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Research Article

Investigation of the Effects of Prebiotics, Probiotics, and Eubiotics on Performance and Intestinal Histomorphometry in Broiler Chickens

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Abstract

This study was designed to investigate the effects of dietary supplementation with Prebiotics, Probiotics, and Eubiotics as alternatives to antimicrobial growth promoters (AGPs) on growth performance and intestinal histomorphometry in Ross 308 broiler chickens. A total of 200 broiler chicks were randomly allocated to five dietary treatments: basal diet (negative control), AGPs (positive control), Prebiotics, Probiotics, and Eubiotics. Key performance indicators such as weight gain (WG), feed intake (FI), feed conversion ratio (FCR), and mortality were recorded over a 42-day trial period, alongside the intestinal histomorphometries. Raw data were collected and analyzed using one-way analysis of variance (ANOVA), followed by the Least Significant Difference (LSD) post-hoc test for pairwise comparisons. Results indicated that broilers that received Prebiotics, Probiotics, and Eubiotics supplementation exhibited significantly improved WG and FCR compared to the negative control group ($p < 0.05$). These improvements were comparable to those observed in the AGP-supplemented group, however, no significant differences were observed between them ($p > 0.05$). Feed intake was significantly influenced ($p<0.05$) across all supplemented groups, while FCR was was significantly affected during the first five weeks. Histomorphometric analysis revealed significantly enhanced villus height and goblet cell density in the Probiotics and Eubiotics supplemented groups. Mortality rates did not differ significantly among the treatment groups. Generally, the findings support the use of Prebiotics, Probiotics, and Eubiotics as effective and safer alternatives to AGPs in Ross 308 broiler production.

Keywords

Broiler, Prebiotics, Probiotics, Eubiotics, Performance, Intestinal- histomorphometry

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1. Introduction

The application of antimicrobial growth promoters (AGPs) in the poultry industry has a long history, dating back to the 1940s, when their growth-promoting potentials were accidentally discovered [1, 2]. From 1947, these promoters became widely adopted in poultry farming across different regions [3]. However, their long-term adverse effects were not anticipated until the emergence of antibiotic resistance (AMR), which was strongly linked to their prolonged use and misuse [4]. Both the World Health Organization and Economic and Social Committee of the European Union have identified the use of antibiotics in food animals as a public health concern [5, 6]. This led to the ban of AGPs on food-producing animals in most regions of the world. With this concern in mind, researchers started to explore safer alternatives to AGP's products that could enhance performance, immune stimulation, and disease prevention without compromising consumer health or animal welfare [7].

Among other alternatives, Prebiotics, Probiotics, and their combinations (Eubiotics) gained attention in the poultry industry due to their potential to outperform AGPs in promoting overall bird health and productivity [8]. It is worth noting that these supplements have not been associated with antibiotic resistance or tissue residues that could threaten human health. Importantly, with the AGPs ban, poultry production systems are shifting toward the use of these alternatives [9-11]. The Food and Agriculture Organization/World Health Organization [12], defines Probiotics as “live microorganisms that, when administered in adequate amounts, confer health benefits to the host”. Prebiotics, on the other hand, are non-digestible food ingredients that selectively support the growth and activity of beneficial gut bacteria [13]. Eubiotics are synergistic combinations of both. Probiotics commonly originate from normal gut-dwelling bacteria of many animals [14], while prebiotics are typically plant-derived complex sugars [15, 16]. The application of Probiotics has shown promise in reducing antibiotic-resistant bacteria in broilers, thus safeguarding public health [7]. Probiotics have also been linked to improved growth, immune response, and gut microbial balance [17], while prebiotics help enrich these beneficial microbes and modulate intestinal ecology [18].

Given the gastrointestinal tract's vulnerability, second only to the skin in exposure to pathogens, its dysbiosis can significantly compromise poultry health [19-21]. Early research demonstrated the benefits of these additives. A study by Samanya M et al. [22], reported increased villus height and crypt depth in broilers fed by *Bacillus subtilis* var. Natto, while that of Timmerman H et al. [23] found that prebiotic- and probiotic-enriched diets significantly improved growth rate, weight gain, and feed conversion ratio (FCR). Other studies found significant responses to disease management. Studies by Lee S et al. and Spring P et al.[24, 25] demonstrated enhanced resistance to coccidiosis and Salmonella infections and that of Alsulami M et al. and Lee S et al.[26, 27] revealed reduced oocyst shedding post-Eimeria infection. Spring P et al. and Zulkifli I et al.[25, 28] also found less severe lesions from E. maxima infections in the supplemented groups, whereas Vesna T et al. [29] reported higher Newcastle disease virus antibody titers in birds receiving supplemented diets.

Despite these promising results, outcome inconsistencies remain and hinder the generalization of results. These inconsistencies are often attributed to strain-specific effects, as observed by Yang Y et al. [30], differences in dosage and delivery [31, 32], environmental variation [33], genetic differences [34], developmental stages [32, 35] and interactions with other dietary additives, as revealed by Amouei H et al.[36]. Additionally, most of the existing research has been conducted in temperate regions, with limited data from tropical settings. This presents a need to investigate the efficacy of these alternatives in a tropical environment. Also, among the widely studied alternatives to AGPs that have demonstrated potential in broilers are the Prebiotics fructo-oligosaccharides (FOS), and mannan-oligosaccharides (MOS) [18, 23, 30] and Probiotics *Lactobacillus acidophilus* and *Bacillus subtilis* [22, 37, 38]. However, their synergistic effects in broiler chickens have never been evaluated elsewhere. Based on this, we hypothesized that synergies between selected Prebiotics (MOS and FOS), Probiotics (*L. acidophilus* and *B. subtilis*), and their combined form may produce more pronounced and additive effects. Therefore, this study was designed to investigate the impact of these additives synergistically on performance metrics and intestinal histomorphometric parameters in Ross 308 broiler chickens of both sexes.

