



## Original research article

# Comparative efficacy of a phytogetic feed additive and an antibiotic growth promoter on production performance, caecal microbial population and humoral immune response of broiler chickens inoculated with enteric pathogens

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## ABSTRACT

The aim of this work was to compare the efficacy of a commercially available phytogetic feed additive (PFA) and an antibiotic growth promoter, which was bacitracin methylene disalicylate (BMD), on performance, nutrient retention, caecal colonization of bacteria and humoral immune responses against Newcastle disease in broiler chickens challenged orally with *Salmonella enteritidis* and *Escherichia coli*. One-day-old male Cobb 400 broiler chicks ( $n = 120$ ) were fed with 1) a negative control (NC) diet, which is the basal diet without any added growth promoter, 2) a positive control (PC) diet, the basal diet supplemented with BMD, 500 mg/kg and 3) a diet supplemented with PFA (150 mg/kg) for 39 days and the birds were inoculated with *S. enteritidis* and *E. coli* on d 28. Supplementation of PFA improved body weight, feed conversion ratio, retention of N and crude fiber, increased fecal moisture content and decreased digesta transit time as compared with the NC and PC groups ( $P < 0.01$ ). Both the PC and the PFA was found to be equally effective in controlling the surge in numbers of *Salmonella* and *E. coli* following oral inoculation of these bacteria as compared with the NC group ( $P < 0.05$ ) at 24 h past inoculation. Caecal content analysis on d 39 indicated lower numbers of *Salmonella*, *E. coli* and *Clostridium* in the PC and PFA groups as compared with the NC group ( $P < 0.05$ ). The number of *Lactobacillus* in the PFA group was higher than those in the NC and PC groups ( $P < 0.05$ ). Humoral immune response, measured as hemagglutination inhibition titer against Newcastle disease, was better in the PC and PFA groups compared with the NC group ( $P < 0.05$ ) at d 21 but the difference did not last till d 39. The heterophil to lymphocyte ratio was narrower ( $P < 0.001$ ) and alkaline phosphatase activity was higher ( $P < 0.01$ ) in the PFA group as compared with the NC and PC groups on d 39. It was concluded that the PFA, which is animal, environment and consumer friendly, may be used as an effective replacement for common in-feed antibiotics like BMD to enhance broiler performance especially when the birds are exposed to heavy infections on fields.

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## 1. Introduction

Phytogetic feed additives (PFA) are the plant derived products used to improve performance of livestock and poultry (Windisch et al., 2008; Jacela et al., 2010). The PFA comprise of a wide variety of herbs, spices and products derived thereof and are mainly essential oils. This class of feed additives is at present used to a great extent as alternatives to the antibiotic growth promoters (AGP) in poultry and swine nutrition. Obviously the ban imposed on the usage of the AGP in diets of food animals is the main driving force that has caused the surge in the use of PFA. Ideally, an

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alternative to the AGP should have the same beneficial effects when included in diets. The dubious mechanism of action of the in-feed antibiotics notwithstanding (Huyghebaert et al., 2011), it is generally accepted that the AGP elicit some antibacterial action and thereby reduce the incidence and severity of subclinical infections. In doing so, they reduce the microbial usage of nutrients and improve their absorption from the intestine by thinning the intestinal mucosa (Brennan et al., 2003; Snyder and Wostmann, 1987). The indirect impact of all these actions is translated into higher growth rate. The basis of this mechanistic explanation is that the AGP do not exert growth promoting effects in germ-free animals. The general industry practice is to feed the livestock with doses of antibiotics at sub-therapeutic levels which is unlikely to exert any growth inhibitory action on the resident bacteria (Niewold, 2007) although a clear shift in the intestinal microbiota was observed when antibiotics were added to broiler diets at levels below the minimum inhibitory concentration (Pedroso et al., 2006; Wise and Siragusa, 2007) and this, at least partly, explains the effects of the AGP. Furthermore, microbiota shifts affect morphology of the gut wall and induce immune reactions, which by affecting energy expenses of the host animal may promote their growth (Teirlynck et al., 2009). Based on the hypothesized mechanism of action of the AGP, the alternative products should have modulatory effects on the gut microbiota and the immune system. Although antimicrobial and growth promoting effects of PFA have been reported by several workers the mode of action of most of the PFA is still not very clear. Possibly, the essential oils present in the PFA promote gut functions by stimulating secretion of digestive enzymes, bile and mucus (Platel and Srinivasan, 2004), the terpenoids and phenolic compounds help the animals to combat with the oxidative stress the animals come across (Aeschbach et al., 1994) and compounds like carvacrol, other terpenoids and aldehydes present in most of the PFA exert substantial antimicrobial effects (Baratta et al., 1998; Burt, 2004; Mathlouthi et al., 2012) thus establishing eubiosis in the gut.

