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Original article

# Enhanced modulation of gut microbial dynamics affecting body weight in birds triggered by natural growth promoters administered in conventional feed

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ABSTRACT

This study explored the effects of natural growth promoters (phytogenic feed additives and organic acids) on animal performance, carcass characteristics, blood parameters, gut microflora composition, and microbe–host interactions in broiler chickens over a 42-day feeding period. Two-hundred-fifty-day-old chicks were randomly assigned to one of five treatments: (i) control diets (CON); (ii) control diets + 40 g/tons antibiotic growth promoter (AB); (iii) control diets + 3 kg/tons organic acids (ORG); (iv) control diets + 3 kg/tons phytogenic feed additives (PHY); (v) control diets + 3 kg/tons organic acids + phytogenic feed additive combination (COM). A non-significant differences ($p > 0.05$) were observed in broiler performance among treatments at 21 days of age; however, a gradually increasing body weight gain and reduced feed conversion ratio were observed at 42 days in treatments versus control group. Biochemical indices were non-significant ($p > 0.05$) except for decreased cholesterol ($p < 0.05$) and increased A/G ratio ($p < 0.05$) recorded in the treatment groups. The addition of PHY and ORG improved total counts of *Enterococcus* spp. and *Lactobacillus* spp. ($p < 0.05$) as well as reduced caecal and ileal *Campylobacter* spp. and *Escherichia coli* ($p < 0.05$). Correlation analysis elucidated beneficial bacteria (*Enterococcus* spp. and *Lactobacillus* spp.) were positively and pathogenic bacteria (*Campylobacter* spp. and *E. coli*) were negatively correlated ($p < 0.05$) with host weight gain. The findings indicated that dietary supplementation of PHY and ORG sustained balanced gut microflora, which in turn improved body weight. This study broadens the significance of using PHY and ORG as safe alternatives to antibiotic growth promoters for achieving healthier and economical broiler production.


## 1. Introduction

Gut microflora plays a character role in host energy, immunity, and metabolism, especially in commercial poultry birds. The gastrointestinal tract (GIT) is densely populated with a variety of organisms such as viruses, fungi, bacteria, and protozoans (Sommer and Backhed, 2013). Over the years, various studies have highlighted the influential impacts of gut bacteria in hosts which include (1) epithelial barrier maintenance; (2) inhibition of pathogenic bacterial adhesion to the intestinal surface; (3) enhancement and maturation of the host's immunity; (4) degradation of non-digestible plant polysaccharides; and (5) production of metabolites such as short chain fatty acids (SCFAs) and vitamins (Al-Asmakh and Zadali, 2015; Sanchez et al., 2017).

In the recent past, tremendous interest in poultry production has been generated. Chicken meat and eggs are optimal sources of quality protein along with crucial vitamins and minerals. Broiler chickens' outstanding performance in terms of feed efficiency and

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feed-to-meat conversion (Vandehaar et al., 2016) within 6 weeks has been found to be linked to intestinal microbiota, as well as various other factors, including bird management, environment, vaccines, and disease control (Kiarie et al., 2013). Modulating the gut ecosystem and functions of farm animals with dietary variable is an effective strategy for achieving desired results in poultry (Madal et al., 2015). Therefore, growth promoters are incorporated into poultry feed to improve chickens' health and establish a stable gut ecosystem.

Natural growth promoters (NGPs) are real, toxin-free, and less residual; thus, they are considered quintessential additives in poultry feeds. Among these alternatives, organic acids and phytogenic feed additives modulate the host immune system through generating antioxidant and anti-inflammatory responses in the gut, which enhance maximum nutrient absorption (Liu et al., 2014; Mueller et al., 2012).

The primary aim in rearing broiler chickens is to increase their body weight in the shortest possible time. In this domain, gut microbiota are key players because they maintain beneficial interactions with the host (Turnbaugh et al., 2009; Dahiya et al., 2017). In the gut, the cecum is densely populated with a variety of bacteria that are responsible for fermentation, which prevents pathogenic bacterial colonization; by contrast, the ileum is the main site for the absorption and digestion of nutrients (Lee et al., 2017). The chicken cecum might harbor obligate anaerobic pathogenic bacteria (*Clostridium* spp., *Campylobacter* spp.), whereas microaerophilic bacteria (*Lactobacillus* spp., *Enterococcus* spp.) are predominant in the ileum (Yin et al., 2010; Boguslawska-Tryk et al., 2015). Gut bacteria were suggested to be positively or negatively correlated with animal performance (Yin et al., 2018). However, investigations of the relationship between performance-related gut microbiota in poultry based on dietary modulation are scarce. In this context, an effective strategy for improved body weight gain is required to identify and modulate relevant gut bacteria (Han et al., 2016).

Prebiotics and probiotics added to poultry feed amplify the number of performance-related bacteria (Murshed and Abudabos, 2015), but the same has not yet been confirmed for organic acids and phytogenic feed additives. Thus, the objective of this study was to assess the influence of phytogenic feed additives and organic acids on animal performance, carcass characteristics, biochemical indices, caecal and ileal microbial populations, and host–microbe interactions of broiler chickens in the quest for alternatives to antibiotics in poultry.