2. Materials and Methods

2.1. Study Area

This study was conducted at Animal, Aquaculture, and Range Sciences department; lower farm poultry houses of the Sokoine University of Agriculture, Morogoro-Tanzania, geographically located at 6.8520 °S, 37.6576 °E.

2.2. Research Design and Experimentation

A total of 200 one-day-old Ross 308 broiler chicks were procured from Silverlands Hatcheries in Iringa, Tanzania. Upon arrival, the chicks were examined, weighed, and randomly allocated into five treatment groups, each consisting of 40 birds, following the fully randomized design as described by Ali A et al. [39]. Each group was further subdivided into four replicates of 10 chicks. Within each replicate, four chicks were randomly selected and tagged on the wings for individual identification.

Group one (negative control) received only a basal diet. Group two (positive control) was fed a basal diet supplemented with 10% Olaquindox (Tanveterina Co. Ltd. Dar es

Salaam, Tanzania) at a dosage of 200 g/ton of feed. Group three received a basal diet enriched with probiotics: Mannan-oligosaccharide (MOS, 99%) at a dosage of 4 kg/ton of feed and Fructo-oligosaccharide (FOS, 95%) at a dosage of 1 kg/ton of feed from Henan Showvet Industrial Co. Ltd (Henan, China). While group four was fed a basal diet containing live, freeze-dried Probiotics: Lactobacillus acidophilus (1×10^{10} CFU/g) at a dosage of 220 g/ton of feed and spore-forming Bacillus subtilis ($\geq1\times10^{11}$ CFU/g) at a dosage of 50 g/ton of feed also from Showvet Industrial Co. Ltd. Lastly, group five received a combination of both Prebiotics and Probiotics at the same dosages.

All experimental diets were formulated based on the nutritional recommendations by Rostagno H et al. [40]. The composition and proximate analysis of the diets are presented in Tables 1 and 2, and were evaluated at the Central Veterinary Laboratory Agency (CVL), Tanzania, using Near Infrared Reflectance Spectroscopy (NIRS). The chicks were raised for 42 days under standard housing conditions. Chlorine-free (boiled) water and feed were provided *ad libitum*. Lighting was supplied via six-watt bulbs, and brooding heat was maintained using 250-watt infrared lamps, starting at 34℃ and gradually reduced to 24℃ by day 28. All birds were vaccinated against Newcastle disease virus (NDV) using Biovac VIR 116 vaccine (LaSota strain, BIOVAC Ltd, Israel) on days 7 and 21, and against Infectious Bursal Disease Virus (IBDV) using Gumboro 1-intermediate strain vaccine (Hester Biosciences Ltd, India) on days 14 and 28.

Table 1. *Composition of the formulated diet.*

Components (kg)	Starter (0-21d)	Finisher (22-42d)
Corn	45.4	52.5
Corn bran	12.5	11.0
Soybean meal	37.0	32.0
Salt	0.5	0.5
Dicalcium phosphate	2.0	1.6
Lysine	0.3	0.2
Methionine	0.3	0.2
Premix[1]	2.0	2.0
Total	100	100

Premix[1] Provides per kg of diet: Vitamin A, 12,000 I. U; Vitamin D3, 4000 I. U; Vitamin E, 12.50 mg; Vitamin K3, 3.60 mg; Vitamin B1 (thiamin), 3.0 mg; Vitamin B2 (riboflavin), 8.0 mg; Vitamin B6, 5..050 mg; Vitamin B12, 18.0 mg; Niacin, 62.0 mg; D-Biotin, 198.0 mg; Calcium D-pantothenate, 20.43 mg; Folic acid, 2.083 mg; manganese, 110.0 mg; iron, 70.0 mg; zinc, 75.0 mg; copper, 9.0 mg; iodine, 2.0 mg; cobalt, 500.0 mg; and selenium, 160.0 mg.

Table 2. *Proximate analysis of the diet.*

Components	Starter (0-21)	Finisher (22-42)
Metabolizable energy (kcal/kgDM)	2919	3172
Crude Protein (%)	25.4	20.8
Methionine /Cystine (%)	1.08	0.88
Lysine (%)	1.55	0.97
Tryptophan (%)	0.3	0.24
Ash (%)	7.8	7.3
Crude fat (%)	3.2	6.1
Crude fiber (%)	6.6	3.7

Components	Starter (0-21)	Finisher (22-42)
Starch (%)	37.8	42.8
Total sugar (%)	4.1	4.0
Dry matter (%)	89.8	89.3

2.3. Performance Metrics

Body weight was recorded weekly throughout the six-week trial using a digital weighing scale (Infitek Co. Ltd, Lixia District, Jinan, Shandong, China). Prior to weighing, feed and water were withdrawn for four hours to reduce potential measurement errors. Thereafter, body weight gain, feed intake, and feed conversion ratio were calculated. Mortality and livability were also monitored and documented daily.

2.4. Intestinal Tissue Sections

At the end of the experimental period (day 42), four wing-tagged chickens from each replicate per treatment group were fasted overnight and humanely sacrificed via exsanguination. The small intestine was then carefully pooled and segmented into the duodenum, jejunum, and ileum based on their anatomical landmarks. As the duodenum was marked from the gizzard to the pancreatic and bile ducts, the jejunum from the ducts to Meckel's diverticulum, and the ileum from the diverticulum to the ileocecal-colonic junction. Tissue samples were collected from the midpoint of each intestinal segment. Two-centimeter sections were rinsed in 0.1 M phosphate buffer (pH 7.3) and fixed in 10% neutral buffered formalin for five days prior to histological processing.

