

1 **Assessing the impact of *Bacillus* strains mixture probiotic on water quality, growth**
2 **performance, blood profile and intestinal morphology of Nile tilapia, *Oreochromis***
3 ***niloticus***

4

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29 **Short Title:** *Bacillus*-based probiotics in Tilapia nutrition

30

31 **Abstract**

32 The aim of this study was to assess the impact of a commercial probiotic, Sanolife PRO-F, on
33 water quality, growth performance, blood profiles and intestinal morphometry of monosex Nile
34 tilapia. A field trial was conducted for 10 weeks in which tilapia fingerlings (20 ± 1.26 g) were
35 randomly distributed into three replicate ponds were sub-divided into three treatment groups,
36 receiving Sanolife PRO-F at 0 (B0), 0.1 (B1) and 0.2 (B2) g kg⁻¹ diet, respectively. The results
37 showed a significant improvement in growth performance, feed conversion ratio and blood
38 profiles in tilapia fed on treated diets. The whole intestinal lengths, anterior and terminal
39 intestinal villi heights and anterior goblet cells count were greater in tilapia fed on treated diets.
40 There were no noticeable differences in growth and intestinal morphology between tilapia fed
41 on B1 and B2 diets. The ammonia concentration in water was lower with B1 diet while electric
42 conductivity, salinity and total dissolved solids were higher with the B2 diet. The pH level of
43 pond water was enhanced by both diets, B1 and B2. In conclusion, application of Sanolife
44 PRO-F at 0.1-0.2 g kg⁻¹ diet might have beneficial effects on growth, immunity, stress
45 responses and gut health and function as well as the water quality of farmed Nile tilapia.

46

47 **KEY WORDS**

48 Nile tilapia, *Bacillus* probiotic, growth performance, intestinal morphology, water quality

49 1 INTRODUCTION

50 Egypt is one of the top ten aquaculture producing countries with an annual production of more
51 than one million tonnes (1,137,000) (FAO, 2016). In 2014, the aquaculture represented about
52 77% of the total fish production in Egypt, of which 85% was produced in a constructed pond-
53 based aquaculture around the Nile Delta lakes (GAFRD, 2016). Tilapia is the most commonly
54 cultivated species, representing more than 65% of the total aquaculture production (Dickson,
55 Nasr-Allah, Kenawy, & Kruijssen, 2016). In the last few years, profit margins decreased due
56 to high costs of production inputs particularly feed, which accounts for 70% of the total costs,
57 in addition to other production challenges (El-Sayed, Dickson, & El-Naggar, 2015; Eltholth,
58 Fornace, Grace, Rushton, & Häsler, 2015; MacFayden et al., 2011, 2012). Probiotics have been
59 used to improve the growth performance and decrease production costs of farmed tilapia in
60 many studies (Ibrahim, 2015; Hai, 2015; Taoka et al., 2006; Welker & Lim, 2011). Probiotics
61 are considered as safe alternatives to antibiotics, with several beneficial effects to the
62 aquaculture industry (Banerjee & Ray, 2017; Dawood & Koshio, 2016; Dawood, Koshio,
63 Ishikawa, El-Sabagh, Esteban, & Zaineldin, 2016; Pérez-Sánchez, Ruiz-Zarzuela, de Blas, &
64 Balcázar, 2014; Zorriehzahra et al., 2016) via different mechanisms such as competitive
65 inhibition of pathogenic bacteria through the production of inhibitory compounds,
66 enhancement of digestive enzymes activities which increase the availability of nutrients to the
67 host, improvement of water quality and enhancement of immune and stress responses of fish
68 (Balcázar et al., 2006; Ibrahim, 2015; Kesarcodi-Watson, Kaspar, Lategan, & Gibson, 2008;
69 Martinez Cruz, Ibanez, Monroy Hermosillo, & Ramirez Saad, 2012).

70 Fish are continuously interacting with the surrounding ecosystems and consequently,
71 the fish gut microbiota and aquatic environments are affected by the composition of the other's
72 microbial populations (Cahil, 1990; Giatsis et al., 2014; Giatsis, Sipkema, Smidt, Verreth, &
73 Verdegem, 2015). Public concerns regarding the use of antibiotics and sanitizers in aquaculture

74 are increasing due to the risk of the development of antibiotic resistance bacteria, a detrimental
75 issue not only for aquaculture but also for the consumers and terrestrial animals and
76 environment (Cabello, 2006; Cabello, Godfrey, Buschmann, & Dölz, 2016; Watts, Schreier,
77 Lanska, & Hale, 2017). Therefore, appropriate prophylactic alternatives to antibiotics should
78 be implemented in aquaculture production to maintain a healthy ecosystem, fish health and
79 immunity while improving the profitability (Defoirdt, Sorgeloos, & Bossier, 2011; Romero,
80 Feijoó, & Navarrete, 2012).

