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Influence of *Bacillus subtilis* and *Bacillus licheniformis* Probiotic Supplementation via the Drinking Water on Performance and Gut Health of Broiler Chickens

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Abstract

The experimental trial was carried out to evaluate the impact of a mixture of *Bacillus subtilis* and *Bacillus licheniformis* probiotic supplementation via the drinking water (AVI-GROW®) on growth performance, carcass traits, gut microbiome, intestinal histomorphology, blood biochemical indices, and litter quality of broiler chickens. A total of 480 one-day-old Ross 308 broiler chicks (as hatch) were randomly allocated into two groups, each with three replicates (80 chicks/replicate). The control group (T1) and the supplemented group (T2) were fed a basal diet, however, T2 was supplemented with (AVI-GROW®) via the drinking water at the rate of 1mL/L drinking water every 12 hours for 2 days after every vaccination and change of diet. The experiment lasted 31 days. Supplementation of (AVI-GROW®) via the drinking water in T2 significantly ($P \leq 0.05$) improved body weight gain and feed conversion ratio (FCR), as well as villus crypt ratio of the small intestine as compared to the control. The dressing percent, breast, thigh and drumstick yields were improved in T2 than in control. Additionally, blood cholesterol, triacylglycerol, ALT, and AST concentrations were reduced, while total protein concentration was significantly ($P \leq 0.05$) increased in T2 as compared to control. Moreover, cecal *Clostridia*l counts were significantly ($P < 0.05$) lower in T2 as compared to control. Supplementation of (AVI-GROW®) in (T2) reduced nitrogen content in birds' excreta and litter. Conclusively, the supplementation of (AVI-GROW®) via the drinking water could improve growth performance, carcass characteristics, gut microbiome, intestinal histomorphology, blood biochemical indices, and litter quality in broiler chickens.

KEYWORDS

Broilers, probiotic, *Clostridia*, Intestinal histomorphology, Blood biochemistry, Litter quality.

INTRODUCTION

Antibiotics that promote growth were commonly used around the world to protect chickens from diseases and thus improve growth performance. Growth-promoting antibiotics have been prohibited in Europe since January 2006 (Xu *et al*., 2021) due to the rising problems brought on by the widespread use of sub-therapeutic doses of antibiotics, such as environmental pollution, the emergence of bacterial antibiotic cross resistance (Mehdi *et al*., 2018), and antibiotic residues in the final product (Andremont, 2000). In order to modify the gut microbiota, supplements like probiotics, prebiotics, organic acids, and exogenous enzymes have been employed as an alternative to antibiotics.

Probiotics are cultures of particular live microorganisms that benefit the host by balancing the bacteria population in the intestine (Lutful Kabir, 2009). To stop pathogenic bacteria from sticking to and infiltrating the gut epithelium, probiotics function through competitive exclusion and antagonistic action (Wine *et al*., 2009). Probiotics can control the composition of the gut microbiota, boost immunity to increase disease resistance, encourage nutrient digestion and absorption, and eventually improve growth performance (Tarradas *et al*., 2020).

Bacillus subtilis and *Bacillus licheniformis* are spore-forming facultative anaerobic bacteria that can endure extreme circumstances found in the gastrointestinal environment, including low pH and bile salts. They can also withstand high temperatures up to 113 °C for 8 min (Shivaramaiah *et al*., 2011). In addition to stimulating the immune system, *Bacillus* spores release antimicrobial peptides (AMP) that are cytotoxic to bacterial pathogens (Sumi *et al*., 2015) and increase the levels of cytokines and chemokines in the chicken gut, such as interleukin-1 β (IL-1 β), interferon- γ, and (IFN -γ) (Lee *et al*., 2013).

Probiotic supplements are most frequently administered in feed and drinking water (Watkins and Kratzer, 1984). The preferred method, which has several advantages over supplementing probiotics into diets, is to supply them in drinking water. According to Karimi Torshizi *et al*. (2010), probiotics in drinking water are able to survive the harsh conditions in the upper gastrointestinal tract because of the water's ability to dilute the effects of gastric acid and digestive secretions, which further benefits probiotic

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microorganism survival in an aqueous environment. This transit time is also shorter than that of solid feed. Supplementation of probiotic products in the drinking water could facilitate the subsequent adaptation process to a gastrointestinal environment by accelerating the revival process of probiotic microorganisms and resulting in a faster colonization of probiotic microorganisms. This is necessary for the majority of the beneficial properties of probiotics (Higgins *et al*., 2007).

Therefore, this study was carried out to evaluate the impact of probiotic (AVI-GROW®) supplementation via the drinking water on growth performance, carcass traits, gut microbiome, intestinal morphology, blood biochemical indices, and litter quality in broiler chickens.

MATERIALS AND METHODS

Birds, diets, and husbandry

This study was conducted in the Animal and Poultry Research Center, Faculty of Veterinary Medicine, Cairo University, Egypt. The Faculty of Veterinary Medicine's Committee for the Institutional Animal Care and Use, Cairo University, Egypt, agreed on the experimental design (Vet CU 23052022452).

