Effects of Dietary *Punica granatum* L. By-products on Performance, Immunity, Intestinal and Fecal Microbiology, and Odorous Gas Emissions from Excreta in Broilers

Sonia T. Ahmed and Chul-Ju Yang

Department of Animal Science and Technology, Sunchon National University,
255 Jungang-ro, Suncheon, Jeonnam 540−950, Republic of Korea

The fruit *Punica granatum* L. has been used for years in traditional medicine owing to the presence of several phytobiotics with antimicrobial and immunomodulatory properties. This study investigated the effects of dietary supplementation with *Punica granatum* L. by-products (PGB) on performance, immunity, intestinal and excreta microflora, and odorous gas emissions from excreta of broiler chickens. Three experimental diets containing 0, 0.5 and 1.0% PGB were fed to 240 one-day-old broiler chicks until 35 days. Dietary PGB linearly reduced the average daily feed intake and feed conversion ratio of broilers. Supplementation with 1% PGB led to a linear increase in the relative weight of the spleen and bursa of Fabricius. The concentration of serum IgA and IgG increased linearly in response to dietary PGB. In the ileal digesta, the concentration of *Saccharomyces cerevisiae* increased linearly and quadratically in response to dietary PGB. Moreover, dietary PGB led to a linear decrease in *Escherichia coli* and *Salmonella* spp. alongside reducing the pH of the ileal digesta. In the cecal digesta, the concentration of *Bacillus* bacteria increased linearly in response to both levels of dietary PGB, while the concentrations of *E. coli* and *Salmonella* decreased when the diet was supplemented with 1% PGB, as did cecal pH. At 35 day, both levels of PGB increased the concentration of fecal *Bacillus*, whereas only 1% PGB increased the concentration of *S. cerevisiae* at 21 day. Increasing levels of PGB induce a linear reduction in fecal *E. coli* at 21 and 35 day, whereas *Salmonella* only at 21 day. Regarding the average of 48 h, dietary PGB effectively reduced the emissions of ammonia and methanethiol from broiler excreta. In conclusion, the results suggest that, dietary PGB improved immunity and the intestinal microbial ecosystem of broilers along with reduced odorous gas emissions from excreta.

Key words: broilers, growth performance, immunity, microbial ecosystem, odorous gas, *Punica granatum* L. by-products

Introduction

Most plants produce secondary metabolites (often called phytobiotics) such as alkaloids, flavonoids, tannins, steroids, phenolics, terpenes and volatile oils as part of their normal growth and development or in response to pathogen attack or stress (Morrissey and Osbourn, 1999). Since use of antimicrobial growth promoters (AGPs) in poultry diets was banned, phytobiotics have received considerable attention as potential alternatives to AGPs owing to their antioxidant, antimicrobial and immunomodulatory activities (Viveros *et al*., 2011). Several scientific studies have demonstrated the

ability of phytogenic feed additives to improve growth performance, nutrient digestibility and immunity while reducing intestinal and fecal pathogenic microorganisms and fecal noxious gas emissions (Wang *et al*., 2008; Yan *et al*., 2011).

Punica granatum L. (commonly known as pomegranate) is a fruit belonging to the *Punicaceae* family that has been used for centuries in traditional medicine for a variety of therapeutic purposes (Ajaikumar *et al*., 2005). Considering its nutritional and health value, pomegranate is now widely consumed as fresh arils or juice, as well as used in the food and food processing industries, and as a raw material for the manufacture of secondary products such as dyes, cosmetics, etc. (Opara *et al*., 2009). Use of pomegranate results in a large amount of by-products (peels, rinds and seeds) most of which are currently discarded. This can lead to environmental pollution; however, there is the potential for these by-

products to be use as feedstuff for livestock (Shabtay *et al.*, 2008). *Punica granatum* L. peels, which represent about 50% of the fruit weight (Aviram *et al.*, 2000), are an important source of several phytobiotics including hydrolysable tannins such as ellagitannin, punicalagin, punicalin and pedunculagin (Seeram *et al.*, 2005), flavonoids, anthocyanins and other phenolic compounds (Ozkal and Dinc, 1994; Li *et al.*, 2006), minerals (Shabtay *et al.*, 2008) and complex polysaccharides (Jahfar *et al.*, 2003). Nuamsetti *et al.* (2012) reported high levels of phenolic content in hot water extract of *Punica granatum* L. peels (166.83 mg GAE/100 g dry weight) and arils with seeds (87.32 mg GAE/100 g dry weight). The seeds, which account for 10% of the total fruit weight (Aviram *et al.*, 2000), are also a rich source of total lipids with high concentrations of punicic acid (C18:3-9*cis*, 11*trans*, 13*cis*) (Melo *et al.*, 2014). A number of in-vitro studies have confirmed the antimicrobial activity of the phenolic and tannin-rich fractions of the *Punica granatum* L. peel and seed extracts against several bacteria and fungi (Nuamsetti *et al.*, 2012; Growther *et al.*, 2012; Dahham *et al.*, 2010). Additionally, Gracious *et al.* (2001) reported that oral administration of aqueous suspension of *Punica granatum* L. fruit rind powder can stimulate humoral and cell-mediated immune responses in rabbits. The immunomodulatory activity of *Punica granatum* L. seed oil (rich in punicic acid) in mice was reported by Yamasaki *et al.* (2006).