In this experiment a commercially available preparation of PFA was compared with a conventional in-feed antibiotic, bacitracin methylene disalicylate (BMD), as a growth promoter for broilers. The PFA under study contained extracts of fennel, Melissa balm, pepper, anise, oak clove and thyme. Since the efficacy of an antimicrobial agent cannot be assessed properly in absence of a real bacterial challenge (Moralez-Lopez et al., 2009), the chickens were inoculated with pathogenic strains of *Salmonella enteritidis* and *Escherichia coli*. It was hypothesized that the infection induced stress (Geraert et al., 1996; Tankson et al., 2001) would facilitate both the growth promoters to elicit their effects more discernibly. The objective of the study was to gauge the performance differences in the birds supplemented the antibiotic or the PFA vis-à-vis a negative control in terms of body weight gain, feed conversion efficiency, nutrient metabolism and colonization pattern of some of the major intestinal bacteria in presence of artificially inoculated gut pathogens.

## 2. Materials and methods

### 2.1. Bird husbandry and measurement of performance traits

A 39-d experiment was conducted with 120 one-day-old male Cobb 400 broiler chicks (average body weight 45 g at 6 h post-hatch) according to the prevailing institutional ethical norms. The chicks were weighed individually and assigned to three treatments each consisting of 8 replicate cages ( $n = 5$  chicks on d 0 and  $n = 4$  chicks from d 8 onwards) following a completely randomized design to minimize the effects of the cages. Immediately after arrival, swabs from the cloacae were collected from all chicks and

analyzed for *Salmonella* and *E. coli* counts. The chicks were found negative for *Salmonella* but *E. coli* was detected in 2 chicks and they were not included in the experiment. Each cage (0.5 × 0.75 m × 0.75 m) was fitted with a feeder, drinker and an excreta collection tray. Feed (starter mash from d 1 to 7, grower mash from d 8 to 21 and finisher mash from d 22 onwards) and water were offered ad libitum. Lighting program was 23 h of light for the first 7 d, 20 h until d 15 and 18 h afterward. The birds were vaccinated against Marek's disease (d 0), Newcastle disease (ND live B1 at d 7 and La Sota at d 21) and infectious bursal disease (d 14). Temperature was maintained around 32 to 34°C during the first week and at 25 to 27°C subsequently.

The chicks were individually weighed for their empty body weight following a 16-h fast at weekly intervals and live weight (LW) and live weight gain (LWG) was calculated cage wise. Cage average for feed intake (FI) was determined every week and feed conversion ratio (FCR) was calculated for each cage as the ratio between feed intake and LWG.

### 2.2. Experimental diets

The dietary treatments included feeding a corn-soybean based negative control (NC) diet devoid of any added growth promoter, a positive control diet (PC) supplemented with BMD (containing 450 mg active BMD/g) 500 mg/kg, and the PFA diet which was supplemented with the phytogenic feed additive (150 mg/kg). The ingredients and chemical composition of the experimental diets are presented in Table 1. The PFA used in this study was obtained from Biomin Phytogenics GmbH, Germany and was included in diet according to the manufacturer's recommendation. The PFA contained extracts from fennel (*Foeniculumvulgarae* var. dulce mil), Melissa balm (*Melissa officinalis* L.), peppermint (*Mentha arvensis* L.), anise (*Pimpinella anisum* L.), oak (*Quercus cortex*), clove (*Syzygium aromaticum* L.), and thyme (*Thymus vulgaris* L.). All the diets were analyzed (AOAC, 1990) for dry matter (DM, method 934.01), N and crude protein (CP, method 968.06; protein-nitrogen determination, Kelplus, Pelican Equipments, Chennai, India), crude fiber (CF, Foss Fiber Cap 2021 Fiber Analysis System, Foss Analytical, Hilleroed, Denmark) and crude fat (petroleum ether extraction; method 920.39; Socsplus, Pelican Equipments, Chennai, India).

### 2.3. Measurement of nutrient retention and digesta transit time

Retention of N and CF was determined through a metabolism trial by total excreta collection method performed between days 36 and 38. During this period excreta were collected daily per cage at every 2 h intervals during 0600 to 2200 h and at 4 h intervals during 2200 to 0600 h and preserved at –20°C. From the pooled excreta a 10% aliquot was preserved for final analysis. Feed samples were collected daily and pooled to produce a single composite of each diet. Both diets and excreta samples were analyzed for N (AOAC, 1990).

Digesta transit time was determined (Afsharmanesh et al., 2010) on d 34 before commencing the metabolism trial. All birds were fasted overnight and force-fed with the respective treatment diets for a period of 15 min. The diets were mixed with chromic oxide (1 g/kg). The transit time was determined as the time from the introduction of the diets to the first appearance of green-colored droppings.