A total of 480 one-day-old Ross 308 broiler chicks (as hatch) were randomly assigned into two different groups, each group was subdivided into three replicates, and each replicate contained 80 birds. The control group (T1) and the supplemented group (T2) were fed a basal diet that was formulated according to Ross 308 broiler nutrient specification manual 2019 (AVIAGEN, 2019) (Table 1). In the supplemented group (T2), chickens were supplemented with (AVI-GROW®) via the drinking water added at the rate of 1mL/L drinking water every 12 hours for 2 days after every vaccination and change of diet, at 1, 7, 12, and 25 days of age. AVI-GROW® (Dana Vet for feed additive, Egypt) is a probiotic supplement solution, supplemented to broilers via the drinking water, and mainly composed of a mixture of *Bacillus subtilis* and *Bacillus licheniformis* (Table 2). Feed and fresh water were provided *ad libitum* during the whole trial.

Table 1. Ingredients composition and chemical analysis of the basal diet.

Ingredients	Basal diet		
	Starter (1-12d)	Grower (12-25d)	Finisher (25-31d)
Yellow corn	58.1	62.69	67.14
Soybean meal 46% CP	34.19	29.6	24.5
Corn gluten meal 60% CP	3.5	3	3
Soya oil	0.5	1.3	2
NaCl	0.35	0.35	0.35
Sodium bicarbonate	0.1	0.1	0.1
DCP	1.3	1.1	1
Limestone	1.3	1.2	1.2
DL-Methionine	0.2	0.18	0.2
L-Lysine	0.2	0.25	0.3
L-Threonine	0.1	0.07	0.05
Ronozyme proact	0.01	0.01	0.01
Ronozyme WX 2000	0.01	0.01	0.01
Hi Phos	0.01	0.01	0.01
Broiler premix[1]	0.13	0.13	0.13
Total	100	100	100
Chemical analysis			
ME (kcal/kg)	3009	3103	3202
CP%	23.06	21.06	19.07
EE%	3.08	3.49	4.77
CF%	2.33	2.27	2.19
Calcium%	1.02	0.93	0.89
Available Phosphorus %	0.49	0.45	0.42

[1]Broiler premix contained per kg: Vit A 12,500,000 IU, Vit D3 5,000,000 IU, Vit E 70,000mg, Vit K3 3,500mg, Vit B1 3,000mg, Vit B2 7,000mg, Vit B6 4,000mg, Vit B12 20mg, Nicotinic acid 50,000mg, Pantothenic acid 15,000mg, Biotin 180mg, Folic acid 2,000 mg, hy D 70mg, Iron 44,000mg, Copper 6,000mg, manganese 70,000mg, Zinc 75,000mg, Iodine 1,300mg, Selenium 230mg.

Table 2. Composition of AVI-GROW® (for each 1 liter of the product).

Components	Amount	Active ingredient
Bacillus subtilis	100g	1.75×10^{11} CFU
Bacillus licheniformis	100g	1.75×10^{11} CFU
DL-Methionine	25.25g	25000mg
L-Lysine HCL	13.15g	10000mg
L-Treptophan	10.31g	10000mg
Sorbitol	50g	

Birds were reared in deep litter floor pens bedded with wood shaving, with a stocking density of 10 birds/m^2. Birds were housed in a semi-closed system. Birds were vaccinated against H5N1 Avian influenza at 7 days old and Newcastle disease at 7, 13, and 20 days old.

Growth performance

The changes in body weights and feed consumption were recorded weekly to calculate the body weight, weight gain, feed intake, feed conversion ratio (FCR), and European Production Efficiency Factor (EPEF) (Marcu *et al*., 2013)

FCR= g of feed / g of weight gain.

EPEF= body weight (kg) × % viability × 100 / feed conversion ratio (g feed/g gain) × Age (d)

Carcass traits

At the end of the experiment (day 31), five representative birds from each replicate (15 birds from each group) were weighted and euthanized after 4 h of fasting for complete evacuation of the gut. Each bird was scalded, de-feathered, and eviscerated after the exclusion of the head, neck, and legs. Weighted carcasses without giblets were expressed as a percentage of their live weight (carcass weight). Breast, thigh, and drumstick weights were recorded after the carcass was dissected (Lai *et al*., 2018).

Sampling and microbiological examinations of cecal content

At 15, 27 and 31 days (after every supplementation of AVI-GROW®), three birds from each group were euthanized and cecal contents were collected. All samples were stored at 4°C till further bacteriological examination. According to the method described by Esmaeilipour *et al*. (2012), 1 g of cecal content was diluted in 9 ml sterile saline solution and homogenized (10−1 dilution). Samples were subjected to 10-fold serial dilution in tubes containing 9ml-sterile saline solution. From the serially diluted tubes, 100µl samples were spread onto Reinforced *Clostridia*l Agar (Oxoid Ltd, Basingstoke, Hants, UK) to enumerate total *Clostridia*. All plates were incubated for 24-48h at 37◦C, and tightly sealed anaerobic jars were used for achieving anaerobic conditions. Finally, the counts of *Clostridia* colonies were reported as mean 10-logarithm colony-forming units (log10 CFU) for each gram of cecal content.