## 2. Methods

### 2.1. Bird management

This trial was conducted at Ideal feeds and experimental units, Karachi, Pakistan under standard operating procedures for broiler housing management after approval of the Ethical Review Board (DG/AA-089) of the Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, in correspondence with the standard protocol from the guidelines "Care and use of agricultural animals in research" (Mcglone, 2010). Two-hundred-fifty-day-old unsexed, healthy, disease-free, Hubbard-strain chicks were purchased from a commercial hatchery and reared from 42 days. The birds were nurtured on the commercial starter (days 1–21) and finisher (days 22–42) diets. The management and feeding practices (vaccination, temperature, humidity, watering, feeding, and lighting) were similar for all chicks as described in the Hubbard Management guide (Aviagen, 2014). The diet was provided in mash form and the birds' access to feeders and water were *ad libitum*. The feed and water were provided in cylindrical hanging feeders and drinkers, respectively. The provision of light during the trial was for 24 h for first 3 days, and subsequently for 23 h/day with 1 h of darkness. Table 1 presents the chemical composition and formula of the experimental diet. Each diet was analyzed for proximate composition on the basis of the Association of Official Analytical Chemists Procedures (AOAC, 2005). The supplementation of the basal diet was in *iso*-caloric and *iso*-nitrogenous forms with the addition of feed additives.

### 2.2. Experimental design and diet

In this experiment, the birds were randomly distributed to five treatments with five replicates per treatment, and a total of 10 birds per replicate were placed in a clean cage (4 X 8 ft.). Experimental groups received a commercial corn-soybean basal diet prepared with the following variations:

(i) Control (CON): basal diet only;
(ii) Antibiotic growth promoter (AB): basal diet + 40 g/tons Enramycin®
(iii) Organic acids (ORG): basal diet + 3 kg/tons Acidifiers

The Acidifiers used in the study were supplied by Kemira Pro GIT SF3 (Shanghai, China) and comprised of 26.5% formic acid; 16% lactic acid; 5% citric acid; 13.1% MCFA (lauric acid-based); and 3.5% mono-, di-, and triglycerides of MCFAs.

**Table 1**
Feed ingredients and chemical composition of experimental diet.

| Ingredients | Composition | |
|---|---|---|
| | Starter (%) | Finisher (%) |
| Corn | 60.1 | 66.2 |
| Soya bean meal (46%) | 21.3 | 22.4 |
| Canola meal | 5.33 | 0.00 |
| Fish Meal (48%) | 0.00 | 4.75 |
| Corn Gluten (60%) | 5.01 | 0.00 |
| APC[a] (50%) | 5.03 | 4.89 |
| Limestone | 1.60 | 1.22 |
| Oil | 0.00 | 0.44 |
| DL-Methionine | 0.19 | 0.14 |
| Lysine HCl | 0.405 | 0.223 |
| Vitamin premix[b] | 0.053 | 0.056 |
| Mineral premix[c] | 0.057 | 0.051 |
| L-Threonine | 0.071 | 0.023 |
| Salt (NaCl) | 0.200 | 0.200 |
| Sodium -bi- carbonate | 0.205 | 0.159 |
| Anti –coccidial[d] | 0.022 | 0.029 |
| DCP 2[e] | 1.12 | 0.658 |
| Choline chloride (60%) | 0.100 | 0.100 |
| Calculated chemical composition (%) | | |
| Metabolize energy[f] (MJ/kg) | 12.1 | 12.9 |
| Crude Protein (%) | 21.5 | 19.1 |
| Calcium (%) | 1.00 | 0.859 |
| Available Phosphorous (%) | 0.400 | 0.395 |
| Dig. Lysine (%) | 1.19 | 1.06 |
| Dig. Methionine (%) | 0.559 | 0.454 |
| Dig. Methionine + Cysteine (%) | 0.890 | 0.743 |
| Dig. Tryptophan (%) | 0.284 | 0.191 |
| Dig. L-Threonine (%) | 0.846 | 0.723 |

[a] Animal protein concentrate (Feather meal).
[b] Vitamin premix composition per kg (Vitamin A 20,000 KIU/kg, Vitamin $D_3$ 5,400 KIU/kg, Vitamin E 48,000 mg/kg; Vitamin $K_3$ 4,000 mg/kg, Vitamin $B_1$ 4,000 mg/kg, Vitamin $B_2$ 9,000 mg/kg, Vitamin $B_6$ 7,600 mg/kg, Vitamin $B_{12}$ 20 mg/kg, Niacin 60,000 mg/kg, Folic acids 1,600 mg/kg, Pantothenic acid 20.000 mg/kg, Biotin 200 mg/kg).
[c] Mineral premix composition per kg (Iron 60.000 mg/kg, Zinc 120,000 mg/kg, Manganese 130.000 mg/kg, Copper 10,000 mg/kg, Iodine 1,800 mg/kg, Selenium 360 mg /kg, Cobalt 400 mg/kg).
[d] Anti –coccidial (Diclazuril 0.5%).
[e] DCP 21(MDCP) Phosphorous 21%, Calcium 17%.
[f] ME (MJ/Kg) = gross energy fed in diet to birds (MJ) – gross energy of excreta collected (MJ) feed intake of diet to each bird (DM based).

(iv) Phytogenic feed additives (PHY): basal diet + 3 kg/tons phytogenics

The phytogenic feed additives contained a mixture of dried powders of *Allium sativa* (garlic) and *Cinnamomum verum* (cinnamon) (10%), dried leaves of *Mentha piperita* (peppermint) *and Camellia sinensis* (green tea) (10%), and seeds of *Nigella sativa* (black cumin) (15%).

(v) Combination (COM): basal diet + 3 kg/tons organic acids + phytogenic feed additives.

### 2.3. Performance parameters

Performance analyses were executed on days 21 and 42, including a daily feed intake (FI) estimation through providing a known amount of feed and measuring the remainder. Additionally, body weight gain (BWG) was recorded daily and weekly for individual birds and per cage, respectively. The feed conversion ratio (FCR) was computed as follows:

$$Feed\ conversion\ ratio(FCR) : \frac{Cumulative\ FI\ (g)}{Total\ BWG\ (g)}$$

### 2.4. Carcass characteristics

At the end of the experiment, the chicks were starved for 8 h prior to slaughtering. Three birds from each replicate with a body weight under one standard deviation of the average treatment weight (15 birds/treatment or 75 birds in total) were randomly selected for estimating biochemical indices, carcass characteristics, and gut microflora count. Birds were slaughtered by having their throats cut with a sharp knife. Carcass characteristics after slaughtering were calculated followed by evisceration. The dressing percentage along with percentages of breast meat, giblets (gizzard, liver, and heart), and abdominal fat were measured as percentages of body weight (cm or g/100 g of body weight).

