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Dietary inclusion of phytogenic feed additives (PFA) as alternative to in-feed antibiotics modulating gut microbial dynamics in broiler chicken

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ABSTRACT

Food safety and quality have been a significant and crucial predicament recently. In poultry, development of novel antimicrobial strategies for drug-resistant pathogens is desired. Here, Phytogenic Feed Additives (PFA) were administered in chicken diet to decipher their impact on animal performance and gut microbiota composition. One-hundred-fifty-day-old chicks were randomly divided into three treatment groups with five replicates/group as (i) Control diets (CON); (ii) Control diets + 0.01% *w/v* enramycin (antibiotic growth promoter, AB); (iii) Control diets + 3% *w/v* blend of garlic, peppermint, cinnamon, black cumin, and green tea (phytogenic feed additives, PFA). Non-significant differences for body weight gain and feed intake (*p>0.05*) were found between AB and PFA groups. Quantitative bacterial analysis of chicken gut revealed supplementation of PFA significantly increased (*p<0.05*) beneficial bacterial (*Lactobacillus* spp. and *Enterococcus* spp.) and reduced (*p<0.05*) pathogenic bacterial (*E. coli* and *Campylobacter* spp.) population versus AB and CON group. Overall, statistical profiling of gut bacteria depicted numerically increased beneficial bacteria in PFA group (79%) followed by AB (72%) and CON (65%) in chicken gut. In conclusion, due to increased animal performance and maintained balanced gut microflora tested phytogenic feed additives of this study might be regarded as potential alternatives to existing antibiotics in poultry for better food animal production.

Keywords: Animal performance, Broiler chicken, Food safety, Gut flora, Phytogenic feed additives

INTRODUCTION

Food safety and quality have been highly significant recently and primary indicator of economic growth in developing countries (Ronquillo & Hernandez, 2017). Food animals emerged as a reservoir of drug-resistant bacteria due to antimicrobials intensification in food industry thus, contributing to the challenge of antibiotic resistance globally (Ma *et al.,* 2019). An increased rate of therapeutic failures in patients infected with antibiotic resistant bacteria has been reported. Besides, extensive use of antibiotic growth promoters (AGP) in food animal production exacerbates the spread and emergence of antibiotic resistance (FAO, 2016). So, emerging antibiotic resistance in food chain is a huge problem in livestock production (poultry, crop and aquaculture) as well as dissemination of antibiotic resistant genes and bacteria in environment poses severe health risks in humans. (FAO, 2016; Chang *et al.,* 2015). Undoubtedly, it leads to the ban on use of antibiotics in food production industry globally. However, it resulted in reduced feed efficiency, animal performance, and rise in occurrence of animal diseases (Toghyani *et al.,* 2010). Hence, there is a dire need to explore promising alternatives without compromising profitability and productivity of poultry industries.

Author's Contribution: Z.R & S.G., Conceived the idea, designed the experiment and finalized manuscript; **S.G.**, Supervised the study; **Z.R.**, Carried out experiments, analyzed data and performed manuscript writing; **S.M.G. & S.Z.**, Helped in chicken's rearing facility; **Z.A.M. & A.A.C.**, Provided technical help; **A.A.**, reviewed manuscript

At present, phytogenic feed additives (PFA) has been gaining considerable attention among other alternatives as they can improve performance through stabilization of a healthy gut environment (Stevanović *et al.,* 2018). PFA encompasses variety of secondary metabolites (herbs, essential oils and spices) having tremendous medicinal properties viz. digestion stimulation, antioxidant, appetizing and antibacterial attributes (Christaki *et al.,* 2012). Nevertheless, PFA thought to be ideal alternatives because of their considerable potential in improving animal performance, increasing feed intake and sustaining immune system (Attia *et al.,* 2017).

