German Journal of Veterinary Research

eISSN:2703-1322

Research article

# The combined effects of probiotic CLOSTAT® and Aviboost® supplement on growth performance, intestinal morphology, and immune response of broiler chickens

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**Article History:**
Received: 01-Aug-2023
Accepted: 10-Sep-2023
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**Abstract**

The present study assessed the effects of dietary supplementation of probiotic CLOSTAT®, alone or in combination with Aviboost® supplement, on growth performance, intestinal histomorphology, and immune response in broiler chickens. A total of 600 one-day-old broiler chicks were divided into three groups: G1 (non-treated negative control group), G2 (probiotic CLOSTAT®- and Aviboost®-treated group), and G3 (probiotic CLOSTAT®-treated group). Feed intake and mean body weight were measured weekly for all groups. Sera were collected for cytokine analysis, and duodenal samples were also collected for histomorphological examination. The results revealed that the mean body weight gain was significantly increased to 2.25 and 2.2 kg/bird in G2 and G3, respectively, compared to 1.95 kg/bird in G1. Similarly, the feed conversion ratio (FCR) was improved to 1.56 and 1.59 in G2 and G3, respectively, compared to 1.8 in G1. Serum interferon-γ (IFN-γ) and interleukin (IL)-6 protein concentrations were significantly increased in G2 and G3 compared to G1. Furthermore, the absorptive cells of the villi revealed structural changes, including hyperplasia and increased goblet cell population and microvilli height, in G2 and G3 compared to G1. The lamina propria of duodenal villi in G2 and G3 showed increased cellularity at 22 days of age. In conclusion, the individual supplementation of CLOSTAT® and Aviboost® led to enhanced performance, intestinal morphology, and immune response, however their simultaneous supplementation slightly improved the body weight gain and FCR but did not exhibit synergistic or additive effects on intestinal morphology and systemic immune response.

**Keywords:** Chicken, *Bacillus*, Probiotic, Nucleotides, Performance, Immunity

**Citation:** Abed, A. H., Radwan, I. A., Orabi, A. and Abdelaziz, K. 2023. The combined effects of probiotic CLOSTAT® and Aviboost® supplement on growth performance, intestinal morphology, and immune response of broiler chickens. Ger. J. Vet. Res. 3 (3): 7-18. https://doi.org/10.51585/gjvr.2023.3.0058

## Introduction

The alarming increase of antibiotic-resistant bacteria and public health concerns surrounding the use of antimicrobial growth promoters (AGPs) in poultry feed have placed increased pressure on researchers around the globe to find ways to substitute these antimicrobials (Kairmi et al., 2022; Helmy et al., 2023; Taha-Abdelaziz et al., 2023). Various feed additives, including probiotics, prebiotics, phytobiotics, enzymes, fatty acids, and immunostimulants, have been suggested as potential alternatives for growth-promoting antimicrobials (Taha-Abdelaziz et al., 2018; Abd El-Hack et al., 2021). Among these additives, the inclusion of probiotics in poultry feed has been found to possess many beneficial effects on the host (Taha-Abdelaziz et al., 2019, 2023).

Indeed, dietary supplementation of probiotics has been shown to improve growth performance (Alagawany et al., 2018; Raheel et al., 2019; Śliżewska et al., 2020), modulate the composition of the gut microbiota, and maintain gut microbial homeostasis (Alizadeh et al., 2021b; Kulkarni et al., 2022). Furthermore, probiotics supplementation has also been found to increase host resistance to a wide range of enteric

pathogens by competing with them for nutrients and adhesion receptors on the intestinal epithelium (competitive exclusion) and by enhancing the intestinal mucosal and systemic immunity (Villagrán-de la Mora et al., 2019; Alizadeh et al., 2021a,b; Shojadoost et al., 2022). In addition to these effects, probiotics have been shown to improve the gastrointestinal tract's (GIT) physiological functions, including digestion and nutrient absorption (Kulkarni et al., 2019; Raheel et al., 2019; Fouad, 2020).

The most frequent microorganisms that are being used as probiotics include several species of bacteria, such as *Bacillus*, *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Pediococcus*, and *Butyricicoccus pullicaecorum* strains (Eeckhaut et al., 2016; Li et al., 2018; Mehdinejad et al., 2018; Villagrán-de la Mora et al., 2019). Other non-bacterial (fungal) probiotics have also been explored as feed additives for chickens, including *Saccharomyces cerevisiae*, *Saccharomyces bourlardii*, *Candida pintolopesii* and *Aspergillus oryzae* (Liu et al., 2018; Fouad, 2020; Villagrán-de la Mora et al., 2019). *Bacillus subtilis* PB6 is a bacterial probiotic recovered from the chicken GIT and was found to improve intestinal health, growth performance, and feed conversion ratio (FCR) (Jayaraman et al., 2017; Fouad, 2020), in addition to producing a heat-stable anti-clostridial factor, which in turn inhibits the growth and proliferation of *Clostridium perfringens*, thereby protecting chickens against necrotic enteritis caused by this pathogen (Jayaraman et al., 2013).

Despite these beneficial effects, there is no consensus on the potential of these probiotics to substitute the in-feed antibiotics (Sugiharto, 2021). Efforts have been made to improve the effects of probiotics by combining them with other feed additives, with prebiotics being the most used additive with probiotics. Prebiotics are non-digestible feed compounds that, when metabolized, provide fermentation substrates that support the growth and activity of probiotic bacteria (Salehimanesh et al., 2016). The combined effects of prebiotics and probiotics (synbiotics) on chickens' performance and health have been extensively studied (Salehimanesh et al., 2016; Fathi et al., 2017; Naghi Shokri et al., 2017; Raheel et al., 2019). The inclusion of synbiotics in poultry diets has been shown to enhance both cellular and humoral immunity (Fathi et al., 2017) by stimulating antibody production (Salehimanesh et al., 2016), enhancing bone marrow cell proliferation, promoting the production of interleukins (IL) and interferons (IFN) and potentiating the cytotoxicity of natural killer cells (Raheel et al., 2019).