### 2.5. Biochemical parameters

For estimating biochemical indices, 2 ml of blood in sterile tubes was collected from the brachial vein using sterile needles and syringes. The blood-containing tubes were placed at room temperature for 6 h in a slanted position and incubated overnight at 4 °C for serum collection. Serum samples were maintained at −20 °C before biochemical analysis was performed. Later, cholesterol, globulin, and albumin levels as well as total protein and albumin/globulin (A/G) ratio were calculated.

### 2.6. Caecal and ileal sample processing

Aseptically whole gastrointestinal tracts (GITs) were removed and the caecum and ileum regions were washed with phosphate-buffered saline (PBS). Later, caecum and ileum contents were squeezed from one end and collected in sterile tubes filled with cryoprotective broth (pre-autoclaved 50 ml of brain heart infusion broth +20% glycerol *v/v*). Samples were immediately stored at −80 °C for further analyses (Ballongue, 1997).

### 2.7. Quantitative bacterial analysis

*Microbial enumeration:* For microbial enumeration, deep frozen cecum and ileum sample per bird were thawed for 20 min; 1 g of sample was macerated in 9 ml of sterilized PBS, of which 1 ml was transferred to 9 ml of sterilized PBS. Later, samples were serially diluted from $10^{-2}$ to $10^{-6}$, and 0.1 ml of each diluted sample was plated in triplicates on appropriate agar plates for a bacterial count of targeted organisms using the spread plate technique. All bacterial counts were denoted as colony-forming units ($\log_{10}$/g of wet digestion) based on the following calculation (Andrews et al., 2014):

$$N = \frac{\sum_C}{[(1x\ n)\ +\ (0.1\ x\ n)\ x\ (d)]}$$

where

N = number of colonies (cfu /g)
$\sum_C$ = sum of colonies on all counted plates
n = number of plates from dilution counted
d = dilution factor

*Bacterial growth media*: Gut microflora was identified using the respective growth media. Total aerobes and anaerobes were enumerated on plate count agar (Oxoid CM0325); Coliforms on MacConkey agar (Oxoid CM0505); *Lactobacillus* spp. on De Man, Rogosa, and Sharpe (MRS) agar (Oxoid CM0359); *Enterococcus* spp. on bile esculin azide agar (Oxoid CM0888); *Escherichia coli* on eosin methylene blue agar (Oxoid 0069); *Campylobacter* spp. on Campylobacter selective agar (Oxoid CM0689) after the addition of laked horse blood (Oxoid SR0048) and *Campylobacter* selective supplements (Oxoid SR0117); *Salmonella* spp. on Hektoen enteric agar (Merck 111681), xylose lysine deoxycholate agar (Oxoid 0469), and bismuth sulphite agar (Oxoid 0201). The plates were incubated at 37 °C for 24–48 h aerobically (plate count, MacConkey, eosin methylene blue, Hektoen enteric, xylose lysine deoxycholate, and bismuth sulphite agar) or 48–72 h anaerobically (plate count, MRS, *Campylobacter* selective agar) and the anaerobic environment was created using an appropriate catalyst (Oxoid™ AnaeroGen™ AN0025A) and an anaerobic gas jar (Oxoid, AG0025A).

*Phenotypic characterization*: The targeted bacterial genera (*Lactobacillus, Enterococcus, Escherichia,* and *Campylobacter*) were phenotypically characterized based on morphological and biochemical parameters. In the morphological analysis, typical colonies on selective growth media were further confirmed by Gram staining, microscopic appearance, and colonial characteristics. For biochemical testing, oxidase, catalase, and indole tests were performed. For *Enterococcus* spp. identification, a 6.5% NaCl test was performed to discriminate between group D streptococci and *Enterococcus* spp.

*Salmonella* spp. *counts:* For *Salmonella* spp. identification, 1 g of sample was pre-enriched in lactose broth for 24 h. The pre-enriched sample (1 ml) was transferred to Rappaport–Vassiliadis broth (Oxoid CM0669) for the selective growth of *Salmonella* spp.; tubes were aerobically incubated at 37 °C for 24 h. Subsequently, 0.1 ml of diluted samples was plated onto selective growth media followed by aerobic incubation for 24 h at 37 °C. The appearance of a typical *Salmonella* colonies on all growth plates was observed (Andrews et al., 2014).

### 2.8. Molecular characterization of Salmonella spp.

Because of the importance of *Salmonella* spp. as a potential poultry pathogen, molecular analysis of samples was conducted to confirm it. Initially, samples were pre-enriched overnight in Rappaport–Vassiliadis broth. Later, DNA extraction was performed using a DNA extraction kit (Promega, USA). Primer sequences of *Salmonella inv A* gene Salm 4 (5′-TCCCGGCAGAGTTCCCATT-3′), Salm 3 (5′-GCTGCGCGCGAACGGCGAAG-3′) were used for identification. Polymerase chain reaction was performed with an initial denaturation for 5 min (95 °C), followed by 35 cycles of denaturation at 95 °C (90 s), annealing at 62 °C (60 s), extension at 72 °C (90 s), followed by a final extension at 72 °C (7 min) in a Thermal

Cycler Bio-Rad (Hercules, California, USA). Amplified products (389 bp) were separated on 2.0% agarose gel and bands were visualized with ultraviolet trans illumination.