Recently, an enormous interest for dietary inclusion of PFA in food-animal production has been generated. Despite the medicinal importance of PFA, research activities evaluating synergistic effect of plant extracts as feed additives on intestinal microbiota and performance in commercial broiler chickens is limited (Vase-Khavari *et al.,* 2019). In the current study, garlic, peppermint, cinnamon, black cumin and green tea were studied as phytogenic feed additives. Garlic (*Allium sativum)*, a well-known herbal remedy in traditional medicine contains several active alkaloids (allicin, allin, ajoene, s-allylcysteine sulphoxide, diallylsulphide), minerals, enzymes, amino acids and vitamins exhibiting antimicrobial, antioxidant, immunomodulatory, anti-inflammatory, and antiparasitic properties (Omer *et al.,* 2019; Singh *et al*., 2017; Karangiya *et al*., 2016). Peppermint (*Mentha piperita*) belongs to Labiatae family and also vastly used in herbal medicine, particularly beneficial as immunity enhancer and protection against various infections. The antibacterial activity of peppermint leaves is due to presence of phenolic compounds menthol, menthone and their derivatives namely neomenthol, isomenthone, pulegone and acetylmenthol (Khurshid *et al.,* 2016). Cinnamon (*Cinnamomum verum*), a medicinal plant found mainly in Sri Lanka and India. The active components present in the bark of cinnamon are cinnamaldehyde, eugenol, carvacrol, cinnamyl acetate, cinnamyl alcohol, benzyl benzoate etc., which possess antimicrobial and anti-inflammatory properties (Yun-feng *et al.,* 2019). Black cumin (*Nigella sativa* L.) is an important medicinal plant found in Asia belongs to family Ranunculaceae and has antioxidant, antimicrobial and anti-cholesterol properties. The pharmacologically important active components present in the seeds of black cumin are thymol, thymohydroquinone, thymoquinone, dithymoquinone and carvacrol (Islam *et al.,* 2016). Green tea (*Camilla sinensis* L.) is a widely consumed beverage globally and gained much attention recently due to its numerous health benefits. Apart from its antioxidant property, polyphenolic compounds present in green tea improves feed efficiency, body weight, exhibit antimicrobial activities against pathogenic bacteria and maintains gut microflora balance in broilers (Khan, 2014).

Considering enormous medicinal attributes, in addition to growth promoting traits, undoubtedly these plants can be implied in broiler production. Therefore, considerable effort was undertaken to estimate the efficacy of garlic, cinnamon, black cumin, peppermint and green tea on animal performance, cecal and ileal microflora composition in broiler chicken in pursuit for alternative growth promoter in poultry industry.

MATERIALS AND METHODS

Ethical approval

This research was approved by The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE) University of Karachi (DG/AA-089) institutional review board. All the birds were reared under standard operating procedures guidelines of broiler housing management as per "Care and use of agricultural animals in research" (McGlone, 2010).

Animal diets and study design

One-hundred-fifty-day old chicks (hubbard) were procured from commercial hatchery and reared for 42 days. The trial was conducted at Ideal feeds and experimental units farm, superhighway Karachi Pakistan. Birds were assigned to three different treatment groups with five replicates (10 birds/replicate). Birds were fed two classical basal diet formulated according to Hubbard's nutrient requirement as starter (0-21 d) and finisher diets (21-42 d) (Table I) (NRC, 1994). Each diet was analyzed for proximate composition as described by AOAC (2005). Basal diet was supplemented in isonitrogenous and iso-caloric form with the addition of feed additives. Treatments groups were control diets (CON); control diets + 0.01% enramycin (antibiotic growth promoter, AB); control diets + 3% phytogenic feed additives (blend of garlic, cinnamon, peppermint, black cumin, green tea, PFA). Chickens were routinely vaccinated against infectious bursal disease (IBD), Newcastle disease (ND), and infectious bronchitis (IB) on day 1, 5, 8, 18, 22, 34 as per scheduled provided by company (Mega Poultry Company Pvt. Ltd). Birds were placed in clean pen with controlled temperature, humidity and ventilation conditions. Chicks were raised on rice hulls litter and access to water and feed was *ad libitum*.

