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

Mabrouk Elsabagh[1*], Radi Mohamed[2], Eman M. Moustafa[3], Ahmad Hamza[4], Foad Farrag[5], Olivier Decamp[6], Mahmoud A.O. Dawood[7], Mahmoud Eltholth[8, 9]

[1]*Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt*

[2]*Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafrelsheikh, Egypt*

[3] *Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt*

[4]*AQUAVET for fish nutrition and health solutions, Alfarouk towers, Zohdy Square, Kafrelsheikh, Egypt*

[5] *Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt*

[6] *INVE Asia Services 471 Bond St., Tambon Bangpood, Amphur Pakkred, Nonthaburi 11120, Thailand*

[7]*Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University, Kafrelsheikh, Egypt*

[8]*Department of Hygiene and Preventive Medicine, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt*

[9] *Institute of Aquaculture, University of Stirling, Stirling, United Kingdom, FK9 4LA*

***Corresponding author:** Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516 Kafrelsheikh, Egypt. E-mail address: mabrouk.elsabagh@vet.kfs.edu.eg (Elsabagh, M.).

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

Abstract

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

KEY WORDS

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

1 INTRODUCTION

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

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

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

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

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

2 MATERIALS AND METHODS

2.1 Experimental design and fish management

This study was carried out at a private tilapia farm in Kafrelsheikh governorate, Egypt. Following two weeks of acclimatization to farm conditions, monosex Nile tilapia, *Oreochromis niloticus*, (20 ± 1.26 g average weight, n = 900) were randomly stocked into 3 separate earthen ponds, of 267 m^2 each and belong to the same farm. Each pond was subdivided into 3 equal replicates using hapa nets, 100 fish each. Fish were fed a commercial tilapia diet (300 g kg^{-1} crude protein and 12.6 MJ kg^{-1} digestible energy) manufactured by ALEKHWA® feed factory (Kafrelsheikh, Egypt). A probiotic blend of *Bacillus* strains (*Bacillus subtilis* 3.25×10^9 CFU g^{-1}, *Bacillus licheniformis* 3.50×10^9 CFU g^{-1} and *Bacillus pumilus* 3.25×10^9 CFU g^{-1}; Sanolife PRO-F, INVE Aquaculture, Belgium, with a total number 1.0×10^{10} CFU g^{-1}) was mixed daily with the basal diet, using sunflower oil (20 ml kg^{-1} diet), at 0 g (B0: control), 0.1 g (B1) and 0.2 g (B2) kg^{-1} diet, respectively. Fish were fed the experimental diets for 10 weeks, with a feeding rate of 4% and 3% of body weight for the first two weeks and the last 8 weeks, respectively.

2.2 Fish performance, feed utilization and biometric indices

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

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

2.3 Water quality analysis

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

2.4 Blood sampling and serum separation

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

2.5 Hematological analysis

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

2.6 Biochemical analysis

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

2.7 Intestinal Morphometry

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

2.8 Statistical analysis

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

3 RESULTS

3.1 Water quality

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

Table 1

3.2 Growth performance, feed utilization and biometric indices

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

Table 2

Figure 1

3.3 Hematological and biochemical parameters

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

Table 3

3.4 Morphometric analysis

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

Table 4

Figure 2

Figure 3

4 DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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Figure legends

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

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

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