### 2.9. Statistical analyses

Data analysis was performed using a one-way analysis of variance through SPSS, version 17.0 (IBM, Armonk, NY, USA). Multiple comparison of means was performed using post-hoc analysis with *Tukey's* HSD test; significance was assumed at $p < 0.05$. The relationship between host weight and targeted bacterial genera was assessed using Pearson's correlation coefficient ($r$) and P *values* through a simple linear regression analysis.

## 3. Results

### 3.1. Performance analysis

Group-wise analyses of performance parameters were recorded during the experiment (Table 2). At 21 days, non-significant differences ($p > 0.05$) in body weight, FI, and FCR were found in all groups; by contrast, at 42 days, significant differences in body weight, FI, and FCR ($p < 0.05$) were noted. Higher FI and FCR were observed in CON among all study groups. At 21 and 42 days, BWG ($p < 0.05$) was remarkably decreased in CON compared with treatment groups. At 42 days, significantly higher BWG ($p < 0.05$) and reduced FCR (1.89) were recorded in PHY among the study groups. In the case of FCR, non-significant differences were noted between ORG and COM groups ($p > 0.05$) at 42 days.

### 3.2. Carcass characteristics

The addition of NGPs in diets significantly enhanced carcass characteristics ($p < 0.05$) in the treatment groups (Table 3). The highest dressing percentage of all groups were noted in the PHY group. All other carcass parameters (liver, heart, gizzard, abdominal fat, and breast meat) remained unaffected ($p > 0.05$) by the dietary changes. Furthermore, the inclusion of phytogenic feed additives alone and combined with organic acids increased carcass and breast meat percentages.

### 3.3. Biochemical parameters

Serum biochemical analysis of dietary changes revealed significant differences in cholesterol and the A/G ratio ($p < 0.05$) for all groups (Table 4). High cholesterol levels were noted in the CON group. The phytogenic feed additive and combination groups showed significantly minimized cholesterol levels ($p < 0.05$) in blood. A/G ratio was significantly higher in the treatment groups ($p < 0.05$) compared with CON. Among the groups, non-significant interactions linked with diet and blood parameters ($p > 0.05$) were observed for total protein, serum albumin, and globulin levels.

### 3.4. Quantitative bacterial analyses

*Microflora composition*: The microbial counts result revealed a higher proportion of total aerobes in the CON and AB groups ($p < 0.05$) in both the caecum and ileum (Fig. 1). Similarly, in the caecum, an eminent number of total anaerobes were noticed in the CON and AB groups (log cfu/g 6.72 and 6.78, respectively), whereas lower counts of total anaerobes were observed in the ORG, PHY, and COM (log cfu/g 6.18, 5.92 and 5.96 respectively) groups. In the ileum, a significantly lower total anaerobe count ($p < 0.05$) was noticed only in the PHY group (log cfu/g 5.84). Coliforms, gram-negative bacteria, indicate a possible presence of harmful, disease-causing bacteria in the gut. In this study, the PHY group had lower coliform counts in the ileum region. Moreover, significantly lower coliform counts in the caecum COM group ($p < 0.05$) indicated the synergistic effects of organic acids and phytogenic feed additives in decreasing gut pathogens.

In addition, targeted beneficial and pathogenic gut bacterial counts of the study groups were performed in the caecum and ileum samples (Table 5). *Lactobacillus* spp. and *Enterococcus* spp. exhibited pronounced increases ($p < 0.05$) in the AB, ORG, PHY, and COM groups compared with the CON group in the caecum. In the ileum, increased counts in the treatment groups were noticed for *Enterococcus* spp., although no significant differences for *Lactobacillus* spp. ($p < 0.05$) were found among all student groups. An increased *E. coli* count was observed in the AB and ORG groups along with CON in both the caecum and ileum. By contrast, *Campylobacter* spp. was decreased significantly in the treatment groups ($p < 0.05$) compared with the CON group. Notably, in the caecum, significantly lower ($p < 0.05$) *E. coli* counts were obtained in the PHY group; however, no *E. coli* populations were detected in the ileum region. In addition, *Campylobacter* spp. was absent in both the PHY and COM groups. Notably, *Salmonella* spp. was not detected through conventional methods in either region, which was then validated through molecular analysis for *Salmonella* spp. detection. A caecum sample gel image (Fig. 2a) showed the presence of *Salmonella* spp. in the CON group with a lower band intensity. In the ileum (Fig. 2b), however, all samples were negative for *Salmonella* spp. in all groups. The lower band intensity in the CON sample compared with the positive control (*Salmonella typhimurium* ATCC 14028) indicated a healthy chicken gut.

**Table 2**
Effect of dietary treatments on performance parameters of broilers at 21 and 42 days.

| Traits | Groups* | | | | | SEM | *P-value* |
|---|---|---|---|---|---|---|---|
| | CON | AB | ORG | PHY | COM | | |
| Initial body weight (g) | 42.3 | 42.4 | 42.6 | 42.5 | 42.8 | 0.012 | 0.970 |
| **0–21 days** | | | | | | | |
| Feed intake (g) | $1040^{b}$ | $1057^{ab}$ | $1056^{ab}$ | $1086^{a}$ | $1079^{a}$ | 6.55 | 0.132 |
| Body weight (g) | $654^{b}$ | $679^{ab}$ | $690^{a}$ | $704^{a}$ | $695^{a}$ | 6.02 | 0.068 |
| FCR (g:g) | $1.66^{a}$ | $1.54^{a}$ | $1.52^{a}$ | $1.53^{a}$ | $1.55^{a}$ | 0.014 | 0.495 |
| **22–42 days** | | | | | | | |
| Feed intake (g) | $3940^{a}$ | $3890^{ab}$ | $3835^{c}$ | $3807^{c}$ | $3843^{bc}$ | 13.1 | <0.001 |
| Body weight (g) | $1870^{c}$ | $1960^{ab}$ | $1940^{b}$ | $2013^{a}$ | $1991^{ab}$ | 14.0 | 0.001 |
| FCR (g:g) | $2.12^{a}$ | $1.99^{b}$ | $1.95^{b}$ | $1.82^{c}$ | $1.90^{bc}$ | 0.013 | <0.001 |

Values are mean of 50 birds/treatment.
SEM: standard error of mean.
$^{a,b,c,d}$ Means with different supersript in the same row differ significantly at $p < 0.05$.
* Groups; CON: control, AB: antibiotic growth promoter, ORG: organic acid, PHY: phytogenic feed additives, COM: combination.