Table I: Composition of commercial starter and finisher diet fed to broilers

Nutrient composition	Starter diet 0 – 21 days	Finisher diet 21 – 42 days
Metabolize energy (kcal/kg)	2900	2989
Crude protein (%)	21.59	19.13
Calcium (%)	1.00	0.96
Lysine (%)	1.19	1.06
Methionine (%)	0.53	0.45
Methionine + cysteine (%)	0.89	0.74

Growth performance analysis

Birds from each pen were weighed weekly for body weight (BW) gain and feed intake (FI) observations based on which feed conversion ratio (FCR) was estimated (FI/BW). Overall, body weight, feed intake, and feed conversion ratio were also calculated during the experiment.

Sample collection

At day 42, five birds from each replicate group were sacrificed through throat cutting with a sharp knife. Subsequently, cecum and ileum contents were scrapped aseptically from intestine and collected in a sterile tube filled with pre-autoclaved broth (20% glycerol v/v in pre-reduced brain heart infusion) to maintain bacterial viability before processing (Mountzouris *et al.*, 2007).

Bacteriological analysis

For Microbial enumeration, 1gm of each deep frozen cecal and ileal sample was macerated in buffered peptone water. Samples were then serially diluted (10^{-2} to 10^{-9}) in phosphate buffer saline. The diluted samples were seeded on freshly prepared bacterial growth media for quantitative analysis. In particular, *Lactobacillus* spp. were enumerated on de Man Rogosa and Sharpe agar (MRS, Oxoid UK), *Enterococcus* spp. on Bile esculin azide agar (Bile Aesculin agar, Oxoid UK), *Escherichia coli* on Eosin methylene blue agar (EMB, Oxoid UK) and *Campylobacter* spp. on *Campylobacter* selective agar (CAMPY agar, Oxoid UK) with the addition of horse laked blood (Oxoid UK) and *Campylobacter* selective supplement containing antibiotics for inhibition of gram-positive bacteria and fungus (Oxoid, UK). Diluted samples (1ml) were plated on growth media using sterile pipettes and bacterial growth was estimated using spread plate technique (Andrews *et al*., 2014). Plates were aerobically incubated for 24-48 h at 37 °C (MRS, EMB and Bile aesculin azide agar) and anaerobically for 48 h at 42 °C (Campy agar). Anaerobic environment was supplied with the use of commercial anaerobic gas jar (Oxoid, UK) and anaerobic catalyst (Oxoid™ AnaeroGen™ , UK). The bacterial colony counts were calculated as colony forming units (base-10 logarithm) per gram of cecal/ileal digesta.

Statistical analysis

The data was analyzed statistically by using statistical package for the social sciences (SPSS 17.0.) software employing ANOVA and Tukey's test (post-hoc analysis) to compare means among treatments and significance at $p<0.05$ was assumed.

RESULTS

Growth performance analysis

The findings of animal performance analysis are shown in Fig. 1. Feed intake and body weight gain were non-significant ($p>0.05$) between PFA and AB group. Birds in CON group showed higher feed intake and lower body weight gain among all groups. Feed conversion ratio was significantly different ($p<0.05$) in all groups. At day 42, FCR of 2.09, 1.98 and 1.88 was attained in CON, AB and PFA group respectively.

