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


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40
41 **Short Title:** Efficacy of *Bacillus* probiotics on *Streptococcus iniae* infected tilapia
42

**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*
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77 **Introduction**

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

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

93 Probiotics are one of the alternatives that have been widely used in the aquaculture industry
94 (Ali et al., 2008; Dawood et al., 2015a,b; Elsabagh et al., 2018). Probiotics are live microbial
95 feed additives which beneficially affect the host by improving its intestinal microbial balance
96 (Fuller, 1989). Previous studies highlighted the use of probiotics in aquaculture to enhance
97 growth performance, digestive enzymes, feed conversion ratio (FCR), immune response and
98 resistance to diseases (Adel et al., 2017; Gobi et al., 2018; Guardiola et al., 2016; Yilmaz et al.,
99 2019; Zaineldin et al., 2018). *Bacillus subtilis* is intensively used in aquaculture and has been
100 reported to promote growth, feed utilization, high antioxidant responses, improve water quality
101 and increase disease resistance (Chiu et al., 2010; Giri et al., 2014; Kumar et al., 2008; Newaj-

102 Fyzul et al., 2007; Yilmaz et al., 2020). In our previous trial, *B. subtilis* was added to Nile
103 tilapia feed and showed improved growth, FCR, health condition and immune response
104 (Elsabagh et al., 2018). In addition, *Bacillus* species enhanced fish health against variable
105 bacterial agents (e.g. *Streptococcus iniae*, *Aeromonas hydrophila* and *Vibrio parahemolyticus*)
106 (Cha et al., 2013; Ramesh et al., 2018; Yi et al., 2018).
107 *Streptococcus iniae* is a major fish pathogen affecting aquaculture (Acar et al., 2015). It
108 possesses many virulence genes that are responsible for entrance, propagation, and evasion of
109 immune defenses of fish and other aquatic species (Vazirzadeh et al., 2019). *Interleukin-8*
110 protease (*IL-8*) is one of the important *S. iniae* virulence genes which is a cell envelope protease
111 that can degrade the chemokine *IL-8* resulting in increased neutrophil resistance and contribute
112 to the pathogenesis of invasive streptococcal infection (Zinkernagel et al., 2008). M-like
113 protein (*simA*) is a surface protein and a major virulence factor of *S. iniae* that protects the
114 bacterium from the phagocytic activity and contributes to adherence to fish epithelial cells. C5a
115 peptidase (*scpl*) is a surface protein that hydrolyses the neutrophil chemoattractant complement
116 factor C5a and impairs the ability of the infected fish to fight *S. iniae* infection. Streptolysin
117 S ”SLS” (*sagA*) is a major virulence factor in the pathogenesis of *S. iniae* that hemolyses
118 erythrocytes and damages host cell membranes. *S. iniae* cytolysin affects erythrocytes,
119 neutrophils, lymphocytes and promotes cerebrovascular trauma. Capsular polysaccharide
120 (cpsD) is one of the most effective *S. iniae* genes that facilitates effective binding to the host
121 epithelial cells and to overcome phagocytosis (Baiano and Barnes, 2009).
122 The main objectives of this study were to identify and characterize *S. iniae* isolates collected
123 from infected Nile tilapia fish farms during summer outbreaks and assess the impact of using
124 the probiotic “*Bacillus* spp. mixture” on the immune response and resistance of Nile tilapia to
125 *S. iniae*.
126

127 **Materials and methods**

128 **Ethical approval**

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

131 **Isolation and identification of *S. iniae* field strain**

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

144 **Molecular Identification**

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

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

167 **Total bacterial count**

168 6 x 6 drop plate method was used for estimating the concentration of *S. iniae* strain and the
169 dose for the experimental studies (CFU/mL) following protocol of Chen et al. (2003) after
170 incubation at 37 °C for 24 h on TSA.

171 **Fish and experimental diet**

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

177 control), 0.1 g (B1) and 0.2 g (B2)/kg diet for ten weeks, respectively (Elsabagh et al 2018).
178 The final feed probiotic concentration for (B1) and (B2) diet, was $1 \times 10^6$ and $2 \times 10^6$ CFU/g
179 of feed, respectively.
180 By the end of the feeding trial, 360 Nile tilapia (100 ± 5.0 g average weight), were divided
181 into six groups; 60 fish/group (three replicates of 20 fish each). Fish were randomly allocated
182 in glass aquariums (50 × 60 × 100 cm); (20 fish/ aquarium) that was equipped with a continuous
183 aeration system to maintain enough dissolved oxygen. Tilapia were fed on the same diet as
184 above for 15 days. Three groups (B0, B1, B2) were identified as a control and were injected
185 intraperitoneal with 0.2 ml of PBS. The other three groups (B0 + *S. iniae*, B1+ *S. iniae* and B2+
186 *S. iniae*) were injected intraperitoneal with *S. iniae* (0.1 mL/fish) at a dose of $3.5 \times 10^7$ CFU
187 that matches a predefined $LD_{50}$ dose (Data not shown). Fish were observed for 15 days post-
188 challenge (dpc), mortalities were recorded daily, according to Cha et al. (2013) and dead fish
189 were moved for further analyses. The relative percentage of mortality determined according to
190 the following equation: Mortality rate = 100 - (test survival/control survival) *100.
191 **Blood sampling and analysis**
192 At 14 dpc, blood samples were collected (Feldman et al. 2000) from 3 fish/replicate. Each
193 sample was divided into two parts; one part in a 5 mL test tube on EDTA for haematological
194 analyses and the second part in a 1.5 mL Eppendorf tube for serum separation. Blood samples
195 were left to clot at 4°C for 60 min then centrifuged at 3000 rpm using an Eppendorf centrifuge
196 for 10 min and serum was collected. Serum samples were stored at -20 °C till analysis.
197 Red blood cells (RBCs) and white blood cells (WBCs) were counted immediately with a
198 hemocytometer after dilution using Hayme's, Natt and Herrick's solutions, respectively
199 (Houston, 1990). Packed cell volume (PCV) was determined according to Karimi et al. (2013).
200 Hemoglobin (Hb) was evaluated using the method of Blaxhall and Daisley (1973). Blood
201 smears were prepared for the determination of differential leukocyte counts (Anderson and

