Effect of treating recycled poultry bedding with tannin extracted from pomegranate peel on rumen fermentation parameters and cellulolytic bacterial population in Arabian fattening lambs

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Abstract

This study was conducted to investigate the effects of recycled poultry bedding (RPB) treated with different levels of pomegranate peel extract (PPE) as a tannin source on cellulolytic bacterial population and rumen fermentation parameters of fattening lambs. For this purpose, twenty-eight Arabian lambs (19.70 ± 2.45 kg body weight, 90 ± 12 days of age) were randomly assigned to four dietary treatments. Recycled poultry bedding was treated with PPE at four levels of 0 (control), 20.00, 25.00 and 30.00% on DM basis. Bacterial populations were enumerated by DNA extraction of samples of rumen liquor followed by real-time polymerase chain reaction analysis. Also, rumen samples were evaluated for pH, volatile fatty acid (VFA) and ammonia nitrogen (AN) concentrations. The populations of total bacteria, Ruminococcus albus and Fibrobacter succinogenes were decreased significantly as the level of PPE in the diet increased, however, the population of Ruminococcus flavefaciens was not affected. Dietary treatments did not have effect on ruminal pH, while AN concentration was decreased in the diets containing RPB treated with PPE compared to the control. Concentrations of total VFA and individual VFA remained unchanged by PPE-treated RPB inclusion in the diet. In conclusion, supplementing RPB with PPE improved nitrogen metabolism of fattening lambs, however, it decreased population of rumen cellulolytic bacteria R. flavefaciens.

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Key words: Cellulolytic bacteria, Pomegranate peel, Real-time PCR, Recycled poultry bedding

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Introduction

Recycled poultry bedding (RPB) is a solid waste from the floor of poultry houses, particularly broiler houses containing the original bedding material, spilled feed, feathers, and excreta. In several countries RPB is used as an inexpensive protein source in ruminant nutrition. The commercial value of RPB as a ruminant feed is based on its crude protein (CP) content including 150-350 g kg⁻¹ dry matter (DM), DM digestibility (650-680 g kg⁻¹) and minerals. This indicated that it has a potential value as ruminant feed. However, due to the presence of pathogenic bacteria it must be processed before feeding the ruminant animals. Most of the N content of RPB is in the form of non-protein N (NPN) which can be rapidly degraded in the rumen by microbes. Up to 96.00% of uric acid, the main component of NPN in RPB, is degraded in the rumen.

Protection of protein from ruminal microbial degradation enhances amino acids flow to the duodenum, thus, the efficiency of microbial protein production and performance of the animal would be improved. Condensed tannin can complex with proteins at common rumen pH of 5.50 to 7.00 thereby slowing down microbial degradation of proteins. The tannin–protein complexes are dissociated in the acidic pH of the abomasum (i.e., pH 2.50 to 3.50) and in the conditions of the distal small intestine (i.e., pH 7.50) release protein for digestion and absorption. It is widely assumed that tannins precipitate only proteins/peptides. However, recently, it was indicated that tannins can react with a wide set of different organic nitrogenous compounds.

Rumen is a complex ecosystem and their microbes are involved in degradation of feed particles. In the rumen, mixed bacterial and fungi population are contributing approximately 80.00% and protozoa 20.00% of plant cell walls degradation. The fibrolytic bacteria *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* are generally considered as the primary organisms responsible for ruminal plant cell walls degradation. Performance of ruminants depends on activity of their microbes in digestion of feed particles. Many studies indicated that tannins selectively inhibit the growth of microorganisms in the gastrointestinal tracts depending on the type of tannins. At high concentration level (i.e., more than 50.00 g kg⁻¹ dietary DM), tannins have significant inhibitory effects on the fibrolytic bacterial in the rumen depended on the type of tannins, thus, it may decrease fiber digestibility. It has been suggested that phenolic monomers decrease cellulose and xylan digestion by inhibiting the attachment of ruminal cellulosytic bacteria such as *F. succinogenes* to fiber particles. Reduction of cellulosytic bacteria population by dietary supplementation with tannins has been shown in vivo and in vitro. Recently the dynamics of cellulosytic bacterial populations (i.e., *F. succinogenes*, *R. albus* and *R. flavefaciens*) in response to dietary changes have been studied using targeting 16S rRNA gene by real-time PCR in ruminants. Thus, the objective of this study was to investigate the effect of RPB treated with tannins extracted from PP (PPE) on changing total and cellulosytic bacteria population by real-time PCR technique and to determine the effect of this process on the rumen fermentation parameters on male Arabian lambs.