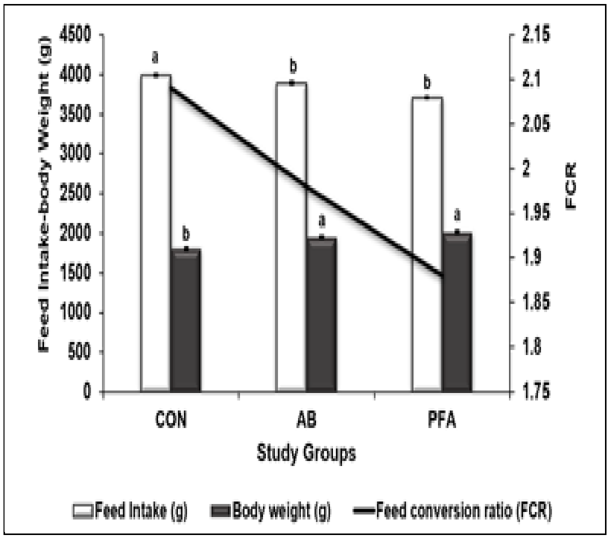

Fig. 1: Estimation of Feed intake (g), body weight (g) and feed conversion ratio (FCR) at 42d in broiler chickens among control (CON), antibiotic growth promoter (AB) and phytogenic feed additive group (PFA). Bars represents mean for five replicates per treatment ± SE. Within the same treatment, bars[a,b,c] with different letters differ significantly (*p<0.05*).

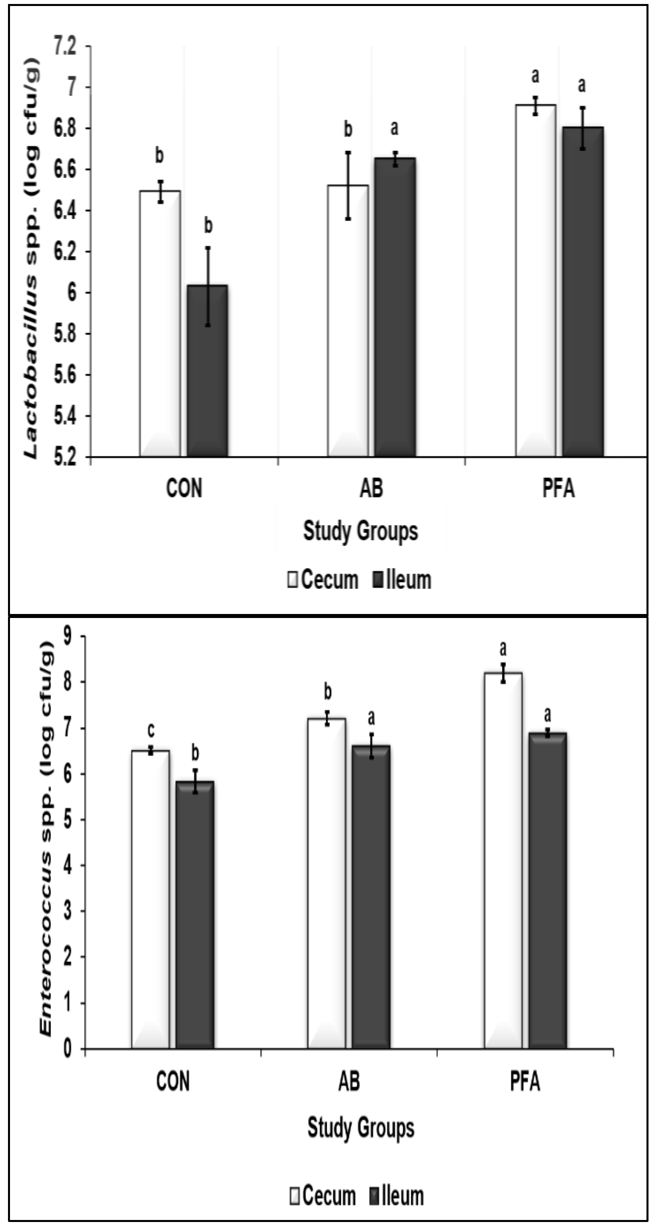

Fig. 2: Enumeration of *Lactobacillus* spp. (a) and *Enterococcus* spp. (b) at 42d in broiler chickens among control (CON), antibiotic growth promoter (AB) and phytogenic feed additive group (PFA). Bars represents mean for five replicates per treatment ± SE. Within the same treatment, bars [a,b,c] with different letters differ significantly (*p<0.05*).