Confirmation and toxin typing of Clostridium perfringens

Genomic DNA of suspected C. perfringens isolates was extracted using an extraction kit (QIA amp mini kit, Qiagen, Hilden, Germany). The multiplex PCR assay was used to detect the presence of genes encoding alpha-toxin (cpa), beta-toxin (cpb), epsilon-toxin (etx), iotatoxin (iap), and CPE (cpe). Primer sequences were published previously (Ghoneim and Hamza, 2017). The multiplex PCR assay was carried out according to (Ghoneim and Hamza, 2017). The PCR reaction mixtures were analyzed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of 100 bp DNA ladder (Fermentas Life Science, USA).

Physical and Chemical examinations of litter

Litter moisture was estimated by drying 10g of litter samples in the hot air oven at 100±5°C for 24-48h (Dumas *et al*., 2011). Moisture % was calculated by subtracting dry weights from the initial weights. Additionally, the total nitrogen content of litter samples was determined as total Kjeldahl nitrogen (Jackson, 1973). Total phosphorous was determined using ascorbic acid (Houba *et al*., 1995) and a spectrophotometer at 880 nm (Spectronic 21D).

Microscopical and histomorphometrical examinations of small intestine

At the end of the experiment, five birds from each group were euthanized. After that, the small intestine was dissected out, sections from the middle of the duodenum, jejunum (at the midpoint between the bile duct entry and Meckel's diverticulum), and ileum (about 0.5 cm in length) were excised and opened longitudinally and gently flushed with 0.1 M phosphate buffered saline (pH 7.4). Then fixed in 10% neutral buffered formalin solution. Slices of 6-7 mm thick were produced, mounted on clean glass slides, and stained with Haematoxylin and Eosin to evaluate general histological structure. Bancroft and Gamble, (2008) recommendations were followed in terms of methods.

For histomorphometrical investigations, the villus length was measured from the villus tip to the bottom, excluding the intestinal crypt, and the depth of the intestinal gland (crypt) was measured. Five stained sections per bird were analyzed using a Leica Quin 500 analyser computer system (Leica Microsystems, Switzerland) in the Histology and Cytology lab, at the Faculty of Veterinary Medicine, Cairo University. The image analyzer was calibrated automatically to transform the image analyzer program's measurement units (pixels) into real micrometer units. For each sample, pictures of each slice were taken at a final magnification of 40x.

Blood biochemical indices

At the end of the experiment, blood samples were collected from 15 birds/group. Samples were permitted to clot at room temperature and then centrifuged for 15 min at 4000 rpm and clear serum was separated and preserved at -20 ˚C until used for biochemical analysis (Ali *et al*., 2022). Determinations of total protein (at wavelength 546nm), albumin (at wavelength 578nm), triacylglycerol (at wavelength 505nm), total cholesterol (at wavelength 500nm), ALT (at wavelength 340nm), AST (at wavelength 340nm), and uric acid (at wavelength 546nm) in sera were performed by using spectrum diagnostics kits (Spectrum Diagnostics Egyptian Company for Biotechnology) according to the manufacturer's instructions (UV-2100 Spectrophotometer, USA).

Statistical analysis

Results were summarized in tables as means and standard errors (SE). Statistical inference was tested using independent sample t test. Significance was indicated at ($P \leq 0.05$). Data analysis was performed using PASW Statistics, Version 18.0 software (SPSS Inc., Chicago, IL, USA). Box plots and bar plots were executed with ggplot2 package (Wickham *et al*., 2016) using R for Statistical Computing (https://www.r-project.org/).

RESULTS

Growth performance

The cumulative growth performance during whole experimental period (1-31 days) of broiler chickens in either control or AVI-GROW® group are illustrated in Tables 3. The data showed that supplementation of AVI-GROW® via drinking water improved the cumulative growth performance in terms of body

weight, body weight gain, feed consumption, feed conversion ratio, and European production efficiency factor (EPEF) in T2 compared to T1, however, the differences were not significant.

Carcass traits

The carcass traits of broiler chickens in either control or AVI-GROW® group are shown in Table 4. The data showed that the dressing percent, breast, thigh, and drumstick yields were improved in T2 compared to T1; however, the differences were not significant.

Microbiological examination of cecal content

The bacterial counts per gram of cecal content of broiler chickens in either control or AVI-GROW® group are shown in (Figure 1). The data showed that the bacterial counts per gram of cecal content were significantly (P<0.05) lower in T2 with an average of 6.14±0.52 (P=0.035) and 8.88±0.29 (P=0.004) log10 CFU of *Clostridia* at days 27 and 31, respectively, as compared to control 8.36±0.47 and 10.72±0.14 log10 CFU of *Clostridia* at days 27 and 31, respectively.