2.5. Intestinal Histomorphometry and Goblet Cells Histochemistry

Following the procedure described by Grozina A et al. [41], about 4 mm-thick segments of the duodenum, jejunum, and ileum were transversely sectioned and processed for histomorphometric and histochemistry analysis at the Histology Laboratory of Sokoine University of Agriculture. Briefly, the fixed tissues were dehydrated in ascending ethanol concentrations (70%, 90%, 95%, and 100%), cleared with chloroform, infiltrated with paraffin wax, and embedded in melted paraffin. Tissue ribbons were sectioned at 3 µm thickness using a microtome, transferred to cold water, then to a 45 ℃ water bath, mounted on microscope slides, and dried in a hot air oven for 12 hours. Thereafter, deparaffinization was performed using three xylene washes, followed by rehydration through descending alcohol concentrations (100%, 95%, 90%, and 70%), and rinsing under running water. Samples were immersed in 3% glacial acetic acid for three minutes, stained with Alcian blue (pH 2.5) for 35 minutes, rinsed, and counterstained with nuclear fast red for eight minutes. After a final rinse, tissues were dehydrated at ascending alcohol concentrations, cleared in xylene (three changes), and mounted with a coverslip using dibutylphthalate polystyrene xylene (DPX) mounting medium. Finally, the stained sections were observed, measured, and photographed using an Olympus light microscope (Model D21-CB, SN 0010842A2, Tokyo, Japan) equipped with a digital camera (Olympus U-TV0.5XC-3, SN OK73198, Tokyo, Japan).

2.6. Measurement of Intestinal Villus Height, Width, Surface Area and Crypt Depth

Villus height (VH), villus width (VW), and crypt depth (CD) were measured according to the method described by Arif M et al. [10], briefly, VH was measured from the tip of a well-defined villus to the opening of the associated crypt. The average villus height for each intestinal section was calculated and used to determine the mean VH for each treatment group. VW was measured at the midpoint of morphologically intact villi, and the mean VW for each section was obtained by averaging the widths of the measured villi. The group mean was then calculated across all chickens within the same treatment. CD was measured from the base of the crypt to the crypt opening, and the mean CD was determined using the same procedure as for VH and VW. All measurements were taken in micrometers (µm) under ×40 magnification. Finally, villus surface area (mm^2) was calculated using the formula described by Asif M et al. [64].

2.7. Measurement of Goblet Cell Numbers

Ten fields of view were selected to ensure full tissue coverage, and goblet cells were counted at ×40 magnification using ImageJ software (version 1.54d/Java 1.8.0_345, 64-bit). Cell counts were performed automatically by the software, applying defined inclusion and exclusion criteria. The average number of goblet cells per tissue was calculated by averaging the counts from the ten fields. The overall average number of goblet cells per treatment group was then determined by averaging the tissue means within each group.

2.8. Data Analysis

Microsoft Excel 2010 was used to clean the raw data, thereafter R software version 4.4.1 implying One-way analysis of variance (ANOVA) was used in the analysis. The significant differences among treatment groups were determined; when a significant effect was detected at $p < 0.05$, post-hoc pairwise comparisons were performed using the Least Significant Difference (LSD) method to validate the findings and improve data interpretation.

3. Results

The effects of dietary Prebiotics, Probiotics and Eubiotics supplements as growth promoters on performance metrics and intestinal histomorphometry in Broiler chickens are presented hereunder.

3.1. Performance Parameters

Table 3 and Figure 1 present the mean performance results for broiler chickens fed a diet supplemented with Antimicrobial growth promoters, Prebiotics, Probiotics, and Eubiotics at different phases of the trial. All AGP alternatives significantly increased FI across the entire trial period compared to both the negative and positive control groups ($p < 0.05$). Among them, the Eubiotics-treated group consistently recorded the highest FI throughout the experiment. In terms of WG, all AGP alternatives resulted in significantly higher gains during the first four weeks and the final week compared to both control groups ($p < 0.05$). Although no statistically significant difference was observed in the fifth week ($p = 0.068$), the Eubiotics group showed a higher WG (304.301 g) than the negative (194.929 g) and positive (219.462 g) control groups, whereas Probiotics and Prebiotics demonstrated a moderate performance, particularly in the early stages. Regarding FCR, all supplemented groups exhibited significantly lower FCR during the first week compared to the controls ($p = 0.027$). However, in the second week, only the Probiotics and Eubiotics groups maintained a significantly lower FCR ($p = 0.038$). From the third week onwards, no statistically significant differences were observed between the supplemented and control groups ($p > 0.05$), although lower FCR values were observed in the Probiotics and Eubiotics groups. Mortality rates were generally comparable across all treatment groups during the first four weeks, with no significant differences observed ($p > 0.05$). However, during the final two weeks, the Eubiotics supplemented group had a higher mortality rate compared to both negative and positive control.

Table 3. *The mean feed intake, weight gain, feed conversion ratio, and mortality of broiler chickens treated with antibiotic growth promoter alternatives.*

Period	Parameters	Treatments					P-value
		Control	AGP	Prebiotics	Probiotics	Eubiotics	
1-7 days	FI (g)	115.005 [a]	130.923 [a]	154.404 [b]	201.943 [c]	236.245 [d]	0.027
	FCR	1.933 [a]	1.873 [a]	1.765 [b]	1.738 [b]	1.712 [b]	0.036
	M (%)	0.800 [a]	0.721 [a]	0.739 [a]	0.875 [a]	0.862 [a]	0.086
	WG (g)	59.502 [a]	69.901 [b]	87.468 [c]	116.193 [d]	137.965 [e]	0.037
8-14 days	FI (g)	152.961 [a]	199.870 [b]	248.843 [c]	266.068 [d]	285.893 [e]	0.022
	FCR	1.924 [a]	1.918 [a]	1.907 [a]	1.800 [b]	1.700 [b]	0.038
	M (%)	0.837 [a]	0.879 [a]	0.789 [a]	1.091 [a]	1.008 [a]	0.471
	WG (g)	79.483 [a]	104.199 [b]	130.495 [c]	147.818 [d]	168.175 [e]	0.017
15-21 days	FI (g)	239.439 [a]	292.572 [b]	325.273 [c]	345.453 [d]	367.365 [e]	0.035
	FCR	2.000 [a]	1.912 [a]	1.850 [a]	1.800 [a]	1.716 [a]	0.335
	M (%)	1.089 [a]	0.893 [a]	0.946 [a]	1.007 [a]	0.952 [a]	0.573
	WG (g)	119.729 [a]	152.988 [b]	175.838 [c]	191.926 [d]	214.094 [e]	0.010
22-28 days	FI (g)	316.997 [a]	345.659 [b]	366.497 [c]	398.926 [d]	423.443 [e]	0.048
	FCR	1.939 [a]	1.859 [a]	1.822 [a]	1.800 [a]	1.728 [a]	0.329
	M (%)	0.929 [a]	0.995 [a]	0.940 [a]	0.869 [a]	1.005 [a]	0.460
	WG (g)	163.500 [a]	185.930 [b]	201.109 [c]	221.628 [d]	245.087 [e]	0.031