Microflora enumeration

Microflora enumeration was performed using conventional technique of growing bacteria on media culture plates and quantification through colony forming unit. Targeted beneficial bacteria (*Lactobacillus* spp. and *Enterococcus* spp.) increased significantly in PFA group (*p<0.05*) as to CON and AB groups. Likewise, lowest counts for pathogenic bacteria (*E. coli* and *Campylobacter* spp.) were observed for PFA and AB groups than CON.

In particular, *Lactobacillus* spp. counts were significantly higher (*p<0.05*) in PFA group whereas, non-significant difference for *Lactobacillus* spp. (*p>0.05*) was noticed between CON and AB groups in cecum (Fig. 2a). In ileum, AB and PFA groups showed significantly increased *Lactobacillus* spp. (*p<0.05*) counts than CON. For *Enterococcus* spp. in cecum, significantly increased pattern was observed from CON, AB and PFA (*p<0.05*) with log cfu/g of 6.51, 7.21 and 8.20 respectively. Similarly, in ileum higher *Enterococcus* spp. counts was observed in PFA group (Fig. 2b).

In case of pathogenic bacteria, increased *E. coli* population was observed in AB group followed by CON and PFA. In ileum, significant difference for lower *E. coli* (*p<0.05*) counts obtained among study groups with lowest counts in PFA group (Fig. 3a). Likewise, decreased pattern for *Campylobacter* spp. counts was recorded in feed additives groups than CON group. PFA group showed significantly lower *Campylobacter* spp. counts (*p<0.05*) in both cecum and ileum regions of broiler chicken at 42 days (Fig. 3b).

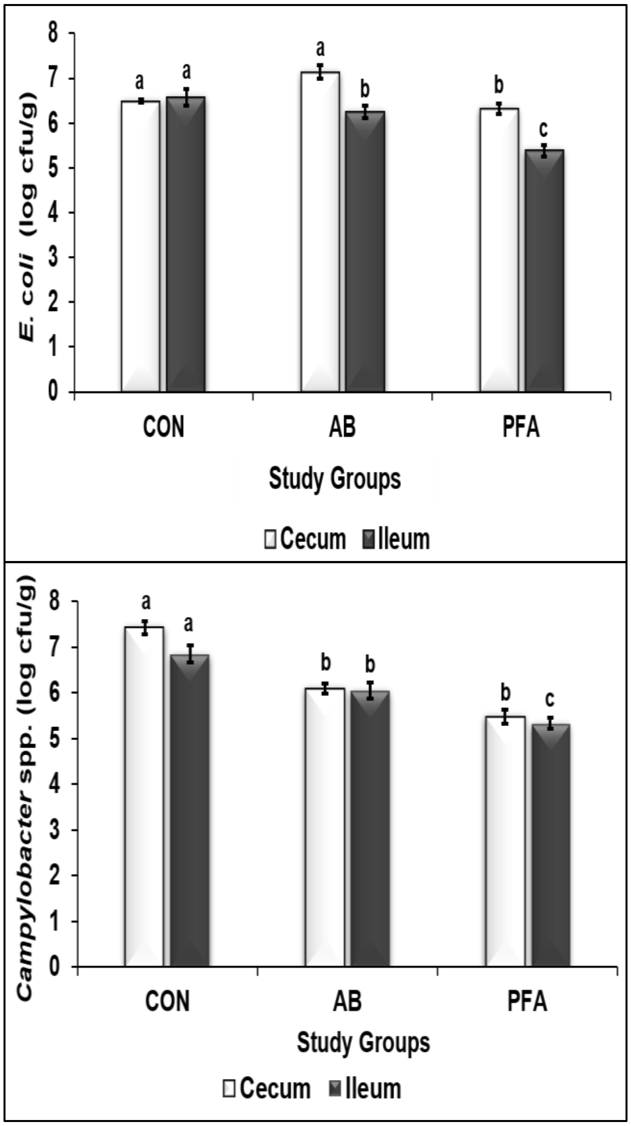

Fig. 3: Enumeration of *E. coli* (a) and *Campylobacter* spp. (b) at 42d in broiler chickens among control (CON), antibiotic growth promoter (AB) and phytogenic feed additive group (PFA). Bars represents mean for five replicates per treatment ± SD. Within the same treatment, bars ^{a,b,c} with different letters differ significantly (*p<0.05*).

