Streptococcus agalactiae* and the up-regulation of inflammatory
609 related genes in infected Nile tilapia (*Oreochromis niloticus*). *Vet. Microbiol.* 172, 265–271.

610 Kind, P. R. N. and King, E. J. (1954): Estimation of plasma phosphatase by determination of
611 hydrolyzed phenol with amino antipyrine. *J. Clin. Pathol.*, 74):322-326.

612 Kong, W., Huang, C., Tang, Y., Zhang, D., Wu, Z. and Chen, X., 2017. Effect of *Bacillus*
613 *subtilis* on *Aeromonas hydrophila*-induced intestinal mucosal barrier function damage and
614 inflammation in grass carp (*Ctenopharyngodon idella*). *Scientific reports*, 7(1), p.1588.

615 Kumar R, Mukherjee SC, Ranjan R, Nayak SK (2008) Enhanced innate immune parameters in
616 *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. *Fish Shellfish Immunol*
617 24(2):168-172.

618 Lee, J. M., W. J. Jang, M. T. Hasan, B. J. Lee, K. W. Kim, S. G. Lim, H.S. Han, and I.S. Kong.
619 2019. Characterization of a *Bacillus* sp. isolated from fermented food and its synbiotic effect
620 with barley β -glucan as a biocontrol agent in the aquaculture industry. *Appl. Microbiol.*
621 *Biotechnol.*, 103: 1429–1439.

622 Liu, X., Chang, X., Wu, H., Xiao, J., Gao, Y. and Zhang, Y., 2014. Role of intestinal
623 inflammation in predisposition of *Edwardsiella tarda* infection in zebrafish (*Danio rerio*). Fish
624 & shellfish immunology, 41(2), pp.271-278.

625 Locke, J. B.; Aziz, R.K.; Vicknair, M. R.; Nizet, V. and Buchanan, J. T. (2008): *Streptococcus*
626 *iniae* M-Like Protein Contributes to Virulence in Fish and Is a Target for Live Attenuated
627 Vaccine Development. Plos One 3(7):

628 Locke, J. B.; Colvin, K. M.; Varki, N.; Vicknair, M. R.; Nizet, V. and Buchanan, J. T. (2007):
629 *Streptococcus iniae* β -hemolysin streptolysin S is a virulence factor in fish infection. Diseases
630 of Aquatic Organisms. 76: 17–26.

631 Ming, C., Rui, W., Liping, L., Huang, T., Weiyi, H., Jian, L., Chao, L., Aiying, L., Honglin, L.
632 and Wanwen, L. (2013): Sequence and evolution differences of *Oreochromis niloticus* CXC
633 contribute to the diversification of cellular immune responses in Tilapia with treatment of
634 *Streptococcus iniae*. J. Animal Vet. Adv. 12 (3): 303- 311.

635 Neamat-Allah, A.N., Mahmoud, E.A. and Abd El Hakim, Y., 2019. Efficacy of dietary Nano-
636 selenium on growth, immune response, antioxidant, transcriptomic profile and resistance of
637 Nile tilapia, *Oreochromis niloticus* against *Streptococcus iniae* infection. Fish & shellfish
638 immunology, 94, pp.280-287.

639 Newaj-Fyzul, A.; Adesiyun, A.A.; Mutani, A.; Ramsubhag, A.; Brunt, J. and Austin, B. (2007):
640 *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*,
641 Walbaum). J. Appl. Microbiol. 103:1699–1706.

642 Pirarat N, Pinpimai K, Endo M, Katagiri T, Ponpornpisit A, Chansue N, Maita M (2011)
643 Modulation of intestinal morphology and immunity in Nile tilapia (*Oreochromis niloticus*) by
644 *Lactobacillus rhamnosus* GG. Res Vet Sci 91(3):92-97.

645 Rahmatullah, M., Ariff, M., and Kahieshesfandiari, M., Daud, H. M., Zamri-Saad, M., Sabri,
646 M. Y., Amal, M.N.A. and Ina-Salwany, M.Y. (2017): Isolation and Pathogenicity of

647 *Streptococcus iniae* in Cultured Red Hybrid Tilapia in Malaysia. J. of Aquatic Animal Health
648 29:208–213.

649 Ramesh, D. and Souissi, S. (2018): Effects of potential probiotic *Bacillus subtilis* KADR1 and
650 its subcellular components on immune responses and disease resistance in *Labeo rohita*.
651 Aquacult. Res. 49: 367–377.

652 Reda, R., & Selim, K., 2015. Evaluation of *Bacillus amyloliquefaciens* on the growth
653 performance, intestinal morphology, hematology and body composition of Nile tilapia,
654 *Oreochromis niloticus*. Aquaculture International, 23, 203–217.