**Table 1**  
Ingredient composition of the basal diet (g/kg, unless stated otherwise).

Item	Pre-starter (d 1 to 7)	Starter (d 8 to 22)	Grower (d 22 to 39)
<b>Ingredient</b>			
Ground corn	605.2	632.9	701
Soybean meal (460 g CP/kg)	349.8	316.8	248.6
Soybean oil	9.7	19.8	22.7
Calcite powder	9	8.4	8
Di-calcium phosphate	12.9	9.9	7.9
DL-methionine	2.5	2.3	2
Lysine hydrochloride	2.2	1.9	1.9
L-threonine	0.8	0.5	0.9
Sodium bi carbonate	1.5	1.5	1.5
Salt	2.8	2.4	1.9
Choline chloride (600 g/kg)	0.8	0.8	0.8
Toxin binder <sup>1</sup>	1	1	1
Mineral premix <sup>2</sup>	1	1	1
Vitamin premix <sup>3</sup>	0.5	0.5	0.5
Antioxidant	0.1	0.1	0.1
Phytase (500 ftu/kg feed) <sup>4</sup>	0.1	0.1	0.1
NSPase enzyme <sup>5</sup>	0.1	0.1	0.1
<b>Nutritive values (calculated unless stated otherwise)</b>			
ME, MJ/kg	12.1	12.55	12.97
Crude protein <sup>6</sup>	210.4	196.7	172.2
Ether extract <sup>6</sup>	36.1	46.9	51.5
Crude fiber <sup>6</sup>	37.2	35.8	33.1
Calcium	9	8	7.2
Available phosphorus	4.5	4	3.6
Dry matter <sup>6</sup>	942.6	942.1	942.2
Total ash <sup>6</sup>	68.9	69.1	67.8
<b>Standardized ileal digestible amino acids</b>			
Lysine	12	11	9.5
Methionine	4.4	4.18	3.8
Methionine + cysteine	8.4	8.03	7.22
Threonine	7.7	7.04	6.46
Tryptophan	1.9	1.9	1.7
Arginine	12.6	11.8	10.45
Isoleucine	7.8	7.37	6.55
Valine	9	8.5	7.5

<sup>1</sup> Containing diatomaceous earth and yeast cell wall (mannan oligosaccharides).

<sup>2</sup> Containing manganese 40 mg, iron 30 mg, zinc 25 mg, copper 3.5 mg (all as sulfate salts), iodine 0.3 mg (as potassium iodide), selenium 0.15 mg (as sodium selenite).

<sup>3</sup> Contained (per kg) retinyl acetate 3.75 mg, 1,25-hydroxy-cholecalciferol 4 mg, DL- $\alpha$ -tocopheryl acetate 30 mg, menadione 4 mg, thiamine propyl disulfide 3 mg, riboflavin tetrabutryrate 8 mg, riboflavin tetrabutryrate 8 mg, methylcobalamin 0.025 mg, sodium pantothenate 15 mg, pyridoxine 5 mg, niacin 60 mg, biotin 0.2 mg, folic acid 2 mg.

<sup>4</sup> *Escherichia coli* phytase with minimum activity of 5,000 ftu/g.

<sup>5</sup> Containing endo 1,4- $\beta$ -xylanase and endo 1,4- $\beta$ -glucanase activity.

<sup>6</sup> Estimated values.

#### 2.4. Measurement of humoral immune response against Newcastle disease

Humoral immune response against ND was measured through hemagglutination inhibition (HI) test (OIE, 2009). Blood samples (approximately 2 mL) were collected from the wing vein of individual birds in vacutainer tubes without anti-coagulant on d 7, 21 and 38. Serum was separated from cells by centrifugation at 2,500  $\times$  g for 10 min and frozen at  $-20^{\circ}\text{C}$  till analyzed. Antibody titer against ND was determined and expressed as  $\log^2$ .

#### 2.5. Measurement of microbiological parameters following inoculation of pathogenic bacteria

To induce enteric challenge, all birds were inoculated on d 28 with  $2.5 \times 10^{10}$  colony forming units (cfu) of *S. enteritidis* 0363P (ATCC 14028) and *E. coli* (ATCC 25922) through oral routes. The freeze dried organisms were retrieved in a sterile nutrient broth

(M 002, Hi Media Laboratories, Mumbai, India) by incubation at  $37^{\circ}\text{C}$  for 48 h. To ensure accuracy of the number of organism per inoculum, both the bacteria from these broth cultures were sub-cultured on specific agar plates (Hi Touch *Salmonella* and *E. coli* Flexi Plates, Hi Media Laboratories, Mumbai, India) and colonies were counted following an incubation period of  $37^{\circ}\text{C}$  for 48 h. The final inoculum for each bacterium was made by diluting 0.5 mL of the stock culture with double the volume of sterile nutrient broth. The inocula were then stored at  $4^{\circ}\text{C}$  for a brief period and inoculated. Prior to inoculation, excreta samples (approximately 100 g) were collected from each cage in sterile containers to enumerate these bacteria in the chickens before inoculation. This was followed by collection of excreta at 24, 48 and 72 h past inoculation. All excreta were stored at  $4^{\circ}\text{C}$  and *Salmonella* and *E. coli* were enumerated within 48 h (Haldar et al., 2011). Duplicate assays were performed and the average value was used for calculation.