Despite the beneficial properties of *Punica granatum* L., there is currently limited research regarding its use in animal nutrition. Previous study of Rajani *et al.* (2011) reported no significant effect of pomegranate peel on the growth performance of broiler. In another study, Shabtay *et al.* (2008) found that *Punica granatum* L. extract led to linearly increased feed intake in beef cattle without any adverse effect on average daily gain. Oliveira *et al.* (2010) showed that supplementation of the diet of preweaned dairy calves with *Punica granatum* L. extract at 5 or 10 g/day improved the immune response and health of animals due to its phenolic content. In this study, we investigated whether supplementation of broiler diets with PGB impacted the performance, serum immunoglobulins, immune organ weight, intestinal, excreta microbiota and pH, and odorous gas emissions from excreta in broiler chickens.

Materials and Methods

Preparation and Analysis of Punica Granatum L. by-product

The by-product of *Punica granatum* L. (PGB) (Goheung-gun cultivar, Korea) was collected from a juice manufacturing company and included the peel, rind and seeds after juice extraction. The by-products were dried in a forced air drying oven (Doori TEC, Doori TEC, FA, Co., Ltd, Seoul, Korea) at 80℃ for 3 days to reduce the moisture content to less than 10%, after which the dried by-products were ground into powder using a milling machine. The samples of PGB were subsequently analyzed in triplicate for crude protein (CP), ether extract (EE), moisture and ash as described by Association of Official Analytical Chemists (AOAC, 2000).

Table 1. **Chemical composition, trace mineral contents, pH, organic acids and secondary metabolite concentration of *Punica Granatum* L. by-products (PGB)**

Item	Concentration in PGB
Chemical composition, g/kg dry matter	
Crude Protein	60.80
Ether extract	20.63
Moisture	90.41
Crude Ash	20.82
Trace minerals, g/kg	
Calcium	10.50
Iron	0.06
Magnesium	0.79
Sodium	0.67
pH	3.56
Total polyphenols, mg/g	145.91
Total flavonoids, mg/g	57.59
Hydrolysable tannins, mg/g	14.26

Each value in the table represented the mean of three replicate analysis.

Trace mineral contents were determined using an Atomic Absorption Flame Emission Spectrophotometer (Model AA-6200, Shimadzu, Japan). The pH of PGB was measured using a digital pH meter (Docu-pH + meter, Sartorius, USA). The concentration of total polyphenols, total flavonoids and hydrolysable tannin contents (quantified by colorimetric analysis) in PGB were analyzed by a commercial analytical company; the Foundation of Agricultural Technology Commercialization and Transfer (FACT, Suwon-si, Gyeonggi-do, Korea). The analytical results of chemical composition, trace mineral contents, pH, total polyphenols, total flavonoids and hydrolysable tannins in PGB are shown in Table 1.

Birds and Experimental Diets

The present study was conducted at the experimental broiler shed of Sunchon National University (Suncheon, Republic of Korea), and all experimental protocols were approved by the Sunchon National University Animal Care and Use Committee. A total of 240 one-day-old male broiler chicks (Ross 308) were purchased from a commercial hatchery (Yangil Farm, Yeosu, Republic of Korea) and utilized in a 5 week experimental period. Broilers were kept in a close ventilated, wire-floor caged broiler house (80 cm long×60 cm wide×40 cm high/cage) at a stoking density of 600 cm^2/bird. The cages had a linear feeder in the front and a nipple drinker in the back to provide feed *ad libitum* and free access to water throughout the experiment. The chicks were randomly divided into three treatment groups with ten replicate cages of eight birds. The cages in each treatment were distributed so that they were located in the front, middle and back of the house. Temperature was maintained at 33℃ for d 1 to 7, after which it was gradually reduced to 24℃ at a rate of 3℃ per week, where it was maintained until the end of the experiment. The relative humidity was maintained at around 50% throughout the experiment, and continuous lighting was provided throughout the experimental period.