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

208 **Phagocytic activity and phagocytic index**

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

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

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

218

219 **Histopathological examination**

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

227 **Statistical analysis**

228 Survival analyses were performed on GraphPad prism version 8 (Graphpad software Inc., San
229 Diego, CA, USA) using Kaplan-Meier method with Mantel-Cox log-rank test (Kaplan and
230 Meier, 1958). Bonferroni correction test was applied for validation of multiple comparisons
231 data set and the alpha value (α) for each comparison was corrected using the current equation
232 α ¼ 0.05/n, where "n" is the total number of comparisons. Variance analysis (one-way
233 ANOVA) and Tukey's multiple comparison test were used to determine the significant
234 variation ($p$<0.05) using GraphPad Prism 5 (Graph-Pad Software, San Diego, CA, USA).
235 Results were expressed in mean ± SE.

236

237 **Results**

238 **Isolates characterization and identification of *S. iniae***

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

246 **Experimental challenge**

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

251 operculum and isthmus area, eye cloudiness and complete loss of scales with tail rot. However,
252 the groups injected with PBS did not show any clinical signs (Figure 3).
253 Different mortality rates were observed with the different concentration of probiotics 15 dpc.
254 The lowest mortality was recorded in group B2 (6.67%), while the highest was in B0+*S. iniae*
255 group (81.67%) (Figure 4).

256 **Haematological and biochemical parameters**

257 Results showed that hemoglobin content and RBCs count were significantly higher in B0, B1,
258 B1+*S. iniae*, B2 and B2+ *S. iniae* compared to B0+ *S. iniae*. Moreover, the RBCs count of B1
259 was significantly higher than that in B2+ *S. iniae*. The PCV% of B0+ *S. iniae* was lower
260 ($p<0.05$) than the other groups. On the other hand, there was no significant variation in PCV%
261 between B1 and B2 groups, but a significant decrease was noticed between B1+ *S. iniae* and
262 B2+ *S. iniae* compared to B1 and B2. The WBCs count was higher ($p<0.05$) in B1+*S. iniae*
263 than B0 but there was no significant elevation in the other groups compared to B0.
264 There was a significant ($p<0.05$) increase in lymphocyte % in all groups compared to B0.
265 Moreover, lymphocyte % of the B2+ *S. iniae* was significantly increased compared to other
266 groups. Neutrophil% was elevated ($p<0.05$) in B2 and B2+ *S. iniae* compared to other groups
267 while there was no significant difference between B0, B1 and B1+ *S. iniae*. Eosinophil% was
268 elevated in all groups compared to B0+ *S. iniae*. The highest degree of elevation was present
269 in B2+ *S. iniae* followed by B1+ *S. iniae*, B1, B0 and B2. Basophil% was significantly
270 increased in all groups compared to B0. There was no significant difference in monocyte%
271 among all groups. The hematological parameters are shown in Figure 5.
272 There were no significant difference in AST level among all groups (Figure 6). The ALT level
273 was significantly higher ($p<0.05$) in B0+ *S. iniae*, B1+ *S. iniae* and B2+ *S. iniae* compared to
274 B0, B1and B2. The alkaline phosphatase and creatinine levels were significantly higher
275 ($p<0.05$) 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).

301 The skin of B0, B1 and B2 groups was composed of intact epidermis and dermis connected
302 with the underlying muscular layers which are characterized by elongated, acidophilic and
303 multinucleated muscle fibers (Figures 10A-C). The *S. iniae* infected fish showed loss of skin
304 layers due to skin ulcer in addition to diffuse degeneration and necrosis of underlying
305 musculature with marked interstitial edema and leukocytic cell infiltration (Figures 10D). The
306 skin of B1+ *S. iniae* fish showed a moderate degree of cutaneous necrosis in addition to
307 interstitial edema and a moderate degree of leukocytic infiltration in the muscle layers (Figures
308 10E). The degree of necrosis and interstitial edema was decreased in B2+ *S. iniae* (Figures
309 10F).
310 The eyeball of B0, B1 and B2 revealed normal architecture of cornea; corneal epithelium,
311 corneal stoma, and corneal endothelium (Figures 11A-C). The eye of the *S. iniae* infected fish
312 showed loss of corneal epithelium and endothelium in addition to interstitial edema in the
313 corneal stroma (Figures 11D). The corneal epithelium appeared vacuolated with interstitial
314 edema in the corneal stroma in B1+ *S. iniae* while the cornea of B2+ *S. iniae* revealed a marked
315 decrease in the vacuolation and interstitial edema (Figures 11E and 11F, respectively).
316 **Discussion**
317 Infection with *S. iniae* is one of the challenges of farmed tilapia intensification in Egypt, where
318 *S. iniae* induces severe mortality in farms and results in a substantial economic loss (Neamat-
319 Allah et al., 2019; Zahran et al., 2019). The irrational use of antimicrobials in livestock
320 production and aquaculture resulted in antimicrobial resistance against infectious bacteria
321 (Garlock et al., 2020). Alternatively, a wide range of functional supplements with
322 immunomodulation potentials is recommended to substitute the antibiotics for sustainable
323 aquaculture (Dawood, 2020). Probiotics are proven to potentiate the immune response of
324 aquatic animals and are recommended as an alternative strategy for enhancing fish immunity
325 (Elsabagh et al., 2018). More specifically, beneficial bacterial cells exert bacteriocins that