At d 39 all birds were killed humanely by cervical dislocation and the small intestine was removed aseptically. The caeca were ligated with light twine, severed out from the small intestine with the contents inside and stored at  $4^{\circ}\text{C}$ . Within 48 h, the caecal contents were collected in sterile polystyrene tubes by applying gentle pressure with a spatula. Approximately 1 g digesta sample was homogenized in a tissue grinder (Remi Motors, Mumbai, India) with double the volume of ice cold phosphate buffered saline. The homogenized samples were decimally diluted and  $1 \mu\text{L}$  of the diluted sample was cultured aerobically on specific agar plates for enumeration of *Salmonella* spp, *E. coli* and *Lactobacillus* spp. The clostridia were cultured anaerobically in reinforced clostridial agar (M 154, Hi-Media Laboratories, Mumbai, India) for 48 h in presence of carbon di oxide and all visible colonies were enumerated in a colony counter.

#### 2.6. Analysis of blood samples

Blood samples were collected at d 38 from the right brachial vein in vacutainer tubes without anticoagulant (one bird per cage, total 8 birds per treatment). Blood was clotted and the serum thus harvested was stored at  $-20^{\circ}\text{C}$ . Activities of enzymes (alkaline phosphatase, acid phosphatase and amylase) and concentrations of metabolites (total protein, albumin and glucose) in serum were measured photometrically using commercial kits (Diasis Diagnostic Sistemier, Istanbul, Turkey). Another aliquot of whole blood was collected with EDTA as anticoagulant and this was analyzed for hemoglobin, total erythrocyte counts as well as total and differential leukocyte counts.

#### 2.7. Calculation and statistical analysis

The cages were the experimental units and all data were pooled cage wise unless specified otherwise and expressed as mean and pooled standard error of means (SEM). The data were subjected to one way analysis of variance (SPSS version 10.1) with the diets as the factor and when found significant the means were separated by Tukey's test. Data involving measurement of the same parameters over a period of time (enumeration of bacteria in faeces and HI titer) were analyzed by repeated measure analysis of variance in the general linear model. The measurements over the time were the 'within subject factors' and the diets were the 'between subject factors'. Interaction between the time/age and the dietary treatments was included in this model. Significant differences were accepted if  $P \leq 0.05$  and values with  $P \leq 0.1$  were described as trends.

**Table 2**  
Live weight change and feed conversion ratio in experimental birds before and after inoculation with *Salmonella enteritidis* and *Escherichia coli* on 28 d of age.

Item	Dietary treatment <sup>1</sup>			SEM	P-value
	NC	PC	PFA		
<b>d 1 to 28 (Pre-challenge period)</b>					
ADG, g/d	41.9	42.5	42.4	0.24	0.655
ADFI, g/d	58.7	59.9	59.7	0.46	0.523
Feed conversion ratio	1.398	1.41	1.407	0.013	0.917
<b>d 29 to 39 (Post-challenge period)</b>					
ADG, g/d	72.4	76.3	82.6	8.45	0.075
ADFI, g/d	169.1 <sup>c</sup>	160.1 <sup>b</sup>	153.1 <sup>a</sup>	1.49	0.0001
Feed conversion ratio	2.336 <sup>b</sup>	2.099 <sup>b</sup>	1.853 <sup>a</sup>	0.072	0.003
<b>d 1 to 398 (Overall period)</b>					
Empty body weight, g	2,016.5 <sup>a</sup>	2,074 <sup>ab</sup>	2,141.8 <sup>b</sup>	23.1	0.04
Feed intake, g	91.8 <sup>b</sup>	89.9 <sup>b</sup>	87.7 <sup>a</sup>	0.52	0.002
Feed conversion ratio	1.816 <sup>b</sup>	1.729 <sup>ab</sup>	1.632 <sup>a</sup>	0.026	0.006

<sup>a,b,c</sup> Within a row, means with dissimilar letters varied significantly ( $P < 0.05$ ).

<sup>1</sup> NC is negative control, the basal diet; PC is positive control, the basal diet supplemented with bacitracin methylene disalicylate, 500 mg/kg; PFA is the basal diet supplemented with a phytogetic feed additive, 150 mg/kg. Means of 8 replicate cages ( $n = 4$  birds in each cage).

### 3. Results

The birds were healthy during the experiment. There was no mortality even after the bacterial inoculations. However, following the inoculation diarrhea and passage of feed particles was observed in the NC group which was accompanied by growth retardation (Table 2). Supplementation of PFA improved FCR during the post-inoculation period ( $P < 0.05$ ) and both LW and FCR ( $P < 0.05$ ) during the overall d 1 to 39 period (Table 2) as compared with the NC group. Retention of N and CF and fecal moisture content increased ( $P < 0.05$ ) while digesta Tt decreased ( $P < 0.05$ ) in the PFA group as compared with the NC and PC groups (Table 3).