81 Previous studies reported that *Bacillus* isolates are promising probiotics candidates for
82 fish (Avella et al., 2010; Banerjee & Ray, 2017; Zorriehzahra et al., 2016). *Bacillus*-based
83 probiotics improved growth and health, digestive enzymes activities, and the intestinal
84 microbiota and morphology of tilapia as. These beneficial effects were demonstrated for
85 *Bacillus subtilis* (Addo et al., 2017; Liu et al., 2017; Standen et al., 2015, 2016; Taoka, Maeda,
86 Jo, & Sakata, 2007) and *Bacillus polyfermenticus* in tilapia broodstock and fry (Lukkana,
87 Jantrakajorn, & Wongtavatchai, 2015). The beneficial effects of *Bacillus amyloliquefaciens*
88 in cage-reared tilapia (Silva et al., 2015) and *Bacillus pumilus* in Nile tilapia reared in captivity
89 and in nature (Srisapoome & Areechon, 2017) were also demonstrated. The impact of a
90 combination of digestive enzymes and *Bacillus*-based probiotics (Adeoye et al., 2016) and a
91 probiotic blend of *Bacillus* with other viable bacteria (Ramos et al., 2017) in tilapia fingerlings
92 has been evaluated. Also, several reports have highlighted that probiotics, including *Bacillus*,
93 provide a more favorable environment for fish through reducing the proliferation of pathogenic
94 bacteria and harmful phytoplankton as well as via the bioremediation of organic wastes in
95 rearing water (Banerjee & Ray, 2017; Fukami, Nishijima, & Ishida, 1997; Ibrahim, 2015;
96 Martinez Cruz, Ibanez, Monroy Hermosillo, & Ramirez Saad, 2012; Zorriehzahra et al., 2016).
97 However, little is known about the impact of commercial probiotics composed of mixed
98 *Bacillus* strains on tilapia reared under the environmental conditions of tilapia farms in Egypt.

99 Therefore, the aim of this study was to investigate the impact of a probiotic blend of *Bacillus*
100 strains (*Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus*) on water quality, growth
101 performance, hemato-biochemical parameters and intestinal morphometry of Nile tilapia
102 (*Oreochromis niloticus*) reared in earthen ponds in Egypt.

103

104 **2 MATERIALS AND METHODS**

105 **2.1 Experimental design and fish management**

106 This study was carried out at a private tilapia farm in Kafrelsheikh governorate, Egypt.
107 Following two weeks of acclimatization to farm conditions, monosex Nile tilapia, *Oreochromis*
108 *niloticus*, (20 ± 1.26 g average weight, $n = 900$) were randomly stocked into 3 separate earthen
109 ponds, of 267 m² each and belong to the same farm. Each pond was subdivided into 3 equal
110 replicates using hapa nets, 100 fish each. Fish were fed a commercial tilapia diet (300 g kg⁻¹
111 crude protein and 12.6 MJ kg⁻¹ digestible energy) manufactured by ALEKHWA[®] feed factory
112 (Kafrelsheikh, Egypt). A probiotic blend of *Bacillus* strains (*Bacillus subtilis* 3.25×10^9 CFU
113 g⁻¹, *Bacillus licheniformis* 3.50×10^9 CFU g⁻¹ and *Bacillus pumilus* 3.25×10^9 CFU g⁻¹;
114 Sanolife PRO-F, INVE Aquaculture, Belgium, with a total number 1.0×10^{10} CFU g⁻¹) was
115 mixed daily with the basal diet, using sunflower oil (20 ml kg⁻¹ diet), at 0 g (B0: control), 0.1
116 g (B1) and 0.2 g (B2) kg⁻¹ diet, respectively. Fish were fed the experimental diets for 10 weeks,
117 with a feeding rate of 4% and 3% of body weight for the first two weeks and the last 8 weeks,
118 respectively.

