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## Saudi Journal of Biological Sciences

journal homepage: [www.sciencedirect.com](http://www.sciencedirect.com)

Original article

## Growth performance, intestinal histomorphology, gut microflora and ghrelin gene expression analysis of broiler by supplementing natural growth promoters: A nutrigenomics approach

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## ARTICLE INFO

## Article history:

Received 14 January 2021

Revised 1 March 2021

Accepted 2 March 2021

Available online 13 March 2021

## Keywords:

Antibiotic resistance

Broiler

Gut bacteria

Intestinal histology

Natural growth promoters

## ABSTRACT

In an epoch of escalating number of antibiotic-resistance bacteria, there is a dire need to develop efficient and novel feeding strategies for animal nutrition as alternatives to antibiotics. Here, implicating nutrigenomic approach, phytobiotics and organic acids were used to evaluate ghrelin gene expression levels, gut microflora composition, performance parameters and intestinal histomorphological changes in broiler chickens. One-day-old chicks ( $n = 315$ ) were reared for 42 days and distributed randomly into five experimental groups; each with three replicates (21 birds per replicate). Experimental groups were control: basal diet only, antimicrobial growth promoter: 40 g/metric ton of basal diet (virginiamycin), organic acids: 4 kg/metric ton of basal diet, phytobiotics: 3 kg/metric ton of basal diet, combination: 7 kg/metric ton of basal diet (organic acids 4 kg and phytobiotics 3 kg metric ton of feed). Growth performance, histological and ghrelin gene expression analysis were executed on 21 and 42 days while, quantitative bacterial analysis of cecum and ileum was performed on day 42. Increased feed intake and body weight ( $p < 0.05$ ) were noticed in phytobiotics group. Addition of phytobiotics significantly improved ( $p < 0.05$ ) villus height and ratio of villus height/crypt depth in ileum, jejunum, and duodenum and down-regulated ghrelin gene expression levels. Total coliform and *Escherichia coli* in cecal and ileal digesta were decreased significantly ( $p < 0.05$ ) in organic acids group. Correlation analysis revealed *Lactobacillus* spp. were positively correlated to villus height/crypt depth ratio in duodenum. The findings indicated the importance of gene-nutrient-microbiota interactions based on nutrigenomics approach. Hence, phytobiotics and organic acids might be suitable alternatives to antibiotics for improved performance and immunity, along with healthier meat production in poultry.

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Peer review under responsibility of King Saud University.



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## 1. Introduction

Nutrigenomics is an emerging science in animal sciences which played a significant role in exploring the effect of individual nutrients on gene expression levels, metabolic processes, and epigenetic factors in the cells. It provides a tool for evaluating the production performance of improved nutrient digestion and absorption in order to maintain animal health and production performance (Nowacka-Woszuik, 2020). Along with their role as building blocks and energy providers, nutrients also have multiple biological func-

<https://doi.org/10.1016/j.sjbs.2021.03.008>

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tions including radical scavenging (antioxidants) and potent signaling molecules (nutritional hormones). Hence, a modified diet is used in livestock systems to infer the role of nutrients in host on gene expression, protein levels and metabolites production (Vilar da Silva et al., 2020).

Antibiotics have been extensively used in animal husbandry for decades. Some are used to treat the bacterial infections as therapeutically for the improvement of health and well-being of food producing animals. Mostly antibiotics used as growth promoting agents for prophylactic purposes to improve growth performance, reduce the bacterial pathogens and increase feed conversion efficiency which are termed as antibiotic growth promoters (AGPs) (Hernández et al., 2006). The continuous use of AGPs in animal production raised the concern associated with emergence of microbial resistance to antibiotics which are used to treat animal human infections (Yakhkeshi et al., 2011). After the ban of AGPs, many alternatives have been searched out and being used such as enzymes, probiotics, prebiotics, organic acids, phytobiotics and antioxidants feed additives. Organic acids (OA) are strong alternatives for AGPs (Smulikowska et al., 2010), in successfully reducing proliferation of acid-tolerant microorganisms such as *E. coli*, *Salmonella* and *Campylobacter* spp. in host gut. OA tend to enhance nutrient absorption and *Lactobacillus* spp. population by increasing height of villus and crypt depth (García et al., 2007; Liu et al., 2014). Phytobiotics contain compounds extracted from medicinal plants with beneficial effect on production performance and animal health. For phytobiotics, whole plant, parts of plants and their essential oils may be used (Stevanović et al., 2018). Plant based feed additives known to exert positive effects on performance parameters, feed consumption, gene expression levels, metabolism, and feed efficiency along with better nutrient absorption in broilers. Natural feed additives extracted from plants are generally considered to be safe, healthy and less hazards to living systems (Ertas et al., 2005). The supplementation of phytobiotics in chicken diet increased villus height with deeper crypt depth and improved digestive enzymes secretions such as proteases and amylases to enhanced growth performance with increased absorptive cell growth in the gastrointestinal tract (Jamroz et al., 2006).

The chicken gastrointestinal tract harbors various population of microorganisms present in symbiotic relationships which ultimately affects the nutrition, metabolism, and immunity in host (Sohail et al., 2012). Nutrient digestion, assimilation and absorption process in broiler depends upon the maturation of small intestine. Mucus production, ecological balance between pathogenic and non-pathogenic bacteria and intestinal epithelial integrity mostly affects the efficacy of intestinal villi. Intestinal villi are considered as natural protection/barrier which prevents entry of any toxic material and pathogenic bacteria in the intestinal lumen (Cheled-Shoval et al., 2011). These factors may cause destabilization of microflora balance and altered intestinal barrier. The altered intestinal barrier expedites the absorption of unwanted substances that reduces absorptive and digestive activities of gastrointestinal tract. Intestinal morphology played a significant role in nutrient absorption by the application of feed additives for increasing the absorptive efficiency of intestine. Since the intestinal morphology is crucial in nutrients absorption thus, certain feed additives are being used for improving intestinal absorptive efficacy (Pelicano et al., 2005; Song et al., 2019a). The current study aimed to analyzed effects of phytobiotics and organic acids on growth performance, gut microflora composition, gut pH, ghrelin gene expression levels, intestinal morphology to decipher their potential as alternatives to antimicrobials in chicken diet.

## 2. Methodology

### 2.1. Experimental design

A total of 315 one day old unsexed chicks were procured from local hatchery. Chicks were randomly distributed among five treatment groups (63 chicks/treatment) with three replicates per treatment (21 birds/replicate). The replicates reared in separate pen (size of each pen was 24 ft<sup>2</sup>). The standard operating procedures of animal experiments were performed according to Care and Use of Agricultural Animals in Research (McGlone, 2010), after approval from institutional ethical committee (DG/AA-089) at The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi.