326 enhance the phagocytosis and fish's innate immunity and increase the resistance against the
327 infections (Dawood and Koshio, 2016; Dawood et al., 2018).
328 In the present study, presumptive identification of *S. iniae* was carried out from colony
329 morphology on blood agar (Rahmatullah et al., 2017; Saleh et al., 2017). Phenotypic characters
330 of the isolates were similar to those reported by Oretega et al. (2018) and Rahmatullah et al.
331 (2017). The biochemical results were similar to those reported by previous studies (Tison et
332 al., 1982; Austin et al., 1997). Seventy-seven isolates were positive for *S. iniae* among 150
333 fish samples that were collected during summer outbreaks. The high incidence of
334 Streptococosis in summer may be attributed to the increase of water temperature (Saleh et al.,
335 2017). High water temperature during summer is considered a stress factor, promoting the
336 expression of various virulent factors, facilitating the invasion of *Streptococcus* to their hosts
337 (Kayansamruaj et al., 2014; Rodkhum et al., 2011). Interestingly, the five virulence genes
338 (*sagA*, *cpsD*, *scpl*, *simA* and *IL-8*) identified in the present study were specific to pathogenic
339 *S. iniae* (Locke et al., 2007; Locke et al., 2008; Baiano and Barnes 2009).
340 The median lethal dose fifty ($LD_{50}$) observed in the current study ($3.5 \times 10^6$) CFU is similar to
341 that used by Oretega et al. (2018) and Rahmatullah et al. (2017). However, Dong et al.
342 (2015) obtained $LD_{50}$ at a concentration of $10^2$ CFU, while Baums et al. (2013) reported that
343 $10^8$ CFU per mL of *S. iniae* could cause mortality in Nile Tilapia. The differences in $LD_{50}$
344 doses may be attributed to the number of virulent genes in *S. iniae* strain, pathogen properties,
345 as well as size, the weight of fish species and variations in water temperature at time of the
346 challenge or natural disease outbreak (Moustafa et al., 2016).
347 Observed signs in the experimentally infected fish may be due to variations of the virulent
348 genes of the selected isolates. Exophthalmia might be attributed to the pressure of accumulated
349 fluid in the abdomen on the eyeballs and/or due to the cytotoxic effect of *S. iniae* on the fish
350 cells (Locke et al., 2007). Ulcerative areas on the abdomen, erosion of the fins, loss of scales

351 and detachment might be attributed to streptolysin S (SLS) virulence factor which has a direct
352 cytotoxic effect against the fish cells (Locke et al., 2007). Haemorrhagic patches over the
353 operculum, mouth and pectoral fins may also attributed to the virulence factors (SLS) produced
354 by *S. iniae* causing blood cells death leading to hemorrhage in different parts of the body
355 (Locke et al., 2007). Eye cloudiness may attributed to *S. iniae simA* virulence factor which
356 facilitates invasion of streptococcus to the fish eyes leading to cloudiness due to bacterial
357 growth. Also streptococcus itself can penetrate the epithelial and endothelial barriers (Locke et
358 al., 2008). Little mortalities in other infected groups may be attributed to the immunostimulant
359 effect of the used probiotics. Similar results were observed in Nile tilapia fed with *Bacillus*
360 probiotic (Addo et al., 2017; Iwashita et al., 2015).
361 Hemato-biochemical variables are simple and applicable diagnostic tools to reveal the immune
362 response and the resistance against infections in many aquatic animals (Fazio et al., 2019). The
363 results showed that tilapia infected with *S. iniae* have impaired RBCs, WBCs, haemoglobin,
364 total protein, globulin, and albumin values; however, incorporating *Bacillus* probiotics into
365 their feed enhanced these values. These results indicate that *Bacillus* could potentiate the
366 immunity and metabolic functions in Nile tilapia and raise its resistance to *S. iniae* (Panigrahi
367 et al., 2010). Our results are supported by previous studies that illustrated that probiotics could
368 enhance the hemato-biochemical indices in fish (Dawood et al., 2019a,b; Elsabagh et al., 2018;
369 Firouzbakhsh et al., 2011; Reda and Selim, 2015; Irianto and Austin, 2002; Firouzbakhsh et
370 al., 2011). A high count of RBCs in tilapia treated with *Bacillus* shows non-anemic features
371 and availability of high Ferrin with the possible capability to scavenge the free radicals (e.g.,
372 ROS) which causes oxidative stress during the infection with *S. iniae*. In addition, the increased
373 count of WBCs indicates the enhanced immune responses (e.g., phagocytosis) to counteract
374 the infection in tilapia treated with *Bacillus*. The improved blood protein profile (total protein,
375 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 secrets 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).