In the context of their impact on gut health, synbiotics supplementation was also found to improve the differentiation and maturation of intestinal epithelial cells, increase the thickness of intestinal mucosa and villi, increase villi surface and crypts depth, and enhance the enzymatic activity of the digestive tract (Raheel et al., 2019; Fouad, 2020). However, while accumulating evidence suggests that the combination of prebiotics and probiotics exert additive and synergistic effects on the gut, there is still no convincing evidence that they could serve as effective alternatives to in-feed antibiotics in poultry production, especially for prevention and control of enteric diseases. Thus, further research is needed to investigate the potential of other feed additives to potentiate probiotics.

In the recent few decades, dietary supplementation of vitamins, minerals, and nucleotides has shown a remarkable ability to exert many beneficial effects on chickens' health and performance (Yu et al., 2002; Chiofalo et al., 2011; Sauer et al., 2011; Taha-Abdelaziz et al., 2018; Raheel et al., 2019). Nucleotides are "intracellular compounds with a low molecular weight involved in many biochemical processes" (Mehdinejad et al., 2018). They are the basic units of nucleic acids (RNA and DNA) that are frequently used to improve disease resistance, diminish mortality, and enhance the growth rate (Frankic et al., 2006) owing to their vital role in many enzymatic reactions involved in carbohydrates, proteins, and fat metabolism (Chiofalo et al., 2011).

Because of a high turnover rate, intestinal and immune cells, such as lymphocytes and bone marrow, may not sufficiently meet their nucleotide requirements (Yu et al., 2002). Thus, they may require nucleotide supplementation for their growth and maturation. We have recently shown that dietary supplementation of Aviboost, which contains vitamins, trace elements, essential fatty acids, essential amino acids, and nucleotides, exhibits growth-promoting effects on the intestinal epithelial cells by enhancing their differentiation, maturation, in addition to improving intestinal morphology by increasing the villus height and crypt depth (Raheel et al., 2019). Moreover, a previous study by Sauer and colleagues demonstrated that nucleotides possess immunomodulatory properties by enhancing bone marrow cell proliferation and interferons and interleukins production and improving the killing activity of natural killer cells (Sauer et al., 2011).

While the beneficial effects of probiotics when combined with a single feed additive, such as prebiotics, organic acids (Fatufe and Matanmi, 2011), vitamins, minerals, enzymes, and phytobiotics (Sugiharto et al., 2018), have been extensively studied, little is known about their effects when combined with multiple additives. Considering the growth-promoting and immunomodulating effects of the multi-nutrient Aviboost supplement (Raheel et al., 2019), the current study investigated the potential synergistic or additive effects of Aviboost and probiotic *Bacillus subtilis* PB6 on broilers' performance, intestinal morphology, and immune response.

## Materials and methods

### Experimental chickens

Six hundred one-day-old Cobb broiler chicks were divided into three treatment groups (G1-G3) (200 chicks per group), each with five replicates and 40 chicks in each replicate. All chicks were reared on a deep litter system and provided ad libitum feed, water, and a continuous light source. All chicks were vaccinated with

**Table 1:** The average body weight and feed conversion ratio (FCR) at different ages in different experimental groups.

| Age (weeks) | Average body weight per gram (g) | | |
|---|---|---|---|
| | Control group | *B. subtilis* PB6 and Aviboost treated group | *B. subtilis* PB6 treated group |
| 1 | 111.4±0.23 | 136.7±0.53* | 132.8±0.22* |
| 2 | 343.4±0.34 | 382.1±0.45* | 375.2±0.32* |
| 3 | 871±0.45 | 950.2±0.34* | 932.2±0.43* |
| 4 | 1470±0.53 | 1665±0.56* | 1600±0.45* |
| 5 | 1950±0.36 | 2250±0.46* | 2200±0.43* |
| FCR | 1.80±0.54 | 1.56±0.23* | 1.59±0.36* |

*indicates a significant increase in body weight ($p<0.05$).

**Table 2:** The average length of the microvilli in different experimental groups

| Age (days) | Average length of the microvilli ($\mu$m) | | |
|---|---|---|---|
| | Control group | *B. subtilis* PB6 and Aviboost treated group | *B. subtilis* PB6 treated group |
| 22 | 1.42± 0.45 | 1.88±0.22* | 1.88±0.12* |
| 31 | 1.42±0.43 | 2.00±0.34* | 2.40±0.14* |

*indicates a significant increase in villus height ($p<0.05$).

Newcastle disease vaccines (MSD, NY, USA), Hitchner B1 at six days of age and Lasota at 18 days of age, and infectious bursal disease vaccine (Intermediate plus strain) (MSD, NY, USA) at 13 days of age.

#### Probiotics and Aviboost supplement

*Bacillus subtilis* PB6 probiotics CLOSTAT (containing $2.0 \times 10^{11}$ CFU/g) was obtained from Kemin Industries, Inc, USA. Aviboost® Poultry Tonic was obtained from Bayer Animal Health, Reg. No. 23083 (Act 36/1947) contains 5000 mg/L nucleotides, 500 mg/L essential fatty acids, 3000 mg/L zinc, multivitamins, minerals, and amino acids.

#### Experimental design

The experimental design was approved according to the guidelines of the Animal Care Committee of Beni-Suef University (approval no. BSU-IACUC 021-195).

Chickens in the first group (G1) received a standard diet with no additives and served as a negative control group. The second group (G2; probiotic CLOSTAT and Aviboost® Poultry Tonic-treated group) received these additives at the manufacturer-recommended dose. Chicken received Aviboost for only 16 h daily in drinking water from day one to day five, 18-20, and 25-31 of age and received probiotic CLOSTAT 0.5 g/kg feed from day one to the last day of the experiment. The third group (G3) received the probiotic CLOSTAT only. Feed intake and mean body weight were measured weekly in all groups until day 31 of age. At 22 and 31 days of age, 15 chickens per group were randomly selected and humanely euthanized for sample collection. Blood samples were collected from all groups and sera were separated to measure IL-6 and IFN-$\gamma$ protein concentrations. Five duodenal samples were collected from each treatment group at 22 and 31 days of age and fixed in 10% buffered formalin or 2.5% buffered glutaraldehyde for histomorphological examination by bright field and electron microscopy, respectively.