Table 2.　**Feed ingredients and nutrient contents of broiler diets**

Item	Starter diet (0 to 21 d)			Finisher diet (22 to 35 d)		
	PGB 0%	PGB 0.5%	PGB 1.0%	PGB 0%	PGB 0.5%	PGB 1.0%
Ingredients (% as fed basis)						
Corn grain	57.58	57.58	57.58	60.64	60.64	60.64
Soybean meal	26.80	26.80	26.80	24.90	24.90	24.90
Corn gluten	5.00	5.00	5.00	3.50	3.50	3.50
Soybean oil	2.20	2.20	2.20	2.20	2.20	2.20
Animal fats	4.50	4.50	4.50	5.00	5.00	5.00
Common salt	0.25	0.25	0.25	0.25	0.25	0.25
Dicalcium phosphate	2.14	2.14	2.14	2.00	2.00	2.00
Limestone	0.92	0.92	0.92	0.88	0.88	0.88
Vitamin-mineral premix[1]	0.30	0.30	0.30	0.30	0.30	0.30
Choline	0.08	0.08	0.08	0.07	0.07	0.07
L-lysine HCl (78%)	0.24	0.24	0.24	0.16	0.16	0.16
DL-Methionine	0.20	0.20	0.20	0.10	0.10	0.10
Pomegranate by-products	0.00	0.50	0.10	0.00	0.50	0.10
Nutrient specifications						
ME (MJ/kg)	13.03	13.01	13.01	13.27	13.25	13.23
Crude protein	20.89	20.79	21.05	19.12	19.07	18.86
Ether extract	5.65	5.57	5.30	2.93	3.18	2.93
Crude fiber	4.42	4.37	4.55	3.71	3.28	3.36
Crude ash	5.63	5.19	5.42	4.61	4.92	4.70
Calcium	1.05	1.05	1.05	0.81	0.81	0.81
Available phosphorus	0.55	0.55	0.55	0.45	0.45	0.45
Lysine	1.42	1.42	1.42	1.10	1.10	1.10
Methionine	0.49	0.49	0.49	0.45	0.45	0.45

[1] Vitamin-mineral mixture provided the following nutrients per kilogram of diet: vitamin A, 15,000 IU; vitamin D3, 1,500 IU; vitamin E, 20.0 mg; vitamin K3, 0.70 mg; vitamin B12, 0.02 mg; niacin, 22.5 mg; thiamine, 5.0 mg; folic acid, 0.70 mg; pyridoxine, 1.3 mg; riboflavin, 5 mg; pantothenic acid, 25 mg; choline chloride, 175 mg; Mn, 60 mg; Zn, 45 mg; I, 1.25 mg; Se, 0.4 mg; Cu, 10.0 mg; Fe, 72 mg; Co, 2.5 mg (Bayer Korea Ltd., Dongjak-Ku, Seoul, Korea).

The dietary treatments evaluated in this study included: (1) basal diet without any supplementation (PGB 0%); (2) basal diet plus 0.5% PGB and (3) basal diet plus 1.0% PGB. Commercially available broiler diets formulated according to the NRC (1994) were used as basal diet, and these were prepared using the same batch of ingredients for starter (0 to 21 d) and finisher (22 to 35 d) periods. The PGB was mixed with basal diet by replacing an equal amount of basal diet on a weight:weight ratio basis. The ingredients and nutrient composition of the experimental diets are shown in Table 2.

Growth Performance Assay

Feed intake and body weight were recorded in replicates at weekly intervals (at 1, 7, 14, 21, 28 and 35 days of age), and the average daily feed intake (ADFI), average daily weight gain (ADWG), and feed conversion ratio (FCR) were calculated per replicate cage from these values for the starter, finisher and entire experimental periods. The mortality was recorded once observed to adjust the FCR data.

Collection and Analyses of Blood Samples

At the end of the feeding trial, three birds per replication were randomly selected and blood samples were collected (10 mL) from the wing veins into a 10 mL anticoagulant-free vacutainer tube (Greiner Bio-One GmbH, Kremsmunster, Austria). The samples were stored on ice during the collection period, after which they were immediately centri-fuged to separate the serum (centrifugation for 15 minutes at 1,610×g at 4℃). Next, the serum samples were carefully transferred to plastic vials and stored at −20℃ until immunoglobulin analysis was performed. The concentrations of serum IgA and IgG were assayed using appropriately diluted samples by a sandwich ELISA with chicken-specific IgA (Cat. No. E30-103) and IgG (Cat. No. E30-104) ELISA kits (Bethyl Laboratories Inc., Montgomery, TX, USA) according to the manufacturer's instructions. Each experiment was conducted in duplicate and the results represent the means of triplicate experiments (Ahmed et al., 2014). The absorbance of each well was measured at 450 nm within 30 min using a microplate autoreader (Thermo Lab Systems Inc., Beverly, MA, USA). The concentrations of IgA and IgG were determined using standard curves constructed from the respective Ig standards and the results were expressed as mg/mL of serum.

Carcass Processing and Immune Organ Weight Measurement

After blood collection, the selected birds were euthanized by cervical dislocation and then subjected to carcass processing. The liver, spleen, and bursa of Fabricius were removed from the carcass and weighed.

Bacterial Population and pH of Ileal and Cecal Digesta

To enumerate the intestinal bacterial population we

followed the method described by Ahmed *et al*. (2014). At first, the ileum (from Meckel's diverticulum to the joint of the ceca) and 10-cm segments from the same area of both ceca were dissected. Approximately 1 g of ileal or cecal digesta was aseptically collected from three birds per replication into a test tube and diluted with 9 mL of sterile sodium chloride saline (Daejung Chemical & Metals Co., Ltd., Siheung, Gyonggi-do, Korea). Next, serial dilutions (1: 10) were prepared, and 20 μL of the diluted samples were plated in duplicate on Difco *Lactobacilli* MRS (Mann, Rogosa and Sharpe) agar to count *Lactobacillus* spp., Difco Mannitol-Egg Yolk-Polymyxin (MYP) agar to count *Bacillus* spp., Difco potato dextrose agar to count *Saccharomyces cerevisiae*, Difco MacConkey sorbitol agar to count *Escherichia coli*, and BBL Salmonella Shigella agar to count *Salmonella* Typhimurium (Becton, Dickinson, and Company, Sparks, MD, 21152 USA). The potato dextrose agar plates were incubated for 48 h at 37℃, while the other agar plated were incubated for 24 h at 37℃. Visible microbial colonies were counted immediately after removal from the incubator based on the color of colonies and expressed as $\log_{10}$ cfu/g.