Materials and Methods

Preparation of RPB and pomegranate peel extract and treating RPB. Recycled poultry bedding was prepared in a factory in Sabzevar (Razavi Khorasan province, Iran). In order to achieve optimal processing and prevent burning materials, RPB was humidified up to 23.00%, and then the material was processed under an indirect thermal operation in a special hot tank (with a capacity of five ton) for 20 min. The tank was comprised of two walls between which a hot steam (80 °C) was flowed. Finally, the produced heat processed broiler litter was ground to pass a 6 mm sieve.

Pomegranate peels was obtained from Baghmalek (Khuzestan province, Iran). A two-step extraction process was performed. In the first extraction step, sun dried PP was ground through a 0.50 mm screen and soaked in water at a ratio of 1:10 (w/v) for 24 hr. In the second step, the aqueous PP was filtered and boiled (at 95 °C) to achieve pomegranate peel extract (PPE). Recycled poultry bedding was treated with PPE at the levels of 0.00, 20.00, 25.00 and 30.00% of DM based on our previous in vitro screening experiment. Tannin extract (87.00% of DM) was diluted in aqueous solution (200.00 g L⁻¹) in distilled water. The solution was added to RPB to obtain levels of 20.00, 25.00 and 30.00% of PPE. The product was then dried at 45 °C for 48 hr to reach a constant weight. In all cases, the control treatment was prepared as described
above, however, using only distilled water instead of tannin solution. The chemical composition of RPB, PPE and RPB treated with different levels of PPE is shown in Table 1. The measurement of the total phenolics and total tannins of PPE was conducted as described in IAEA manual.22 Total phenol was determined using Folin–Ciocalteu’s reagents, and the concentration was measured as tannic acid equivalent using standard tannic acid (Merck, Darmstadt, Germany). Total tannins were measured as described in IAEA manual.22

Animals and experimental diets. The experiment was approved by the Animal Care and Ethics Committee of Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Iran.

Twenty-eight male fat-tailed Arabian lambs (19.70 ± 2.45 kg body weight) of 90 ± 12 days of age were randomly assigned into the four dietary groups using completely randomized design (7 lambs per dietary treatment). Animals were individually housed in concreted floor pens (1.30 × 1.20 m) in a close shed building. The feeding trial lasted 64 days preceded by 14-day adaptation period to the individual pens, diets and the experimental conditions (total 78 days). Within the adaptation period, the amount of RPB treated with different levels of PPE was gradually elevated in diet offered to each animal to reach the levels considered as the experimental levels in dietary treatments. At the start of the adaptation period, all the animals were treated for external (1.00 mL of Azantole 10.00% per 7.00 L of water, as spraying method; Bayer, Leverkusen, Germany) and internal (Triclabendazole + levamisole 7.00 L of water, as spraying method; Bayer, Leverkusen, Germany) and vaccinated against enterotoxaemia (3.00 12.00 mL per lamb; Darou-Pakhsh Co., Tehran, Iran) parasites and vaccinated against enterotoxaemia (3.00 12.00 mL per lamb; Darou-Pakhsh Co., Tehran, Iran). At the end of the adaptation period, all the animals were treated for external (1.00 mL of Azantole 10.00% per 7.00 L of water, as spraying method; Bayer, Leverkusen, Germany) and internal (Triclabendazole + levamisole 7.00 L of water, as spraying method; Bayer, Leverkusen, Germany) and vaccinated against enterotoxaemia (3.00 12.00 mL per lamb; Darou-Pakhsh Co., Tehran, Iran) parasites and vaccinated against enterotoxaemia (3.00 12.00 mL per lamb; Darou-Pakhsh Co., Tehran, Iran)