The PCR of *Clostridium perfringens* revealed alpha toxin in all samples that represented type A.

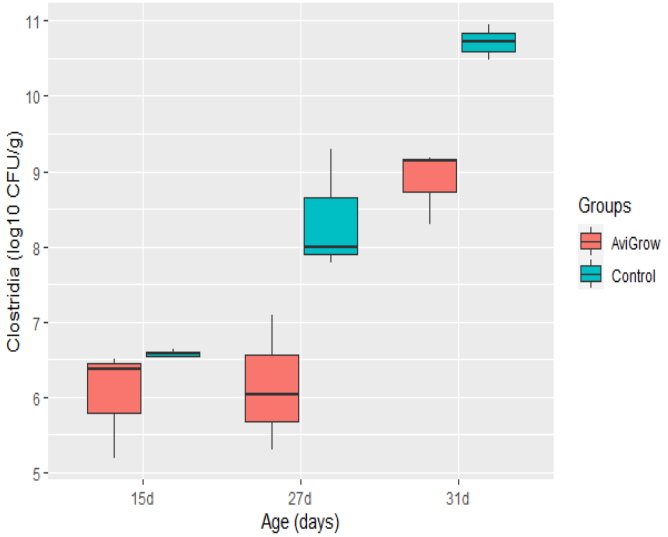

Fig. 1. Influence of AVI-GROW® supplementation via the drinking water to broiler chickens on cecal *Clostridia*l count at the end of different phases (starter 15d, grower 27d, and finisher 31d).

Table 3. Influence of AVI-GROW® supplementation via the drinking water on broilers' cumulative performance (days 1-31) (means±SE1).

Groups	Body weight (g)	Weight gain (g)	Feed intake (g)	FCR (g/g)	EPEF
T1	1949.39±20.10	1907.79±20.10	2811.59±49.63	1.47±0.04	309.09±10.73
T2	2076.94±48.38	2035.34±48.38	2860.50±53.71	1.41±0.03	339.05±15.26
p- value	NS[2]	NS	NS	NS	NS

Data are expressed as mean±Standard error (SE)
T1: Control (basal diet); T2: basal diet + AVI-GROW® (1mL/L drinking water/12h for 2days after every vaccination and change of diet); FCR: Feed Conversion Ratio; EPEF: European Production Efficiency Factor; NS: Not significant

Table 4. Influence of AVI-GROW® supplementation via the drinking water on broilers' carcass traits.

Groups	Dressing (%)	Breast (%)	Thigh (%)	Drum (%)
T1	73.23±0.48	36.51±0.19	25.91±0.52	12.88±0.15
T2	73.14±0.56	37.29±0.68	24.93±0.33	13.00±0.19
p- value	NS[2]	NS	NS	NS

Data are expressed as mean±Standard error (SE)
T1: Control (basal diet); T2: basal diet + AVI-GROW® (1mL/L drinking water/12h for 2days after every vaccination and change of diet); NS: Not significant

Table 5. Influence of AVI-GROW® supplementation via the drinking water on broilers' small intestine histomorphometry.

Groups	Villus length	Crypt depth	Villus crypt ratio
Duodenum			
T1	1602.61±36.07	109.584 ± 3.38[a]	14.65 ± 0.39[b]
T2	1880.63 ± 31.16	107.662 ± 6.73[b]	17.66 ± 0.80[a]
p- value	NS[2]	0.01	0.04
Jejunum			
T1	1084.36 ± 22.07	125.10 ± 2.75	8.67±0.12
T2	1148.28 ± 47.65	106.22 ±3.70	10.82±0.41
p- value	NS	NS	NS
Ileum			
T1	665.55±25.10	94.33±1.12	7.06±0.29
T2	839.93±26.54	100.42±2.20	8.36±0.23
p- value	NS	NS	NS

Data are expressed as Mean±SE
[a,b] Mean values with different superscripts in the same column indicate significant difference (P≤0.05).
T1: Control (basal diet); T2: basal diet + AVI-GROW® (1mL/L drinking water/12h for 2days after every vaccination and change of diet); NS: Not significant