Before inoculation the number of *Salmonella* was less ( $P < 0.05$ ) in the PFA group as compared with the NC group (Fig. 1) but *E. coli* count was similar ( $P > 0.05$ ) across the diets (Fig. 2). A surge in the number of both the bacteria was observed following the inoculation (time effect  $P < 0.05$ ) and this occurred irrespective of the dietary treatments. At 24 h past the inoculation counts for both *Salmonella* and *E. coli* were lower in the PC and PFA groups ( $P < 0.01$ ).

Enumeration of bacteria in the caecal content at d 39 (Table 4) indicated significant reduction in *Salmonella*, *E. coli* and *Clostridium* numbers in the PC and the PFA groups ( $P < 0.01$ ) as compared with the NC group. *Lactobacillus* count was higher in the PFA group compared with the NC and PC groups ( $P < 0.01$ ).

Humoral immune response against ND (Fig. 3) was identical across the diets at 7 d of age and increased with time similarly in

**Table 3**  
Retention of nutrients, fecal moisture content and digesta transit time in the challenged chickens.

Item	Dietary treatment <sup>1</sup>			SEM	P-value
	NC	PC	PFA		
<b>Nutrient retention, g/g (intake)</b>					
Nitrogen	0.731 <sup>a</sup>	0.739 <sup>a</sup>	0.779 <sup>b</sup>	0.007	0.011
Crude fiber	0.705 <sup>a</sup>	0.721 <sup>ab</sup>	0.749 <sup>b</sup>	0.008	0.049
<b>Fecal moisture, g/g (feces)</b>					
	0.691 <sup>a</sup>	0.717 <sup>a</sup>	0.754 <sup>b</sup>	0.011	0.043
<b>Digesta transit time, min</b>					
	178.6 <sup>b</sup>	179.5 <sup>b</sup>	115.4 <sup>a</sup>	6.66	0.001

<sup>a,b</sup> Within a row, means with dissimilar letters varied significantly ( $P < 0.05$ ).

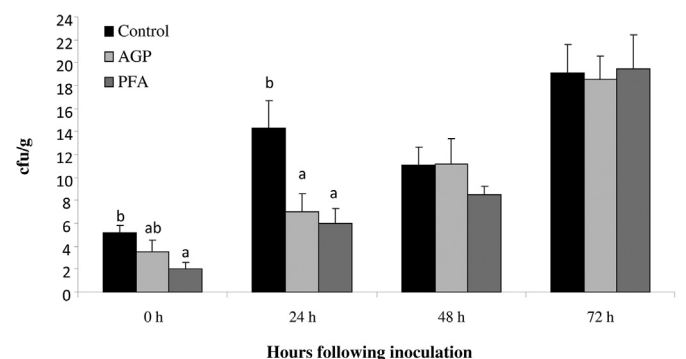
<sup>1</sup> NC is negative control, the basal diet; PC is the basal diet supplemented with bacitracin methylene disalicylate, 500 mg/kg; PFA is the basal diet supplemented with a phytogetic feed additive, 150 mg/kg. Means of 8 replicate cages ( $n = 4$  birds in each cage). Measurement was performed on 34 d of age.

all dietary groups (time effect  $P < 0.01$ ). At d 21, the ND titer was better in the PC and the PFA groups relative to the NC group ( $P < 0.001$ ) although the difference did not last when measured on d 38 ( $P > 0.05$ ).

Hematological parameters (Table 5) indicated subtle effect ( $P > 0.05$ ) of BMD and PFA on hemoglobin and total erythrocyte counts. Compared with the NC group, total leukocyte and lymphocyte counts were greater in the PC and PFA groups ( $P < 0.001$ ). Across the diets heterophils were lower in number in the PFA group ( $P < 0.001$ ). The heterophil-lymphocyte ratio was comparatively wider in the NC and PC groups as compared with the PFA group ( $P < 0.001$ ). Alkaline phosphatase activity in serum was higher in the PFA group ( $P < 0.01$ ) but that of acid phosphatase and amylase did not change due to dietary treatments ( $P > 0.05$ ). Total protein, albumin and glucose in serum did not change due to dietary treatments ( $P > 0.05$ ).