### 2.2. Experimental feeds

Two experimental rations were formulated including starter and finisher diets according to hubbard nutritional requirements of broilers and each diet was analyzed for proximate analysis (AOAC, 2005). Experimental ration with ingredients and nutrients composition is mentioned in Table 1. The starter and finisher diets were formulated and calculated to contain crude protein 21.3% and 19.02%, respectively. Rations were formulated in *iso*-nitrogenous and *iso*-caloric form with the growth enhancers and divided into five treatments:

- Control: (basal diet without additives)
- Antimicrobial growth promoter (AGP): (basal diet + virginiamycin, 40 g/metric ton)
- Organic acids: (basal diet + blend of formic acid, (45%), propionic acid (11.5%) and citric acid (15%); 4 kg/metric ton)
- Phytobiotics: (basal diet + mixture of dried powder of rhizome of ginger (10%), liquorice (10%), ashwagandha roots (10%), black seeds (10%) and leaves of green tea (15%); 3 kg/metric ton)
- Combination: (basal diet + combination of phytobiotics and organic acids; 4 kg/metric ton)

### 2.3. Growth performance analysis

All experimental diets were administered to birds *ad libitum*. The growth performance analysis including body weight, feed intake, and feed conversion ratio were executed at 21 and 42 days during the trial.

### 2.4. Sample collection

On day 21 and 42, three chicks from every replicate pen were randomly selected and slaughtered for histomorphological and microflora analysis. The distal portion of ileum, jejunum and duodenum were collected for histomorphological analysis. The cecal and ileal digesta of slaughtered birds on day 42, were aseptically collected in cryo-protective broth. All samples were stored at –80 °C for further analyses.

### 2.5. Histological analysis

Three chicks from every replicate were selected for histomorphological analysis and slaughtered for collection of distal portions of ileum, jejunum, and duodenum at 21- and 42 days. The portions were initially fixed in buffered formalin saline (10%) followed by dehydration in various alcohol concentrations in a range from 70–100%. Later, samples were infiltrated with xylene and then embedded in paraffin wax. Section cutting of samples were

**Table 1**  
Composition of starter and finisher diets.

Feed ingredients	Inclusion (kg)		Nutrients	Calculated composition	
	Starter	Finisher		Starter Diet	Finisher Diet
Corn	590	620	ME <sup>8</sup> , Kcal /kg	2900	3000
Soya bean meal (44%)	194.3	280	Crude protein (%)	21.5	19.17
Canola meal	100	0	Crude fiber (%)	4.34	3.98
Fish meal (55% crude protein)	30	0	Ether extract (%)	3.97	4.71
Corn gluten (60% crude protein)	30	25	Ash (%)	3.99	3.7
PPM <sup>1</sup> (50% crude protein)	20	20	Calcium (%)	1	0.9
Limestone	13	14	Available. Phosphorous (%)	0.47	0.4
Vegetable oil	3.6	16	Dig. Lysine (%)	1.19	1.07
DL-Methionine	1.8	2.6	Dig. Methionine (%)	0.54	0.56
Lysine sulphate 55%	4.7	3.9	Dig. Methionine + Cysteine (%)	0.88	0.88
Vitamin premix <sup>2</sup>	0.5	0.5	Dig. Tryptophan (%)	0.023	0.2
Mineral premix <sup>3</sup>	0.5	0.5	Dig. L-threonine (%)	0.9	0.8
L-threonine	1.6	1.5			
Salt (NaCl)	2	2	Proximate analysis		
Sodium-bi-carbonate	2.5	2.3	ME <sup>8</sup> , Kcal /kg	2898	2996
*Anti -coccidial <sup>4</sup>	0.2	0	Crude protein (%)	21.3	19.02
*Anti -coccidial <sup>5</sup>	0	0.5	Crude fiber (%)	4.21	3.76
* MDCP <sup>6</sup> (21%)	3	9.9	Ether extract (%)	3.99	4.78
Choline chloride 60%	2	1	Ash (%)	3.87	3.73
Quantum blue (Phytase)	0.2	0.2			
Seldox <sup>7</sup> (antioxidant)	0.15	0.15			
Total	1000	1000			

PPM<sup>1</sup> = Poultry by Product Meal, Vitamin premix<sup>2</sup>: composition per kg (vitamin A, 20,000 KIU/kg; vitamin E, 48,000 mg/kg; vitamin D3, 5,400 KIU/kg; vitamin B1, 4,000 mg/kg; vitamin K 34,000 mg/kg; vitamin B2, 9,000 mg/kg; vitamin B6, 7,600 mg/kg; vitamin B12, 20 mg/kg; Niacine, 60,000 mg /kg; Folic acids, 1,600 mg/kg; Pantothenic Acid, 20,000 mg/kg; Biotin, 200 mg/kg); Mineral premix<sup>3</sup>: composition per kg (Manganese, 130,000 mg/kg, Iron , 60,000 mg/kg; Iodine, 1,800 mg/kg; Zinc, 120,000 mg/kg; Copper, 10,000 mg/kg; Selenium, 360 mg/kg ; Cobalt , 400 mg/kg); \*Anti-coccidial<sup>4</sup>: maduramicin 1%; \*Anti -Coccidial<sup>5</sup> Diclazuril 0.5%; \* MDCP<sup>6</sup> 21% mono dicalcium phosphate; Seldox<sup>7</sup>: contained butylated hydroxy anisole, butylated hydroxy toluene, ethoxyquin and citric acid; ME<sup>8</sup>: metabolizable energy.

performed through microtome (section size 5 μm). The sections were placed on a glass slide, fixed in wax ribbon and stained with hematoxylin-eosin. Slides were analyzed under a microscope and pictures were taken. These images were analyzed using the (Image J, 1.50i) software. The depth of the crypt was calculated by measuring the distance between the basolateral membrane and individual villi. (Baurhoo et al., 2007).

2.6. Microflora composition

For bacterial enumeration, 1 g sample was transferred in 9 mL phosphate buffer saline (PBS) and serially diluted. The *Lactobacillus* spp., *Escherichia coli*, *Salmonella* spp., total coliform, and total viable bacteria (aerobes and anaerobes) were quantified on de Man Rogosa and Sharpe (CM0359, Oxoid UK), xylose lysine deoxycholate (CM0469, Oxoid UK), eosin methylene blue (CM0069, Oxoid UK), MaConkey’s (CM0505, Oxoid UK) and plate count agar (CM0325, Oxoid UK), respectively. The colonial counts were estimated through log cfu/g using spread plate technique (Andrews et al., 2014).