Around 0.1 g of ileal and cecal contents were suspended in 0.9 mL of distilled water, after which the pH was measured with a Uni pH testa (Trans Instruments (S) PTE Ltd., 5 Jalan Kilang Barat, Petro Centre, Singapore) at 25℃.

Bacterial Population and pH of Excreta

To observe the effects of dietary PGB on excreta microbiology during the starter and finisher period we followed the method described by Ahmed *et al*. (2014). Briefly, fresh fecal samples were collected from the bottom tray of each wire-floor cage into zipper plastic bags at d 21 and 35 of the experimental period. One gram of the fecal sample was then diluted to a 1:9 weight : volume ratio in sterile sodium chloride saline, which was subsequently homogenized for 2 min, after which 10-fold serial dilutions were prepared. The microbial plating, incubation and counting methods were the same as for the intestinal digesta.

To measure fecal pH, 4 g of fecal sample was weighed and diluted to a 1:9 weight : volume ratio in 36 mL of DW, after which the pH was measured directly using a Uni pH testa (Trans Instruments (S) PTE Ltd, 5 Jalan Kilang Barat, Petro Centre, Singapore) at 25℃.

Measurement of Odorous Gas Emissions from Excreta

About 500 g of excreta sample were collected in plastic zipper bags from the bottom tray of each replicate cage and placed in 2 liter plastic boxes in triplicate to measure the emission of ammonia (NH_3), hydrogen sulfide (H_2S) and methanethiol (methylmercaptans) from broiler excreta (Ahmed *et al*., 2014). The boxes were equipped with a cover containing two holes. One hole was used to insert a tube with a cap to facilitate gas measurement and the other was sealed with an Advantec® membrane filter (pore size 1.0 μm, Toyo Roshi Kaisha Ltd., Otowa, Tokyo, Japan) to facilitate insertion of fresh air to offset the negative pressure created during drawing of headspace air during sampling. Following sampling at 0 h, the samples were allowed to ferment at room temperature (average 27℃), with subsequent samples collected at 3 h, 6 h, 12 h, 24 h and 48 h. A Gastec (model AP-20) gas sampling pump (Gastec Corp., Ayase, Kanagawa, Japan) and Gastec detector tube (3 LA, 3M for NH_3; 4 LB, 4LK for H_2S; 70L for methanethiol) were used to measure the odorous gas emissions. During measurement, the tube was opened and 100 mL of headspace air was sampled from approximately 3.0 cm above the sample surface. The concentration of lethal gases was expressed as ppm/100 mL.

Statistical Analyses

All experimental data were analyzed in accordance with the General Linear Model (GLM) procedure established by the Statistics Analysis Systems Institute (version 9.1; SAS Ins. Inc., Cary, NC, USA). Individual cages served as the experimental units for growth performance, excreta microbiota, pH and odorous gas emissions, whereas individual birds served as the experimental units for organ weight, serum immunoglobulins, ileal and cecal microbita and pH. An orthogonal polynomial contrast test was performed to determine linear and quadratic effects of increasing levels of PGB in diets on each measurement. Treatment means were separated using a Student's *t* test at a probability level of $p < 0.05$.

Results

Performance Parameters

The effects of dietary supplementation with PGB on broiler growth performance parameters during different phases are shown in Table 3. Orthogonal contrast revealed that inclusion of PGB in the broiler diet had no significant effect on ADWG in any periods throughout the experiment. Conversely, increasing the levels of PGB in the broiler diet linearly reduced the ADFI during the finisher ($p = 0.028$) and overall ($p = 0.033$) experimental periods, whereas it led to a linear improvement in FCR during the starter ($p = 0.029$) and overall ($p = 0.040$) periods.

Relative Immune Organs Weight and Serum Immunoglobulins

The effects of dietary PGB on relative immune organs weight and serum immunoglobulin concentrations are shown in Table 4. The relative weight of the liver was not affected by dietary supplementation with PGB. However, supplementation with 1% PGB led to a linear increase in the relative weight of the spleen ($p = 0.038$) and bursa of Fabricius ($p = 0.005$). Increasing levels of PGB in broiler diet increased the concentration of both IgA (Linear, $p = 0.003$; quadratic, $p = 0.006$) and IgG (linear, $p = 0.023$; quadratic, $p = 0.073$) relative to the control.