#### Performance parameters

The daily feed intake was recorded for each group, and the body weight of individual birds was measured weekly from the first week of age until five weeks of age. At five weeks of age, the average feed intake and average body weight for chickens in all groups were used to calculate the feed conversion ratio (FCR) using the following formula: FCR = average feed intake/average live weights of birds per group.

#### Histomorphological examination using light and transmission electron microscopy (TEM)

Histomorphological changes of the duodenal mucosa in different experimental groups were assessed using light and electron microscopy at 22 and 31 days of age, according to Bozzola and Russell (1999). Following sample processing, paraffin-embedded duodenal sections (5 $\mu$m) were stained with hematoxylin and eosin and examined by light microscope, according to the method described by Salehimanesh et al. (2016). For TEM, duodenal samples were treated, and semi-thin sections were prepared and examined according to the protocol described by Raheel et al. (2019).

#### Enzyme-linked immunosorbent assay (ELISA)

Protein levels of chicken IFN-$\gamma$ and IL-6 were measured in the serum of all groups at 22 and 31 days of age using ELISA as per the manufacturer's instructions (Novatein Bio, Massachusetts, USA).

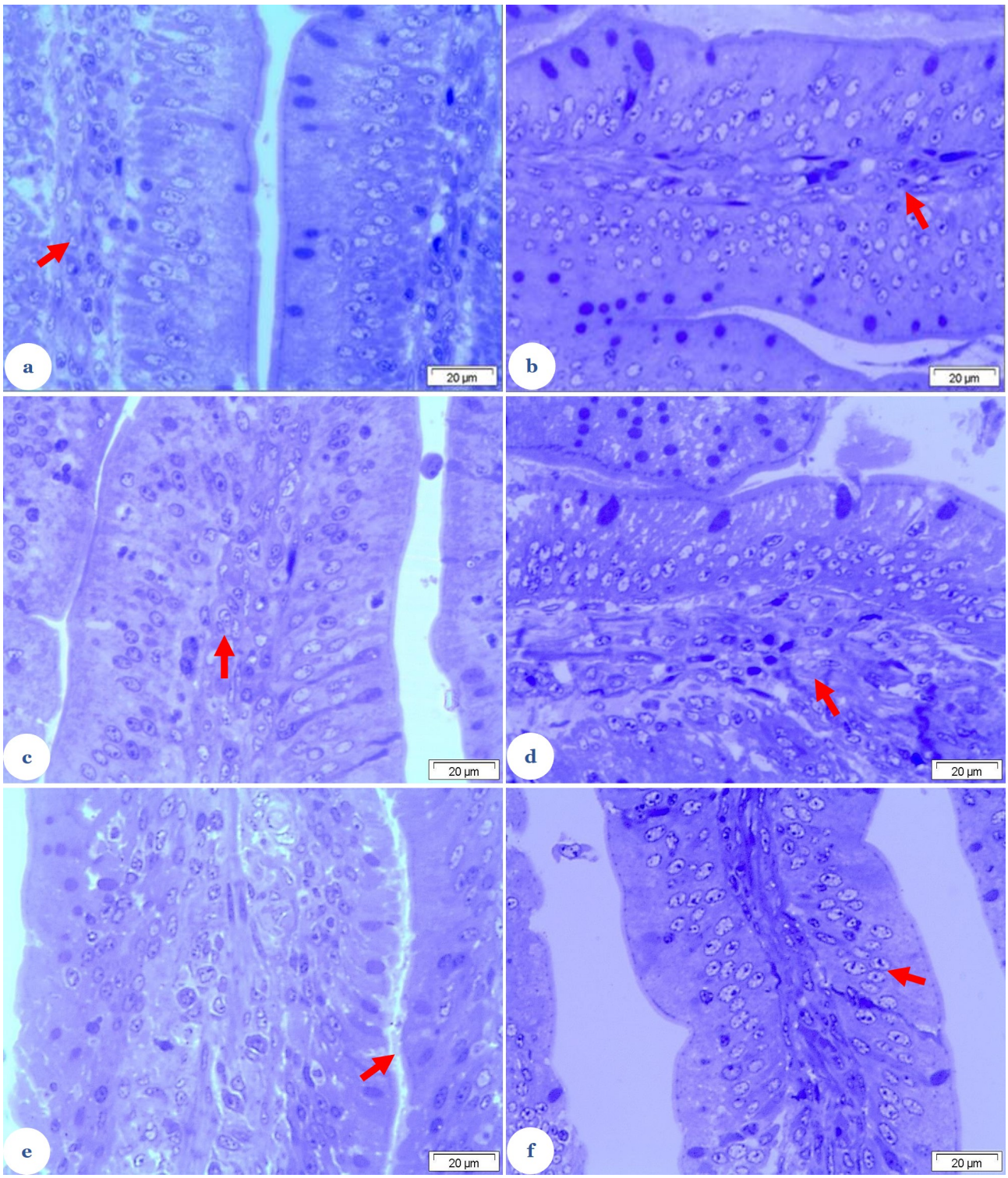


**Figure 1:** Light microscopic histological examination of the duodenum (semi-thin section of duodenal villi). Group 2 (Aviboost and *B. subtilis* PB6) at 22 days of age showed increased thickening of the villus core due to increased cellular reaction, mostly of lymphocytes and the covering epithelium showing hyperplasia with increased goblet cell population in between **(a)**, and slight congestion of the blood vessels with cellular reaction at 31 days of age **(b)**. Group 3 (*B. subtilis* PB6) at 22 days showed increased cellularity of the connective tissue core, mostly of lymphocytes, and numerous vacuolated cells containing deeply stained bodies near the luminal surface of the covering columnar absorptive cell layers and focal hyperplasia in the covering epithelium with vacuolation or intercellular edema, increased goblet cell population, thickened core of the villi due to cellular reaction **(c)**, and slight edema congestion of the blood vessels at 31 days of age **(d)**. Group 1 (Control group) at 22 days showed thin connective tissue cores covered by high columnar absorptive epithelial cells with oval vesicular nuclei situated mainly on the basement membrane, Numerous goblet cells were found, mostly in the upper part between the absorptive cells **(e)**, and mild focal hyperplasia of the absorptive cells at 31 days of age **(f)**.