655 Reitman, S., Frankel, S. 1957. A colorimetric method for the determination of serum glutamic
656 oxaloacetic and glutamic pyruvic transaminases. *Amer. J. Clin. Pathol.* 28:56-63.

657 Ren, P., Xu, L., Yang, Y., He, S., Liu, W., Ringø, E. and Zhou, Z., 2013. *Lactobacillus*
658 *planarum* subsp. *plantarum* JCM 1149 vs. *Aeromonas hydrophila* NJ-1 in the anterior intestine
659 and posterior intestine of hybrid tilapia *Oreochromis niloticus*♀× *Oreochromis aureus*♂: An
660 ex vivo study. *Fish & shellfish immunology*, 35(1), pp.146-153.

661 Rodkhum, C., Kayansamruaj, P., Pirarat, N., 2011. Effect of water temperature on
662 susceptibility to *Streptococcus agalactiae* serotype In infection in Nile tilapia *Oreochromis*
663 *niloticus* (L.). *Thai J. Vet. Med.* 41, 309–314.

664 Saleh, H.A.; Sabry, N. M.; Abd Al-Razik, M.; Mohamed, F. A.S. and Ibrahim, M. S. (2017):
665 Pathogenicity and Characterization of Streptococcosis in Egyptian Nile Tilapia (*Oreochromis*
666 *niloticus*) in Kafr Elshikh Governorate. *Alexandria Journal of Veterinary Sciences* Vol. 52 (1):
667 173-180.

668 Schalm, O. W.; Jain, N. C. and Carroll, E. J. (1975): *Veterinary hematology*, 3rd edn. Lea &
669 Febiger.

670 Shah, D., Shringi, S., Besser, T. and Call, D. (2009): *Molecular detection of foodborne*
671 *pathogens*, Boca Raton: CRC Press, In Liu, D. (Ed). Taylor & Francis group, Florida, USA,

672 Pp. 369-389.

673 Tison DL, Nishibuchi M, Greenwood JD and Seidler RL (1982). *Vibrio vulnificus* biogroup 2:
 674 new biogroup pathogenic for eels. Applied Environmental Microbiology, 44(3): 640-646.

675 Turner, J. R. 2006. Molecular Basis of Epithelial Barrier Regulation: From Basic Mechanisms
 676 to Clinical Application. American Journal of Pathology 169, 1901–1909.

677 Vazirzadeh, A., Jalali, S. and Farhadi, A., 2019. Antibacterial activity of *Oliveria decumbens*
 678 against *Streptococcus iniae* in Nile tilapia (*Oreochromis niloticus*) and its effects on serum and
 679 mucosal immunity and antioxidant status. Fish & shellfish immunology, 94, pp.407-416.

680 Yi, Y.; Zhang, Z.; Zhao, F.; Liu, H.; Yu, L.; Zha, J. and Wang, G. (2018): Probiotic potential
 681 of *Bacillus velezensis* JW: antimicrobial activity against fish pathogenic bacteria and immune
 682 enhancement effects on *Carassius auratus*. Fish Shellfish Immunol. 78:330–322

683 Yilmaz, S., Yigit, N.Ç.S.E.M. and Çelik, E.Ş., 2019. Combined Effects of Dietary *Bacillus*
 684 *subtilis* and Trans-cinnamic Acid on Growth Performance, Whole Body Compositions,
 685 Digestive Enzymes and Intestinal bacteria in Rainbow Trout (*Oncorhynchus mykiss*). Journal
 686 of Zoological Research| Volume, 1(02).

687 Yılmaz, S., Ergun, S., Yigit, M. and Çelik, E.Ş., 2020. Effect of combination of dietary *Bacillus*
 688 *subtilis* and trans-cinnamic acid on innate immune responses and resistance of rainbow trout,
 689 *Oncorhynchus mykiss* to *Yersinia ruckeri*. Aquaculture Research, 51(2), pp.441-454.

690 Zahran, E., Risha, E., Elbahnaswy, S., Mahgoub, H.A. and Abd El-Moaty, A., 2019. Tilapia
 691 piscidin 4 (TP4) enhances immune response, antioxidant activity, intestinal health and
 692 protection against *Streptococcus iniae* infection in Nile tilapia. Aquaculture, 513, p.734451.

693 Zaineldin AI, Hegazi S, Koshio S, Ishikawa M, Bakr A, El-Keredy AM, Dawood MA, Dossou
 694 S, Wang W, Yukun Z (2018) *Bacillus subtilis* as probiotic candidate for red sea bream: Growth
 695 performance, oxidative status, and immune response traits. Fish Shellfish Immunol 79:303-
 696 312.