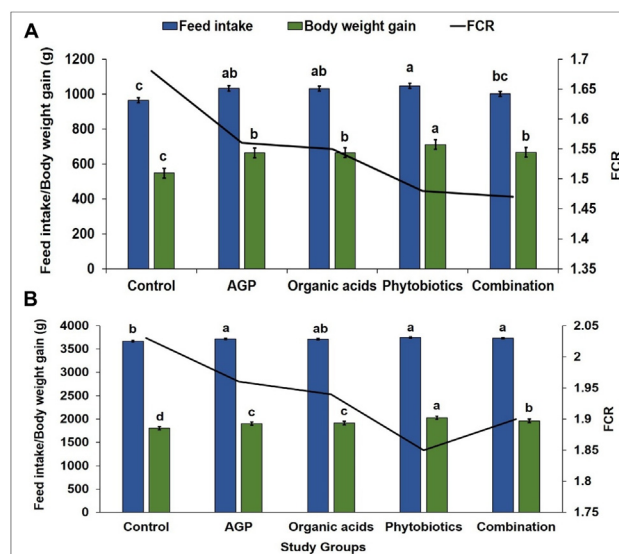
2.7. Gastrointestinal tract pH analysis

For determination of pH, 10 g of gut contents (crop, proventriculus, duodenum, jejunum, ileum and gizzard) were taken and diluted in PBS to make 1:10 dilution and pH was observed using pH meter (Al-Natour and Alshawabkeh, 2005).

2.8. Gene expression analysis

The expression of ghrelin gene was determined using real-time quantitative polymerase chain reaction (RT-qPCR). Primers used in the study are forward 5’ CCTGGGACAGAACTGCTC 3’ and reverse 5’ CACCAATTTCAAAGGAACG 3’ designed using Primer 5.0 software and manufactured from commercial company (Promega, USA). Total RNA was extracted from proventriculus tissue

(100 mg) using TRIzol ((Invitrogen/Life Technologies, Isogene Co, Russia). The quantitative and qualitative analysis of extracted RNA was performed using Nanodrop P 360 (Implen, Germany) and agarose gel electrophoresis, respectively. RNA (2 μg) was initially reverse-transcribed using commercial kit (Thermo Fisher Scientific, UK) and real-time PCR was performed using SYBR green master mix (Applied Biosystems, Warrington, UK) on ABI 7300 system (Applied Biosystems, USA). The reaction conditions were denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The ghrelin and actin (housekeeping)



**Fig. 1.** Growth performance of broiler chickens at 21 (A) and 42 days (B). Significance assumed at  $p < 0.05$ . Bars<sup>a,b,c</sup> with similar letter represents non-significant differences ( $p > 0.05$ ). Error bars represents standard error of mean. FCR: Feed conversion ratio.

gene expressions were analyzed in all samples through ddCt relative quantification plate study software.

### 2.9. Statistical analysis

Analysis of variance (one-way ANOVA) was performed for statistical analysis of data and Duncan multiple range (DMR) test was used for comparing means. The significance was assumed at  $p < 0.05$ . Pearson's test was conducted to find correlation among changes associated with gut microflora composition and correlation coefficient ( $r$ ) was indicated with significance at  $p < 0.05$ .

## 3. Results

### 3.1. Performance analysis

The performance parameters were significantly different ( $p < 0.05$ ) in all groups (Fig. 1.). The highest feed intake, weight gain and improved feed efficiency were observed in phytobiotics at 21

(Fig. 1A) and 42 days (Fig. 1B) followed by combination group. Similarly, elevated body weight gain ( $p < 0.05$ ) was also observed in combination and phytobiotics. Phytobiotics group also showed feed conversion ratio of 1.85 which indicated better nutrient efficiency.

### 3.2. Histological analysis

Non-significant effects ( $p > 0.05$ ) of different feed groups for villus height (Fig. 2A) and crypt depth (Fig. 2B) in jejunum and duodenum at 21 days were noted. In ileum, highest villus height (620.5  $\mu\text{m}$ ) was observed in phytobiotics supplemented group. Higher depth of crypt in duodenum (140.4  $\mu\text{m}$ ) and ileum (120.4  $\mu\text{m}$ ) was recorded in organic acids and combination groups, respectively. Phytobiotics displayed significantly ( $p < 0.05$ ) different crypt depth (109.7  $\mu\text{m}$ ) in all feed groups. Furthermore, phytobiotics and combination groups showed highest ileum (5.6  $\mu\text{m}$ ) and jejunum (3.6  $\mu\text{m}$ ) ratio (Fig. 2C). At 21 days, villus height to crypt ratio was unaffected ( $p < 0.05$ ) in duodenum among feed