**Statistical analysis**

All analyses were carried out using one-way ANOVA (Graphpad prism v6.07, Graphpad Software, San Diego, CA, USA) followed by Dunnett's multiple com-

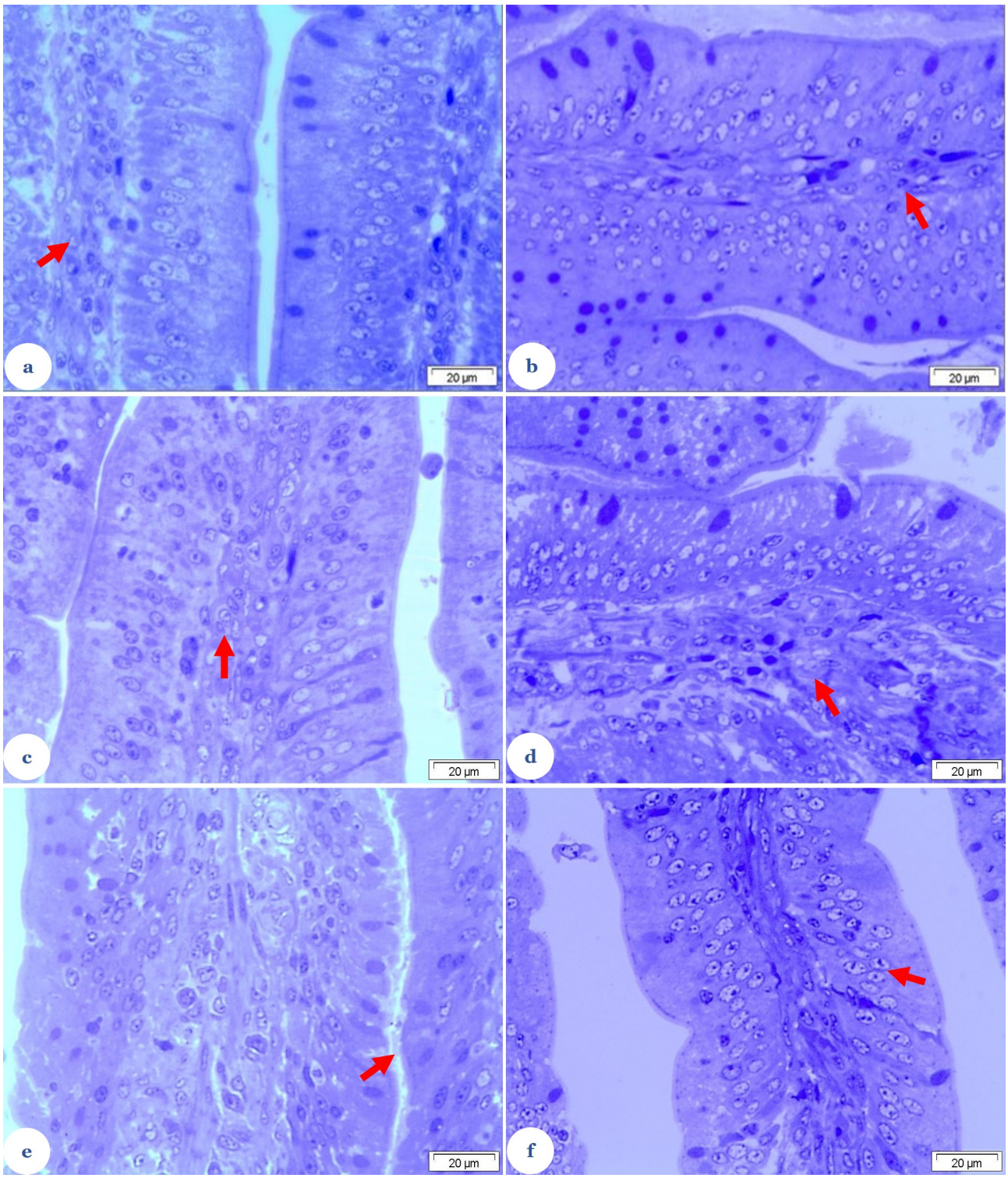


**Figure 2:** Electron microscopic examination of the duodenum. Group 2 (Aviboost and *B. subtilis* PB6) at 22 days of age showed absorptive cells (A) having erected microvilli (arrow) with average lengths of 1.88 and 2.0 $\mu$m, respectively. The cytoplasm of the cells contained mitochondria, free ribosomes, rough endoplasmic reticulum (RER), small electron-dense granules (g), and absorptive vacuoles (V) **(a)**, and the presence of small fat vacuoles (F) in the cytoplasm at 31 days of age **(b)**. Group 3 (*B. subtilis* PB6) showed absorptive cells (A) having erected microvilli (arrow) with average lengths of 1.88 and 2.4 $\mu$m, respectively. The cytoplasm of the cells showed vacuolation containing mitochondria, free ribosomes, RER, and a few electron-dense granules (g). There are numerous membrane absorptive vacuoles (V) variable sizes between and inside the cells at 22 days **(c)** and 31 days of age **(d)**. Group 1 (Control group) showed absorptive cells (A) having microvilli (arrow) with an average length of 1.42 $\mu$m. The cytoplasm of the cells contained mitochondria, free ribosomes, RER, absorptive vacuole (V), and a few electron-dense granules (g) at 22 days **(e)**, and goblet cells (G) destined with mucus globules embedded between the absorptive cells at 31 days of age **(f)**.

parisons to examine the significant differences between the treatment groups compared to the control group within the same time point. Cytokine graphs were created using GraphPad Prism v6.07. Data are presented

as means ± standard deviation (SD). Differences were considered significant if $p \leq 0.05$.

## Results

### Feed efficiency (feed intake and FCR) and body weight gain

The mean total feed intake was 708 kg per group at the 5$^{th}$ week of age. The mean body weight significantly increased from 1.95 kg /bird in G1 to 2.25 and 2.2 kg/bird in G2 and G3, respectively ($p<0.05$). A significant improvement of the FCR was also observed in G2 and G3 (1.56 and 1.59) as compared to G1 (1.8) ($p<0.05$; Table 1). The combination of CLOSTAT and Aviboost improved the body weight and FCR better than using each individually.