400 Bacterial infection can be diagnosed by histopathological examinations, which present a clear
401 image of the possible inflammatory features induced by the infection (Chen et al., 2006; Turner,
402 2006). The intestinal function is disturbed by the pathogenic bacteria due to impaired barriers
403 immunity and mucosal integrity, followed by the overexpression of inflammation-related
404 cytokines. Once the inflammation occurred in the intestine, the remaining tissues in the body
405 are also damaged and lose their regular function. During the infection, disturbance of the
406 intestinal barrier's function is often associated with inflammation of the intestine and other
407 tissues (Liu et al., 2014). In the present study, the tissue damage was detected in the skin, liver,
408 spleen, and cornea of Nile tilapia infected with *S. iniae* and fed with or without *Bacillus*. The
409 results showed severe inflammation in these tissues, which were attributed to the impaired
410 function induced by toxins produced by *S. iniae*. These results also associated with the
411 dysfunction in the hepatic tissue (high ALT and ALP levels). Notably, incorporating *Bacillus*
412 in tilapia diets revealed a protective effect against the impact of *S. iniae* on liver, spleen, skin,
413 and cornea tissues.
414 As the skin is the first line of defence (Guardiola et al., 2014), it is crucial to determine the
415 impact of *S. iniae* and the expected defensive role of *Bacillus* in tilapia. The results showed
416 normal features in the tilapia skin fed on *Bacillus* and infected with *S. iniae,* but inflammation
417 and tissue damage were observed in infected tilapia that did not receive any probiotic
418 supplement in their feed. These results clearly showed the possible anti-inflammatory role of
419 *Bacillus* against *S. iniae* infection (Kong et al., 2017). The *S. iniae* infected tilapia showed loss
420 of skin layers due to skin ulcer in addition to diffuse degeneration and necrosis of underlying
421 musculature with marked interstitial edema and leukocytic cell infiltration (Das et al., 2013).
422 However, further studies are required to investigate the role of anti-inflammatory genes in
423 tilapia fed on *Bacillus* probiotics.
424

425 **Conclusion**

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

432

433 **Data Availability Statement**

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

436 **Author Contributions**

437 Authors contributed to the current study equally.

438 **Conflict of interest**

439 No conflict of interest.

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443

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

| Biochemical Tests | *Streptococcus iniae* strain |
|---|---|
| Gram | +ve |
| Morphology | Cocci |
| Motility | -ve |
| Catalase | -ve |
| Oxidase | -ve |
| Aesculin | -ve |
| Gelatinase | -ve |
| Haemolysis | *β-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.

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

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

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

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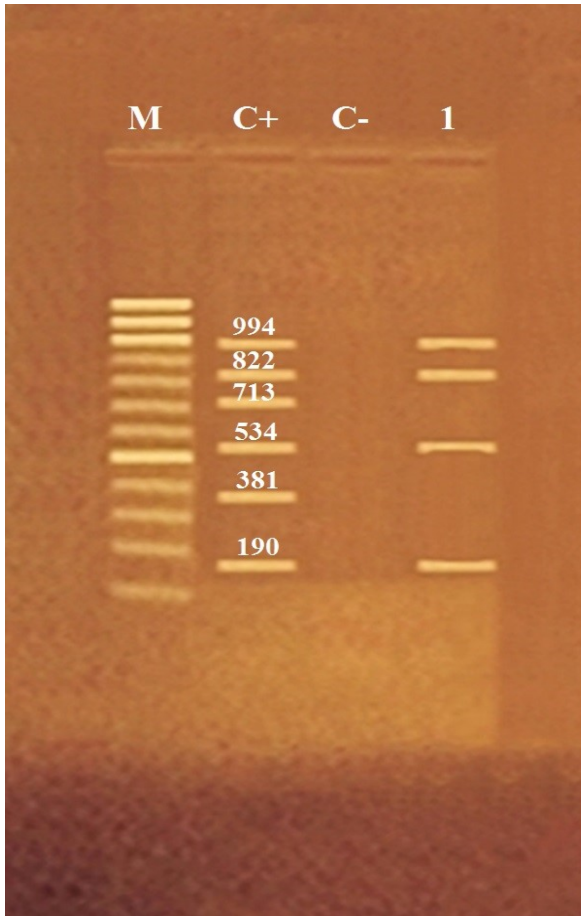


737 **Figure 1.** 1% agarose gel showing multiplex PCR results of 7 virulence-associated genes (*saga*
738 (190 bp), *pdi* (381 bp), *cpsD* (534 bp), *pgmA* (713), *scpl* (822) and *simA* (994 bp) of *S. iniae*
739 isolated in the current study. Lane M: 100 bp DNA marker, Lane C+: Positive control, Lane
740 C-: Negative control (RNAse free water), Lanes 1: DNA from selected *S. inaie* isolated in this
741 study.

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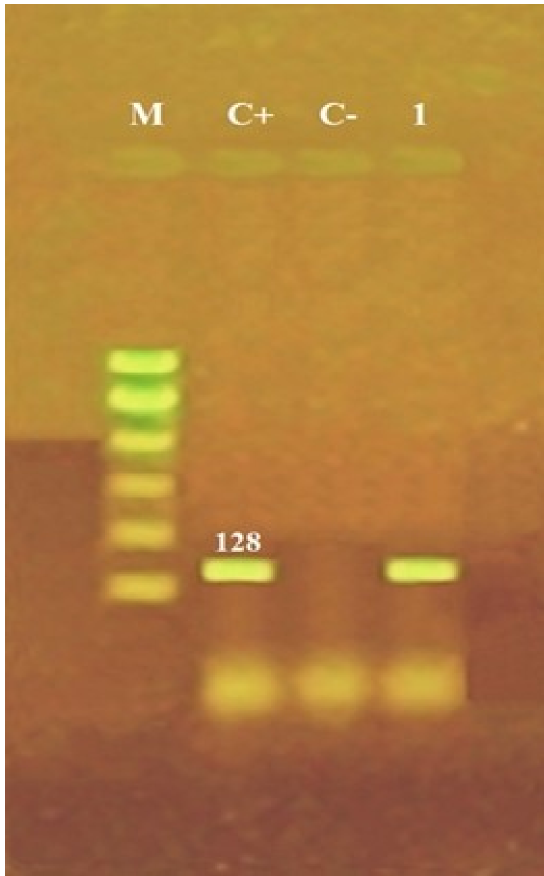


747 **Figure 2. 1%** Agarose gel showing PCR result of *IL-8* gene (128 bp) of *Streptococcus iniae*.
748 Lane M: 100 bp DNA marker, Lane C+: positive control, Lane C-: negative control (RNAse
749 free water only), Lanes 1: DNA from selected *S. iniae* from the current study.