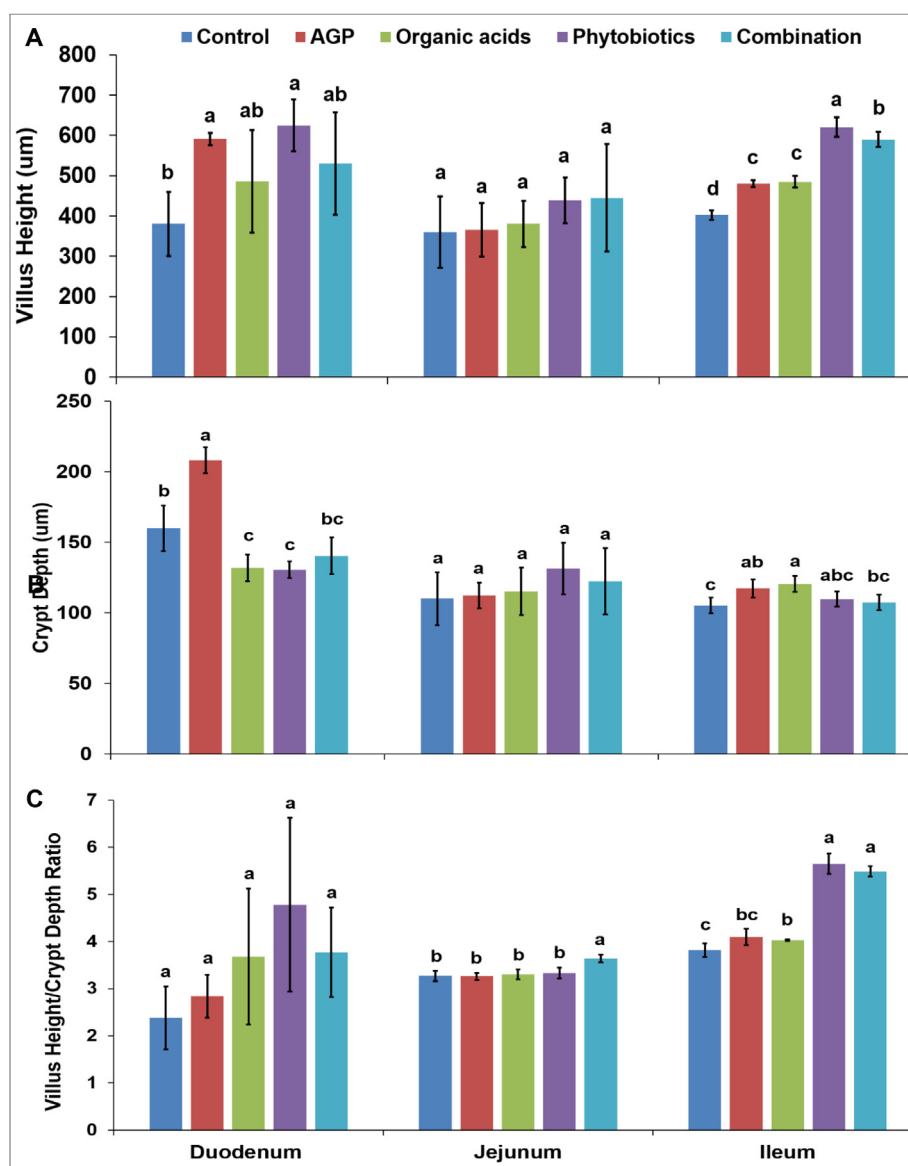


Fig. 2. Villus height (A), crypt depth (B) and villus height/crypt depth ratio (C) of broiler chickens at 21 days. Significance assumed at  $p < 0.05$ . Bars<sup>a,b,c</sup> with similar letter represents nonsignificant differences ( $p > 0.05$ ).



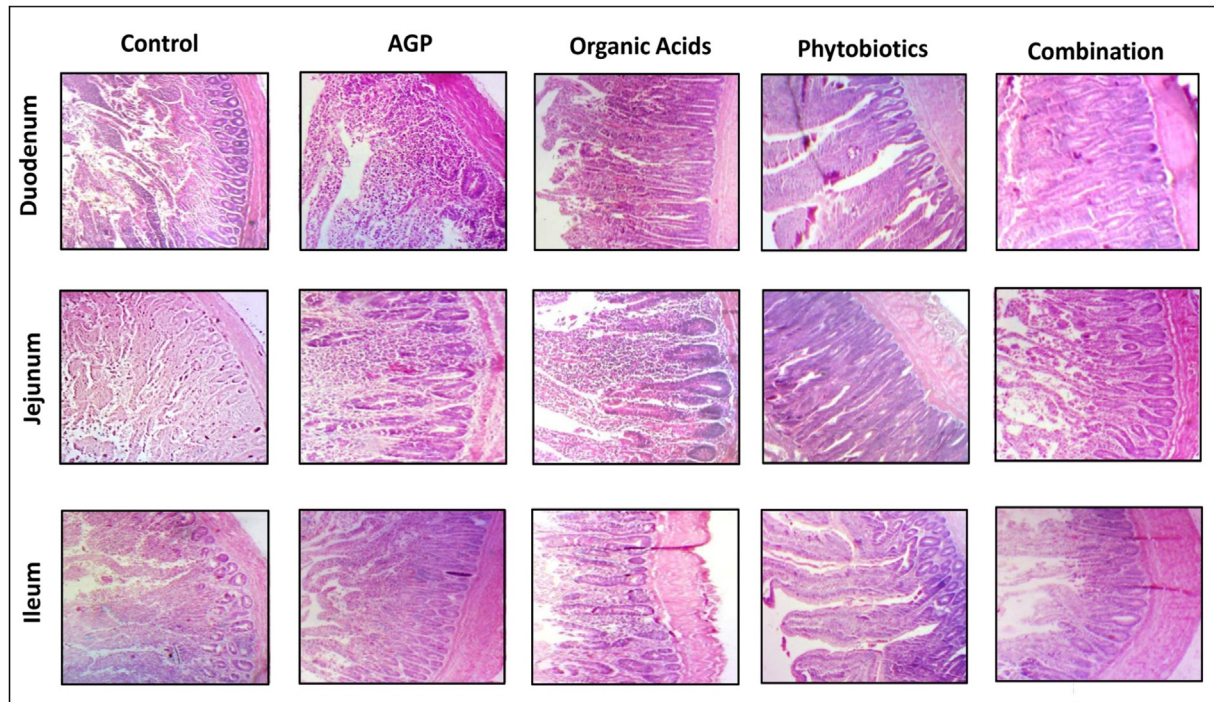


Fig. 3. Histomorphology of jejunum, duodenum, and ileum showing villus height at 21 days in broiler chickens.

groups. Among natural growth enhancers, non-significant ( $p > 0.05$ ) effects on ileum villus height were exhibited (Fig. 3.).

On the other hand, villus height (Fig. 4A) and crypt depth (Fig. 4B) of jejunum, duodenum and ileum affected significantly ( $p < 0.05$ ) by different feed groups at 42 days. Organic acids fed group exhibited highest duodenum crypt depth (188.2  $\mu\text{m}$ ). Combination group showed highest crypt depth of jejunum (182.1  $\mu\text{m}$ ) and ileum (170.0  $\mu\text{m}$ ) among all groups. The higher ratio of jejunum (7.4  $\mu\text{m}$ ) and ileum (7.3  $\mu\text{m}$ ) was observed in phytobiotics supplemented group. Villus height and crypt depth ratio of duodenum was non-significant ( $p > 0.05$ ) among feed groups at 42 days (Fig. 4C). Phytobiotics showed highest duodenum (1390.4  $\mu\text{m}$ ), jejunum (1345.0  $\mu\text{m}$ ) and ileum (1235.9  $\mu\text{m}$ ) villus height compared to other groups (Fig. 5).