The overall efficiency of PFA and AB were showed in Fig 4. In CON (Fig. 4a), the percentage of pathogenic bacteria is 35% and beneficial bacteria is 65%. In AB group (Fig. 4b), the percentages of beneficial and pathogenic bacteria are 72% and 28% respectively. Notably, PFA group showed (Fig 4c) higher percentages of beneficial bacteria (79%) with lowest pathogenic bacteria (21%) in gut of broiler chickens.

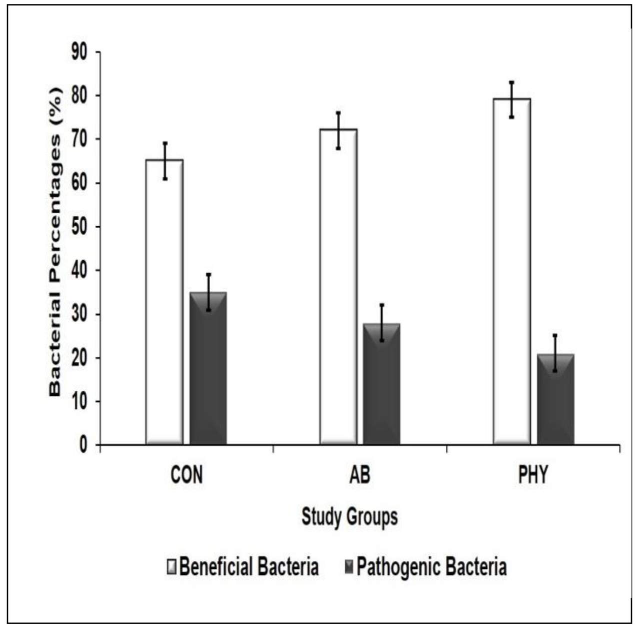

Fig. 4: Bar graphs showing targeted beneficial and pathogenic bacteria population in chicken gut among control (CON), antibiotic growth promoter (AB) and phytogenic feed additive group (PFA).

DISCUSSION

Phytobiotics, phytochemicals or phytogenic feed additives comprises combination of organic molecules and active components which might contain nutritional values (Mohammadi Gheisar *et al*., 2018). They may possess antibacterial, anti-inflammatory, antioxidant properties and enhances nutrient absorption and digestion in gut (Suresh *et al.*, 2018). Nowadays, a variety of phytogenic feed additives available in market which makes the comparison of scientific investigation difficult. In this study, blend of five plants (garlic, black cumin, cinnamon, peppermint and tea leaves) were included in the chicken diet hence, the interpretation of current finding requires attention.

The current study showed statistically non-significant differences for body weight and feed intake (*p<0.05*) in both feed additives groups (AB and PFA). These results are no different than