Results illustrated in Table 1 show the mean body weight gain and FCR in G1, G2, and G3. In the 1$^{st}$ week of age, the mean body weight significantly increased from 111.4 g/bird in G1 to 136.7, 132.8 g/bird in G2 and G3, respectively ($p<0.05$); in the 2$^{nd}$ week, from 343.4 g/bird in G1 to 382.1 and 375.2 g/bird in G2 and G3, respectively ($p<0.05$); in the 3$^{rd}$ week, from 871 g/bird in G1 to 950.2 and 932.2 g/bird in G2 and G3, respectively ($p<0.05$); in the 4$^{th}$ week, from 1470 g/bird in G1 to 1665 and 1600 g/bird in G2 and G3, respectively ($p<0.05$); and in the 5$^{th}$ week, from 1950 g/bird in G1 to 2250 and 2200 g/bird in G2 and G3, respectively ($p<0.05$). Meanwhile, the FCR improved to 1.56 and 1.59 in G2 and G3 compared to G1, respectively ($p<0.05$).

### Duodenal histomorphology using light and electron microscopy

Microscopic examination of the absorptive cells of the duodenal villi of chickens in the probiotic and Aviboost-treated group (G2) at 22 and 31 days of age revealed structural changes compared with G1, including epithelial hyperplasia with increased goblet cell population and increased microvilli length reaching 1.88 $\mu$m and 2 $\mu$m at 22 and 31 days, respectively (Table 2). The absorptive villus epithelium contained light electron-dense cells containing numerous membranes bounded fat globules in both groups. The endocrine cells were recorded at both ages, with many electron-dense polymorph secretory granules in their cytoplasm (Figure 1a&b and Figure 2a&b). The lamina propria of the duodenal villi contained numerous lymphocytes, macrophages, and plasma cells at 22 days (Figure 3a and Table 3), while the plasma cell population increased in number at 31 days (Figure 3b and Table 3). The activity of plasma cells was enhanced, as demonstrated by the dilation of the rough endoplasmic reticulum (RER) in the cytoplasm.

Microscopic examination of the duodenal villi of chickens in the probiotic-treated group (G3) at 22 and 31 days of age revealed structural changes compared with G1; probiotic treatment resulted in epithelial hyperplasia with increased goblet cell population and increased microvilli length up to 1.88 $\mu$m and 2.4 $\mu$m at 22 and 31 days, respectively ($p<0.05$; Table 2). The absorptive villus epithelium contained light electron-dense cells containing numerous membranes bounded fat globules (absorbed fat) in their cytoplasm (Figure 1c&d and Figure 2c&d). The lamina propria of the duodenum contained numerous lymphocytes, macrophages, and plasma cells at 22 days (Figure 3c&d and Table 3), while the plasma cell population increased in number at 31 days (Figure 3d and Table 3).

Microscopic examination of duodenal tissues of chickens in the control group (G1) at 22 and 31 days of age revealed standard morphological structure of the duodenal mucosa. The epithelial surface of the duodenal villi consisted of high columnar absorptive cells with microvilli in the luminal surface with an average length of 1.42 $\mu$m at 22 and 31 days (Table 2). Numerous goblet cells were found between the absorptive cells, especially in the upper part. At 31 days, mild focal hyperplasia of the absorptive epithelial cells and the appearance of a few small endocrine cells having small numbers of electron-dense granules and situated mainly on the basement membrane (Figure 1e&f and Figure 2e&f). The lamina propria of the duodenal villi showed a normal morphological appearance, small vasculature, and a few connective tissue cells and fibers without any pathological reaction at 22 days of age (Figure 3e&f and Table 3), while a few small plasma cells appeared at 31 days of age (Figure 3f and Table 3). No significant differences in the duodenal morphology and cellularity were observed between the treatment groups (G2 and G3).

### Villus cell population at different ages in the different experimental groups

The villus cell populations at 22 and 31 days of age in different experimental groups are illustrated in Table 3. Compared with G1, both G2 and G3 showed a moderate increase in epithelial cells and absorbed fat cells. The endocrine cell population showed a mild and moderate increase in G2 and G3, respectively, at 22 days of age, and a higher increase in their population was observed at 31 days of age. Moreover, lymphocytes and macrophage populations showed a mild increase in G2 and G3 at 22 and 31 days of age. The plasma cell population showed a moderate and high increase in G2 and G3, respectively. No significant differences in the villus cellular population were observed between the treatment groups (G2 and G3).

### Protein levels of IFN-$\gamma$ and IL-6

The results of serum cytokine concentration revealed an increase in both IFN-$\gamma$ and IL-6 protein levels in the probiotics-only group (G3) and Aviboost and probiotics group (G2) compared to the non-treated negative control group (G1). Results illustrated in Figure 4a show the average mean IFN-$\gamma$ protein concentration in the control and different treatment groups of broiler chickens at 22 and 31 days of age. At 22 days of age, the mean IFN-$\gamma$ concentration significantly increased from 125±0.56 pg/mL in the control

**Table 3:** The average length of the microvilli in different experimental groups.

| Cells | Average length of the microvilli ($\mu$m) | | | | | |
|---|---|---|---|---|---|---|
| | Day 22 of age | | | Day 31 of age | | |
| | Control group | *B. subtilis* PB6+ Aviboost group | *B. subtilis* PB6 group | Control group | *B. subtilis* PB6+ Aviboost group | *B. subtilis* PB6 group |
| Epithelial cells | – | ++ | ++ | + | ++ | ++ |
| Absorbed fat cells | – | ++ | ++ | – | ++ | ++ |
| Endocrine cells | – | ++ | + | – | ++ | ++ |
| Plasma cells | – | +++ | ++ | – | +++ | ++ |
| Lymphocytes | + | + | + | + | + | + |
| Macrophages | + | + | + | + | + | + |

*Scores; –; no cell changes, +; low cell changes, ++; moderate cell changes, +++; high cell changes.

group (G1) to 187±11 pg/mL and 183±7 in the probiotics and Aviboost-treated group (G2) and probiotics-treated group (G3), respectively ($p$<0.05). At 31 days of age, the mean IFN-$\gamma$ concentration significantly increased from 290±20 pg/mL in G1 to 443±32 and 436±43 pg/mL in G2 and G3, respectively ($p$<0.05).