Bacterial Population and pH of Ileal and Cecal Digesta

Orthogonal contrast revealed that inclusion of PGB in the diet did not alter the *Lactobacillus* populations of the ileal and cecal content of broilers (Table 5). However, increasing levels of PGB resulted in higher concentrations of *S. cerevisiae* in the ileal digesta (linear, $p = 0.003$; quadratic, $p = 0.007$). In contrast, supplementation with 1% PGB led to a linear decrease in the number of *E. coli* ($p = 0.006$) and *S.* Typhimurium ($p = 0.031$), together with a reduced pH ($p =$

Table 3. **Effects of dietary *Punica Granatum* L. by-products (PGB) on the growth performance of broilers**

Performance parameter	PGB (% of diet)			Probabilities		
	0	0.5	1.0	SEM	Linear	Quadratic
Average daily weight gain (g/bird)						
Starter (0 to 21 day)	42.08	44.93	43.14	1.21	0.570	0.171
Finisher (21 to 35 day)	75.05	72.46	72.16	1.06	0.093	0.413
Overall (0 to 35 day)	55.26	55.94	54.74	0.89	0.702	0.430
Average daily feed intake (g/bird)						
Starter (0 to 21 day)	62.21	59.86	57.82	1.70	0.123	0.956
Finisher (21 to 35 day)	146.86[a]	136.45[b]	136.82[b]	2.45	0.028	0.137
Overall (0 to 35 day)	96.08[a]	90.53[ab]	89.45[b]	1.80	0.033	0.353
Feed Conversion Ratio (g Feed/g Gain)						
Starter (0 to 21 day)	1.48[a]	1.33[b]	1.34[b]	0.04	0.029	0.118
Finisher (21 to 35 day)	1.96	1.89	1.90	0.03	0.319	0.405
Overall (0 to 35 day)	1.74[a]	1.62[b]	1.64[b]	0.03	0.040	0.087

Each value represents the means of 10 replications with 8 birds/replication.
[a, b] Means not sharing a common superscript in the same row differ significantly ($p < 0.05$).

Table 4. **Effects of dietary *Punica Granatum* L. by-products (PGB) on immune organ weight and serum immunoglobulins of broiler**

Parameter	Pomegranate by-product (% of diet)			Probabilities		
	0	0.5	1.0	SEM	Linear	Quadratic
Immune organ relative weight (%)						
Liver	2.17	2.10	2.12	0.12	0.770	0.773
Spleen	0.06[b]	0.06[b]	0.08[a]	0.01	0.038	0.195
Bursa of Fabricius	0.11[b]	0.12[b]	0.17[a]	0.01	0.005	0.141
Serum immunoglobulin (mg/mL)						
IgA	0.75[b]	0.84[a]	0.82[a]	0.01	0.003	0.006
IgG	1.70[b]	1.78[a]	1.77[a]	0.02	0.023	0.073

Each value represents the means of 10 replications with 3 birds/replication.
[a, b] Means not sharing a common superscript in the same row differ significantly ($p < 0.05$).

Table 5. **Effects of dietary *Punica Granatum* L. by-products (PGB) on ileal and cecal microbiology and pH of broilers**

Parameter	Pomegranate by-product (% of diet)			Probabilities		
	0	0.5	1.0	SEM	Linear	Quadratic
Ileum microbiota (log^{10} CFU/g)						
Lactobacillus spp.	5.68	5.64	5.06	0.40	0.317	0.311
Bacillus spp.	5.03	4.68	5.23	0.58	0.829	0.578
Saccharomyces cerevisiae	3.32[b]	5.21[a]	4.80[a]	0.25	0.003	0.007
Escherichia coli	5.97[a]	5.26[ab]	4.73[b]	0.22	0.006	0.765
Salmonella Typhimurium	1.61[a]	1.25[ab]	0.68[b]	0.25	0.031	0.736
Ileal pH	6.64[a]	6.50[ab]	6.26[b]	0.10	0.031	0.685
Cecum microbiota (log^{10} CFU/g)						
Lactobacillus spp.	6.65	6.50	6.78	0.22	0.708	0.489
Bacillus spp.	5.58[b]	6.31[a]	6.25[a]	0.15	0.014	0.066
Saccharomyces cerevisiae	5.69	5.57	6.31	0.37	0.335	0.439
Escherichia coli	6.57[a]	6.91[a]	5.73[b]	0.22	0.029	0.025
Salmonella Typhimurium	2.16[a]	2.39[a]	0.91[b]	0.40	0.002	0.009
Cecal pH	6.82[a]	6.60[ab]	6.44[b]	0.11	0.041	0.805