Period	Parameters	Treatments					P-value
		Control	AGP	Prebiotics	Probiotics	Eubiotics	
29-35 days	FI (g)	381.831 ^{a}	409.370 ^{b}	438.723 ^{c}	460.564 ^{d}	520.725 ^{e}	0.022
	FCR	1.959 ^{a}	1.865 ^{a}	1.835 ^{a}	1.800 ^{a}	1.711 ^{a}	0.498
	M (%)	0.944 ^{a}	1.112 ^{a}	1.041 ^{a}	0.980 ^{a}	1.132 ^{a}	0.374
	WG (g)	194.929 ^{a}	219.462 ^{a}	239.138 ^{a}	255.878 ^{a}	304.301 ^{a}	0.068
36-42 days	FI (g)	431.955 ^{a}	471.016 ^{b}	520.939 ^{c}	533.597 ^{d}	548.468 ^{e}	0.023
	FCR	1.955 ^{a}	1.900 ^{a}	1.854 ^{a}	1.800 ^{a}	1.705 ^{a}	0.359
	M (%)	1.179 ^{a}	1.043 ^{a}	1.148 ^{a}	1.046 ^{a}	1.717 ^{a}	0.362
	WG (g)	220.986 ^{a}	247.902 ^{b}	281.049 ^{c}	296.444 ^{d}	321.622 ^{e}	0.018

Different superscripts in the same row indicate statistically significant differences ($P < 0.05$) between treatments on the same trial period. The same superscripts indicate no significant different.

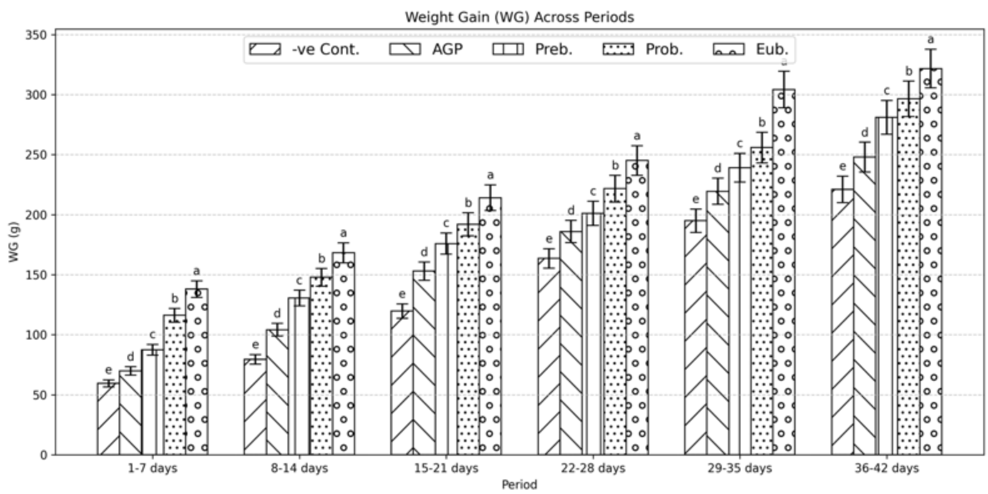

Figure 1. *The weight gain trends of Broiler chickens over time for different treatment groups across six-week period.*

3.2. Intestinal Histomorphometry and Goblet Cells Histochemistry

Table 4 and Figures 2-4 present the mean villus morphology and goblet cell counts in different intestinal segments (duodenum, jejunum, and ileum) of Broiler chickens administered various growth promoter alternatives over a 42-day period. Additionally, Figure 5 illustrates the distribution of goblet cells around the intestinal villi.

In the duodenum, VH, (p = 0.065) and VH: CD ratio (p = 0.068) showed no statistically significant differences among treatment groups and the control. However, numerically higher VH values were observed in the Probiotics (2358.93 µm) and Eubiotics (2407.82 µm) groups compared to the control (1860.91 µm), suggesting enhanced nutrient absorption. CD was significantly greater in the Prebiotics group (368.30 µm) compared to the control (354.61 µm; p = 0.029). A significant difference was found in the VH: VW ratio (p = 0.019), with Probiotics (8.84) and Eubiotics (9.51) indicating more slender, absorption-efficient villi. Villus surface area (VSA) showed no significant differences, although AGPs and supplemented groups displayed slightly higher numerical values. Goblet cell count (GCC) did not differ significantly (p = 0.080), but Eubiotics had the highest mean count (15,987.71). In the jejunum, significant differences were observed in VH (p = 0.043) and CD (p = 0.016), with Prebiotics showing the highest VH (1316.93 µm) and Probiotics the lowest CD (219.37 µm). The VH: VW ratio was highly significant (*p* = 0.001), with Prebiotics (8.72) displaying the most favorable ratio. VW was significantly higher in the Prebiotics group, matching the AGPs group. Although VSA was not statistically significant, higher values were recorded in the Prebiotics and Eubiotics groups. GCC was significantly elevated in the Prebiotics group (32,180.63; $p = 0.037$).