Table 1. Chemical composition of recycled poultry bedding (RPB) and pomegranate peel extract (PPE; g kg⁻¹ DM or as stated), (n = 4, composite samples for the four 16-day periods).

| Parameters                              | PPE          | 0  | 20 | 25 | 30 |
|----------------------------------------|--------------|----|----|----|----|
| Dry matter (g kg⁻¹ fresh weight)       | 875.00       | 900.00 | 885.00 | 884.00 | 879.00 |
| Crude protein                          | 203.00       | 224.00 | 209.00 | 207.00 | 202.00 |
| Neutral detergent fiber                | 75.00        | 350.00 | 291.00 | 289.00 | 288.00 |
| Acid detergent fiber                   | 57.50        | 195.00 | 175.00 | 173.00 | 170.00 |
| Ash                                    | 253.00       | 147.00 | 164.00 | 167.00 | 169.00 |
| Total phenolic                         | 151.00       | -    | 30.20 | 37.80 | 45.50 |
| Total tannins                          | 111.00       | -    | 22.20 | 27.80 | 33.50 |

*RPB treated with PPE at the levels of 0 (control), 20.00, 25.00 and 30.00% of DM.

Table 2. Feed ingredients (g) and chemical composition (g kg⁻¹ DM or as stated) of the experimental diets containing recycled poultry bedding (RPB) treated with different levels of pomegranate peel extract (PPE).

| Ingredients (g)                      | PPE supplement (%) |
|-------------------------------------|--------------------|
| Alfalfa hay                         | 150.00             |
| Wheat straw                         | 50.00              |
| Corn silage                         | 50.00              |
| Treated RPB                         | 180.00             |
| Soybean meal                        | 40.00              |
| Corn grain, ground                  | 169.00             |
| Barley, ground                      | 250.00             |
| Wheat bran                          | 100.00             |
| Urea                                | 2.00               |
| Salt                                | 3.00               |
| Magnesium oxide                     | 1.00               |
| Sodium bicarbonate                  | 2.00               |
| Minerals and vitamins               | 3.00               |