**Table 3**
Effect of dietary treatments on carcass characteristics of broilers at 42 days.

| Traits[†] (%) | Groups[*] | | | | | SEM | *P-values* |
|---|---|---|---|---|---|---|---|
| | CON | AB | ORG | PHY | COM | | |
| Dressing | 61.0[d] | 62.6[cd] | 63.5[bc] | 66.3[a] | 64.9[ab] | 0.533 | 0.004 |
| Liver | 1.92[c] | 2.23[b] | 2.35[b] | 2.51[a] | 2.56[a] | 0.050 | 0.212 |
| Heart | 0.433[b] | 0.443[b] | 0.523[a] | 0.576[a] | 0.577[a] | 0.018 | 0.620 |
| Gizzard | 2.09[c] | 2.35[b] | 2.44[b] | 2.82[a] | 2.61[a] | 0.077 | 0.439 |
| Breast meat | 20.2[d] | 22.4[c] | 22.6[c] | 24.8[a] | 23.3[b] | 0.377 | 0.163 |
| Abdominal fat | 1.34[a] | 1.38[a] | 1.35[a] | 1.42[a] | 1.41[a] | 0.015 | 0.867 |

SEM: standard error of mean.
[†] These are mean values of five replicates (cages).
[*] Groups; CON: control, AB: antibiotic growth promoter, ORG: organic acid, PHY: phytogenic feed additives, COM: combination.
[a,b,c,d] Means with different superscript in the same row differ significantly at $p < 0.05$.

**Table 4**
Effect of dietary treatments on biochemical parameters of broilers at 42 days.

| Traits (mg/dl)[†] | Groups[*] | | | | | SEM | *P-values* |
|---|---|---|---|---|---|---|---|
| | CON | AB | ORG | PHY | COM | | |
| Cholesterol | 76.1[a] | 72.4[b] | 73.2[b] | 69.0[c] | 68.7[c] | 0.799 | 0.033 |
| Total protein | 3.55[a] | 3.22[a] | 3.37[a] | 3.55[a] | 3.68[a] | 0.298 | 0.221 |
| Serum albumin | 1.67[b] | 1.53[c] | 1.56[c] | 1.66[b] | 1.75[a] | 0.010 | 0.241 |
| Serum globulin | 1.60[a] | 1.35[c] | 1.27[d] | 1.48[b] | 1.34[c] | 0.047 | 0.103 |
| [**]A/G ratio | 0.91[c] | 1.13[b] | 1.20[a] | 1.15[b] | 1.26[a] | 0.034 | 0.021 |

SEM: standard error of mean.
[†] The are mean values of five replicates (cages).
[ab,c] Means with different superscript in the same row differ significantly at $p < 0.05$.
[*] Groups; CON: control, AB: antibiotic growth promoter, ORG: organic acid, PHY: phytogenic feed additives, COM: combination.
[**] A/G ratio: albumin/globulin ratio.

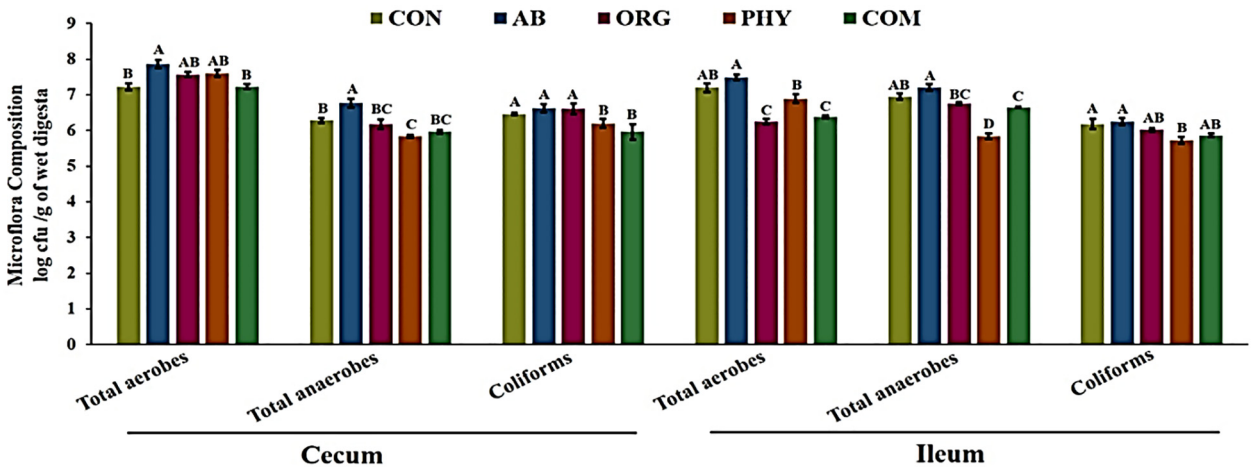


**Fig. 1.** Microflora composition (log cfu/g) of study groups in broiler cecum and ileum regions at 42 days. Groups: CON: control, AB: antibiotic growth promoter, ORG: organic acid, PHY: phytogenic feed additives, COM: combination. [A,B,C] Bars (Means ± standard error of mean) with different letters within the same microbial group differ significantly at $p < 0.05$.