Zhang XY, Fan HP, Zhong QF, Zhuo YC, Lin Y, Zeng ZZ (2008) Isolation, identification and pathogenicity of *Streptococcus agalactiae* from tilapia. Journal of Fisheries of China 5:772-779.

Zinkernagel, A. Z.; Timmer, A. M.; Pence, M. A.; Locke, J. B.; Buchanan, J. T.; Turner, C. E.; Mishalian, I.; Sriskandan, Sh.; Hanski, E. and Nizet, V. (2008): The IL-8 Protease SpyCEP/ScpC of Group A Streptococcus Promotes Resistance to Neutrophil Killing. Cell Host & Microbe 4: 170–178.

722 **Table 1.** Phenotypic and biochemical characteristics of the isolated *S. iniae* in the current
723 study.

Biochemical Tests	<i>Streptococcus iniae</i> strain
Gram	+ve
Morphology	Cocci
Motility	-ve
Catalase	-ve
Oxidase	-ve
Aesculin	-ve
Gelatinase	-ve
Haemolysis	<i>β</i> -haemolytic
Tryptic soy agar	+ve
Blood agar	+ve
Nutrient agar	+ve
Brain heart infusion broth	+ve
Oxidative–fermentative (OF) test	F
Voges–Proskauer test	+ve
α-galactosidase	-ve
β-galactosidase	-ve
β-glucuronidase	+ve
Alkaline phosphatase	+ve
Arginine dihydrolase	-ve
Acidification (ribose)	+ve
Acidification (arabinose)	-ve
Acidification (mannitol)	+ve
Acidification (sorbitol)	-ve
Acidification (lactose)	-ve
Acidification (trehalose)	+ve
Acidification (inulin)	-ve
Acidification (raffinose)	-ve
Acidification (amidon)	+ve
Acidification (glycogen)	+ve

724 +ve= positive, -ve= negative.

725

726 **Table 2.** Primer sequences used in the study

Primer	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
<i>sagA</i> (F)	5' AGGAGGTAAGCGTTATGTTAC '3		
<i>sagA</i> (R)	5' AAGAAGTGAATTACTTTGG '3	190	Baums et al. (2013)
<i>pdi</i> (F)	5' TTTCGACGACAGCATGATTG '3		
<i>pdi</i> (R)	5' GCTAGCAAGGCCTTCATTTG '3	381	Baums et al. (2013)
<i>cpsD</i> (F)	5' TGGTGAAGGAAAGTCAACCAC '3		
<i>cpsD</i> (R)	5' TCTCCGTAGGAACCGTAAGC '3	534	Baums et al. (2013)
<i>pgmA</i> (F)	5' TATTAGCTGCTCACGGCATC '3		
<i>pgmA</i> (R)	5' TTAGGGTCTGCTTTGGCTTG '3	713	Baums et al. (2013)
<i>scpl</i> (F)	5' GCAACGGGTTGT CAAAAATC '3		
<i>scpl</i> (R)	5' TCTCCGTAGGAACCGTAAGC '3	822	Baums et al. (2013)
<i>simA</i> (F)	5' TTTCGACGACAGCATGATTG '3		
<i>simA</i> (R)	5' AACCATAACCGCGATAGCAC '3	994	Baums et al. (2013)
<i>IL-8</i> (F)	5' GCACTGCCGCTGCATTAAG '3		
<i>IL-8</i> (R)	5' GCAGTGGGAGTTGGGAAGAA '3	128	Ming et al. (2013)

727 F: Forward, R: Reverse, bp: base pair.

728

729

730

731

732

733

734

735

Figure 1. 1% agarose gel showing multiplex PCR results of 7 virulence-associated genes (*saga* (190 bp), *pdi* (381 bp), *cpsD* (534 bp), *pgmA* (713), *scpl* (822) and *simA* (994 bp) of *S. iniae* isolated in the current study. Lane M: 100 bp DNA marker, Lane C+: Positive control, Lane C-: Negative control (RNase free water), Lanes 1: DNA from selected *S. iniae* isolated in this study.

Figure 2. 1% Agarose gel showing PCR result of *IL-8* gene (128 bp) of *Streptococcus iniae*. Lane M: 100 bp DNA marker, Lane C+: positive control, Lane C-: negative control (RNase free water only), Lanes 1: DNA from selected *S. iniae* from the current study.

Figure 3. Signs of *Streptococcus iniae* infection in experimentally challenged Nile tilapia. (A) exophthalmia (pop-eye) (black arrow) and hemorrhages on the skin and base of pectoral fin (black arrow head); (B) erosion of tail fin (black arrow head), skin ulceration and scale detachment (white arrow head); (C) hemorrhages at the operculum, bottom of mouth and abdomen (white arrow); (D) corneal opacity (black arrow).