Results illustrated in Figure 4b show the average IL-6 protein concentrations in the control and different treatment groups of broiler chickens at 22 and 31 days of age. At 22 days of age, the mean IL-6 concentration significantly increased from 16±0.76 pg/mL in G1 to 28±6 and 32±4 pg/mL in G3 and G2, respectively ($p$<0.05). At 31 days of age, the mean IL-6 concentration significantly increased from 21±3 pg/ml in G1 to 43±8 and 47±7 pg/mL in G3 and G2, respectively ($p$<0.05). No significant differences in the IFN-$\gamma$ and IL-6 protein levels were observed between the treatment groups (G2 and G3).

## Discussion

Besides their hazardous effects on the environment, long-term use of antibiotics as therapeutic agents or growth stimulators may result in the emergence of drug-resistant microorganisms that threaten both animal and human health (Śliżewska et al., 2020; Helmy et al., 2023). The discovery of the antimicrobial and immunomodulatory potentials of probiotics and other feed additives played a crucial role in enabling poultry farmers to phase out medically important antibiotic growth promoters (Markowiak and Śliżewska, 2018).

Although dietary supplementation of probiotics has been shown to modulate the immune response in chickens and improve their intestinal health (Fathi et al., 2017; Raheel et al., 2019); however, there is a consensus that the administration of probiotics alone cannot "completely" compensate for antibiotic effects and thus, the inclusion of other feed additives is needed to augment their impact (Sugiharto, 2021). We have recently shown that dietary inclusion of Aviboost modulates immune response and improves broiler chickens' body weight and intestinal morphology (Raheel et al., 2019). However, whether concurrent supplementation of Aviboost would augment the immunomodulatory and growth-promoting effects of probiotic *B. subtilis* PB6 remains to be determined.

Numerous studies have highlighted the role of probiotics, particularly *B. subtilis*, in improving poultry performance and health. For example, Teo and Tan (2006) demonstrated a significant improvement in the FCR in *B. subtilis* PB6-supplemented broilers raised under normal conditions. When supplemented with broiler chickens raised under heat stress conditions, Bioplus 2B probiotic (containing *B. subtilis* CH201 and *B. licheniformis* CH200) has also been shown to significantly improve their FCR (Rahimi and Khaksefidi, 2006). In another study, Abramowicz et al. (2019) indicated that administering 0.25 g/L of *B. subtilis* PB6 probiotic to chickens enhances their antioxidant defense mechanisms and growth performance. In a disease challenge model, Jayaraman et al. (2013) found that *B. subtilis* PB6 supplementation could mitigate necrotic enteritis caused by *C. perfringens* and improve broiler chickens' intestinal health.

Similar beneficial effects were observed for Aviboost supplementation. In addition to acting as a growth factor on the gut cells, supplementing Aviboost to chickens has also been shown to enhance the activity of digestive enzymes and gut absorptive capacity (Domeneghini et al., 2006). Additional reports suggest that yeast extract supplementation (rich in nucleotides, amino acids, vitamins, and minerals) improves chickens' performance, FCR, and productivity indices (Silva et al., 2009; Doo et al., 2019). Jung and Batal (2012) have also highlighted the critical role of nucleotides in maintaining the growth performance of heat-stressed chickens. The present study's findings are generally consistent with these observations; dietary inclusion of commercial *B. subtilis* PB6, either alone or combined with Aviboost, significantly improved performance-related parameters, including weight gain and FCR.

The integrity of the intestinal mucosa is regarded as an essential determinant of intestinal function. Therefore, any damage to the intestinal mucosa will negatively impact nutrient absorption and poultry growth performance. Increasing villus height, on the other hand, results in an increase in the surface area for nutrient absorption (Afsharmanesh and Sadaghi, 2014). We have recently shown that Avisboost supplementation increases villus height and provides adequate nucleotide pools that support the development and proliferation of intestinal cells, especially those with rapid turnovers such as epithelial cells and lymphocytes, and may, therefore, compensate for any decrease in de novo nucleotides synthesis by body organs in sick or heat-