119

120 **2.2 Fish performance, feed utilization and biometric indices**

121 Fish feed intake (FI) was recorded daily and fish growth was monitored biweekly for ten weeks.
122 At the end of the experiment, six fish were randomly sampled from each hapa, 18 fish per
123 treatment. Fish were harvested using 0.5 cm mesh size net and placed in separate polypropylene

124 containers then transported to the laboratory. Fish samples were dried using a clean and sterile
125 filter paper to remove the excess water before weighing. Fish were weighed using digital
126 balance (PW Balance, ADAM equipment Co., USA). The length and width of fish were
127 measured using a measuring board as described by Lagler (1978). The length was measured as
128 the distance from the snout to the beginning of the caudal fin. The length and weight of fish
129 were recorded to the nearest mm and 0.1 g, respectively. The length-weight relationship (LWR)
130 was calculated using the logarithmic regression formula: $W = a \times L^b$ while condition factor (K)
131 was calculated as $K = 100 \times W/L^3$, where W is the total weight (g) and L is the total length
132 (cm) whereas a and b are the regression slope and intercept (regression coefficient),
133 respectively, as reviewed by Froese (2006). Other growth assessment variables were calculated
134 as follows: body weight gain (BWG) = $(W_t - W_0)$, specific growth rate (SGR, % body
135 weight/day) = $100[(\ln W_t - \ln W_0)/t]$, weight gain rate (%) = $(W_t - W_0)/W_0 \times 100$, where W_0 and
136 W_t are the initial and final weights of live fish (g), respectively, and (t) is the feeding period in
137 days. Feed conversion ratio (FCR) was calculated as $FI (g)/BWG (g)$.

138

139 **2.3 Water quality analysis**

140 Dissolved oxygen (DO) was determined in each pond at 50 cm below the pond water surface
141 using a dissolved oxygen meter (AQ 600 Milwaukee, Romania). Three water samples were
142 collected from each pond by inverting 250 mL sterilized glass bottle 15 cm below the pond
143 water surface. Physio-chemical analysis of water samples was carried out to determine the total
144 ammonia (NH_3) using a portable colorimeter (Martini MI 405), pH, temperature, salinity,
145 electrical conductivity (EC) and total dissolved solids (TDS) using Multiparameter probe
146 apparatus according to Eaton, Clesceri, Rice, Greenberg, and Franson (2005).

147

148 **2.4 Blood sampling and serum separation**

149 Blood samples were taken from the caudal blood vessels (v. caudalis) from 18 fish per
150 treatment (6 fish per replicate) using a sterile syringe. Each sample was divided into two parts;
151 the first part was transferred into a 2-mL sterile test tube with EDTA for hematological assay
152 and the second part was kept in a 2-mL plain Eppendorf tube for serum separation. Blood was
153 left to clot at 4°C for 60 min. After that, tubes were centrifuged at 3000 rpm using an Eppendorf
154 centrifuge for 10 min for serum separation. The serum was collected in Eppendorf tubes and
155 stored at -40 °C until analyses.

156

157 **2.5 Hematological analysis**

158 The following blood parameters were measured: red blood cells (RBCs), hemoglobin,
159 hematocrit and total leukocytes count using an automatic blood cell counter (Exigo-Vet., Boule
160 Medical AB Inc., Stockholm, Sweden). Differential leukocytes count for the calculation of
161 heterophils to lymphocytes (H/L) ratio and monocytes were performed according to Anderson
162 & Siwicki (1995).

163

164 **2.6 Biochemical analysis**

165 Serum total protein was determined colorimetrically by using commercial kits (TP0100,
166 Sigma-Aldrich, USA). Serum albumin was measured using bromocresol green binding method
167 (Doumas, Watson, & Biggs, 1971). Serum globulin was calculated by subtracting albumin
168 values from total protein. Albumin/globulin (A/G) ratio was calculated by dividing albumin
169 values by globulin ones. Serum alkaline phosphatase (ALP), glutamic pyruvic transaminase
170 (GPT), glutamic oxaloacetic transaminase (GOT) and creatinine assays were performed as
171 described by Palti et al. (1999).