Lastly, the ileum, VH demonstrated no significant differences ($p = 0.093$), though the Eubiotics group had the highest numerical VH (1945.48 µm), followed by Prebiotics, both close to AGPs. VW was slightly higher across all treated groups without significant differences. A significant effect on the VH: VW ratio (p = 0.045) was observed in the Prebiotics (8.76) and Eubiotics (10.30) groups. GCC was significantly different (p = 0.002), with Prebiotics, Probiotics, and Eubiotics groups showing elevated counts, reflecting improved mucosal health. While CD showed no significant variation, it was numerically higher in the Eubiotics and Prebiotics groups. The VH: CD ratio showed a non-significant trend favoring Eubiotics (10.30), indicating taller and more efficient villi. VSA was significantly higher in the Prebiotics group (1.01 mm², p = 0.015), supporting its role in enhancing nutrient absorption.

Table 4. *Villus height: Crypt depth, Villus height to Crypt depth ratio, Villus width, Villus height to Villus width ratio, Villus surface area, and Goblet cell count of the duodenum, jejunum, and ileum in Broiler chickens treated with growth promoter substitutes for 42 days.*

Intest. part	Parameters	Treatments					P-value
		Control	AGP	Prebiotics	Probiotics	Eubiotics	
Duodenum							
	VH (µm)	1860.91 [a]	2102.61 [a]	1950.06 [a]	2358.93 [a]	2407.82 [a]	0.065
	CD (µm)	354.61 [a]	343.83 [a]	368.30 [b]	307.86 [a]	345.16 [a]	0.029
	VH: CD	5.25 [a]	6.12 [a]	5.29 [a]	7.66 [a]	6.98 [a]	0.068
	VW (µm)	245.62 [a]	281.43 [a]	302.43 [a]	266.87 [a]	253.18 [a]	0.085
	VH: VW	7.58 [a]	7.47 [a]	6.45 [b]	8.84 [c]	9.51 [c]	0.019
	VSA (mm^2)	1.20 [a]	1.54 [b]	1.52 [a]	1.63 [a]	1.59 [a]	0.077
	GCC	15232.97 [a]	15944.15 [a]	14335.65 [a]	15362.18 [a]	15987.71 [a]	0.080
Jejunum							
	VH (µm)	1165.23 [a]	1175.21 [a]	1316.93 [b]	1153.61 [a]	1266.06 [b]	0.043
	CD (µm)	256.86 [a]	203.44 [b]	230.16 [a]	219.37 [a]	204.08 [b]	0.016
	VH: CD	4.54 [a]	5.78 [b]	5.72 [a]	5.26 [a]	6.20 [a]	0.076
	VW (µm)	144.07 [a]	150.56 [b]	151.06 [b]	142.32 [a]	157.87 [b]	0.015
	VH: VW	8.09 [a]	7.81 [a]	8.72 [b]	8.11 [a]	8.02 [a]	0.001
	VSA (mm^2)	0.44 [a]	0.46 [a]	0.53 [a]	0.41 [a]	0.53 [a]	0.079
	GCC	28072.10 [a]	23831.04 [b]	32180.63 [c]	25247.65 [b]	26508.72 [b]	0.037
Ileum							
	VH (µm)	1768.77 [a]	1789.35 [a]	1900.90 [a]	1790.80 [a]	1945.48 [a]	0.093
	CD (µm)	306.50 [a]	300.96 [a]	313.05 [a]	260.98 [a]	322.9 [a]	0.087
	VH: CD	5.77 [a]	5.95 [a]	6.07 [a]	6.86 [a]	6.03 [a]	0.075
	VW (µm)	186.97 [a]	184.58 [a]	216.91 [a]	200.40 [a]	188.88 [a]	0.089
	VH: VW	9.46 [a]	9.69 [a]	8.76 [b]	8.94 [a]	10.30 [c]	0.045
	VSA (mm^2)	0.85 [a]	0.85 [a]	1.01 [b]	0.94 [a]	0.97 [a]	0.015
	GCC	22635.9 [a]	19941.90 [b]	25405.02 [c]	20112.28 [b]	23678.80 [b]	0.002

Different superscripts in the same row indicate statistically significant differences ($P < 0.05$) between treatments on the same trial period. The same superscripts indicate no significant difference.

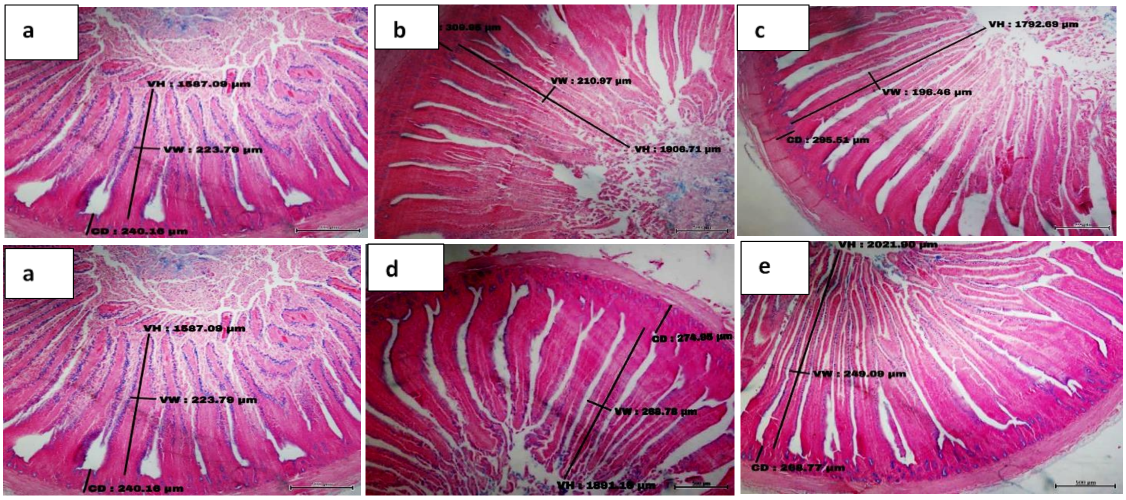

Figure 2. Photomicrographs of the cross-section of duodenum from Broiler chickens fed on Basal feed (a); AGP (b); Prebiotics (c); Probiotics (d) and Eubiotics (e) treatments showing differences in villi height, crypt depth, and villi width (stained with Alcian blue-Nuclear fast red; x40).