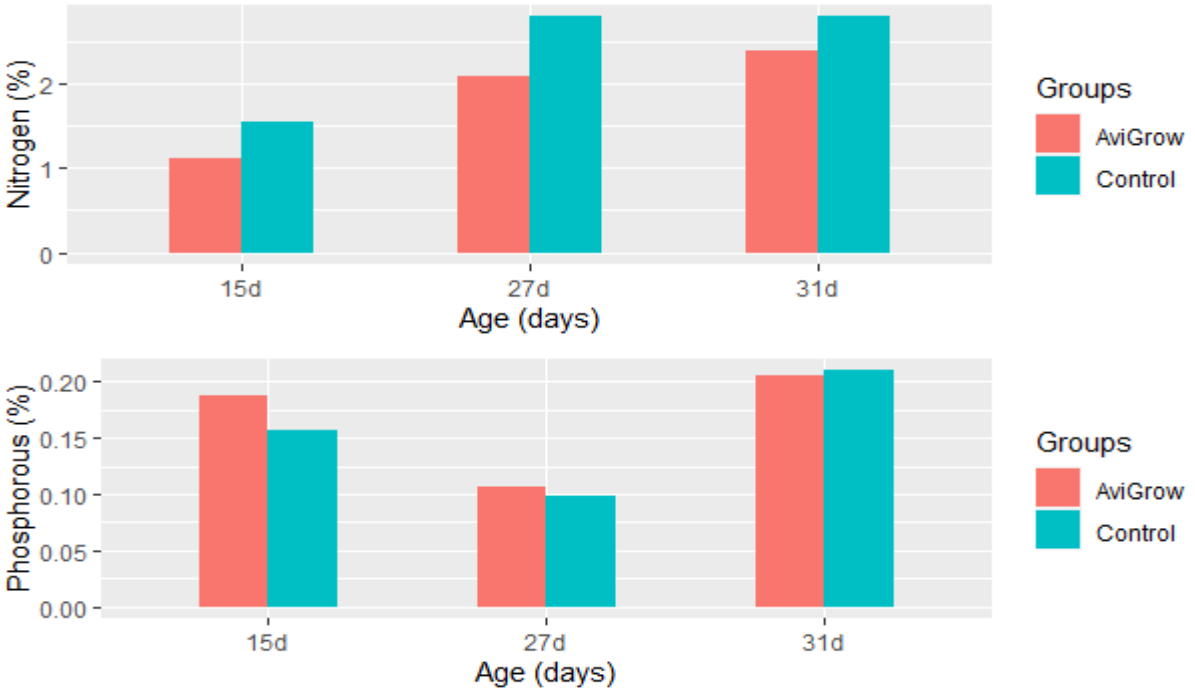

Fig. 2. Influence of AVI-GROW® supplementation via the drinking water to broiler chickens on nitrogen (A) and phosphorous (B) contents (%) of deep litter at the end of different phases (starter 15d, grower 27d, and finisher 31d).

Table 6. Influence of AVI-GROW® supplementation via the drinking water on broilers' blood biochemical indices.

Groups	TAG (mg/dL)	Cholesterol (mg/dL)	Albumin (g/dL)	Total protein (g/dL)	ALT (U/L)	AST (U/L)	Uric acid (mg/dL)
T1	72.53±1.52	110.63±3.03	1.25±0.09	2.12±0.11[b]	17.94±0.70	253.6±1.30	6.55±0.39
T2	65.70±1.23	98.13±2.00	1.48±0.11	2.22±0.16[a]	13.59±0.66	238.5±7.09	6.67±0.41
p- value	NS[2]	NS	NS	0.01	NS	NS	NS

Data are expressed as Mean±SE

[a,b] Mean values with different superscripts in the same column indicate significant difference (P≤0.05).

T1: Control (basal diet); T2: basal diet + AVI-GROW® (1mL/L drinking water/12h for 2days after every vaccination and change of diet); TAG: Triacylglycerol; ALT: Alanine amino transferase; AST: Aspartate amino transferase; NS: Not significant

Physical and Chemical examinations of litter

Nitrogen, phosphorus, and moisture contents for litters collected from broiler chickens in either control or AVI-GROW® group are shown in (Figures 2 and 3). Birds supplemented with AVI-GROW® in the drinking water in (T2) exhibited lower litter moisture than control during starter phase (15d) (T2=38%; T1=42%), although higher litter moistures than control were reported during grower (27d) (T2=44%; T1=41%) and finisher phases (31d) (T2=33%; T1=29%), however significant differences were not indicated (Figure 2). Additionally, nitrogen contents were decreased for litters collected from T2 with an average of 1.12, 2.10, and 2.40% at days 15, 27, and 31, respectively, as compared to control (1.55, 2.80, and 2.80% respectively). Litter phosphorus contents didn't show any significant differences between T1 and T2.

The small intestine histomorphometry

The changes in villus length, crypt depth, and villus crypt ratio, as well as normal histological structure of the three parts of the small intestine of broiler chickens in either control or AVI-GROW® groups were demonstrated in Table 5 and Figure 4. Histomorphometrical data revealed that AVI-GROW® supplementation via the drinking water in (T2) increased villi length and V: C ratios along different intestinal sections, compared to control, with a significant enhancement (P<0.05) was observed in duodenum of T2.