172

173 **2.7 Intestinal Morphometry**

174 Ten fish were randomly selected from each treatment. After deep anesthesia using 40% ethyl
175 alcohol, the abdomen was dissected, the total length of intestine was measured and specimens
176 from anterior (hepatic loop), middle and terminal parts of the intestine were sampled. The
177 samples were fixed in Bouin's solution for 18-24 hr, dehydrated in ascending concentrations
178 of ethanol and prepared for histological investigations. Sections of 4-5 μm thickness were
179 stained with hematoxylin and eosin for general morphometry and with periodic acid-Schiff
180 (PAS) for goblet cell staining according to Bancroft, Stevens, and Turner (1996). The length
181 of intestinal villi was measured by using image analysis software (NIH, Bethesda, MD).

182

183 **2.8 Statistical analysis**

184 After normality verification, data were analysed by a one-way ANOVA followed by Duncan's
185 multiple range test using GLM PROC of SAS (v. 9.4, SAS Institute Inc., Cary, NC, USA).
186 Results are presented as means \pm SE. The LWR was calculated by linear regression analysis of
187 SAS using the log-transformed data of weight and length. The level of significance and
188 tendency was set at $P < 0.05$ and $P < 0.1$, respectively.

189

190 **3 RESULTS**

191 **3.1 Water quality**

192 Water quality parameters are shown in Table 1. Ammonia concentration was significantly
193 lower ($P < 0.05$) in B1 pond than B2 and the control ponds while pH was higher ($P < 0.05$) in
194 both B1 and B2 ponds than the control. Water EC, TDS and salinity were significantly higher
195 ($P < 0.05$) in B2 than B0 and B1.

196

197 **3.2 Growth performance, feed utilization and biometric indices**

Table 1

198 In general, all growth performance parameters (fish final weight, BWG, SGR, WGR, length
199 and width) were improved by feeding B1 and B2 diets compared with B0 diet, Table 2. There
200 were significant differences ($P < 0.05$) for all parameters except for the length ($P < 0.1$). There
201 was no significant difference between B1 and B2 diets. For all performance parameters, the B2
202 group showed the highest values followed by B1 then B0 except for FCR, where B0 showed
203 the highest value followed by B1 then B2. There were no significant differences among
204 treatments regarding feed intake and condition factor ($P > 0.1$). The logarithmic regression of
205 LWR and determination coefficient values (R^2) are demonstrated in Figure 1. There was a
206 significant correlation ($P < 0.05$) between the length and the weight among all experimental
207 groups with an R^2 value of 0.48, 0.63 and 0.77 and regression slopes of 2.17, 2.55 and 2.96 for
208 B0, B1 and B2 treatments, respectively.

Table 2
Figure 1

209

210 **3.3 Hematological and biochemical parameters**

211 Results of hematological analysis are summarised in Table 3. The total leukocyte count was
212 significantly higher ($P < 0.05$) in fish fed on B1 and B2 diets than those fed on B0 diet, but
213 there was no significant difference between B1 and B2 diets. RBCs ($P < 0.1$), hematocrit ($P <$
214 0.05) and monocytes ($P < 0.1$) were higher in fish fed on B2 diet than those fed on B0 and B1
215 diets. Hemoglobin was higher while both of heterophils and H/L ratio were lower in fish fed
216 on B1 and B2 diets than those fed on B0 diet. Globulin was higher ($P = 0.054$) while A/G ratio
217 was lower ($P < 0.05$) in fish fed on B1 and B2 diets than those fed on B0 diet (Table 3).

Table 3

218

219 **3.4 Morphometric analysis**

220 The results of the morphological analysis are summarised in Table 4 and Figures 2 and 3. The
221 total length of the intestine was significantly increased ($P < 0.05$) by feeding B1 (95 cm) and
222 B2 (93 cm) diets compared with B0 diet (65 cm), but there was no significant difference
223 between B1 and B2 diets. The lining epithelium of the intestine was simple columnar cells,
224 which contain enterocytes, goblet cells and scattered ciliated cells. The length of the intestinal
225 villi in the anterior and terminal parts of the intestine was significantly increased ($P < 0.05$)
226 with probiotic feeding, but no significant changes were observed in the middle part of the
227 intestine. The number of PAS-positive goblet cells was significantly increased ($P < 0.05$) in
228 the anterior part of the intestine of fish fed B1 and B2 diets than that fed B0 diet.

Table 4
Figure 2
Figure 3

229

230 **4 DISCUSSION**

231 In Egypt, aquaculture industry, especially tilapia farming, is growing steadily making a
232 significant contribution to income and food security. Intensive fish farming is associated with
233 a high incidence of stress-related diseases which may lead to the use of antibiotics. The later
234 may result in developing antimicrobial resistance and/or the public health hazards. Probiotics
235 are considered a safe alternative to antibiotics. To the best of our knowledge, this is the first
236 trial to evaluate the effect of *Bacillus*-based probiotic on tilapia production in Egypt.