### 3.3. Quantitative analysis of gut bacteria

Microflora composition of cecum and ileum were enumerated to examine the effects of various feed groups. Total viable anaerobes, total viable coliform bacteria, *Escherichia coli* and *Lactobacillus* spp. counts were considerably affected ( $p < 0.05$ ) by feed variations in both cecal and ileal regions. The highest total anaerobes and *Lactobacillus* spp. counts were evident in both phytobiotics and combination groups whereas, decreased population of *Escherichia coli* and total coliform was noted in organic acids group with 5.3 and 5.4 log cfu/g respectively (Table 2). In particular, *Salmonella* spp. was not detected from cecal and ileal digesta in any study group.

#### 3.3.1. Gastrointestinal pH

Dietary variation affected ( $p < 0.05$ ) gastrointestinal pH at 21 and 42 days in crop and gizzard (Table 3). The pH of proventriculus and small intestine (jejunum, duodenum, and ileum) remained unaffected ( $p < 0.05$ ) with the changes in diet.

### 3.4. Gene expression analysis

Avian ghrelin gene expression was carried by RT-qPCR. real time polymerase chain reaction at 21 and 42 days of age. The ghrelin gene expression was significantly ( $p < 0.05$ ) different among all treatment groups at both stage of broiler production; 21 (Fig. 6A) and 42 (Fig. 6B) days. The expression level of ghrelin gene was up-regulated in control and organic acids groups among all treatments at 21 days whereas, down-regulation of gene was observed only in phytobiotics at 42 days.

### 3.5. Correlation analysis

The association of cecal/ileal microflora composition with ghrelin gene expression, growth performance parameters and small intestinal histomorphology was executed using Pearson's correlation test. Correlation analysis showed cecal *Lactobacillus* spp. were positively correlated to villus height/crypt depth ratio in duodenum ( $r = 0.8$ ,  $p < 0.05$ ) (Table 4). Moreover, cecal and ileal total anaerobes are also positively correlated to villus height/crypt depth ratio of duodenum, jejunum, and ileum. Interestingly, cecal *Lactobacillus* spp. population correlated positively ( $r = 0.9$ ,  $p < 0.05$ ) with body weight of broiler chickens (Table 5).

## 4. Discussion

The findings of current study evinced significantly increased ( $p < 0.05$ ) body weight in chickens fed with organic acids and phytobiotics in diet. The histomorphological changes revealed increased villus height in phytobiotics and combination groups. It also affected the gut microbial dynamics of broiler chickens with numerically increased population of *Lactobacillus* spp. and decreased total coliform and *Escherichia coli* growth. Inclusion of natural feed additives maintained pH in various regions of gastrointestinal tract similar to antibiotic growth promoter. Organic acids exhibited up-regulated levels of ghrelin gene in broiler at 21 and 42 days whereas, down-regulated levels of ghrelin were

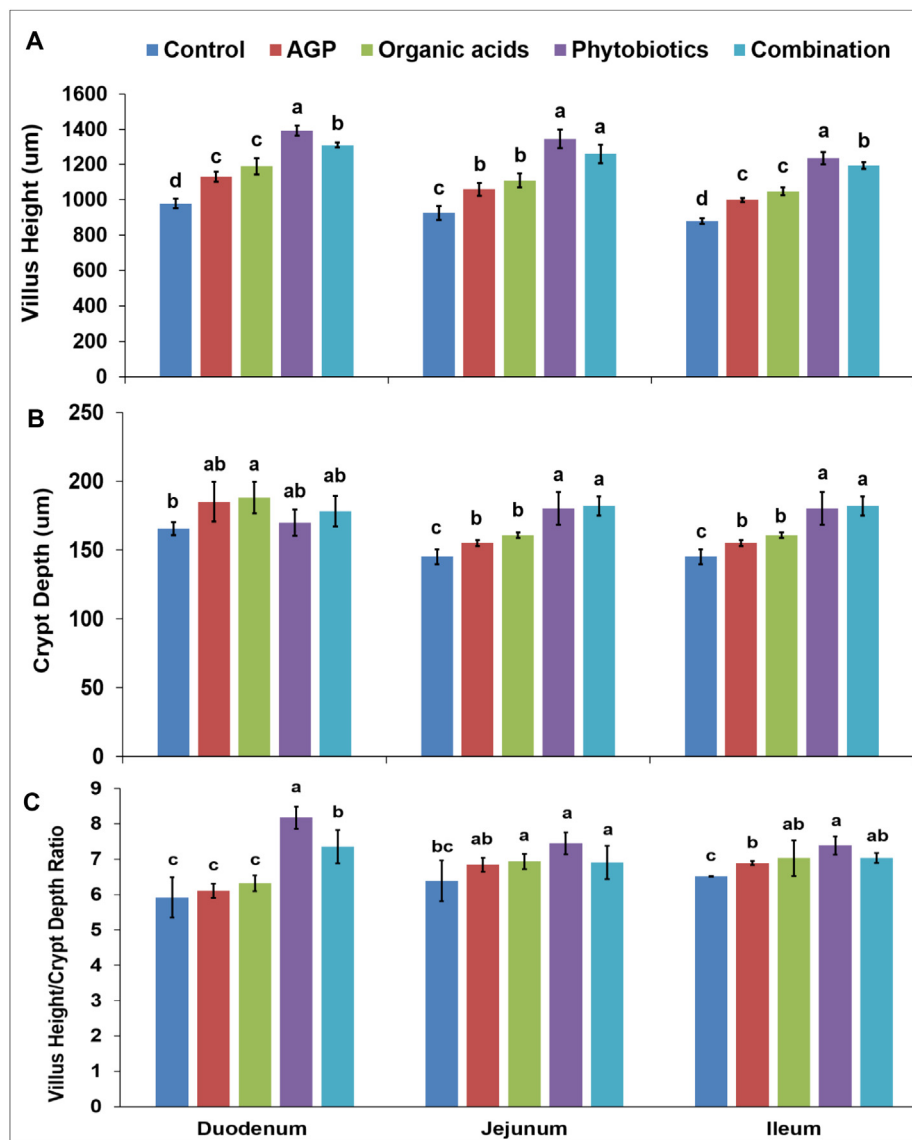


Fig. 4. Villus height (A), crypt depth (B) and villus height/crypt depth ratio (C) of broiler chickens at 42 days. Significance assumed at  $p < 0.05$ . Bars<sup>a,b,c</sup> with similar letter represents nonsignificant differences ( $p > 0.05$ ).

noticed in phytobiotics on both days. Correlation analysis further validated that increased *Lactobacillus* spp. population is positively correlated to villus height/crypt depth ratio in broiler chickens and body weight gain in broiler.