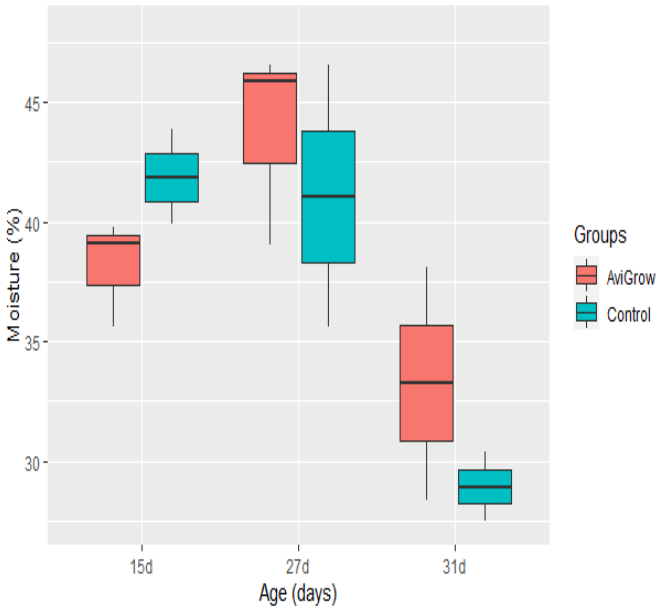

Fig. 3. Influence of AVI-GROW® supplementation via the drinking water to broiler chickens on moisture content (%) of deep litter at the end of different phases (starter 15d, grower 27d, and finisher 31d).

Blood biochemical indices

The blood biochemical indices of broiler chickens in either control or AVI-GROW® groups are shown in Table 6. The data

showed that triacylglycerol, blood cholesterol, ALT, and AST concentrations were reduced, while uric acid concentration was increased in T2 as compared to control, however, the differences were not significant. Moreover, total protein concentration was significantly (P≤0.05) increased in T2 as compared to control.

DISCUSSION

Bansal *et al.* (2011) demonstrated that probiotic supplementation may enhance chicken growth performance and feed consumption. Due to its capacity to increase broiler productivity and efficiency, *Bacillus subtilis* and *Bacillus licheniformis* have received extensive attentions as an antibiotic alternatives (Rhayat *et al.*, 2017).

In the current study, the supplementation of AVI-GROW® via drinking water in T2 improved the cumulative growth performance in term of birds' body weight, body weight gain, feed consumption, feed conversion ratio (FCR), and European production efficiency factor (EPEF) but did not differ significantly. These supported the findings of Zhang *et al.* (2013), who found that supplementing 105 and 108 cfu/kg of probiotics based on the *Bacillus* species improved average daily gain (ADG). According to Zeng *et al.* (2021), supplementing with compound probiotics significantly raised the ADG in birds from days 1–42, but did not significantly alter it during the early growth stages. According to this justification, these probiotics' growth-promoting properties mostly benefit later growth phases. According to Amerah *et al.* (2013), supplementing broiler chicken fed with 1.5×108 cfu/kg of *B. subtilis* could improve the feed conversion ratio. Beneficial metabolites produced by *B. licheniformis* or *B. subtilis*, including extracellular digestive enzymes, lysozyme, antifungal proteins, and different antibiotics, among others, may be responsible for the improvements in body weight gain and feed conversion ratio (Kim and Yoon, 2008). Additionally, administration of *B. subtilis* or *B. licheniformis* may improve the broilers' immunity (Guo *et al.*, 2020) and regulate intestinal microbiota composition and metabolic function (Chen and Yu, 2020). Moreover, chickens in T2 (AVIGROW® group) recorded the best values for FCR and the European production efficiency factor (EPEF). Various measures of boiler performance are included in the EPEF, such as BW, survival rate, FCR, and production management. Recently, EPEF had become increasingly recognized by practitioners as an essential performance measurement method, as well as a significant profitability index (Śliżewska *et al.*, 2020). Additionally, probiotics were shown to have little to no impact on the cumulative growth performance of broiler chickens (Lee *et al.*, 2010), and these findings were in agreement with ours.

The primary metrics used to assess the meat production of broiler chickens are the dressing percent, breast, thigh, and drumstick muscle yields in the carcass characteristics (Ahmat *et al.*, 2021). In the current study, the dressing percent, breast, thigh, and drumstick yields were improved in T2 compared to control; however, significance was not indicated. These findings were consistent with those of Sarangi *et al.* (2016), who discovered that supplementing probiotics to broiler chicks did not significantly increase carcass yield when compared to the control. In contrast to our findings, Salehizadeh *et al.*, (2019) observed that birds supplemented with *B. subtilis* had higher carcass yields and individual meat cuts than control birds. These discrepancies in the outcomes could be caused by the probiotic strains, administration dosage, preparation techniques, bird age, and hygiene status (Zhang *et al.*, 2012).