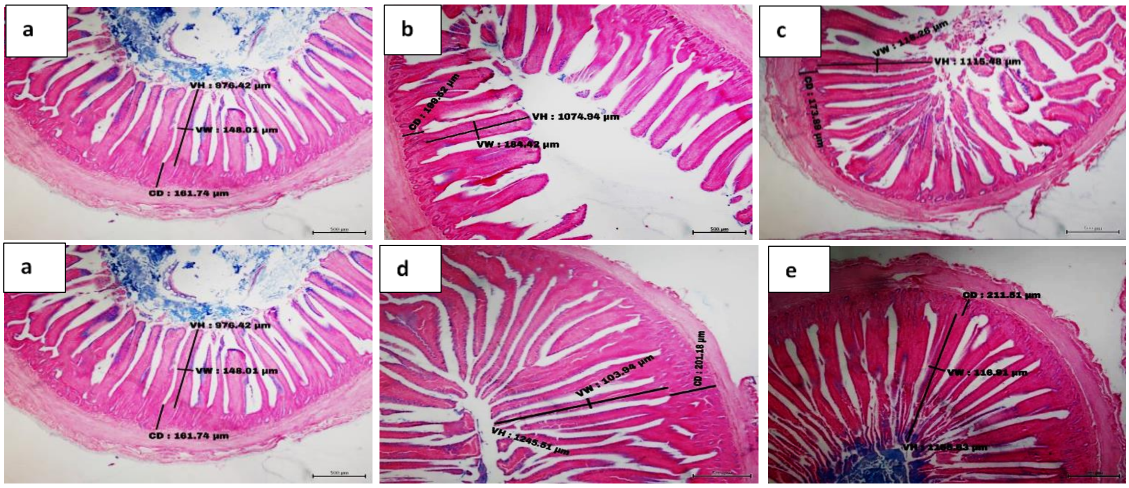

Figure 3. Photomicrographs of the cross-section of jejunum from Broiler chickens fed on Basal feed (a); AGP (b); Prebiotics (c); Probiotics (d) and Eubiotics (e) treatments showing differences in villi height, crypt depth, and villi width (stained with Alcian blue-Nuclear fast red; x40).

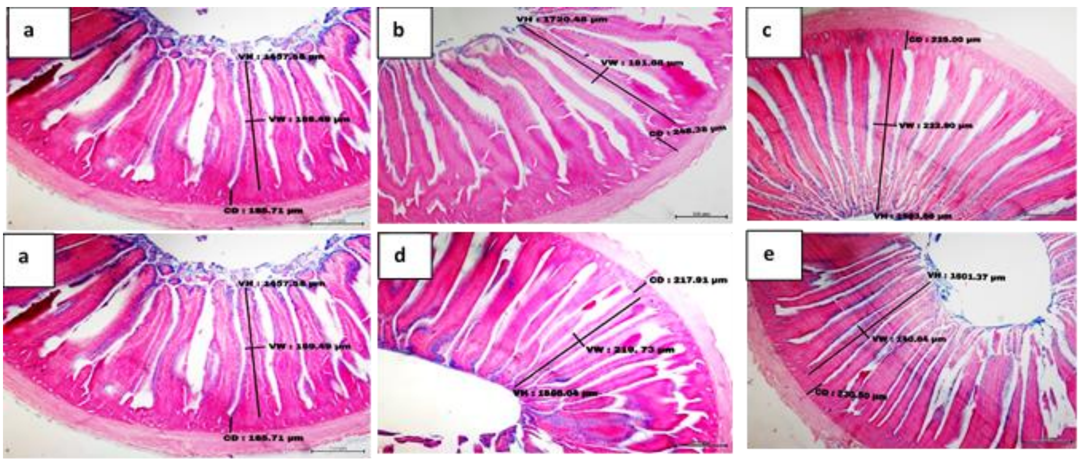

Figure 4. Photomicrographs of the cross-section of ileum from Broiler chickens fed on Basal feed (a); AGP (b); Prebiotics (c); Probiotics (d) and Eubiotics (d) treatments showing differences in villi height, crypt depth, and villi width (stained with Alcian blue-Nuclear fast red; x40).

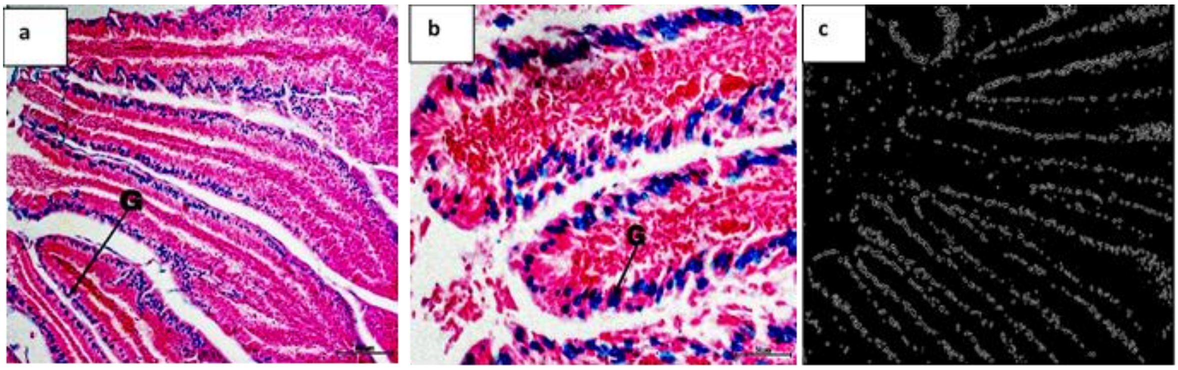

Figure 5. Photomicrographs of the small intestine villi (a & b) showing goblet cells (G) Alcian blue-Nuclear fast red stain, at x100 and x400 respectively; (c) showing goblet cells (whitish color) captured with Image J software. (Image J 1.54d/Java 1.8.0_345, 64-bit).