Each value represents the means of 10 replications with 3 birds/replication.
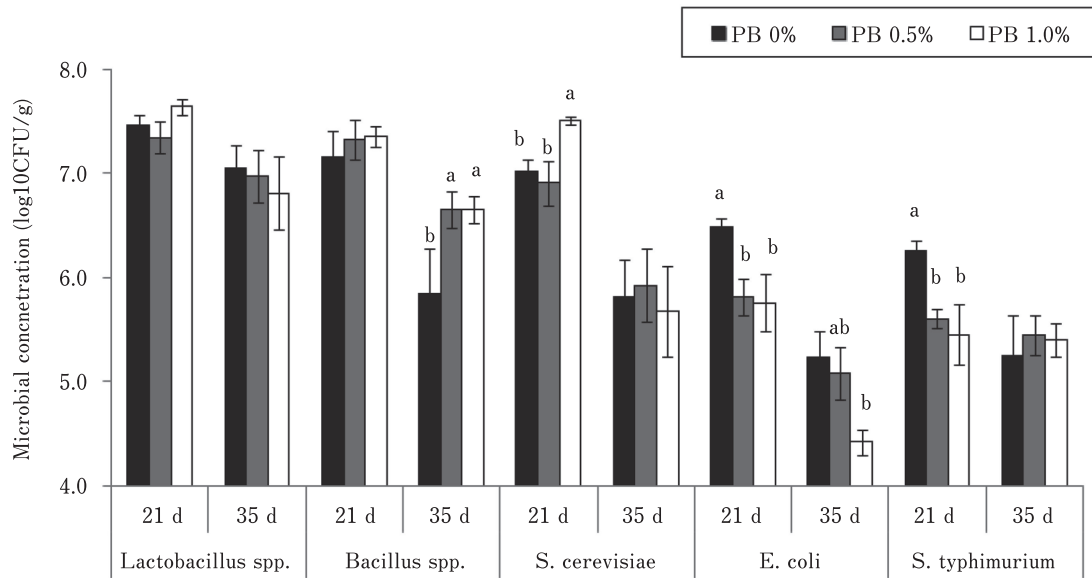[a, b] Means not sharing a common superscript in the same row differ significantly ($p < 0.05$).

Fig. 1. **Effects of dietary *Punica Granatum* L. by-products (PGB) on fecal microflora at d 21 and 35 of the experiment.** Data are presented as the mean$\pm$SE. Bars at a particular time point not sharing a common letter differ significantly ($p < 0.05$).

0.031) in the ileal digesta. In the cecal digesta, dietary supplementation with PGB resulted in higher levels of *Bacillus* spp. (linear, $p = 0.014$) and lower levels of *E. coli* (linear, $p = 0.029$; quadratic, $p = 0.025$) and *Salmonella* ($p = 0.002$; quadratic, $p = 0.009$). A linear reduction in cecal pH was observed ($p = 0.041$) when the diet was supplemented with 1% PGB.

Bacterial Population and pH of Broiler Excreta

Dietary PGB had no significant effect on the *Lactobacillus* concentration of broiler excreta (Fig. 1). The excreta *Bacillus* concentration was not affected by PGB supplementation within the first 21 d of the experiment; however, it linearly increased in response to supplementation by 35 d ($p < 0.05$). Supplementation of the diet with 1% PGB led to a linear increase in *S. cerevisiae* by 21 d ($p = 0.037$), but no significant difference was recorded at d 35. Increasing level of PGB linearly reduced the number of *E. coli* from broiler excreta both in starter ($p = 0.026$) and finisher ($p = 0.024$) period. On the other hand, linear reduction in the number of excreta *Salmonella* was found on d 21 ($p = 0.013$), whereas no differences were observed at d 35 relative to the control. No significant differences were observed in excreta pH among treatment groups (data not shown).

Odorous Gas Emission from Excreta

Figure 2 presents the effects of dietary PGB on the emission of odorous gases (NH_3, H_2S, and methanethiol) from broiler excreta. At 0 and 6 hour of incubation, NH_3 emission was significantly lower in the 0.5% PB supplemented group (Fig. 2, a), whereas at 12, 24 and 48 h both levels of PGB led to effective reductions in NH_3 emissions from broiler excreta ($p < 0.05$). Lower emission of H_2S from broiler excreta was only observed at 0 h ($p < 0.05$) in response to 1% PGB supplementation (Fig. 2, b). Methanethiol emissions from broiler excreta were significantly lower at 0 h, 3 h and 48 h (Fig. 2, c) in response to increasing levels of PGB ($p < 0.05$).