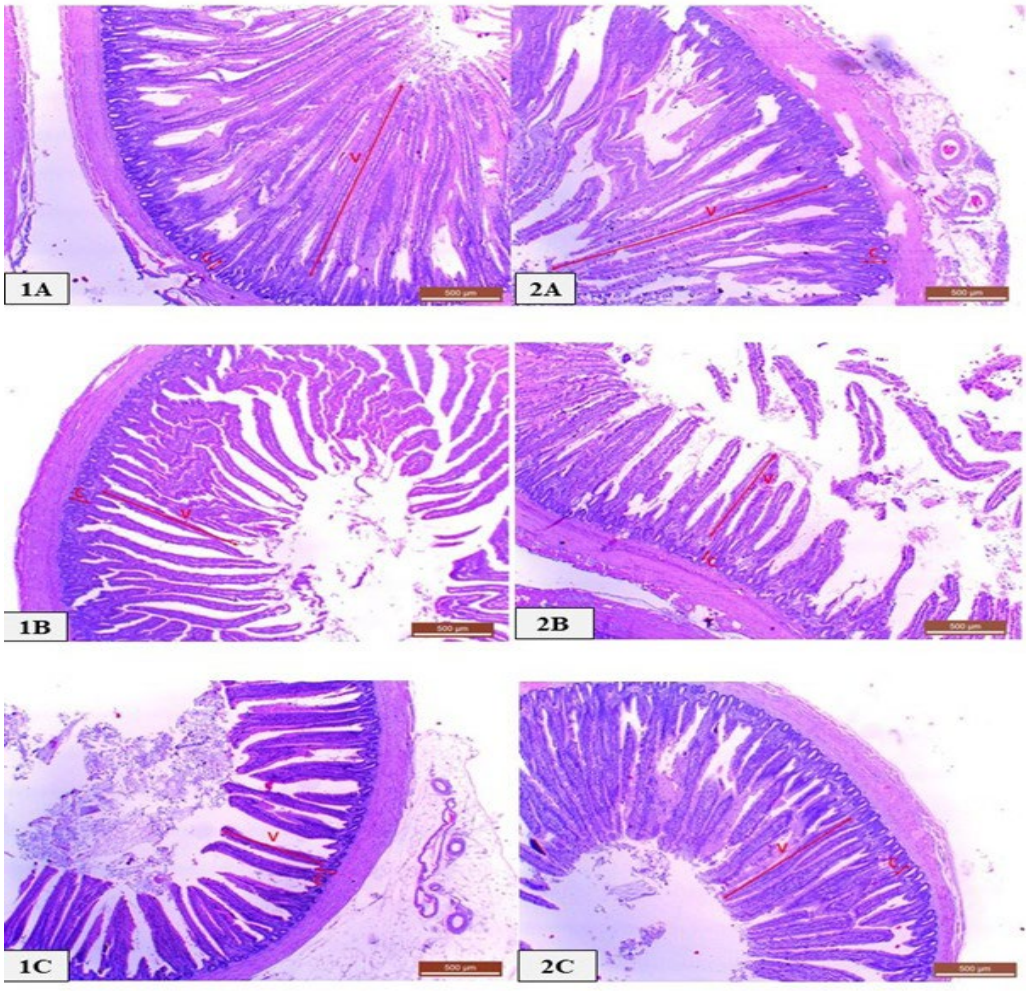

Figure 4. Influence of AVI-GROW® supplementation via the drinking water on broilers' small intestine histomorphometry. Photomicrographs of the small intestine. 1A,1B, and 1C showing normal histological picture of duodenum, jejunum and ileum in the control group. While 2A,2B, and 2C showing normal histological picture of duodenum, jejunum and ileum in the AVI-GROW® group. V: villus length; C: crypt depth.

In the current study, the *Clostridia* counts per gram of cecal content were significantly (P<0.05) lower in T2 as compared to control. According to research by Bortoluzzi *et al*. (2019), *Bacillus subtilis* supplementation improved broiler performance by lessening the negative impacts of necrotic enteritis (NE) brought on by *Clostridium perfringens*. *Clostridium perfringens* was able to be stopped from growing *in vitro* by *Bacillus licheniformis* (Lin *et al*., 2019). Numerous studies have suggested that supplementing *Bacillus subtilis* helps prevent necrotic enteritis in broiler chickens by competitive exclusion of *Clostridium perfringens* in the gastrointestinal tract (Cheng *et al*., 2018). *Bacillus licheniformis* isolated from the gastrointestinal tract of broilers has demonstrated *in vitro* antibacterial action against a wide range of pathogens, including *Clostridium perfringens* (Barbosa *et al*., 2005). According to several investigations, *Bacillus licheniformis* can create antibacterial substances that resemble bacteriocin (Guo *et al*., 2012).