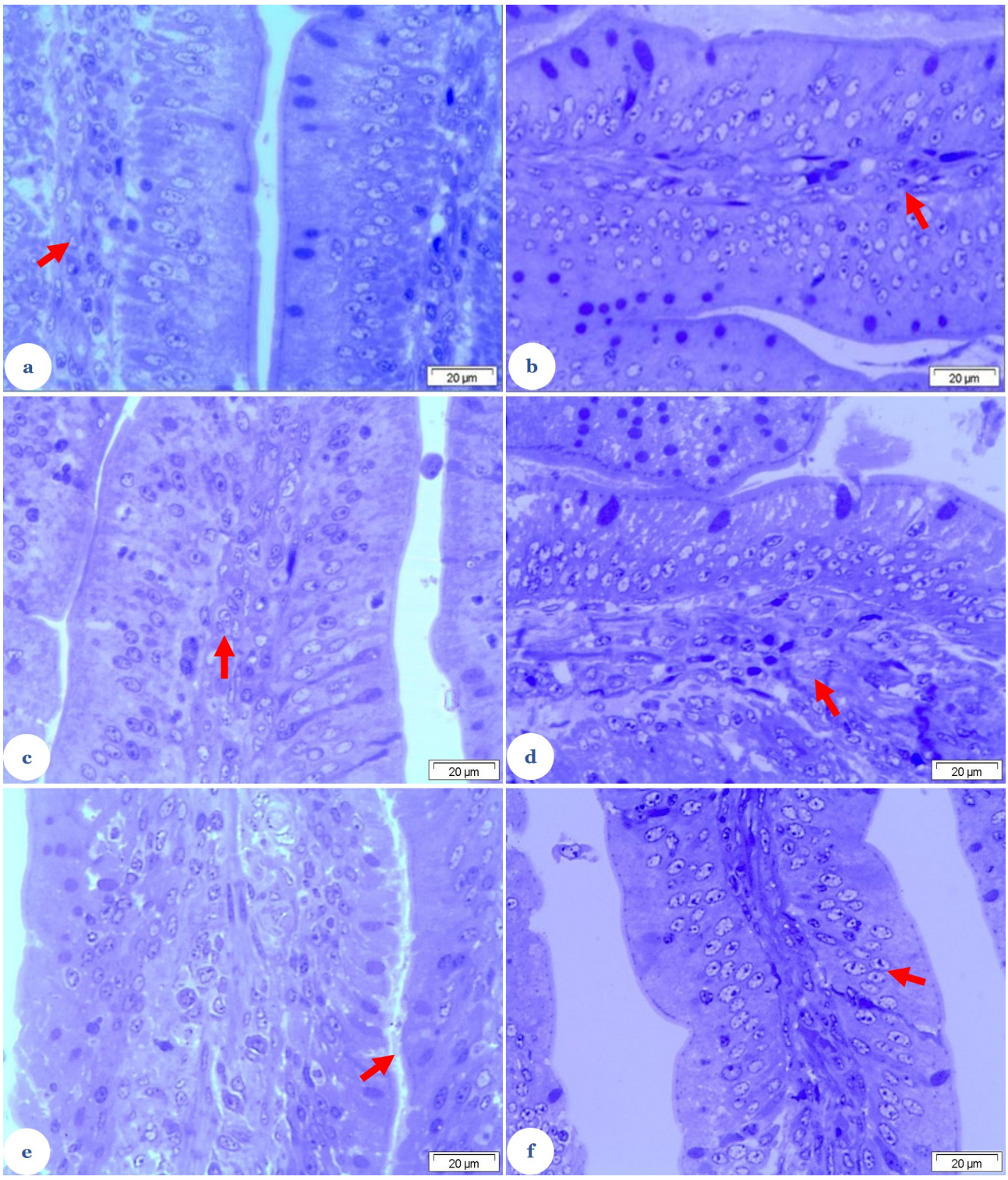


**Figure 3:** Electron microscopic examination of the duodenum. Group 2 (Aviboost and *B. subtilis* PB6) duodenal villus core showing the presence of a large number of well-active plasma cells (P), macrophage cells (M), lymphocytes (L), and collagen fibers (Co) at 22 days **(a)** and 31 days of age **(b)**. Group 3 (*B. subtilis* PB6) duodenal villus core showing the presence of numerous active plasma cells (P), macrophages (M), and lymphocytes (L) at 22 days of age **(c)**, and mild edema in the form of empty spaces at 31 days of age **(d)**. Group 1 (Control group) duodenal villus core containing collagen fibers (Co), macrophages (M), and lymphocytes (L) at 22 days of age **(e)**, and fewer lymphocytes (L) and plasma cells (P) at 31 days of age **(f)**.

stressed chickens (Raheel et al., 2019).

The present study's findings align with those recorded by Jayaraman et al. (2013) in that supplementation of *B. subtilis* PB6 with or without Aviboost to broiler chickens improved their intestinal morphology by increasing villus height and cellularity, making it a promising alternative to antibiotic growth promoter. Al-Fataftah and Abdelqader (2014) have also suggested using *B. subtilis* to ameliorate the adverse effects of heat stress on gut health by restoring intestinal microbial colonization and the impaired villus-crypt structure.