Discussion

The biological significance of dietary tannins in poultry nutrition is related to their adverse effects on feed intake (Armstrong *et al*., 1974) and nutrient utilization (Smulikowska *et al*., 2001). Since tannins are astringent, bitter plant polyphenols, they react with salivary muco-protein or directly with taste receptors in the mouth, reducing palatability (McLeod, 1974; Ashok and Upandhyaya, 2012). This study showed that the inclusion of PGB in the broiler diet reduced the ADFI and improved FCR values, while the ADWG remained unaffected. The reduction in feed intake can be explained by reduced diet palatability due to the presence of a considerable amount of tannin in the experimental PGB (hydrolysable tannin 14.26 mg/g on a DM basis, Table 1). Consistent to our result, Rajani *et al*. (2011) also reported reduction in feed intake without any significant effect on weight gain in broiler fed diet supplemented with pomegranate peel. However, Shabtay *et al*. (2008) reported a significant increase in feed intake with a positive tendency toward increased weight gain of bull calves fed diet supplemented with pomegranate peel. This could have been because non-ruminants are usually more sensitive to anti-nutritional factors in their diets than ruminants (Ahn *et al*., 1989; Norton, 1994). Reduction of ADFI and improvement of the FCR value in response to dietary supplementation with PGB credited to the presence of high polyphenol in PGB (145.91
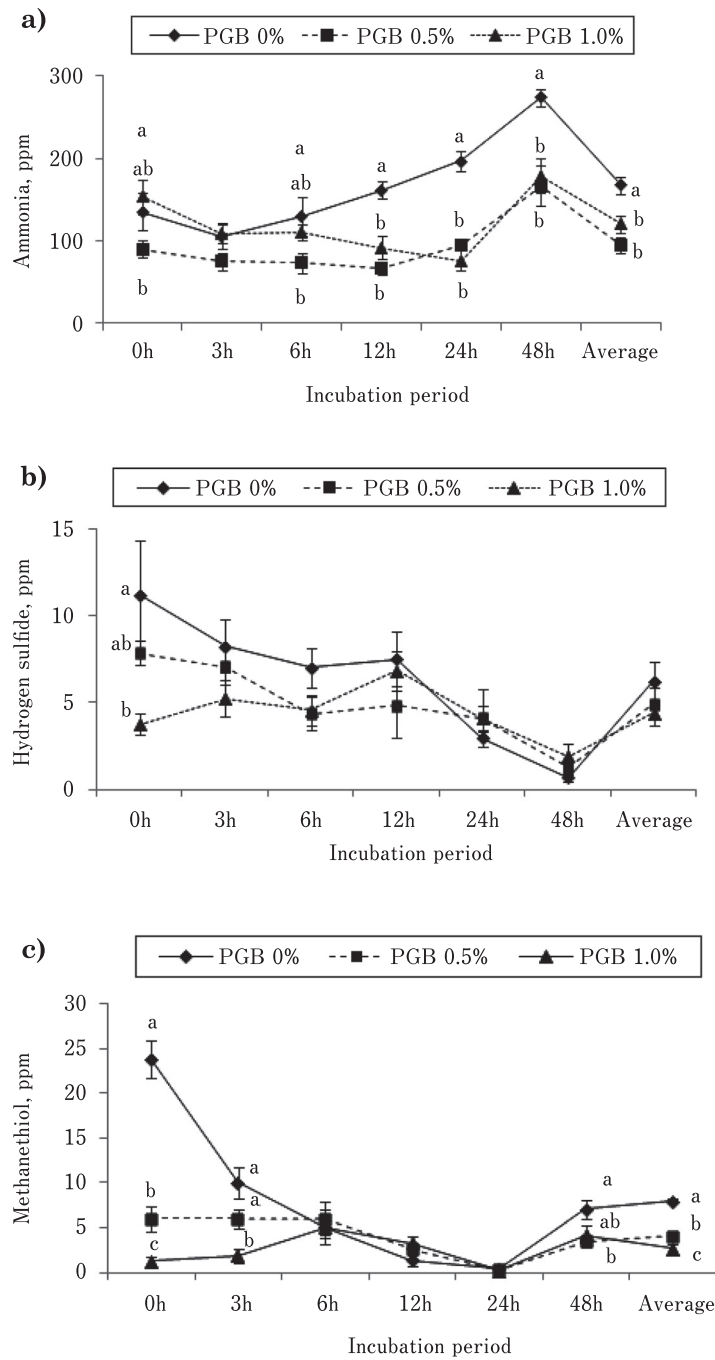
Fig. 2. **Effects of dietary *Punica Granatum* L. by-products (PGB) on (a) ammonia, (b) hydrogen sulfide and (c) methanethiol emissions from broiler excreta after different incubation periods.** Data are presented as the mean±SE. Points at a particular time not sharing a common letter differ significantly ($P < 0.05$).

mg/kg) which can improve utilization of diet energy by manipulating the gut microflora to achieve better feed conversion.

The main immune organs in poultry are the liver, spleen, and bursa of Fabricius. During an immune response, mature lymphocytes and other immune cells interact with antigens in these tissues. Consequently, immune tissue mass can indicate immune status in some cases (Grasman, 2002; Smith and Hunt, 2004). In the current study, PGB did not affect the growth of the liver, but increased the weight of the spleen (the site of immune response to blood borne antigens) and bursa of Fabricus (the site of B cells maturation in birds). In addition, dietary supplementation with PGB led to a significant increase in serum IgA and IgG concentration. Wang *et al*. (2000) reported that dietary supplementation with polyunsaturated fatty acid (PUFA), especially n-3 fatty acid, increased the growth of immune organs. Pomegranate seeds are a rich source of PUFA, particularly *α*-linolenic acid, linoleic acid and CLA (Fadavi *et al*., 2006; Melo *et al*., 2014), which may be responsible for the increased weight of the spleen and bursa of Fabricius observed in the present study. Dietary CLA is also reportedly a potent enhancer of Ig production (Kohno *et al*., 2004; Yamasaki *et al*., 2004). Yamasaki *et al*. (2006) reported significantly enhanced IgG and IgM production in spleen lymphocytes of rats fed diet supplemented with pomegranate seed oils. In addition, ellagitannin (Ramstead *et al*., 2013) and a polysaccharide (PSP001) (Joseph *et al*., 2012) isolated from pomegranate peel was also found to have immunomodulatory activity via stimulation of the growth of normal lymphocytes.