* MCal kg⁻¹ DM, calculated according to NRC (2007).23

Rumen fluid sampling. Rumen liquor (RL) samples (40-50 mL) were taken by stomach tube from all experimental lambs on day 54 of the trial 3 hr after the morning feeding. The first 10.00 to 20.00 mL of RL collected form each lamb was discarded to avoid saliva contamination.24 The pH was measured immediately and samples were strained through two layers of muslin. For determination of rumen ammonia concentration, 5.00 mL or the sample was collected into 1.00 mL of 0.20 N HCl, transported to the laboratory and frozen at –20 °C. For VFA analysis, 1.00 mL acid orthophosphoric acid (200 mL L⁻¹) containing 20 mM 2-ethyl-butyric acid was added to 4.00 mL of RL (1:4 ratio) and stored at –20 °C. Another sub-sample of RL was stored at –20 °C for DNA extraction.
Laboratory analysis. The RPB, PPE and experimental diets were oven-dried at 55 °C and ground to pass a 1 mM sieve (Wiley mill; Thomas Scientific, Swedesboro, USA). The DM, ash and nitrogen (N) were analyzed following AOAC procedure numbers of 930.15, 924.05, 984.13 and 954.02, respectively. The neutral detergent fiber (NDF) was determined without sodium sulphite and amylase treatment, and expressed as inclusive of residual ash. The determination of acid detergent fiber (ADF) was performed and expressed as inclusive of residual ash. Lignin was determined by solubilization of cellulose with 720 g kg⁻¹ sulphuric acid, according to the procedure described by Robertson and Van Soest. Lignin was determined by Shimadzu GC-14 B gas chromatography (GC) machine (Shimadzu, Tokyo, Japan) equipped with a Carboxen™1000, 45/60, 2.00 m × 1/80 column (Supelco, St. Louis, USA) and a flame ionization detector. An internal standard (2-ethyl-n-butyric acid) was used to help quantify VFA concentrations. DNA extraction and real-time polymerase chain reaction. After thawing, RL samples were shaken and transferred to 1.50 mL micro tubes containing glass beads and vortexed twice for 5 min with incubation on ice between shakings. This work allowed disruption of bacterial cell wall and for separation of bacteria from feed particles. Tubes were centrifuged at 200 g for 5 min at 4 °C for the sedimentation of feeds particles. The supernatants (200 μL) were transferred to fresh 1.50 mL micro tubes and DNA extraction was performed using a genomic DNA purification with NucleoSpin blood kit (containing 10.00 mL buffer B3, 6.00 mL wash buffer BW, 6.00 mL wash buffer B5, 13.00 mL elution buffer, 6.00 mg proteinase K, 1.80 mL proteinase buffer and 10.00 mL NucleoSpin Blood) equipped with spin columns. Total bacterial, F. succinogenes, R. flavefaciens and R. albus, rDNA concentrations were measured using real time PCR and the SYBR Green PCR Master Mix Kit (SYBR Green I qPCR Master Mix, Syntol, Russia) according to Valizadeh et al. The 16S rRNA gene-targeted primer sets used in the present study are described in Table 3.

| Target species | Primer sequence | Annealing temperature (°C) | PCR product size (bp) |
|---------------|-----------------|---------------------------|----------------------|
| Total bacteria | F: GTGCTGCAAGGGTTGTCGTCA | 61 | 120 |
|              | R: ACAGTCRTCCMCACTTCCCT | 61 | 175 |
| F. succinogenes | F: GTCCTGCAAGGGTTGTCGTCA | 61 | 155 |
|              | R: CGCTCTGCAAGGGTTGTCGTCA | 61 | 122 |
| R. flavefaciens | F: CCCTAAAAACAGCCAGCCGTCCGAT | 61 | 100 |
|              | R: CCTCCTGCCGTTAGAACA | 61 | 175 |
| R. albus      | F: CGCTCTGCAAGGGTTGTCGTCA | 61 | 120 |
|              | R: CCTCCTGCCGTTAGAACA | 61 | 175 |

Relative fold change in genomic DNA = 2^{ΔCt}
where, $\Delta Ct = Ct_{treated} - Ct_{untreated}$, and $Ct$ is the cycle number at which the fluorescence generated within a reaction crosses the threshold. To achieve optimal relative expression results, all the relative comparisons were made on a constant basis of extracted DNA. Change in cellulolytic species was reported as fold change in genomic DNA per $\mu$L of extracted DNA compared to control. All post-run data analyses were performed using sequence detector software (SDS; version 1.4; Applied Biosystems).

**Statistical analysis.** Data was subjected to ANOVA using the MIXED procedure of SAS (version 9.2; SAS Inst., Cary, USA). The model included the fixed effect treatment and the random effect of animal within treatment. Autoregressive covariance structure was the best fit for the data as determined by the lowest Akaike’s information criterion. The model used was:

$$Y_{ijk} = \mu + T_i + A_j + e_{ijk}$$

where, $Y_{ijk}$ is observation parameters, $\mu$ is the general mean, $T_i$ is the fixed effect of treatment on the assessed parameters (i.e., bacterial population and ruminal parameters), $A_j$ is the random effect of animal within the treatment and $e_{ijk}$ is the random error associated with the observation $ijk$.

For all statistical analyses, significance was declared at $p \leq 0.05$, unless otherwise stated. The Fisher’s protected least significant difference (LSD) test was used for multiple treatment comparisons using the LSMEANS of SAS. The residual analysis was carried out to test the model assumptions using the UNIVARIATE procedure of SAS with NORMAL and PLOT options.