*Phenotypic characterization of gut bacteria:* The targeted bacterial genera in this study were also phenotypically characterized (Table 6). *Lactobacillus* spp. colonies appeared round and opaque on MRS agar. *E. coli* was observed as characteristic metallic green sheen colonies, indicating lactose fermentation on eosin methylene blue agar. *Campylobacter* spp. Showed gray, watery colonies on *Campylobacter* selective agar. The microscopic analysis confirmed phenotypic identification to be gram-positive (*Enterococcus* spp. and *Lactobacillus* spp.) and gram-negative (*E. coli* and *Campylobacter* spp.). Biochemical analysis revealed the growth of *Enterococcus* spp. in 6.5% NaCl. Furthermore, *E. coli* produced catalase enzyme, which lacks cytochrome *c* oxidase, whereas *Campylobacter* spp. were positive for both enzyme production, respectively. *E. coli* has the ability to break down amino acid tryptophan to form indole, which after 24 h and the addition of Kovac's reagent turns a pink colour, indicative of a positive result.

### 3.5. Relationship between bacterial genera and body weight in chickens

To elucidate the relationship between targeted bacterial genera and host weight gain, a simple linear regression analysis was performed (Fig. 3). Gut bacteria count either positively or negatively affected host weight gain. In particular, the beneficial bacteria *Lactobacillus* spp. ($r = 0.782$, $p = 0.01$) and *Enterococcus* spp. ($r = 0.892$, $p = 0.04$) were correlated positively with BWG (Fig. 3a and b),

**Table 5**
Effect of dietary treatments on targeted bacterial genera composition (log cfu/g) in broiler at 42 days.

| Targeted bacteria | Groups* | | | | | Statistics | |
|---|---|---|---|---|---|---|---|
| | CON | AB | ORG | PHY | COM | SEM | *P-values* |
| *Lactobacillus* spp. | | | | | | | |
| Cecum | 6.49[b] | 6.52[b] | 6.77[ab] | 6.91[ab] | 7.13[a] | 0.077 | 0.019 |
| Ileum | 6.03[a] | 6.65[b] | 6.31[b] | 6.80[b] | 6.47[b] | 0.095 | NS |
| *Enterococcus* spp. | | | | | | | |
| Cecum | 6.51[b] | 7.21[a] | 7.21[a] | 7.30[a] | 7.37[a] | 0.096 | 0.015 |
| Ileum | 5.83[b] | 6.61[a] | 6.52[a] | 6.48[a] | 6.58[a] | 0.094 | 0.015 |
| *Escherichia coli* | | | | | | | |
| Cecum | 6.49[b] | 7.13[a] | 7.14[a] | 6.31[b] | 6.38[b] | 0.114 | 0.013 |
| Ileum | 6.57[a] | 6.25[b] | 6.12[bc] | ND | 6.01[c] | 0.665 | <0.001 |
| *Campylobacter* spp. | | | | | | | |
| Cecum | 6.43[a] | 6.79[a] | 6.54[a] | 6.48[a] | 6.03[b] | 0.073 | 0.012 |
| Ileum | 6.85[a] | 6.95[a] | 5.66[b] | ND | ND | 0.854 | <0.001 |

SEM: standard error of mean.
ND: Not detected.
[a,b,c] Means with different superscript in the same row differ significantly at $p < 0.05$.
* Groups; CON: control, AB: antibiotic growth promoter, ORG: organic acid, PHY: phytogenic feed additives, COM: combination.

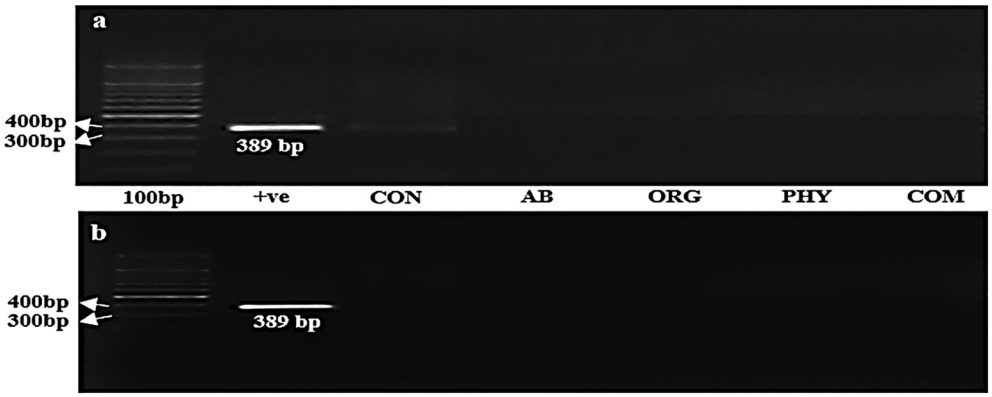


**Fig. 2.** Gel images of *Salmonella* spp. detection in cecum (a) and ileum (b) regions of broiler chickens. Groups: CON: control, AB: antibiotic growth promoter, ORG: organic acid, PHY: phytogenic feed additives, COM: combination. "+ve" = Positive control (*Salmonella typhimurium*, ATCC 14028).

**Table 6**
Phenotypic characteristics of targeted bacterial genera.

| Targeted bacteria | Morphological identification | | | Biochemical identification | | | |
|---|---|---|---|---|---|---|---|
| | Gram stain | Morphology | Macroscopic appearance | Growth with 6.5% NaCl | Catalase test | Oxidase test | Indole test |
| *Lactobacillus* spp. | + | Slender rods | Round, white | + | – | – | – |
| *Enterococcus* spp. | + | Spherical cocci | Smooth, black | + | – | – | – |
| *Escherichia coli* | – | Short rods | Round, metallic green | – | + | – | + |
| *Campylobacter* spp. | – | Curved rods | Flat, mucoid, irregular edges | – | + | + | – |

"+" = Growth/positive, "– "= No growth/negative.

whereas the pathogenic bacteria, *E. coli* ($r = -0.585$, $p = 0.04$) and *Campylobacter* spp. ($r = -0.102$, $p = 0.03$) were negatively correlated with BWG in hosts (Fig. 3c and d).