Numerous methods have been developed to improve birds' nutrient utilization, which in turn reduces the excretion of components into the environment (Awaad *et al*., 2019). Lin *et al*., (2017) stressed the significance of integrating techniques into manufacturing systems to achieve environmental safety and economic viability. Microbes break down urea and uric acid in poultry litter, causing an 80% loss of nitrogen (N) as ammonia (NH3) (Ritz *et al*., 2004). In the current study, nitrogen content in litters collected from T2 was lower than in controls. Ismael *et al*. (2022) reported that the supplementation of probiotics could help reduce ammonia emissions in the environment and reduce ammonia's negative impacts on poultry as well as decrease the total nitrogen content of litter compared to birds fed the basal diet, which is in agreement with our findings. Furthermore, too much litter phosphorus applied to the soil causes runoff and phosphorus pollution of surface and groundwater (Dankowiakowska *et al*., 2013). In the current study, litter phosphorus content didn't show any significant differences between T2 and control. Ismael *et al*. (2022) observed that supplementing probiotics to a low-energy diet enhanced phosphorus consumption and decreased its excretion in broiler droppings, both of which decreased environmental pollution. When the frequency of expelled water (urine and feces) exceeds the rate of evaporation, poultry litter becomes wet (Collett, 2007). In the current study, birds supplemented with AVI-GROW® in the drinking water (T2) showed lower litter moisture than the control during the starter phase. According to Ismael *et al*. (2022), probiotic-supplemented birds produced the least wet litter, which is consistent with our findings. But higher litter moisture than the control was reported during the grower and finisher phases. However, significant differences were not indicated.

Intestinal health and recovery were assessed using the villi length and crypt depth as key indicators. A lengthy villus with a functionally active epithelium and a shallow crypt with ongoing cell renewal are both indicators of a high villi length to crypt depth ratio (Mohammadagheri *et al*., 2016). In the current study, AVI-GROW® supplementation via the drinking water in (T2) increased villi length and V: C ratios along different intestinal sections, compared to control, with a significant enhancement was observed in duodenum of T2. These findings agreed with Abudabos *et al*. (2013) who reported that, in broiler chicks, *B. subtilis* had a beneficial effect on the villi's histomorphology and gut integrity. Moreover, according to Zhang *et al*. (2021), the villi height/crypt depth ratios were significantly higher in the probiotics-supplemented groups than in the control groups following experimentally induced necrotic enteritis (NE) in broiler chickens, increasing the surface area for effective nutrient absorption. Villi lengthening increases the surface area of the intestine, which promotes nutrient absorption to promote broiler development and production (Mohammadagheri *et al*., 2016). Additionally, supplementing with *Bacillus licheniformis* significantly enhanced the small intestine's morphology, and this result was superior to that of using commercial antibiotics (Lin *et al*., 2019).

Serum biochemical indices, in particular serum total protein, albumin, uric acid, triacylglycerol, and total cholesterol, partially reflect the bird's metabolism and state of health. In the current study, triacylglycerol, blood cholesterol, ALT, and AST concentrations were reduced, while uric acid concentration was increased in T2 as compared to control, however, the differences were not significant. Moreover, total protein concentration was significantly (P≤0.05) increased in T2 as compared to control. These results agreed with Abudabos *et al*., (2019) who reported that several probiotics, including *Bacillus subtilis* significantly boosted serum total protein in broilers. A high blood total protein concentration suggests a vigorous protein metabolism and good nutritional performance in chickens, and it is a useful biomarker of protein metabolism in poultry (Grisoni *et al*., 1991). The greater protein profile level of birds supplemented with probiotics may have been partially attributed to superior growth performance and carcass quality. Supplementation of *Bacillus* spp. and its metabolites could raise serum total protein and albumin levels (Ahmat *et al*., 2021). Although probiotics such as *B. subtilis* and *B. licheniformis* were supplemented to chicks, there was no discernible difference in the blood total protein, albumin, or globulin levels (Abaza *et al*., 2008) and this disagree with our findings. In addition to enhancing protein synthesis and encouraging protein deposition in broilers, supplementation of *Bacillus* spp. also relieves renal pressure and lowers serum uric acid levels (Ahmat *et al*., 2021). Numerous studies have also shown that probiotic supplementation reduces non-protein nitrogen in chicken blood, such as urea, ammonia, and uric acid (Park *et al*., 2016).

Although no significant change was seen, the cholesterol level in the birds supplemented with *Bacillus* spp. was lower than the control, and it has been discovered that probiotics' enzymatic conversion of cholesterol to coprostanol in the intestines encourages its elimination through droppings (Ahmat *et al*., 2021). By preventing cholesterol synthesis, probiotics may have an impact on blood cholesterol levels (Fukushima and Nakano, 1995). Gong *et al*., (2018) found that *B. subtilis* and in vitro supplementation significantly decreased serum levels of uric acid, total cholesterol, and triacylglycerols. The liver plays a key role in the metabolism of several substances, and ALT and AST are vital enzymes in these metabolic procedures. As a result, rising serum levels of ALT and AST could be used as toxicity indicators of liver injury (Ramesh *et al*., 2012). In the current study, ALT and AST concentrations were reduced in T2 as compared to control; however, the differences were not significant. These findings confirmed those of Zhang *et al*. (2017), who noted that the levels of ALT and AST in the *Bacillus subtilis* group were much lower than those in the control group, indicating that supplementing *Bacillus subtilis* to the diet may have reduced liver damage from oxidative stress.

CONCLUSION

Conclusively, the supplementation of (AVI-GROW®) via the drinking water could modulate gut microbiota and enhance intestinal health and integrity of broiler chickens.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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