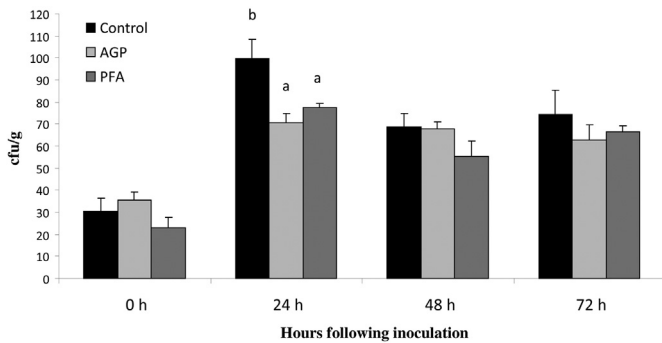
### 4. Discussion

Results of the present investigation revealed that supplementation of the PFA improved body weight and FCR of broilers inoculated with enteric pathogens. Plant extracts and essential oils reportedly improved broiler performance (Ciftci et al., 2005; Jamroz et al., 2005; Mountzouris et al., 2011) which corroborate the present findings. Gut acting growth promoters may not elicit



**Fig. 1.** Total *Salmonella* (colony forming units/g) in excreta of broiler chickens fed diets supplemented with either an antibiotic growth promoter (AGP) or a phytogetic feed additive (PFA) and orally inoculated with *S. enteritidis* 0363P (ATCC 14028) and *E. coli* (ATCC 25922) at 28 d of age (mean  $\pm$  SEM). Bars with dissimilar letters varied significantly ( $P = 0.03$  at 0 h and  $P = 0.007$  at 24 h). Time (hours past the infection) effect  $P = 0.0001$ ; diet  $\times$  time interaction  $P > 0.05$ .





**Fig. 2.** Total *E. coli* (colony forming units/g) in excreta of broiler chickens fed diets supplemented with either an antibiotic growth promoter (AGP) or a phytochemical feed additive (PFA) and orally inoculated with *S. enteritidis* 0363P (ATCC 14028) and *E. coli* (ATCC 25922) at 28 d of age (mean  $\pm$  SEM). Bars with dissimilar letters varied significantly ( $P = 0.005$  at 24 h). Time (hours past inoculation) effect  $P = 0.0001$ ; diet  $\times$  time interaction  $P > 0.05$ .

**Table 4**

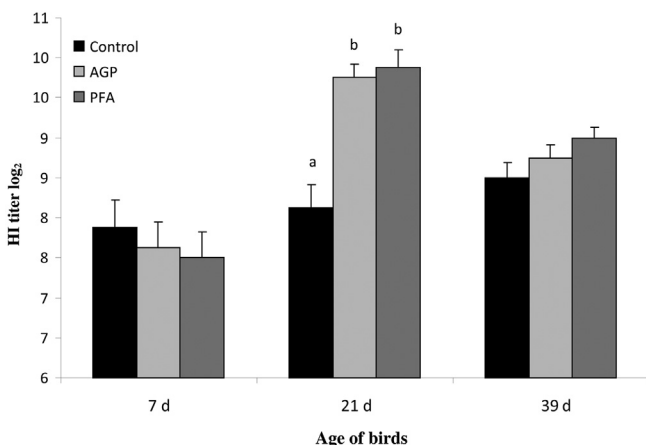
Caecal populations (colony forming units/g caecal digesta) of selected microorganisms in broilers supplemented with an antibiotic growth promoter or a phytochemical feed additive (PFA) and infected with *Salmonella* and *Escherichia coli* at 28 d of age.

Parameter	Dietary treatments <sup>1</sup>			SEM	P-value
	NC	PC	PFA		
<i>Salmonella</i> spp.	30.18 <sup>b</sup>	18.48 <sup>a</sup>	10.97 <sup>a</sup>	2.15	0.0001
<i>Escherichia coli</i>	50.79 <sup>b</sup>	20.17 <sup>a</sup>	13.57 <sup>a</sup>	4.03	0.0001
<i>Lactobacillus</i> spp.	10.44 <sup>a</sup>	10.92 <sup>a</sup>	25.5 <sup>b</sup>	1.89	0.001
<i>Clostridium</i> spp.	15.87 <sup>b</sup>	9.97 <sup>a</sup>	10.21 <sup>a</sup>	0.88	0.003

<sup>a,b</sup> Within a row, means with dissimilar letters varied significantly ( $P < 0.05$ ).

<sup>1</sup> NC is negative control, the basal diet; PC is the basal diet supplemented with bacitracin methylene disalicylate, 500 mg/kg; PFA is the basal diet supplemented with a phytochemical feed additive, 150 mg/kg. Means of 8 replicate cages ( $n = 4$  birds in each cage). Enumeration was performed at 39 d of age.

any effect in absence of a real enteric challenge (Bedford, 2000) and unlike the antibiotics, PFA may require some time to bring any shift in gut microbiota (Giannenas et al., 2003; Hernandez et al., 2004; Jamroz et al., 2005; Mountzouris et al., 2011). The PFA do this by decreasing the production of growth depressing microbial metabolites such as ammonia and biogenic amines (Jamroz et al.,



**Fig. 3.** Hemagglutination inhibition (HI) titer against ND in broiler chickens fed diets supplemented with either an antibiotic growth promoter (AGP) or a phytochemical feed additive (PFA) and orally inoculated with *S. enteritidis* 0363P (ATCC 14028) and *E. coli* (ATCC 25922) at 28 d of age (mean  $\pm$  SEM). Bars with dissimilar letters varied significantly ( $P = 0.0001$ ) at 21 d. Age effect  $P = 0.0001$ ; age  $\times$  diet interaction  $P = 0.004$ .