**Results**

Processing RPB with increasing PPE levels enhanced crude ash, total phenolic and total tannin content of RPB, while it decreased DM, CP, NDF and ADF (Table 1).

**Population of total and cellulolytic bacteria in the rumen.** Data generated from real-time PCR assays for total bacteria are expressed as ng per mL of extracted DNA, while quantity of cellulolytic bacteria (R. albus, F. succinogenes, R. flavefaciens and R. albus) are expressed as fold changes in genomic DNA compared to the control. As shown in Table 4, populations of total bacteria, R. albus and F. succinogenes were significantly decreased ($p < 0.05$) and the level of PPE increased in the diet, while the population of R. flavefaciens was not influenced ($p > 0.05$) by the experimental diets.

**Ruminal parameters.** Dietary treatments did not have effect ($p > 0.05$) on ruminal pH, while AN concentration was decreased ($p < 0.05$) in the diets containing RPB treated with PPE compared to the control treatment (Table 5). Treatment of RPB with PPE did not have effect ($p > 0.05$) on total VFA concentration and individual VFAs compared to those of the control group.

**Discussion**

Reduction of total bacteria, R. albus and F. succinogenes in the diets containing RPB treated with PPE might be due to binding tannins of PPE to substrate cell wall resulting in a reduction in the availability of binding sites on cell wall for rumen microbes. Moreover, previous report suggested that tannins may form strong complexes with substrates and reduce adhesion of microbes. Tannins also are known for their antimicrobial activity either on cellulolytic or proteolytic bacteria, as reduction of microbial attachment caused by tannin is supported by the reduction ruminal microbial population. Bae et al. indicated that addition of tannin in pure cultures in vitro resulted in the formation of tannin–protein complexes on the cell surface of F. succinogenes, suggesting interference of tannin with the adhesion process. Therefore, based on Molan et al., it is likely that the reduction in microbial attachment is related to binding of tannin to bacterial cell surface. Similar to results obtained in the present study, tannins have reduced cellulolytic bacteria population in vivo and in vitro by inhibiting microbial growth. Bento et al. observed reduction of microbial attachment when supplementing cellulose with mimosa tannin compared to cellulose alone. Filter paper digestion and endoglucanase activity of F. succinogenes, a predominant ruminal cellulolytic bacterial species, also were inhibited in a dose dependent manner by purified condensed tannins (CT). In the present study, both extracellular and cell-associated endoglucanase activities were completely inhibited by 400 µg CT per mL. Filter paper digestion by this bacterium also was decreased with increase in concentration of CT. Further work showed that CT caused detachment of bacteria from cellulose fibres, and it was concluded that CT reduce fiber digestion by inhibiting fibrilolytic enzymes and preventing bacterial attachment.

**Table 4.** Effects of recycled poultry bedding (RPB) treated with different levels of pomegranate peel extract (PPE) on total and some cellulolytic bacteria population in the rumen of male-Arabian lambs, *(indicates Fold change compared to control)*.

| Bacteria                      | PPE supplement (%) | SEM | $p$ value |
|-------------------------------|--------------------|-----|-----------|
|                               | 0                  | 20  | 25        | 30        |       |
| Total bacteria (ng µL$^{-1}$) | 514.00$^a$         | 472.00$^a$ | 392.00$^b$ | 365.00$^c$ | 26.10  | 0.01  |
| *F. succinogenes*             | 1.03$^a$           | 0.72$^a$ | 0.81$^a$  | 0.45$^c$  | 0.09   | 0.01  |
| *R. flavefaciens*            | 1.20               | 1.07  | 0.87      | 0.82      | 0.11   | 0.12  |
| *R. albus*                   | 1.05$^a$           | 0.59$^b$ | 0.49$^c$  | 0.18$^c$  | 0.06   | < 0.01|

$^a$, $^b$, $^c$ Means with different letters are significantly different within rows ($p < 0.05$).
Min et al. demonstrated that the major fiber degrading bacteria in the rumen such as F. succinogenes, R. albus have been found to be inhibited by tannins although degree of inhibition varied among the studies depending upon the dose and type of tannins.46 Such interactions are likely to have a substantial effect on the ability of ingested tannins to influence attachment of microflora to substrates as well as their effect on enzyme activities.