Figure 4. Kaplan-Meier (Log-rank Mantel Cox) representation of cumulative survival of tilapia fingerlings at 15 dpc with 10^6 CFU/mL of *S. iniae*. Each curve represents the average results of three parallel tanks holding 20 fish/tank/challenge group. Groups that do not share letters are significantly different ($p < 0.05$). Stars (****) denotes statistical significance ($p < 0.0001$) as determined by Log-rank (Mantel Cox) test.

Figure 5. Haematological parameters of the different treatments post feeding with *Bacillus* probiotic mixture and post-challenge with *S. iniae*. Three groups (B0, B1, B2) were identified as a control, for which 0.2 ml of PBS were injected intraperitoneal. The other three groups (B0 + *S. iniae*, B1+ *S. iniae* and B2+ *S. iniae*) were intra-peritoneally injected with *S. iniae* (0.2 ml/fish). Values are expressed as mean $\pm$ SE from triplicate groups. Bars with different letters are significantly different from those of control group ($P<0.05$).

Figure 6. Blood biochemical parameters of the different treatments post feeding with *Bacillus* probiotic mixture and post-challenge with *S. iniae*. Three groups (B0, B1, B2) were identified as a control, for which 0.2 ml of PBS were injected intraperitoneal. The other three groups (B0 + *S. iniae*, B1+ *S. iniae* and B2+ *S. iniae*) were intra-peritoneally injected with *S. iniae* (0.2 ml/fish). Values are expressed as mean $\pm$ SE from triplicate groups. Bars with different letters are significantly different from those of control group ($P < 0.05$).

Figure 7. Phagocytosis of the different treatments post feeding with *Bacillus* probiotic mixture and post-challenge with *S. iniae*. Three groups (B0, B1, B2) were identified as a control, for which 0.2 ml of PBS were injected intraperitoneal. The other three groups (B0 + *S. iniae*, B1+ *S. iniae* and B2+ *S. iniae*) were intra-peritoneally injected with *S. iniae* (0.2 ml/fish). Values are expressed as mean $\pm$ SE from triplicate groups. Bars with different letters are significantly different from those of control group ($P < 0.05$).

Figure 8. Histomicrograph of hepatopancreas of Nile tilapia received different concentration of probiotic and challenged with *S. iniae*. (A), (B), (C) hepatopancreas of B0, B1 and B2 groups showing normal hepatic and pancreatic portions (arrowhead and arrow respectively). (D) hepatopancreas of *S. iniae* infected group showing severe diffuse hepatic and pancreatic necrosis (arrow). (E) hepatopancreas of B1+ *S. iniae* group showing mild to moderate fatty change within hepatocytes (arrow). (F) hepatopancreas of B2+ *S. iniae* group showing mild fatty changes within hepatocytes (arrow). H&E. Bar= 100 μ m.

Figure 9. Histomicrograph of spleen of Nile tilapia. (A), (B) spleen of B0 and B1 groups showing normal white pulp (arrow head) consisting from the lymphoid elements and normal red pulp. (C) spleen of B2 group showing increase the white pulp (arrowhead) and melanomacrophage centers (arrow). (D) spleen of *S. iniae* infected group showing wide necrotic area with marked depletion of lymphoid tissue (arrow). (E) spleen of B1+ *S. iniae* group showing mild to moderate lymphoid depletion (arrow). (F) spleen of B2+ *S. iniae* group showing normal white (arrow) and red pulps. H&E. Bar= 100 μ m.

Figure 10. Histomicrograph of skin of Nile tilapia. (A), (B), (C) skin of B0, B1 and B2 groups showing intact skin (arrow) and normal muscle architecture (arrow head). (D) Skin of *S. iniae* infected group showing muscular degeneration (hyalinosis) (arrow) with interstitial edema associated with marked leukocytic infiltration (arrowhead). (E) skin of B1+ *S. iniae* group showing mild to moderate cutaneous necrosis with moderate degree of leukocytic infiltration (arrow). (F) skin of B2+ *S. iniae* group showing mild cutaneous necrosis with moderate degree of myolysis (arrow). H&E. Bar= 100 μ m.

Figure 11. Histomicrograph of cornea of Nile tilapia. (A), (B), (C) cornea of B0, B1 and B2 groups showing intact corneal epithelium (arrow head), stroma (S) and corneal endothelium (arrow). (D) cornea of *S. iniae* infected group showing loss of corneal epithelium and edema and degeneration in corneal stroma (S). (E) cornea of B1+ *S. iniae* group showing moderate interstitial edema in corneal stroma (S) and vacuolation in corneal epithelium (arrow head). (F) cornea of B2+ *S. iniae* group showing mild interstitial edema in corneal stroma (S) and vacuolation in corneal epithelium (arrow head). H&E. Bar= 100 µm.