In current study the mixture of phytobiotics such as *Camellia sinensis* (green tea), *Withania somnifera* (ashwagandha), *Zingiber officinale* (ginger), *Nigella sativa* (black seed) and *Glycyrrhiza glabra* (licorice) were used which showed improved feed efficiency. It may be due to the numerous biologically active compounds present in the phytobiotics which has antimicrobial, immunomodulatory, antioxidative properties along with growth promoting effects which ultimately improved host weight gain (Reis et al., 2018). Aswagandha contains withanine and withanolides, these compounds may stimulate the gastrointestinal tract secretions which help to increase nutrient digestion and absorption to improve weight gain (Jyotsana and Berwal, 2019). Ginger, a potential phytobiotics, having bioactive compounds such as shogals, ginerdiol, and gingerdione acting as pharmacological, antioxidant, and antimicrobial agent (Teles et al., 2019; El-Hack et al., 2020). Addition of ginger powder improved nutrient digestion and absorption in the

gastrointestinal tract of chicken by stimulating the disaccharidase, lipase and maltase secretions which may lead to improved feed intake and feed efficiency (Idoko et al., 2020). The feeding of phytobiotics and organic acids in combination proved to enhance body weight gain and feed conversion efficiency in broiler compared to AGPs. The synergistic effect of these two additives is linked with gut physiology because mostly organic acids are active in crop, gizzard and upper portion of digestive tract while, essential oils are active in later segment of the intestinal tract or distal portion of the gut. Both of these additives exhibited promising results in broiler gut when administered as alternatives to diet (Gole et al., 2020).

The findings of current research regarding gut microflora populations proved that the dynamics of intestinal bacteria are influenced by the supplementation of organic acids in chickens feed and responsible for significantly reduced growth of harmful bacterial populations such as *Clostridium perfringens*, *Campylobacter* & *E. coli* (Markowiak and Śliżewska, 2017). The application of propionic acid suppresses *E. coli* and *Salmonella* growth in cecal and fecal microorganism populations without exerting negative effects on the growth and counts of *Lactobacillus* spp. in chickens (Ding

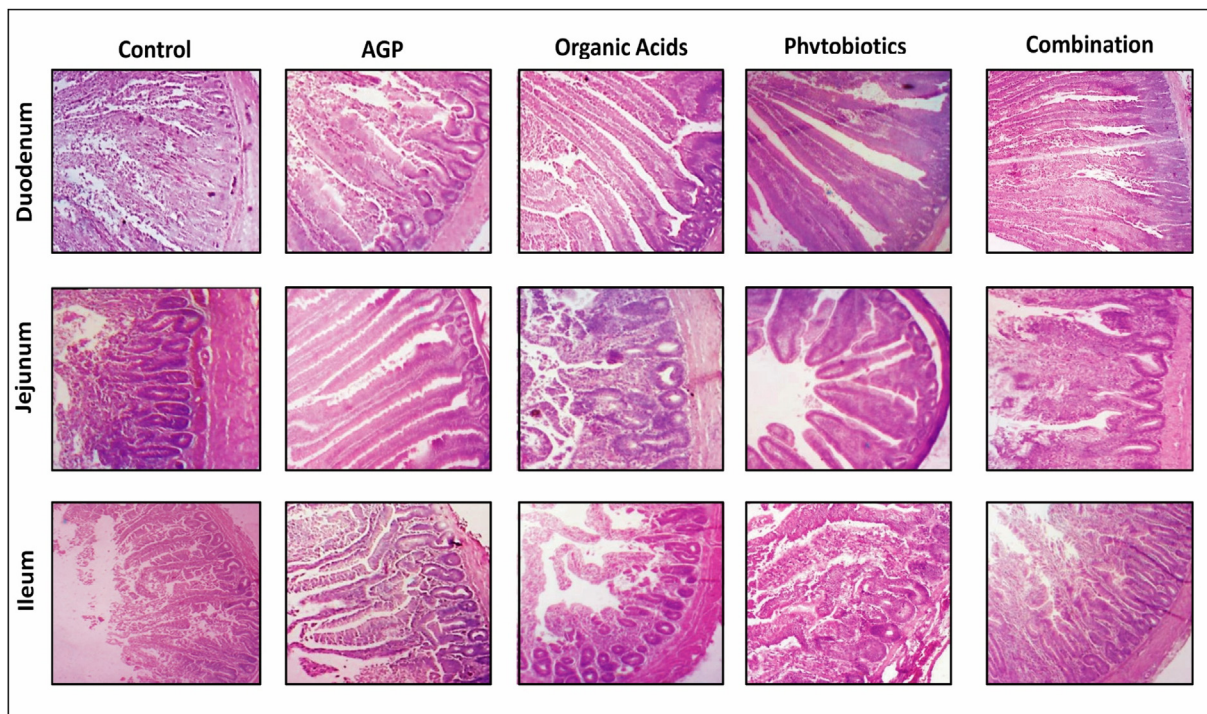


Fig. 5. Histomorphology of jejunum, duodenum, ileum showing villus height at 42 days in broiler chickens.

Table 2  
Microbial enumeration (log cfu/g) of cecal and ileal digesta of broilers at 42 days.

Study groups	Total aerobes	Total anaerobes	Total coliform	<i>Lactobacillus</i> spp.	<i>Escherichia coli</i>	<i>Salmonella</i> spp.
Cecal digesta						
Control	6.5 <sup>a</sup>	5.9 <sup>d</sup>	5.9 <sup>a</sup>	5.2 <sup>d</sup>	6.1 <sup>a</sup>	ND
AGP	6.4 <sup>ab</sup>	6.1 <sup>c</sup>	5.6 <sup>b</sup>	5.3 <sup>cd</sup>	5.5 <sup>bc</sup>	ND
Organic acids	6.2 <sup>ab</sup>	6.4 <sup>b</sup>	5.4 <sup>c</sup>	5.5 <sup>bc</sup>	5.3 <sup>c</sup>	ND
Phytobiotics	6.2 <sup>b</sup>	6.9 <sup>a</sup>	5.4 <sup>bc</sup>	5.9 <sup>ab</sup>	5.6 <sup>b</sup>	ND
Combination	6.4 <sup>a</sup>	6.7 <sup>a</sup>	5.5 <sup>b</sup>	5.8 <sup>a</sup>	5.6 <sup>b</sup>	ND
SEM	0.05	0.05	0.05	0.09	0.08	
<i>p</i> -value	0.16	<0.001	0.01	<0.001	<0.001	
Ileal digesta						
Control	6.3 <sup>a</sup>	5.9 <sup>b</sup>	6.1 <sup>a</sup>	5.1 <sup>d</sup>	5.2 <sup>a</sup>	ND
AGP	6.3 <sup>a</sup>	6.1 <sup>b</sup>	5.2 <sup>b</sup>	5.3 <sup>cd</sup>	4.6 <sup>b</sup>	ND
Organic acids	6.1 <sup>a</sup>	6.5 <sup>a</sup>	4.9 <sup>c</sup>	5.6 <sup>bc</sup>	4.5 <sup>b</sup>	ND
Phytobiotics	6.2 <sup>a</sup>	6.9 <sup>a</sup>	4.9 <sup>c</sup>	5.8 <sup>ab</sup>	4.7 <sup>b</sup>	ND
Combination	6.2 <sup>a</sup>	6.6 <sup>a</sup>	5.1 <sup>bc</sup>	5.7 <sup>a</sup>	4.7 <sup>b</sup>	ND
SEM	0.09	0.1	0.07	0.09	0.12	
<i>p</i> -value	0.54	<0.001	<0.001	<0.001	0.01	