237 Values of water quality parameters reported in this study were within the range
238 desirable for tilapia farming (Boyd & Tucker, 1998). Ammonia was decreased by B1 diet while
239 EC, TDS and salinity were increased by B2 diet and pH was enhanced by both diets, B1 and
240 B2. These alterations might contribute to improving water quality and, consequently, fish
241 health and performance and could be attributed to the enhanced growth of beneficial bacteria
242 and planktons in ponds where tilapia were fed *Bacillus* supplemented diets (El-Haroun, Goda,
243 & Chowdhury, 2006; Fukami, Nishijima, & Ishida, 1997). Recently, it was reported that
244 *Bacillus* can displace *Vibrio* and colonize the gut of shrimp (Hostins et al., 2017). Accordingly,

245 bacteria shed with fish excreta might change the bacterial community in favor of water quality
246 improvement (Balcázar et al., 2006; Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000).
247 However, the Sanolife probiotic was delivered via feed and not directly added to pond water,
248 and we have no evidence regarding the abundance of the Sanolife probiotic in pond water in
249 our study. Effects of *Bacillus* probiotics on water quality, bacterial community and plankton
250 population of pond water deserve further research in a comparative approach, Sanolife
251 probiotic applied to feed and/or added to water.

252 Growth performance and feed utilization efficiency were significantly improved by
253 feeding *Bacillus* supplemented diets, implying a potential role of *Bacillus* probiotic in
254 mitigating stress factors and promoting fish welfare. Similar findings have been observed in
255 tilapia (Adeoye et al., 2016; Liu et al., 2017; Lukkana, Jantrakajorn, & Wongtavatchai, 2015;
256 Silva et al., 2015), gilthead sea bream (*Sparus aurata*) (Avella et al., 2010) and Eurasian perch
257 (*perca fluviatilis* L.) (Mandiki et al., 2011) fed *Bacillus*-based probiotics. Many studies
258 (Adeoye et al., 2016; Avella et al., 2010; El-Haroun, Goda, & Chowdhury, 2006; Liu et al.,
259 2017; Lukkana, Jantrakajorn, & Wongtavatchai, 2015; Mandiki et al., 2011; Silva et al., 2015;
260 Taoka, Maeda, Jo, & Sakata, 2007) demonstrated the ability of *Bacillus* to colonize the gut of
261 fish and accordingly enhance the production of organic acids, activation of digestive enzymes
262 and detoxification of the harmful constituents of feeds and collectively maintain a healthy gut
263 with a subsequent improvement in nutrient digestibility and absorption. Recently, it was
264 demonstrated that *Bacillus* can displace pathogenic bacteria from the gut and accordingly
265 enhance disease resistance and improve fish performance (Addo et al., 2017; Hostins et al.,
266 2017; Srisapoom & Areechon, 2017).

267 Importantly, feeding B2 diets resulted in an isometric growth pattern (i.e. proportional
268 increases in weight and length that give fish ideal shapes) as indicated by the slope value of
269 logarithmic regression of weight-length data (2.96), which approaches the value of ideal

270 growth (3.0) suggested by Froese (2006). The slope value of B1 (2.55) diet was lower than the
271 ideal growth value but still within the range of 2.5 to 3.5 estimated by Froese (2006) for several
272 fish species. On the contrary, the estimated value of B0 diet i.e. 2.17, was markedly lower than
273 the mean value of ideal growth, implying slender growth of fish in B0 group, i.e. length
274 increases more than weight. These findings further indicate the beneficial effects of probiotics
275 towards a more favorable growth form in fish farms (Froese, 2006).