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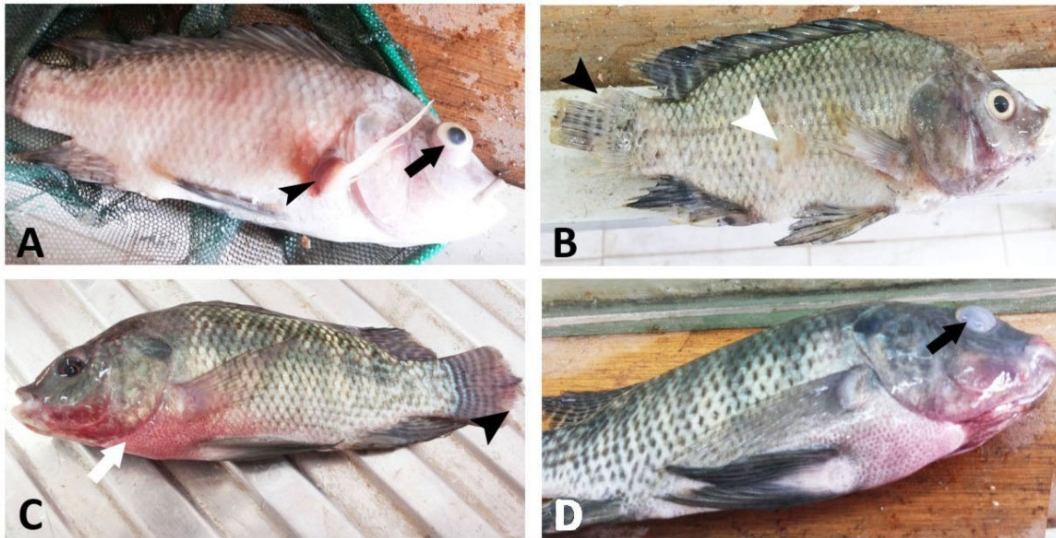


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760 **Figure 3.** Signs of *Streptococcus iniae* infection in experimentally challenged Nile tilapia. (A)
761 exophthalmia (pop-eye) (black arrow) and hemorrhages on the skin and base of pectoral fin
762 (black arrow head); (B) erosion of tail fin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

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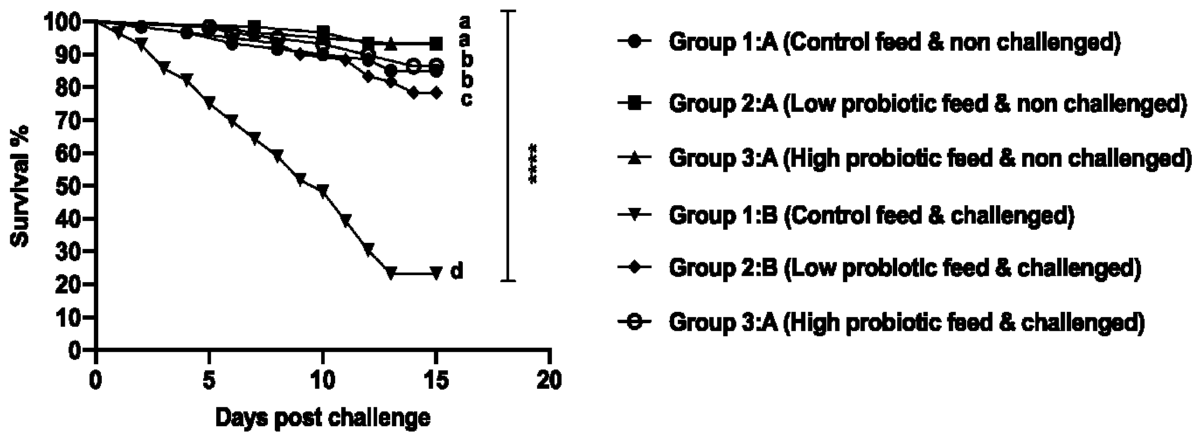


793 **Figure 4.** Kaplan-Meier (Log-rank Mantel Cox) representation of cumulative survival of
794 tilapia fingerlings at 15 dpc with $10^6$ CFU/mL of *S. iniae*. Each curve represents the average
795 results of three parallel tanks holding 20 fish/tank/challenge group. Groups that do not share
796 letters are significantly different ($p < 0.05$). Stars (****) denotes statistical significance ($p <$
797 $0.0001$) as determined by Log-rank (Mantel Cox) test.