2003; Windisch et al., 2008), selection of healthier microbial groups (Castillo et al., 2006) and increasing nutrient availability to the host (Anderson et al., 1999). This is evidenced by the higher numbers of *Lactobacillus* in the caeca of the PFA supplemented group in this study. A depression in the intra-epithelial leukocyte numbers of the intestinal mucosa of pigs supplemented with essential oils was reported which indicates that PFA might be capable of controlling the inflammatory reactions the intestinal mucosa is always exposed to (Nofrarias et al., 2006). Controlling of inflammatory processes should spare nutrients for absorption (Huang et al., 2010; Ahmed et al., 2013). Moreover, due to their antibiotic effects essential oils in PFA influence intestinal microbiota and facilitate production of digestive enzymes to increase nutrient digestibility (Stoni et al., 2006; Mountzouris et al., 2011). The cumulative effect might have had improved retention of N and CF in the PFA group during this study.

Inoculation with enteric pathogens pushed the birds to more of a 'diseased' state and allowed both BMD and the PFA to elicit their effects since both of them are gut modulators. Retention of N and CF was measured during this so called 'diseased' state and the results indicate that despite an enteric challenge the PFA could support the process of digestion more efficiently.

Generally essential oils are more active against gram-positive than gram-negative bacteria (Brenes and Roura, 2010) although they may be equally effective against the Gram negative bacteria too (Helander et al., 1998; Smith-Palmer et al., 1998; Chao et al., 2000; Xu et al., 2008). Thyme was one of the constituents of the PFA employed in the present study and, therefore, a considerable activity against the gram-negative bacteria was expected. Intriguingly, the PFA was found to be as effective as BMD against *Clostridium*. There are reports that essential oils from thymol, eugenol, curcumin, piperin, carvacrol and cinnamaldehyde might reduce proliferation of *Clostridium perfringens* in broiler gut, which is in agreement with the present findings (Jamroz et al., 2003; Mitsch et al., 2004; McReynolds et al., 2009). This looks promising because this is an area where the AGP like BMD generally enjoy an edge over the non-antibiotic growth promoters. However, unlike BMD the PFA did not hamper with the growth of *Lactobacillus* as it was reported earlier in pigs (Manzanilla et al., 2004; Castillo et al., 2006; Ahmed et al., 2013). As such, carvacrol, which was one of the components of the PFA used in this study, is reportedly less active against *Lactobacillus* and *Bifidobacteria* (Si et al., 2006) in comparison to pathogens like *E. coli* and *Salmonella* spp. Possibly with a reduction in the numbers of *E. coli* and *Salmonella*, *Lactobacillus* got more room for proliferation and the PFA supported this process while BMD, being an antibiotic, hindered it in the PC group. Lower counts of *Clostridium*, *Salmonella* and *E. coli* and a higher count of *Lactobacillus* had a stabilizing effect on gut microbiology and improved digestibility of nutrients (Brenes and Roura, 2010). Apart from the effects on intestinal microbiology the essential oils in PFA reportedly stimulate secretion and activities of digestive enzymes like trypsin, lipase and amylase (William and Losa, 2001; Lee et al., 2003; Wenk, 2003; Jang et al., 2004; Jamroz et al., 2005; Jang et al., 2007) and may increase villus height (Cardoso et al., 2012). A higher faecal moisture content and shorter digesta Tt in the PFA supplemented group is suggestive of greater secretory activities of the small intestinal mucosa.

The generally subtle effects of supplemental BMD and PFA on serum metabolites and enzyme activities notwithstanding, the PFA increased alkaline phosphates in serum which is suggestive of a higher osteoblastic activity (Leung et al., 1993). Therefore, it is possible that the PFA stimulated the process of bone growth as well.

The differential leukocyte count indicated towards the anti-inflammatory and immune-modulation effects of the PFA. The absence of clinical manifestation notwithstanding, inoculation of

**Table 5**  
Hematological parameters, serum activity of enzymes and metabolite concentrations in serum of broilers supplemented with an antibiotic growth promoter or a phytogetic feed additive (PFA) and infected with *Salmonella* and *Escherichia coli*.