4. Discussion

The use of various forms of Prebiotics and strains of Probiotics in the poultry industry has gained attention as promising alternatives to AGPs. This has been driven by the growing demand for antibiotic-free poultry products and the rising prevalence of antimicrobial-resistant (AMR) bacteria in both humans and animal [42, 43]. This study aimed to investigate the synergistic effects of dietary Prebiotics, Probiotics, and Eubiotics as growth promoters on Ross 308 broiler chickens' performance and intestinal histomorphometry. Overall, the findings revealed that these supplements improved both performance and intestinal structure compared to the negative control and AGP-treated (positive control) groups, showcasing their potential as AGP alternatives.

Chickens supplemented with Prebiotics, Probiotics, and Eubiotics exhibited significantly increased feed intake across all trial periods compared to the non-supplemented groups. Similar findings were observed by El-kahal Hassanien A et al. [45], when a probiotic blend containing *Enterococcus faecium*, *Lactobacillus acidophilus*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*, along with prebiotics containing β-glucan and mannan-oligosaccharides were supplemented. Abdel-Raheem S et al. [42] also reported enhanced feed intake in chickens fed diets supplemented with Prebiotics and Probiotics. Likewise, Raksasiri B et al. [46] found significant improvements when the Jerusalem artichoke prebiotic and BACTOSAC-P® (a multi-strain probiotic) were used in combination. Conversely, Ali A et al. [39] reported no significant increase in feed intake with MOS Prebiotics, *Bacillus subtilis* Probiotics, or their combination. Leblebicier Ö et al. [47] also found no influence of MOS Prebiotics on feed intake. Likewise, Gurram S et al. [48] observed no significant differences in feed intake among chickens supplemented with a multistrain probiotic, chicory powder, and coriander powder. However, feed intake naturally increases as chickens grow due to rising nutritional demands. The observed increase in feed intake in our study likely resulted from improved palatability and gut health due to the supplements, promoting faster growth, muscle development, and earlier attainment of market weight.

Another key observation was a significantly lower feed conversion ratio in all supplemented groups during the first week compared to the control. In the second week, Prebiotics and Eubiotics continued to show a significant reduction in FCR, and although the difference was not statistically significant in the following weeks, all supplemented groups maintained numerically lower FCRs. These results are supported by Samli H et al., Nakpiran H et al., Ahmed H et al., Khomayezi R et al. and Popova T et al. [44, 49-52], who reported improved FCRs with prebiotic and probiotic supplementation. Fallah R et al.[53] also found that synbiotic supplements enhanced FCR. Conversely, Dela Cruz P et al. [54], noted higher FCRs in chickens supplemented with Eubiotics during the initial two weeks, Murshed M et al. [55] reported a contrary finding that chickens supplemented with Prebiotics, Probiotics, or AGPs outperformed those receiving Eubiotics. FCR reflects the efficiency of converting feed into body mass; a lower ratio indicates better feed utilization. Prebiotics serve as a substrate for Probiotics, improving nutrient digestibility and absorption through enzymes such as proteases, peptidases, amylase, and bile salt hydrolase produced by Probiotics.

Weight gain was significantly higher in chickens receiving AGPs alternatives during the first four weeks and again in the sixth week compared to controls. Although no statistical difference was noted in the fifth week, supplemented groups consistently showed numerically higher weight gain. Abudabos A et al. [56] observed similar improvements using *B. subtilis* at 2×10^7 cfu/g in infected chickens. Roy B et al. and Al-Baadani H et al. [37, 57] also reported increased weight gain with prebiotic, probiotic, and eubiotic supplementation. In contrast, Ali A et al. [39] found significant weight gain only in chickens fed synbiotics, and Al-Khalaifa H et al. [58] observed minimal effects with prebiotic and probiotic combinations at 28 days. Weight gain observed in this study correlates with improved feed intake and lower FCR, contributing to muscle growth and earlier market readiness. Notably, performance was comparable between AGP and supplement-treated groups, suggesting that AGPs may no longer be essential for optimal production.

Mortality rates showed no significant differences among groups throughout the study, although the positive control had numerically lower mortality during the first week, likely due to AGPs' protection against pathogens. Prebiotics also demonstrated lower early mortality, potentially due to mannose and fructose oligosaccharides activities. A study by Ali A et al.[39] found similar results with MOS in Arbor Acres chickens. Prebiotics, once fermented, produce postbiotics such as lactic acid and short-chain fatty acids, which lower gut pH and inhibit pathogens like *Clostridium perfringens* and *Salmonella enteritidis* [10, 59]. However, from the third week onward, mortality was numerically higher in all supplemented groups, potentially linked to Sudden Death Syndrome (SDS), a condition in which healthy, heavy broilers die suddenly without clinical signs. Post-mortem examinations revealed ascites, indicating right heart failure, often associated with pulmonary hypertension. Basaki M et al. and Nisar H et al.[60-61] also linked SDS to higher mortality in broilers. To mitigate SDS, farmers may need to reduce feed rations or phase out chickens during peak of SDS periods.

Regarding intestinal histomorphometry, chickens supplemented with Prebiotics had significantly taller villi in the jejunum compared to controls. Duodenum and ileum villi were numerically taller in Eubiotics-fed chickens. These results align with Alsulami M et al., Roy B et al. and El Jeni R et al. [26, 37, 62] but differ from Murshed M et al.[55] who found higher villi height in the ileum with Prebiotics. Salim H et al. [63] reported increased ileal villi height with Probiotics, while Asif M et al. [64] found improvements in both jejunum and ileum using MOS. Abdel-Raheem S et al. [42], however, observed higher villi across all segments in symbiotics-fed chickens. AGP-treated birds also showed slightly improved villi height, consistent with their role in enhancing intestinal health and nutrient absorption.