Ahmed *et al.* (2016), who found that administration of plant extracts (black tea, black cumin and fenugreek) did not improve the growth performance of chickens alongside antibiotic lincomysin. However, birds fed PFA and AB showed lower feed intake and increased body weight gain compared to diet without feed additives which indicated better feed efficiency (input/output ratio). Murugesan *et al.* (2015) discussed that supplementation of phytogenic feed additives and antibiotic growth promoter enhances body weight in chicken than control. Oleforuh-Okuleh *et al.* (2015) reported that garlic as feed additive in basal diet (1g/kg) increases host weight and feed efficiency in broilers. The beneficial role of garlic on animal performance depends on dose and availability of commercial garlic such as allicin rich (raw garlic) or non-allicin rich (synthetic garlic) which differs in active substances present in them (Khan *et al.,* 2012). Similar results for peppermint (Asadi *et al*., 2017), cumin (Habibi *et al.*, 2016), cinnamon (Tajodini *et al.,* 2015) alone and in combination as feed additives revealed enhanced feed efficiency and host weight gain in broilers. PFA mode of action for improved animal performance is remain unclear however, they may involve in feed components stabilization, maintaining balanced gut bacteria, and stimulate digestive and pancreatic enzymes secretion (Reyer *et al.,* 2017). This property of phytogenic feed additive is apparent in birds of PFA group in current study compared to CON birds, which shows increased feed intake and decreased body weight gain. Enhanced feed efficiency in birds of PFA could be a result of increased secretion of pancreatic enzymes which enables the adaptive mechanism of birds in PFA group for better utilization of nutrients from diet that ultimately optimizes nutrient absorption and digestion. It mainly depends on the selection of plants which contain bioactive compounds, and parts of plants used. The bioactive compounds vary in different parts of plants and few studies demonstrated administration of single plant as feed additive (Ahsan *et al.*, 2018). Additionally, the effectiveness of PFA depends on inclusion level, diet composition and genetic potential of broilers.

There is ample literature illustrating the antibacterial role of PFA (Yap *et al.*, 2014; Wati *et al.*, 2015), but studies focusing on exploring PFA's mode of action for facilitating beneficial bacteria proliferation in gut is limited (Mountzouris *et al.*, 2011; Yang *et al.*, 2015). In the current experiment, inclusion of PFA in feed significantly reduced cecal and ileal coliforms (*E. coli* and *Campylobacter* spp.) along with fortification of chicken gut with beneficial bacteria (*Enterococcus* spp. and *Lactobacillus* spp.). This might attribute to the property of *Lactobacillus* spp. in excluding pathogens adhesion to mucosal wall due to their rapid colonization, acidification, and proliferation in gastrointestinal tract (Aoudia *et al.,* 2016). The reduction of *Campylobacter* spp. using PFA is inspiring and paves the way for safe alternatives to AGP in poultry. The alkaloids and essential oils present in PFA causes inhibition of *Campylobacter* spp. via hydrolyzation of bacterial cell wall (Naseri *et al.*, 2012). Jamroz *et al.* (2005) opined that stabilization of gut microbiota by PFA causes reduction of *E. coli* and *Clostridium* spp. in broiler. The biosynthetic machinery of bacterial cell wall tends to be a target site for antimicrobial activity of various compounds. PFA attacks both bacterial cell wall and cytoplasm, often changes cell morphology (Faleiro, 2011). Nazzaro *et al.* (2013) reported that PFA and their derivatives contains active compounds of lipophilic nature, which enables them to penetrate cell membrane and mitochondria of bacteria. The membrane permeability of bacterial cell by essential oils resulting in leakage of cell content and eventually killing bacteria. Additionally, PFA inhibits the energy metabolism, membrane bound electron flow, coagulates and denatures cytoplasm proteins which leads to proton pump collapsing and ATP pool draining (Reis *et al*., 2018). In this study, cecal and ileal microflora estimation revealed that PFA and AB comparably reduced total pathogen composition in gut. This property of PFA in inhibition of bacterial load might alleviate immune system pressure, thus allowing energy reallocation towards improved performance. So, from gut perspective, PFA purportedly maintained healthy gut, which could potentially be concomitant with enhanced growth (Umatiya *et al*., 2018).

Briefly, supplementation of PFA in based diet increased the body weight and decreased feed intake in broiler which in turn maintained better feed conversion ratio comparable to antibiotic used in this experiment. Furthermore, supplementation of PFA aided establishment of healthy gut flora comprised of high counts of *Lactobacillus* spp. and *Enterococcus* spp. and less *Campylobacter* spp. and *E.coli*. The current study demonstrated the effectiveness of PFA utilization in considering them as potential alternative to antibiotics in poultry diet.

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