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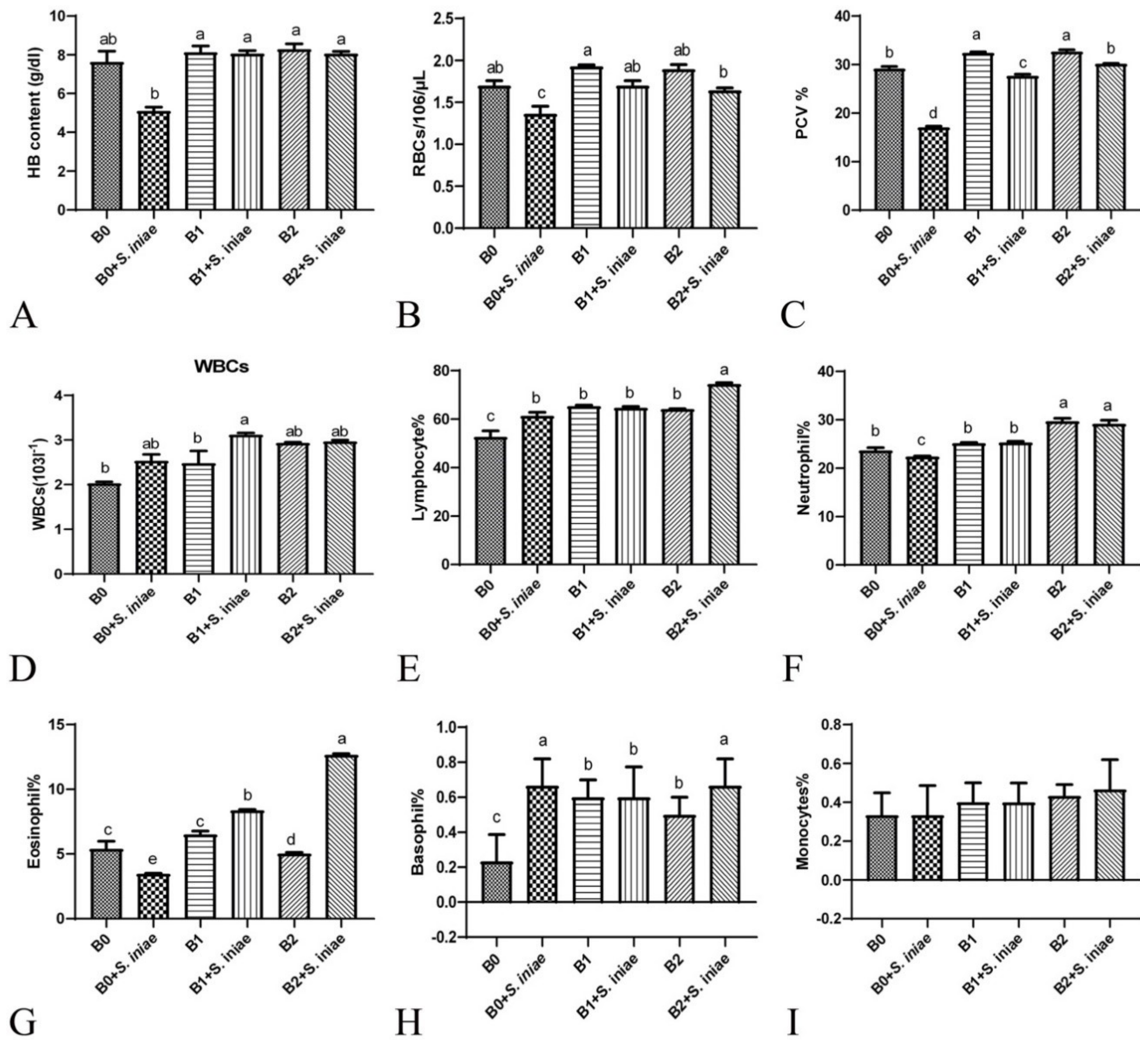


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802 **Figure 5.** Haematological parameters of the different treatments post feeding with *Bacillus*
803 probiotic mixture and post-challenge with *S. iniae*. Three groups (B0, B1, B2) were identified
804 as a control, for which 0.2 ml of PBS were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

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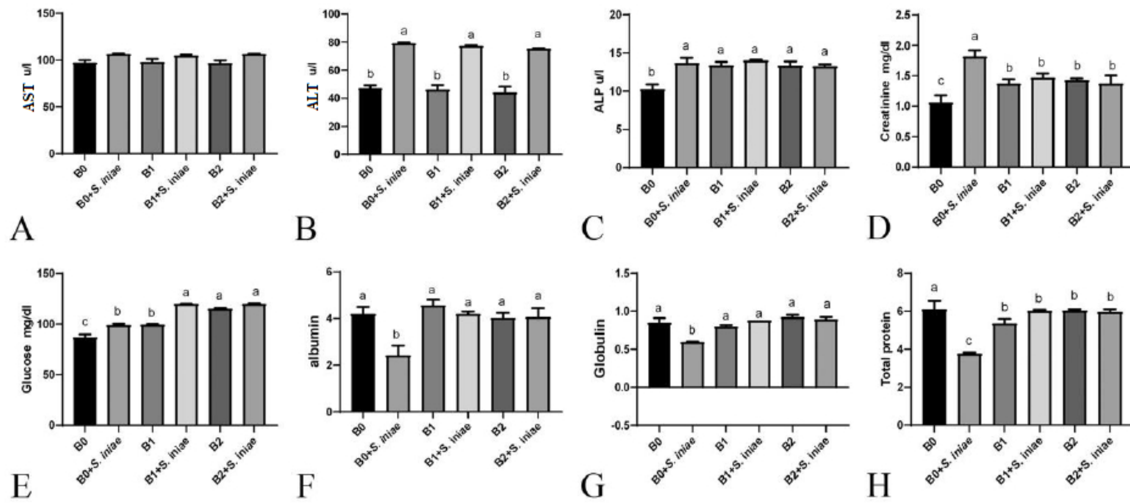


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811 **Figure 6.** Blood biochemical parameters of the different treatments post feeding with *Bacillus*
812 probiotic mixture and post-challenge with *S. iniae*. Three groups (B0, B1, B2) were identified
813 as a control, for which 0.2 ml of PBS were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