Villi width was significantly increased in the jejunum of Prebiotics-fed chickens. Duodenal villi were numerically wider in both Prebiotics and AGP-treated groups, while no statistical differences were observed in the ileum. However, numerical increases in width were again highest in Prebiotics-fed birds. Prebiotics enrich the substrate for Probiotics, which in turn generate postbiotics that help sterilize the intestine and improve digestibility [13]. This broader villus base enhances nutrient absorption by increasing surface area. These findings contradict those of Yaqoob M et al. and Eugenio Bahule C et al. [38, 65], who found increased villi width with Probiotics and synbiotics instead.

Crypt depth was significantly higher in the duodenum of Prebiotics-fed chickens and in the jejunum of those fed Eubiotics. Ileal crypt depth was numerically highest in Eubiotics-fed birds, followed by those on Prebiotics. These results highlight how Prebiotics and Eubiotics can enhance crypt depth in different intestinal regions. Neveling D et al. [66] found increased crypt depth in the duodenum, jejunum and ileum of chickens fed with Probiotics, while Khomayezi R et al. [51] observed a decrease in CD of the duodenum. Gurram S et al. [48] supported our findings with significant increases in duodenal crypt depth with Prebiotics. Deep crypts signify healthier villi, improved immune modulation, and enhanced nutrient uptake [67]. No statistical differences were observed in the villi height-to-crypt depth ratio across all segments, although Probiotics-fed chickens showed numerically higher ratios in the duodenum and ileum. Eubiotics-fed birds had the highest ratio in the jejunum. This indicates that different AGP alternatives affect intestinal segments differently, with Probiotics having the strongest influence. Rodjan P et al. [68] observed similar trends with probiotic and organic acid synergies. Fernandes B et al. [69], however, found no significant differences with combined prebiotic-probiotic supplementation. Gurram S et al. [48] reported improved ratios with probiotic, chicory, and coriander supplements.

Villi surface area in the ileum from chickens fed Prebiotics supplements was significantly higher. However, no statistically significant different of the villi surface area in the duodenum and jejunum from chickens supplemented with either treatment. Numerical higher surface areas in these segments were observed with Eubiotics and equally Prebiotics and Eubiotics in the duodenum and jejunum of the chickens' groups respectively. Villi surface area is crucial indicator of intestinal health and is directly proportional to the nutrient absorption efficiency of the intestine [70, 71]. The significant and numerical increase of the surface area observed in this study was associated with the effects of the supplements used. Ali A et al. [39], similarly found duodenal villi surface increase in chickens fed with synbiotics supplements. However, Solis de los Santos F et al. [72] found significant increase in illeal surface area of the Prebiotics-AM-treated birds on day 3, 7, and day 21 in birds housed in local altitude compared to the control, the surface area was reduced on day 3 in the simulated high altitude compared to the local altitude. Contrarily, a study by Shah M et al. [7] found no significant different in villi surface area at any segment of the intestine when Zinc and Protexin® probiotic was used in broiler chickens.

Furthermore, this study observed an increased number of goblet cells in chickens fed diets supplemented with AGPs alternatives. The increase was statistically significant in the jejunum and ileum of the supplemented groups compared to the negative control. Interestingly, the AGP group exhibited similar effects in these intestinal segments. These findings are consistent with previous studies in broiler chickens, including Grozina A et al. [41] who reported increased goblet cell numbers following Probiotic and Synbiotic supplementation; Seryakova A et al. [73] who used the phytobiotic Butitan at varying levels (E1-0.25%, E2-0.5%, E3-0.75%) and Bacillus subtilis-based probiotics (0.006%); and Martins A et al. [74] who evaluated Prebiotic and antibiotic supplementation. Additionally, our study found a numerically higher number of goblet cells in the jejunum at day 42 compared to other intestinal segments. This contrasts with the findings of Martins A et al. [74] who reported higher goblet cell counts in the ileum, suggesting that different supplements may stimulate goblet cell proliferation in specific regions of the gastrointestinal tract. Goblet cells play a key role in intestinal defense by secreting mucus that forms a protective barrier against pathogenic microorganisms. While an increase in goblet cells in the respiratory tract typically indicates infection, in the gastrointestinal tract, such an increase does not always imply a negative response. Under normal physiological conditions, goblet cell proliferation may occur in response to pathogenic exposure; however, changes resulting from dietary or environmental modulation may reflect a positive adaptation. In our study, the increased number of goblet cells was interpreted as a beneficial outcome, as no histopathological lesions were observed in the small intestine. This suggests that the dietary supplements may have stimulated mitotic activity in intestinal epithelial cells, promoting mucosal development and enhancing gut health.

This study highlights the advantages of incorporating dietary Prebiotics, Probiotics, and Eubiotics as effective alternatives to antibiotic growth promoters in broiler chicken production. However, some discrepancies between our results and previous studies may stem from differences in supplement strains, dosages, delivery methods (feed vs. water), environmental conditions, broiler strain genetics, age, or development stage [25, 30, 32, 33]. This alert on cautionary extrapolation of these supplements.

5. Conclusions

Overall, these natural additives improved growth performance, enhanced feed conversion efficiency, and positively influenced intestinal histomorphology particularly villus height and width, as well as goblet cell density. Among the supplements tested, Eubiotics demonstrated the most notable improvements in live weight, villus height, and goblet cell count, underscoring their strong potential as natural growth promoters. Based on these findings, these supplements are recommended as promising substitutes for AGPs, offering a sustainable approach to reducing the risks associated with antibiotic resistance from prolonged AGP use. However, further research is needed to optimize dosing especially when combining these supplements, as this study applied doses intended for individual use. Additional investigations should also assess the economic viability of large-scale application and evaluate their effectiveness under field conditions or experimentally induced stress, where environmental challenges are less controlled.

Abbreviations

AGP	Antimicrobial Growth Promoter
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
CD	Crypt Density
DPRTC	Directorate of Research, Technology Transfer and Consultancy
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio
FOS	Fructo-oligosaccharide
LSD	Least Significant Difference
MNUAT	Mwalim Nyerere University of Agriculture and Technology
MOS	Mannan-oligosaccharide
SUA	Sokoine University of Agriculture
WHO	World Health Organization

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Author Contributions

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Data Availability Statement

The data is available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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