Parameter	Dietary treatment <sup>1</sup>			SEM	P-value
	NC	PC	PFA		
Hemoglobin, g/L	102.8	106.6	106.3	0.4	0.717
Total erythrocytes, 10 <sup>12</sup> /L	3.54	3.66	3.64	0.01	0.724
Total leukocytes, 10 <sup>9</sup> /L	46.6 <sup>a</sup>	69.4 <sup>b</sup>	59.8 <sup>b</sup>	2.66	0.0001
<b>Differential leukocyte counts, 10<sup>9</sup>/L</b>					
Heterophils	11.9 <sup>b</sup>	11.2 <sup>b</sup>	5.4 <sup>a</sup>	0.74	0.0001
Lymphocytes	29.3 <sup>a</sup>	48.6 <sup>b</sup>	47.7 <sup>b</sup>	2.41	0.0001
Monocytes	0.87	1.23	0.81	0.09	0.098
Eosinophils	4.41 <sup>a</sup>	7.24 <sup>b</sup>	4.09 <sup>a</sup>	0.54	0.025
Basophils	0.14 <sup>a</sup>	1.09 <sup>ab</sup>	1.71 <sup>b</sup>	0.22	0.007
Heterophil:lymphocyte ratio	0.413 <sup>c</sup>	0.234 <sup>b</sup>	0.113 <sup>a</sup>	0.03	0.0001
<b>Serum enzyme activity, µ/L</b>					
Alkaline phosphates	2.5 <sup>a</sup>	3.79 <sup>a</sup>	6.23 <sup>b</sup>	0.49	0.003
Acid phosphatase	4.86	3.91	5.24	0.34	0.268
Amylase	643.3	677.1	727.5	32.65	0.591
<b>Serum metabolites</b>					
Total protein, g/L	33.19	34.19	35.59	0.58	0.243
Albumin, g/L	18.98	18.99	20.44	0.44	0.303
Glucose, mmol/L	9.86	10.03	10.28	0.08	0.131

<sup>a,b,c</sup> Within a row, means with dissimilar letters varied significantly ( $P < 0.05$ ).

<sup>1</sup> NC is negative control, the basal diet; PC is the basal diet supplemented with bacitracin methylene disalicylate, 500 mg/kg; PFA is the basal diet supplemented with a phytogetic feed additive, 150 mg/kg. Means of 8 replicate cages ( $n = 4$  birds in each cage). Mortality was not there in any of the treatments and hence is not mentioned (means of 8 birds from each dietary group).

pathogens increased total leukocyte count in the PC and the PFA groups to a greater extent compared to the NC group. This might be induced by *Salmonella* as reported by Garcia et al. (2009). *Salmonella* induced leukocytosis occurs due to fast multiplication of the organism within the phagocytes which causes cell lyses and release of the organism into the blood stream triggering an antigen-antibody reaction (Berchieri et al., 1991). Presence of the bacterium in liver, spleen, kidneys, thymus, and heart might also stimulate production and release of leukocytes into the blood stream. The lower leukocyte count in the NC group may be due to depletion or poor functioning of the organs responsible for production and storage of leukocytes under the influence of *Salmonella* (Garcia et al., 2009). Literature depicting positive effects of PFA containing sanguinarine alkaloids or black cumin seeds on leukocyte counts and T-cell mediated immune responses are available (Swami and Tan, 2000; Islam et al., 2004; Vrublova et al., 2010; Khan et al., 2012) which explains the better antibody titer against Newcastle disease virus in this study. The birds receiving supplemental BMD also showed comparable improvement in titre against Newcastle disease which is in contrary to the findings of Al-Beitawi et al. (2009). The narrow heterophil-lymphocyte ratio, which is considered as an indicator of stress in avians, indicated that the magnitude of stress in the PFA group was less in comparison to the NC and the PC groups. Although not measured in this study, the inoculation of the pathogens obviously triggered release of corticosterones following the general rule of inflammation (Geraert et al., 1996). Being catabolic hormone corticosterones blunt humoral immune responses (Tankson et al., 2001). The higher heterophil counts in the NC and PC group supported our hypothesis that despite absence of any clinical manifestation per se the birds experienced a 'diseased' state which the PFA supplemented birds did not. Plausibly the PFA depended less on the phagocytic activities of the heterophils while BMD acted through some other routes to improve the immunity.

The growth promoting effect of BMD and PFA notwithstanding, body weight in all groups was lower than the breed standard and this may question about the validity of the study. However, factors like weighing following a 16 h fasting, contrary to the breed standards where birds are weighed with feed, mash diets rather

than pelleted ones and housing in cages with restricted movements rather than in floor pens might explain the lower body weight.

## 5. Conclusions

Data of the present study indicated that supplementation of broilers with PFA (150 mg/kg) improved body weight gain and feed conversion efficiency especially when the birds were inoculated with enteric pathogens. At this dose level the PFA influenced the microbial ecology of the cecum and was as effective as antibiotic growth promoters like BMD in controlling the numbers of *Clostridium*, *Salmonella* and *E. coli*. It was concluded that the PFA, which is animal, environment and consumer friendly, may be used as an effective replacement for common in-feed antibiotics like BMD to enhance broiler performance especially when the birds are exposed to heavy infections under field conditions.

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