## 4. Discussion

The results obtained in the current study illustrated that the addition of NGPs (organic acids and phytogenic feed additives) in the diets of chickens significantly enhanced BWG, decreased pathogenic bacterial load, maintained blood cholesterol levels and A/G ratio, and promoted the growth of beneficial bacteria, which was positively correlated with enhanced host weight gains without affecting the carcass yield.

Indeed, the profound role of dietary growth promoters on gut bacteria for enhancing broiler weight gain is the subject of intensive research. It is claimed that AGPs are being used in poultry to improve feed efficiency, body weight, and carcass yield; reduce mortality; and eventually enhance growth (Kumar et al., 2019). Similarly, AGPs tended to increase the FCR and body weight in broilers compared with a control group (Kumar et al., 2019). Interactions with gut microbial populations provide the basis for antibiotics to exert growth-promoting effects on bird health (Gadde et al., 2018). A germfree approach employing animal models suggested that antibiotics may alter the divergence and composition of microbial ecosystems in the gut, resulting in stable and balanced microbiota, which in turn improves growth performance (Lin, 2011). After the EU ban on in-feed antibiotics, various strategies have been considered as alternatives over the last decade. To achieve healthier and more economical poultry meat, equivalent results have been achieved using phytogenic feed additives and organic acids as growth promoters in broiler chicken diets (Ghaly et al., 2017; Karangiya et al., 2016).

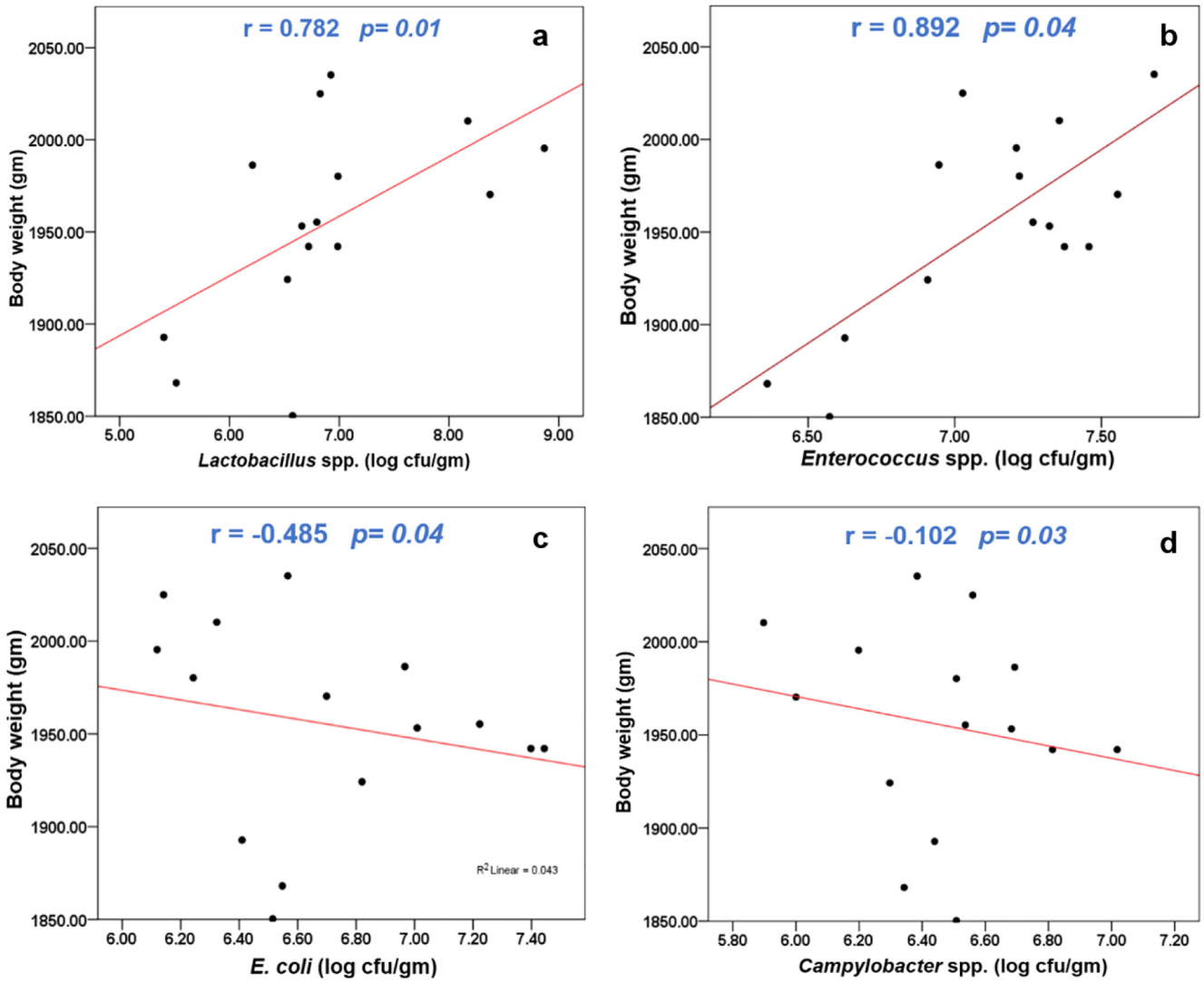


**Fig. 3.** The relationship between body weight and targeted bacteria (a) *Lactobacillus* spp., (b) *Enterococcus* spp., (c) *E. coli*, and (d) *Campylobacter* spp. in chickens. Simple linear regression was performed to assess relationship based on Pearson's correlation coefficient (*r*) and *P* values.