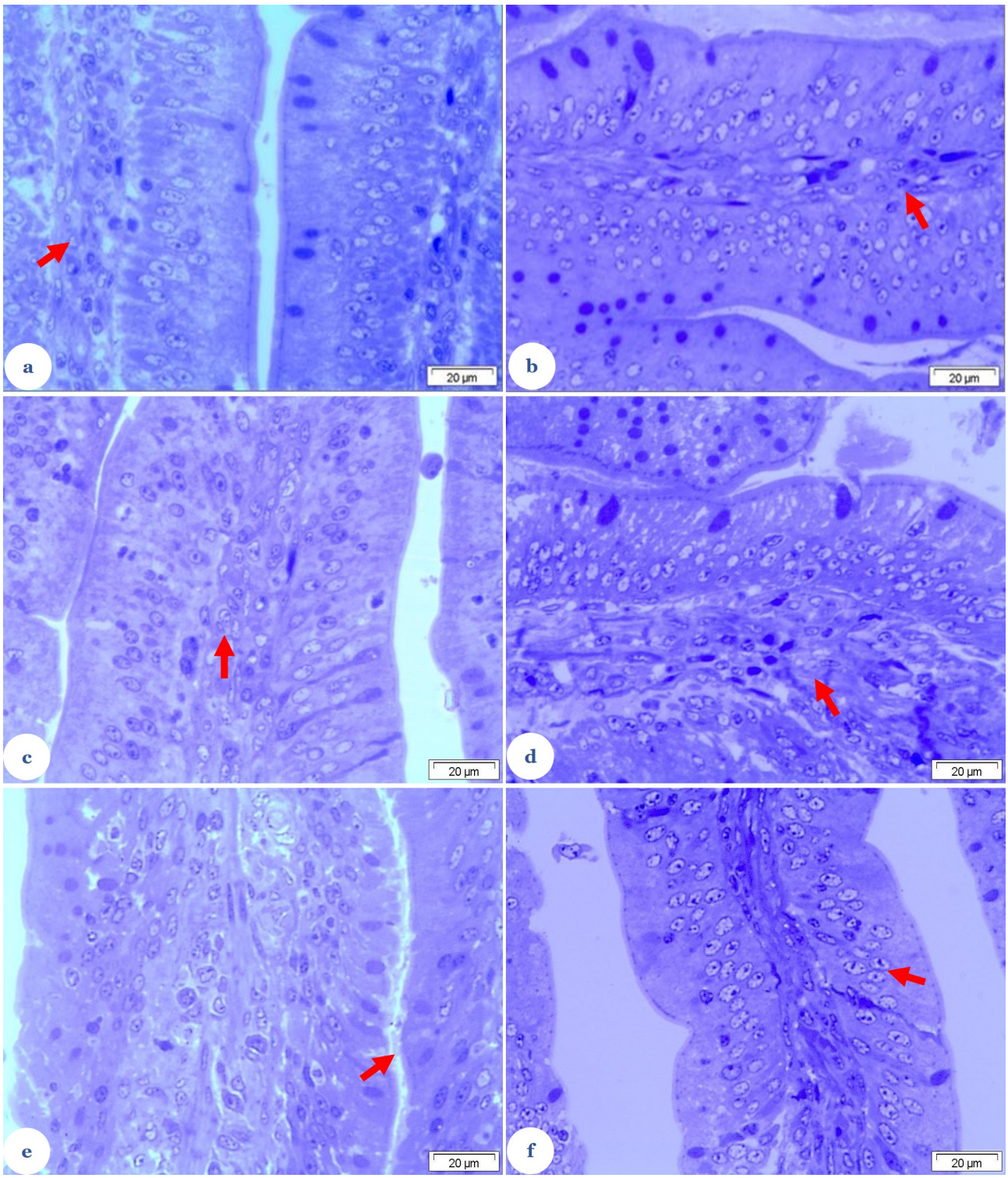


**Figure 4:** Interferon (IFN)-$\gamma$ **(a)** and Interleukin (IL)-6 protein **(b)** concentrations in the sera of all treatment groups (Group 3: *B. subtilis* PB6 treated group; Group 2: Aviboost and *B. subtilis* PB6 treated group; Group 1: Control group) at 22 and 31 days of age using ELISA. (*) indicates a significant increase in IFN-$\gamma$ and IL-6 protein levels ($p<0.05$).

In the present study, the quantitative histomorphological analysis of the intestinal mucosa revealed an increase in villus height and hyperplasia of villus epithelium with an increase in the number of goblet cells in the groups treated with probiotics alone or with Aviboost. Similarly, the lamina propria of the duodenal villi in both the probiotics group and probiotics and Aviboost group had numerous lymphocytes, macrophages, and plasma cells at 22 days, while at 31 days of age, the plasma cells population increased in number and activity as demonstrated by dilatation of RER in their cytoplasm. Notably, the increase in villi height and cellularity correlates with the enhanced growth and FCR of the supplemented chickens. These results are consistent with previous studies conducted by our group and others (Dell'Orto et al., 2002; Raheel et al., 2019) in that nucleotide or Aviboost supplementation to chickens improved enterocyte integrity, development, and turnover, with significant crypt cell proliferation. Along similar lines, supplementation of nu-

cleotides in chickens has been found to increase intestinal villi length and improve nutrient absorption and weight gain (Yu et al., 2002). In addition to increasing villus height and crypts depth, dietary nucleotides also act as growth factors by promoting the differentiation and maturation of the enterocytes, increasing the thickness of the intestinal mucosa, and promoting the activities of some digestive enzymes (Domeneghini et al., 2006).

The noticeable increase in the population of epithelial cells, absorbed fat cells, and endocrine cells in both the probiotics group and probiotics and Aviboost group may explain the improvement in growth performance and FCR compared to the non-treated negative control group. While supplementation of probiotics alone or with Aviboost induced a mild alteration in lymphocytes and macrophage populations, moderate and high alterations in plasma cell population were observed in the probiotics group and probiotics and Aviboost group, respectively, which is indicative of their role in modulation of the intestinal immune cells' population.

IFN-$\gamma$, a pluripotent cytokine, is secreted by T cells and natural killer cells (Sachdeva et al., 2015) and plays a crucial role in T cell differentiation and macrophage activation (Taha-Abdelaziz et al., 2020). IL-6 is also a pluripotent cytokine produced by various immune cells. It plays a vital role in promoting lymphocyte growth, differentiation, and activation, acute-phase protein production by hepatocytes and is also essential in initiating and regulating the inflammatory process (Kaiser et al., 2000, 2006; Taha-Abdelaziz et al., 2016; Raheel et al., 2019). Findings from our earlier investigation and studies conducted by other researchers indicated that Aviboost supplementation influences intestinal cytokine levels (Hess and Greenberg, 2012; Raheel et al., 2019). Yeast extract, an essential source of nucleotides, has been found to play a role in various biological processes, exert beneficial effects on the immune system, and enhance host resistance to colonization by pathogenic microorganisms' colonization (Świątkiewicz et al., 2014). (Alizadeh et al., 2016, 2017) observed an increase in the expression of Toll-Like Receptors and cytokines, such as IL-4 and IL-18, in birds receiving nucleotides, which indicates their immunomodulatory properties.

It is worth mentioning that the results of the probiotics-alone group and probiotics-and Aviboost-treated group were also compared to the results of the Aviboost-supplemented group, which had been published separately (Raheel et al., 2019), and no significant differences were observed. Interestingly, while the growth-promoting and immunomodulatory effects of *B. subtilis* PB6 were comparable to those of Aviboost, their combination did not exhibit additive or synergistic effects. Nonetheless, it should be noted that only one treatment modality of *B. subtilis* PB6 and Aviboost have been investigated in this study. Thus, further studies are needed to determine whether different treatment modalities would result in more desirable outcomes.

## Conclusion

Supplementation of probiotic CLOSTAT with or without Aviboost supplement to chickens exerted several beneficial effects, including i) improved growth performance as demonstrated by an increase in body weight gain and a reduction in FCR, ii) improved intestinal morphology as demonstrated by an increase in villus height and crypts depths as well as an increase in the populations of epithelial, endocrine and various immune cells in the intestinal mucosa, iii) enhanced systemic immune responses as demonstrated by elevated levels of IFN-$\gamma$ and IL-6 in the sera of treated chickens. The combination of CLOSTAT and Aviboost slightly improved the body weight gain and FCR of broiler chickens but did not exert additive effects on the intestinal morphology and systemic immune response. These findings suggest using CLOSTAT and Aviboost as alternatives to the in-feed antibiotics to improve chickens' performance and gut health and modulate their immune system. Further research is also required to investigate the role of these supplements in enhancing intestinal mucosal responses and protecting chickens against enteric infections. The mechanisms by which these supplements modulate chickens' immune system also requires further investigation.

**Article Information**

Ethical Approval. The experimental design was approved according to the guidelines of the Animal Care Committee of Beni-Suef University (approval no. BSU-IACUC 021-195).

Funding. The authors declare no funding was received for this study.

Conflict of Interest. The authors declare no conflict of interest.

Authors Contributions. Methodology; AHA and AO, formal analysis; AHA, AO, IAR, and KTA, investigation; AAHA, AO, IAR, and KTA, writing—original draft preparation; AHA, AO, and IAR, writing—review and editing; KTA.

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