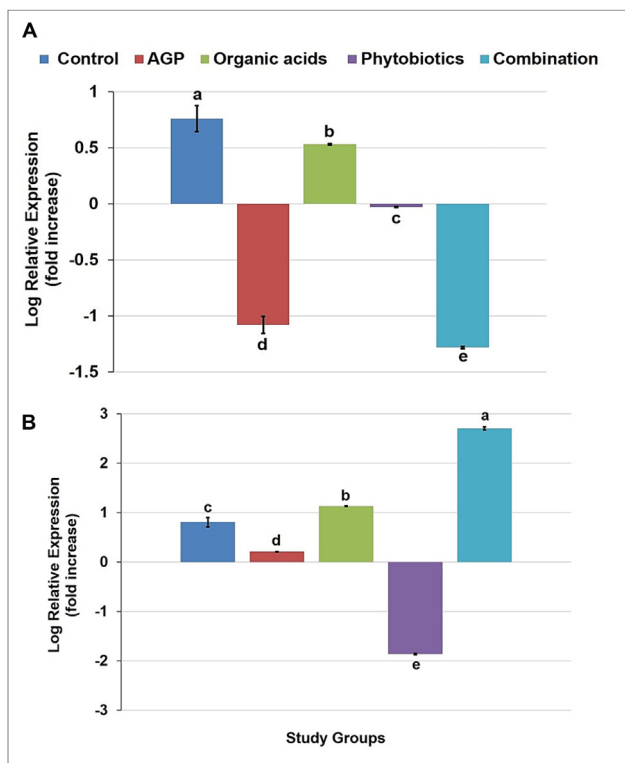
<sup>a,b,c,d</sup> Means bearing different letters in a row differ significantly (*p* < 0.05). SEM: Standard error of mean. ND: Not detected.

Table 3  
Gastrointestinal pH of broiler chickens at 21 and 42 days.

Study groups	Crop	Proventriculus	Gizzard	Duodenum	Jejunum	Ileum
0–21 days						
Control	5.1 <sup>a</sup>	2.9 <sup>a</sup>	2.8 <sup>a</sup>	5.7 <sup>a</sup>	6.7 <sup>a</sup>	7.5 <sup>a</sup>
AGP	5.3 <sup>a</sup>	2.9 <sup>a</sup>	2.6 <sup>ab</sup>	5.5 <sup>ab</sup>	6.5 <sup>ab</sup>	7.3 <sup>ab</sup>
Organic acids	4.0 <sup>b</sup>	2.7 <sup>b</sup>	2.7 <sup>c</sup>	5.4 <sup>b</sup>	6.4 <sup>b</sup>	7.2 <sup>b</sup>
Phytobiotics	5.0 <sup>a</sup>	2.8 <sup>ab</sup>	2.5 <sup>b</sup>	5.6 <sup>ab</sup>	6.6 <sup>ab</sup>	7.3 <sup>ab</sup>
Combination	4.2 <sup>b</sup>	2.7 <sup>ab</sup>	2.3 <sup>c</sup>	5.5 <sup>b</sup>	6.5 <sup>ab</sup>	7.4 <sup>ab</sup>
SEM	0.19	0.05	0.06	0.07	0.07	0.10
<i>p</i> -value	<0.001	0.10	<0.001	0.06	0.10	0.31
21–42 days						
Control	5.1 <sup>a</sup>	3.0 <sup>a</sup>	2.9 <sup>a</sup>	5.8 <sup>a</sup>	6.8 <sup>a</sup>	7.7 <sup>a</sup>
AGP	4.8 <sup>b</sup>	2.8 <sup>ab</sup>	2.8 <sup>ab</sup>	5.7 <sup>a</sup>	6.6 <sup>ab</sup>	7.5 <sup>ab</sup>
Organic acids	4.1 <sup>d</sup>	2.7 <sup>b</sup>	2.1 <sup>d</sup>	5.3 <sup>b</sup>	6.0 <sup>d</sup>	7.3 <sup>b</sup>
Phytobiotics	4.7 <sup>bc</sup>	2.9 <sup>ab</sup>	2.6 <sup>bc</sup>	5.7 <sup>a</sup>	6.4 <sup>bc</sup>	7.5 <sup>ab</sup>
Combination	4.5 <sup>c</sup>	2.8 <sup>b</sup>	2.3 <sup>cd</sup>	5.6 <sup>ab</sup>	6.2 <sup>cd</sup>	7.4 <sup>ab</sup>
SEM	0.06	0.07	0.10	0.10	0.09	0.12
<i>p</i> -value	<0.001	0.06	<0.001	0.06	0.01	0.28

<sup>a,b,c,d</sup> Means bearing different letters in a row differ significantly (*p* < 0.05). SEM: Standard error of mean.





**Fig. 6.** Ghrelin gene expression at 21 (A) and 42 (B) days in broiler chickens. Significance assumed at  $p < 0.05$ . Bars<sup>a,b,c</sup> with similar letter represents nonsignificant differences ( $p > 0.05$ ).

**Table 4**

Correlation analysis of cecal/ileal microflora and villus height/crypt depth ratio of broilers at 42 days of age.