Phytogenics are still in their infancy in terms of being used as feed additives, but we attempted to unravel their effects on maintaining gut microbiota composition and improving animal performance. This study revealed that phytogenic feed additives selectively inhibited *E. coli* and *Campylobacter* spp. with increased *Lactobacillus* spp. One possible mode of action of phytogenic feed additives is modulating the immune and oxidative defense systems along with promoting digestive enzyme secretion (amylase and proteases) to improve animal health (Kaschubek et al., 2018; Brenes and Roura, 2010). Hashemi and Davoodi (2010) reported that supplementation of phytogenic feed additives improved body weight, increased carcass response, and maintained feed efficiency in broilers. Najafi and Taherpour (Najafi and Taherpour, 2014) asserted that the immunomodulatory effects of cinnamon in broiler chickens on dietary inclusion levels of 0.4% (4 g/kg) and 0.8% (8 g/kg) improved FCR as well as enhanced hemoglobin concentration and lymphocyte proportions in the blood. Phytogenic feed additives increase the hydrophobicity of pathogenic bacterial species through modulating their cellular membrane, consequently affecting the surface properties of microbial cells. In this manner, they not only affect the virulence properties of harmful bacteria, but also play a crucial role in bacterial adhesion to mucosal cells, which is dependent on the hydrophobicity of microbial surface cells (Mohiti-Asli and Ghanaatparast-Rashti, 2018). The present study used a blend of different phytogenics, which were recognized to deferentially inhibit pathogenic bacteria and promote beneficial bacterial growth in broiler gut (Munir, 2015). Additionally, phytogenic feed additives prevent host gut inflammation, which might lead to reduced animal performance as well as economic losses. A blend of phytogenics (cinnamaldehyde, carvacrol, and capsicum) modified the Nrf2 and NF-κB pathways, provided protection against oxidative stress, and reduced inflammation, which in turn improved host health and growth performance (Yang et al., 2015).

Organic acids (also known as acidifiers) are weak acids with lower dissociation constants. Blends of organic acids have been used as feed additives in poultry as an alternative to antibiotics for a long time. They have distinct roles in enhancing immunity and nutrient digestibility, sustaining the gastrointestinal tract, and improving animal performance in broilers (Rodriguez-Lecompte et al., 2012; Brzoska et al., 2013). In this study, the ORG group exhibited inhibition of *E. coli* and *Campylobacter* spp. in the caecum and ileum. In their non-dissociated form (MCFA; e.g., lauric acids), they penetrate bacterial cell walls and disrupt usual physiological processes in the gut (Dhama et al., 2014). Mostly, the pathogenic bacteria *E. coli, Campylobacter*, and

*Salmonella* spp. Is pH-sensitive; they are unable to tolerate pH gradients (internal and external), and hence, including organic acids in diets cause a reduction in pathogenic colonization in the gut and increases beneficial bacteria (Khan and Iqbal, 2016). The combination of acidifier complexes used in this study was highly enriched with lauric acid (C12) and contained both SCFAs and MCFA. MCFA exerts potential antibacterial effects in the gut against gram-positive bacteria (*Clostridium* spp.), unlike SCFAs, which mainly control gram-negative bacteria. The addition of SCFAs complements the activity of MCFA by creating pores in the cell membranes of gram-positive bacteria, which facilitates the penetration of MCFA (Ding et al., 2017). These findings are also related to the fact that the predominant bacterial genus in the ileum is *Lactobacillus* spp. *Lactobacillus* spp. was proven to dominate *Campylobacter* spp. in the ileum as a consequence of the production of inhibitory organic acids (Andreopoulou et al., 2014). In the gut, phytogenic feed additives and organic acids tend to increase the *Lactobacillus* spp. count and decrease *E. coli* and *Campylobacter* spp.

Regarding the relationship of body weight and gut microbiota, studies are limited; hence, the correlation between them (positive or negative) is ambiguous (Clarke et al., 2014; Delzenne and Cani, 2011). In broiler chickens, several bacterial genera are identified as performance-related bacteria based on dietary modulation (Torok et al., 2011). The findings of the present study attribute to the property of *Lactobacillus* spp. of producing SCFAs as a result of bacterial fermentation (Meimandipour et al., 2010). These SCFAs are involved in (1) lowering the pH, which changes the gut microbiota composition; and (2) preventing pH-sensitive pathogenic bacteria. These variations in the gut are significantly linked to increased host weight gain (He et al., 2019). In addition, *Enterococcus* spp. is positively correlated with increased BWG because it prevents pathogenic bacterial adhesion to mucosal walls through the "competitive exclusion" phenomenon. *Campylobacter* spp. is a well-known pathogen causing campylobacteriosis in broilers. In humans, it can cause food-borne illnesses; infected poultry has been shown to be the principal source of this zoonosis (Kashoma et al., 2019). The present study observed that *Campylobacter* spp. and *E. Cole* were negatively correlated with host weight gain along with variation in the feed.

## 5. Conclusion

In short, microbial diversity in the caecum and ileum regions of broiler chicken's gut is positively responsive to changes in diet. Growth performance, carcass characteristics, and biochemical and quantitative bacterial analyses revealed that variation in diet enhanced performance-related bacteria in the gut. In chicken guts, phytogenic feed additives and organic acids tend to increase the *Lactobacillus* spp. count and decrease *E. coli* and *Campylobacter* spp. Decreased targeted pathogenic bacterial growth in the NGP (ORG, PHY, and COM) groups together with an increased host weight gain with increased feed efficiency. Hence, safe, healthier, and economically broiler production can be achieved using these feed additives without compromising body weight. However, the underlying relationships of these additives with increased beneficial gut bacteria remain a subject for elucidative research in the future.

## 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.

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