276 The overall improvement in hematological characteristics reported in this study by
277 feeding *Bacillus* probiotics might indicate a role of *Bacillus* in stimulating certain immune and
278 stress responses of fish (Nayak, 2010). Similarly, leukocyte count, hematocrit and hemoglobin
279 were increased in Nile tilapia fed *Bacillus amyloliquefaciens* (Reda & Selim, 2015) and
280 monocytes were increased in *Labeo rohita* (Ham.) fed *Bacillus subtilis* (Kumar, Mukherjee,
281 Ranjan, & Nayak, 2008). Further, probiotic use has been associated with increased RBC and
282 leukocyte count in rainbow trout (Irianto & Austin, 2002) and increased RBCs, leukocytes,
283 hemoglobin with a reduction in heterophils in Oscar, *Astronotus ocellatus* (Firouzbakhsh,
284 Noori, Khalesi, & Jani-Khalili, 2011). In addition to enhancing fish immune and stress
285 responses through improving the hematological parameters, probiotics have also been reported
286 to improve the fish environment quality by interacting with harmful phytoplankton, resulting
287 in enhanced fish welfare (Fukami, Nishijima, & Ishida, 1997).

288 The results of the fish serum biochemical analysis in this study reflected a significant
289 increase in globulin accompanied by a significant decrease in A/G ratio in B1 and B2 groups,
290 potentially indicating a contribution of probiotic administration in promoting the immune
291 response of Nile tilapia. Similar increases in globulin were demonstrated in Nile tilapia fed
292 *Bacillus*-based probiotics (Reda & Selim, 2015; Zhou, Tian, Wang, & Li, 2017). The absence
293 of changes in ALP, GPT and GOT indicate that the probiotic used was safe for the fish
294 metabolic health. The roles of *Bacillus*-based probiotics in enhancing immune status of Nile

295 tilapia have been described in detail elsewhere (Addo et al., 2017; Liu et al., 2017; Srisapoome
296 & Areechon, 2017; Wang et al., 2017).

297 The current study revealed that the heights of the intestinal villi in the anterior and
298 terminal parts of the intestine, as well as the number of PAS-positive goblet cells in the anterior
299 part of the intestine, were significantly increased in the probiotic-treated groups compared with
300 the control group. Similar findings were described previously in Nile tilapia (Mello et al., 2013;
301 Ramos et al., 2017; Reda & Selim, 2015). Goblet cells secrete mucus with bactericidal effects
302 and facilitate transport through the intestinal epithelium (Smirnov, Perez, Amit-Romach, Sklan,
303 & Uni, 2005). Higher counts of PAS-positive goblet cells form a protective mucus layer
304 maintaining the integrity of the intestinal epithelium in addition to preventing the entry of
305 pathogens into the intestinal tract (Ellis, 2001). Despite there is no evidence of mucus
306 production markers in the current study, enhanced mucus secretion with increasing the activity
307 of gut mucosal immunity has been associated with probiotics administration in fish (Lazado &
308 Caipang, 2014; Nayak, 2010). The role of the gut in nutrient digestion and absorption is well-
309 known in fish (Grosell, Farrell, & Colin, 2010). In addition, the intestinal villi height, muscular
310 layer thickness and the goblet cells count are good indicators of a healthy intestine (Khojasteh,
311 2012). Therefore, the increased intestinal absorptive area, with a subsequent increase in nutrient
312 absorption and retention, and the enhanced goblet cells count highlight the observed
313 improvement in growth performance, immune response and stress resistance in Nile tilapia of
314 our study.

315 In conclusion, the results demonstrated that dietary supplementation of *Bacillus* strains
316 probiotic improved the growth performance and feed utilization of farmed tilapia. It also
317 enhanced certain markers of immune and stress responses particularly the hematocrit, RBC,
318 total leukocyte count, monocytes and globulin. Moreover, the total length of the intestine,

319 heights of intestinal villi and the numbers of the intestinal goblet cells were improved, and the
320 fish's environment was more favorable with *Bacillus* probiotics administration.

321

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514 **Figure legends**

515 **FIGURE 1** Logarithmic regression of weight (W) and length (L) data of Nile tilapia fed
516 *Bacillus* strains mixture probiotic at 0, 0.1 and 0.2 g kg⁻¹ diet; B0, B1 and B2, respectively.

517 **FIGURE 2** Hematoxylin-eosin-stained photomicrograph of the anterior, middle and terminal
518 parts of the intestine of Nile tilapia fed *Bacillus* strains mixture probiotic at 0, 0.1 and 0.2 g kg⁻¹
519 diet; B0, B1 and B2, respectively.

520 **FIGURE 3** Periodic acid–Schiff -stained photomicrograph of the anterior part of the intestine
521 showing the difference in the number of goblet cells in the intestinal villi of Nile tilapia fed
522 *Bacillus* strains mixture probiotic at 0, 0.1 and 0.2 g kg⁻¹ diet; B0, B1 and B2, respectively.

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