Parameters	Villus height/Crypt depth ratio		
	Duodenum	Jejunum	Ileum
Cecal digesta			
Total aerobes	-0.5	-0.8	-0.8
Total anaerobes	0.8*	0.8	0.9*
Total coliform	-0.5	-0.8	-0.8
<i>Lactobacillus</i> spp.	0.8*	0.7	0.8
<i>Escherichia coli</i>	-0.1	-0.5	-0.5
Ileal digesta			
Total aerobes	-0.1	-0.3	-0.4
Total anaerobes	0.8	0.8*	0.9*
Total coliform	-0.5	-0.7	-0.8
<i>Lactobacillus</i> spp.	0.7	0.7	0.8
<i>Escherichia coli</i>	-0.2	-0.5	-0.6

\* Correlation significant at  $p < 0.05$  level.

**Table 5**

Correlation analysis among microflora, ghrelin gene expression and growth parameters in broiler chickens.

Parameters	Feed intake	Body weight gain	FCR
Cecal coliform	-0.8	-0.7	0.7
Cecal <i>Lactobacillus</i> spp.	0.8	0.9*	-0.9*
Ileal coliform	-0.9*	-0.8	0.8
Ileal <i>Lactobacillus</i> spp.	0.8	0.8	-0.8*
Ghrelin gene expression	-0.2	-0.3	0.3

\* Correlation significant at  $p < 0.05$  level. FCR: Feed conversion ratio.

et al., 2019). Morphological changes of gastrointestinal tract are influenced by variation and composition of feeds. Morphological parameters i.e. villus height and crypt depth are responsible for

vigorous functioning of intestine by facilitating larger surface area for better absorption of nutrient (Awad et al., 2009). Higher villus height/crypt depth ratio is linked with epithelial cell turn over while decreased ratio is associated with decreased intestinal bacterial population along with reduced surface area for nutrients absorption (Laudadio et al., 2012). Present work demonstrated that application of organic acids enhanced villus height of duodenum and jejunum and reduced growth of pathogenic bacterial population with increased beneficial bacterial counts. It has already been reported that the application of formic and citric acids enhanced villus height and crypt depth width and enhanced the growth performance of broilers (Ragaa and Korany, 2016). Chaveerach et al. (2004) (Chaveerach et al., 2004) reported that propionic, acetic and formic acids are considered to improve villus height/length and crypt depth and ultimately improved the production performance. These short chain fatty acids (SCFAs) may be used as antibacterial agents in poultry feed to decrease the proliferation of *Salmonella*, *Campylobacter*, *E. coli* and total coliform in the broiler gut. The application of phytobiotics in poultry diets increases villus height with deeper crypt and enhanced the digestive enzymes secretion such as proteases and amylases, to promote the growth and production performance of chickens along with enhanced absorptive cell growth in the gastrointestinal tract (Abudabos et al., 2018). Phytobiotics or plant derived compounds alone and in combinations have been proved to enhance growth performance, production, and health of animals (Rahimi et al., 2011). Phytobiotics having antimicrobial activity came in contact with the microbial cell membrane, alters permeability for H<sup>+</sup> and K<sup>+</sup> cations resulting in decreased entero-pathogens population. Moreover, phytobiotics also help in maintaining microflora balance that will involve in modifying digestive organs functioning, nutrient absorbance and weight gain of chicken (Ferdous et al., 2019). Phytobiotics significantly modulates gastrointestinal microflora and intestinal epithelium integrity by reducing the pathogenic bacterial load and stabilizing intestinal health (Talbot et al., 2018). Antibacterial activity of black cumin against *E. coli*, *Shigella sonnei* & *Streptococcus mutans* has already been established (Khan et al., 2012). Black seed contains essential oils (carvacrol and thymol) and fatty acids which enhanced digestive secretions and maintained intestinal integrity. These effects eventually improved weight gain in host (Alagawany et al., 2015). Ginger and green tea supplementation in chicken diet improved villus height surface area for nutrient absorption and cell division processes in various sections of intestine along with decreased pathogenic load (Cao et al., 2005; Gadang et al., 2008). It may be associated with the presence of bioactive molecules in green tea against numerous bacteria. The polyphenols present in green tea known to increased *Lactobacilli* with reduced population of *Proteus* spp. and *Enterobacteriaceae* at 56 days of age in poultry. Moreover, green tea leaves contain L-theanine which might be responsible for improved weight gain in birds (Williams et al., 2016)).

In current study, we analyzed avian ghrelin gene expression among treatment groups. Physiological status including feed consumption was different among all treatment groups in broiler chicken during experimental period of research which shown noteworthy role of avian ghrelin gene expression through the different satiety level. The supplementation of organic acids group depicted up-regulation of avian ghrelin gene expression in broilers which revealed enhanced nutrients digestion and absorption process in gastrointestinal tract with noticeable hunger signs in broilers. At the result, more feed consumed to attain body weight gain and eventually improved feed conversion ratio (Song et al., 2019b). Down-regulation of avian ghrelin gene expression was observed in phytobiotics group. It may be due to enhanced amino acids, vitamins, mineral absorption, protein digestibility, carbohydrate metabolism and fat digestibility through optimum bile acid production



which displayed postprandial satiety response in gastrointestinal tract to attain increased body weight and improved feed conversion (Khwatenge et al., 2020; Shahryar and Lotfi, 2020). However, the combination fed group showed up- and down-regulation of avian ghrelin gene expression at 21 and 42 days of age of broilers. It might be due to irreconcilability of doses, smell, and taste of certain organic acids and phytobiotics. Phytobiotics and organics acids has antimicrobial effects to maintain beneficial microflora populations, improvements in histomorphology of intestine and ultimately improved digestion and absorption of nutrients produced competitive exclusion resulting metabolic energy may intervene with energy homeostasis to illuminate up- and down-regulation in combination fed group as well.

## 5. Conclusion

In conclusion, phytobiotics and organic acids can be supplemented as dietary substitutes to antibiotics for preventing possible emergence of microbial resistance in humans. These alternatives might reduce intestinal pathogenic bacteria and increased population of *Lactobacillus* spp. and maintained satiety levels through regulation of ghrelin gene with improvement in intestinal histological changes attributed to increased digestion and nutrient absorptions resulted in increased performance and host health. It is assumed that phytobiotics meet the biological safety requirements of poultry products without developing resistance in human and poultry microorganisms.

## 6. Ethics approval

The study protocol was approved by ethical committee of KIBGE, University of Karachi (Pakistan) and performed by the owner of poultry farm-house. Every effort was made to minimize animal suffering.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The authors (SM, KAG) express their sincere appreciation to the Deanship of Scientific Research at the King Saud University for its funding of this research through the Research Group Project No. 1440-0138.

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