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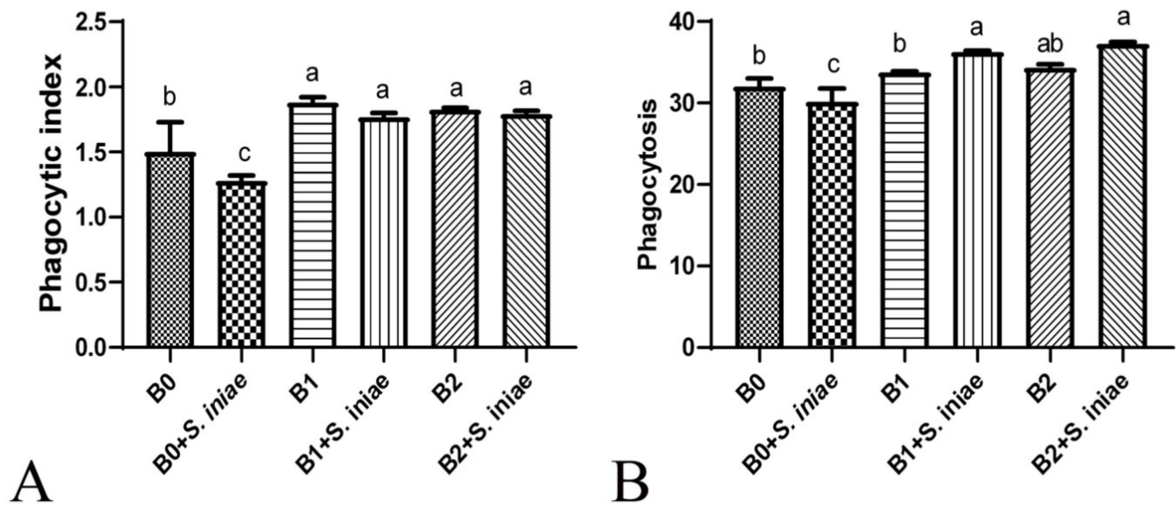


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833 **Figure 7.** Phagocytosis of the different treatments post feeding with *Bacillus* probiotic mixture
834 and post-challenge with *S. iniae*. Three groups (B0, B1, B2) were identified as a control, for
835 which 0.2 ml of PBS were injected intraperitoneal.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

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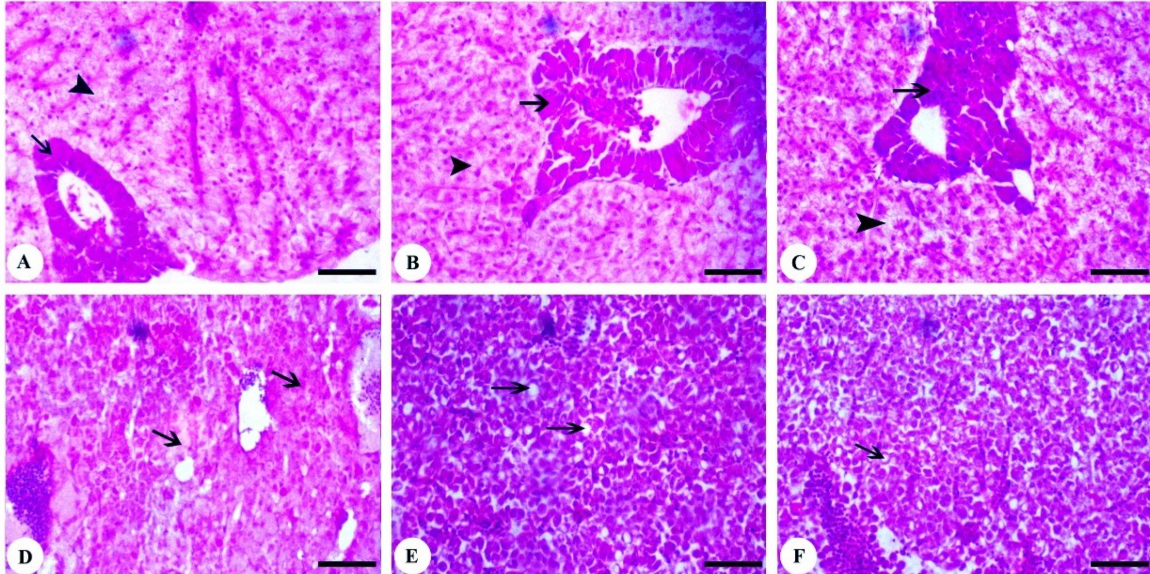


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845 **Figure 8.** Histomicrograph of hepatopancreas of Nile tilapia received different concentration
846 of probiotic and challenged with *S. iniae*. (A), (B), (C) hepatopancreas of B0, B1 and B2 groups
847 showing normal hepatic and pancreatic portions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

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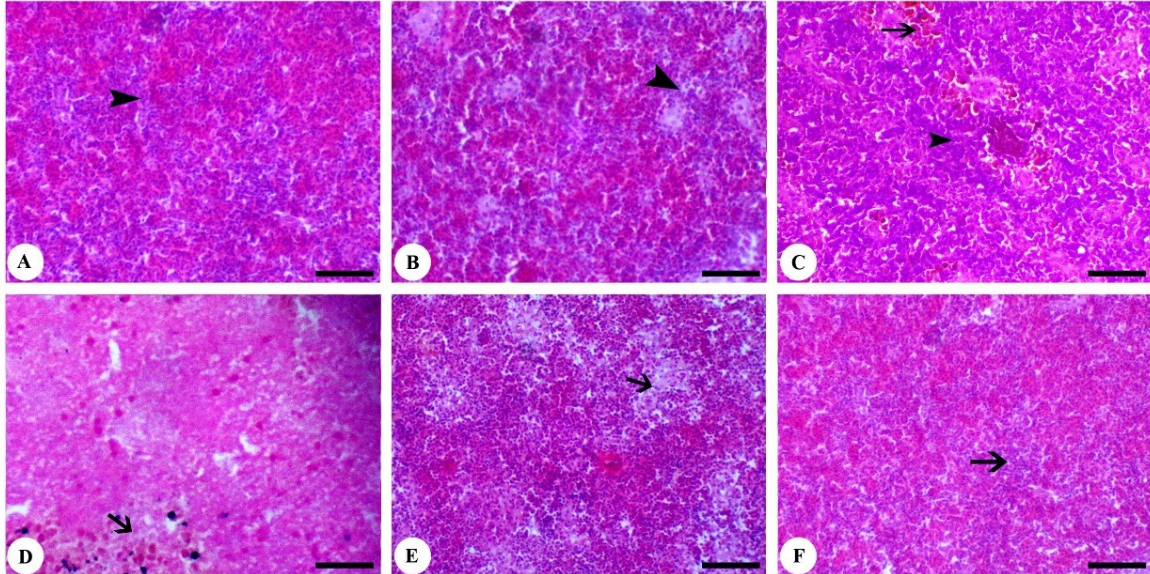