The results of this study demonstrated that, increasing levels of PGB in the diet leading to decreasing amounts of *E. coli* and *Salmonella* in the ileal and cecal digesta. Dietary supplementation with PGB also resulted in dissipation of the ileal and cecal pH. A number of *in vitro* studies have confirmed the antimicrobial activity of pomegranate fruit peel against pathogenic *E. coli* and *Salmonella* (Prashanth *et al*., 2001; Voravuthikunchai *et al*., 2005; Nuamsetti *et al*., 2012). The antimicrobial effects of PGB in the current experiment could be attributed to their high contents of polyphenols (145.91 mg/g), total flavonoids (57.59 mg/g) and hydrolysable tannins (14.26 mg/g). The low pH of PGB (pH 3.56) may also create an acidic condition in the digestive tract of broiler which prevents the growth of pathogenic bacteria. Previous studies of rats and broilers have shown that dietary plant polyphenols induce a shift in the microbial populations of the intestinal tract (Dolora *et al*., 2005; Viveros *et al*., 2011). However, the exact mechanism by which these effects occur is not clear. According to Scalbert (1991), tannins exert antimicrobial activity by inhibiting extracellular microbial enzymes and removing substrates required for microbial growth, resulting in disruption of the membrane structure and function. In this study, dissipation of the intestinal pH gradient together with the antimicrobial activity of phenolic tannins may have been responsible for the reduction in *E. coli* and *Salmonella*. Conversely, the inclusion of PGB in the broiler diet resulted in an increase in the *S.*

cerevisiae population in the ileum and *Bacillus* population in the cecum, which are considered beneficial bacteria for intestinal function (Teo and Tan, 2007; Lei *et al*., 2009). A possible explanation for this is that yeast such as *Candida* sp. and *S. cerevisiae* and bacteria such as *Bacillus* spp. produce tannase (tannin-degrading enzyme), which enables them to use the breakdown product of tannin (gallic acid and glucose) as nutritional substrates (Mondal *et al*., 2001). The antimicrobial activity of PGB against excreta microbiology was more pronounced during the starter period (by d 21) than in the finisher period (by d 35), when increasing dietary levels of PGB decreased the number of *E. coli* and *Salmonella*. This may have occurred because the effects of antimicrobials are more pronounced in young growing animals (Wenk, 2000). The observation of higher concentrations of *Bacillus* spp. and lower *E. coli* in the excreta by d 35 can be explained as a reflection of cecal microbiology.

According to Ferket *et al*. (2002), fecal noxious gas emissions of non-ruminants are related to nutrient utilization and intestinal microbial ecosystems. Therefore, improvement of nutrient utilization and manipulation of gut microbiota with feed additives can be important tools for reducing odorous gas from broiler excreta. In poultry, most non-utilized feed nitrogen is excreted as uric acid with urine, which is readily converted into NH_3 by microbial urease enzyme present in feces. Many viable intestinal organisms such as *Bacteroides*, *Bifidobacteria*, *Clostridia*, *Proteus* spp., and *Klebsiella* spp. possess urease activity (O'Grady, 1966). *E. coli* itself has no demonstrable urease activity; therefore, it releases ammonia via deamination of protein and other nitrogenous substances (Vince *et al*., 1973). The antimicrobial effects of tannin rich plant extract against NH_3 producing *E. coli*, *Klebsiella* spp. and *Pseudomonas* spp. was reported by Ferreira *et al*. (2010). Powell *et al*. (2011) reported that feeding tannin extract can reduce urine N excretion and urease activity in dairy feces. In this study, the effectiveness of dietary PGB at reducing emissions of NH_3 from broiler excreta can be attributed to a reduction in the concentration of both intestinal and excreta ureolytic bacteria including *E. coli*, together with a reduction in fecal urease activity due to the presence of high concentrations of tannins in PGB (hydrolysable tannins, 14.26 mg/g; Table 1).

Hydrogen sulfide and methanethiol (methylmercaptan) represent 70 to 97% of the total volatile S in animal manure, and the amount of methanethiol exceeds the amount of H_2S produced for pigs and poultry (Banwart and Brenmer, 1975). Bacterial sulfate reduction and/or decomposition of sulfur-containing organic compounds are mainly responsible for the production of these compounds. Several scientists have found sulfate-reducing pathways in E. coli (Fujimoto and Ishimoto, 1961; Tsang and Schiff, 1976) and Salmonella (Dreyfuss, 1964). Tannins have been shown to be effective at reducing the emissions of H_2S from animal waste and reducing the population or metabolic activity of anaerobic sulfate reducing bacteria (Whitehead *et al*., 2012). The lower emission of excreta H_2S and methanethiol observed in the present study can be explained by the presence of con-

siderable amount of tannin in PGB (hydrolysable tannins, 14.26 mg/g; Table 1), which may reduce the population of sulfate reducing bacteria including *E. coli* and *Salmonella*.

Based on the results of the present study, dietary supplementation with PGB was effective at improving the immune response and intestinal microbial ecology of broilers by reducing the pathogenic bacteria. These facts may have an important influence on the improved feed conversion in broilers. Moreover, dietary PGB also significantly reduced the emissions of NH_3, H_2S and methanethiol from broiler excreta. Based on these findings, *Punica granatum* L. by-products can be used as natural feed additives in broiler diet to improve immunity, microbial safety and housing environment without affecting weight gain.

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