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859 **Figure 9.** Histomicrograph of spleen of Nile tilapia. (A), (B) spleen of B0 and B1 groups
860 showing normal white pulp (arrow head) consisting from the lymphoid elements and normal
861 red pulp. (C) spleen of B2 group showing increase the white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

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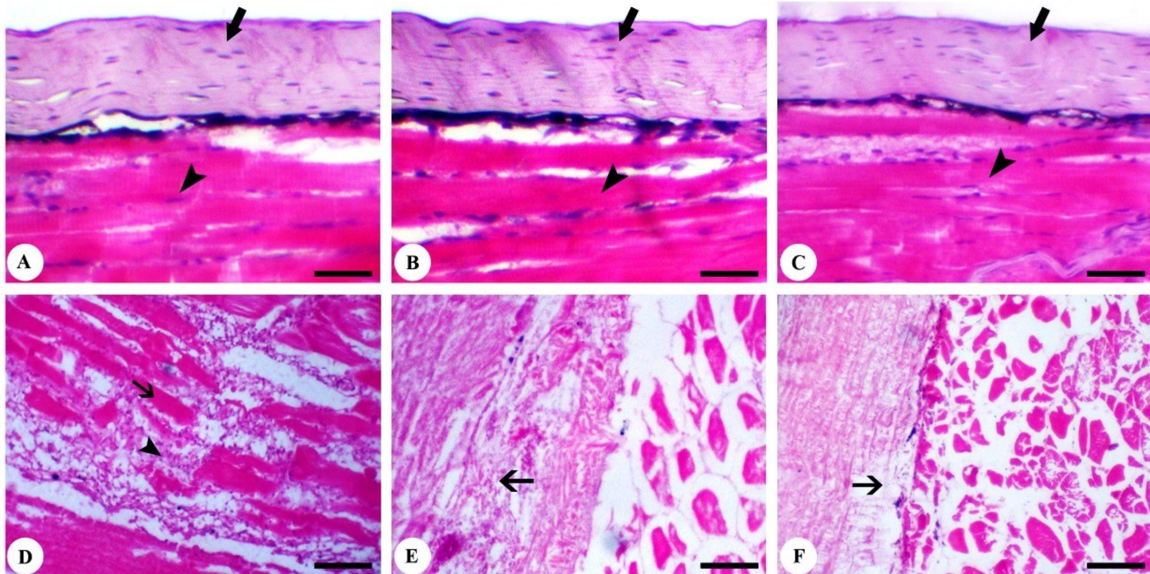


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871 **Figure 10.** Histomicrograph of skin of Nile tilapia. (A), (B), (C) skin of B0, B1 and B2 groups
872 showing intact skin (arrow) and normal muscle architecture (arrow head). (D) Skin of *S. iniae*
873 infected group showing muscular degeneration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

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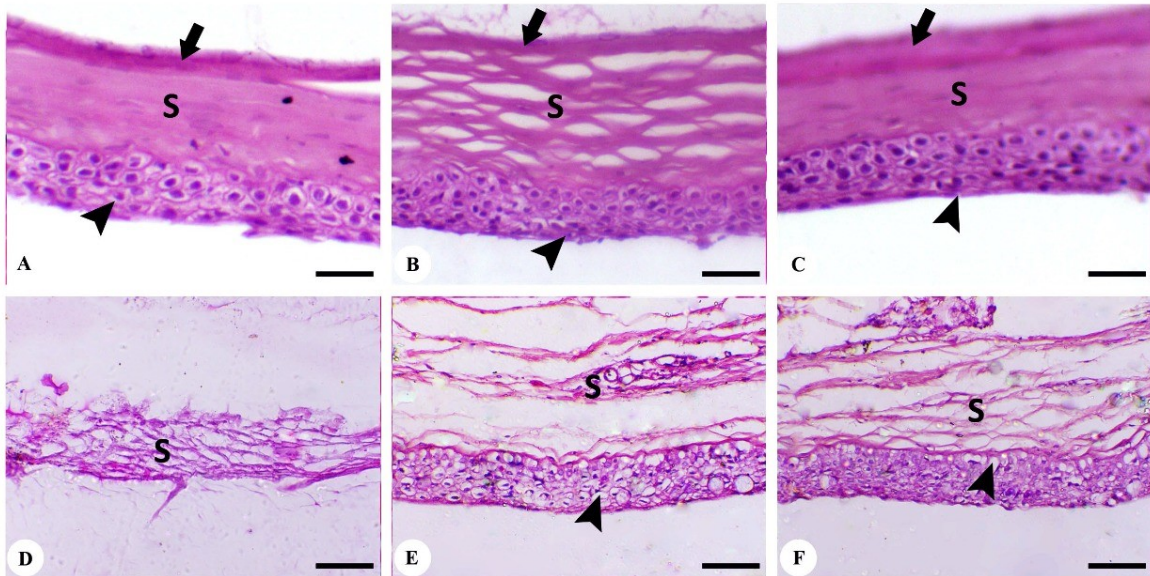


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883 **Figure 11.** Histomicrograph of cornea of Nile tilapia. (A), (B), (C) cornea of B0, B1 and B2
884 groups showing intact corneal epithelium (arrow head), stroma (S) and corneal endothelium
885 (arrow). (D) cornea of *S. iniae* infected group showing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

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