In the present study, population of R. flavefaciens remained unchanged by level of PPE in the diet. The different bacterial susceptibility to tannins probably has resulted from the different mechanisms of attachment to substrate.36 It has been reported that R. flavefaciens adheres intimately to cellulose fibers, where they tend to produce "pits" because of substrate digestion and progressively deeper pits in the cellulose are formed.41 These pits hold R. flavefaciens close to the substrate, where they continue digestion of cellulose via their cellulases. Singh et al. showed that supplementing diets of goats with Ficus infectiria leaves at 50% of DM did not change the number of F. Succinogenes, while number of R. flavefaciens was reduced.42 Similarly, Ghasemi et al. studied the effects of using pistachio hulls (contain tannin) as a replacement for alfalfa hay in the diet of Baluchi sheep on their rumen cellulolytic bacterial population. In their work, populations of total bacteria, F. succinogenes and R. albus were significantly decreased as the dietary level of pistachio hulls elevated, while R. flavefaciens counts remained unchanged in the rumen liquor.17

In all experimental lambs, the mean ruminal pH values were within the normal physiological range of 6.10 - 6.80 required for optimum microbial growth as reported by Van Soest.43 Similarly, Jolazadeh et al. and Yildiz et al. observed unchanged rumen pH with supplementation of ruminant diets with different kind of tannins.5, 44

Concentrations of ruminal AN were reduced by treatment of RBP with PPE. The effect of tannins on ruminal protein metabolism has been attributed to their ability to bind plant protein, to reduce the activity of microbial enzymes, and to reduce the growth rate of bacteria,36 and finally to decrease ruminal ammonia.45 The reduction in cellulolytic bacteria population supports these findings (Table 4). Lower ruminal AN concentration with increasing PPE level in the diet may have resulted from a greater concentration of tannins that bound to proteins and decreased proteolysis of feed protein and subsequently lowered the concentration of ruminal NH3–N.46 As reported by Ghasemi et al., some part of decrease in bacterial population might be related to this fact that tannins inhibit rumen microbial function by reducing the availability of ruminal AN for microbial growth.17 Similarly, Jolazadeh et al. indicated that supplementing soybean meal with different levels of tannins extracted from pistachio hulls in the diets of Holstein bulls decreased their ruminal AN concentration and subsequently improved growth performance and average daily gain (ADG)5 Results of our in vivo study also indicated that supplementation of RBP with 25.00% PPE in the finishing diets of Arabian lambs improved growth performance and N metabolism without affecting feed intake and gain efficiency.

The concentrations of total and individual VFA in the rumen of lambs was unaffected by feeding experimental diets. Researchers have shown various patterns of ruminal VFA depend on the level of dietary tannins used. Consistent with our results, Krueger et al. reported no effect on VFA in steers fed with high-grain diet when using mimosa and chestnut extracts as sources of tannins.46 Theodoridou et al. also found that VFA production was not affected by tannins.47 Yildiz et al. reported no difference in VFA concentration in lambs receiving oak leaves.44 In contrast to our finding, Tan et al. using various rates of condensed tannins of Leucaena leucocephala reported that total VFA concentration was decreased with increase in level of tannin.48 Different results obtained in the present study compared to others could be related to difference in type of tannins, concentrations and dietary ingredient.8

The results of the current study indicated that among three examined ruminal cellulolytic bacteria, F. succinogenes and R. albus were most sensitive to tannin content of PPE, and their population as well as total bacteria counts were decreased in the rumen of Arabian fattening lambs by treatment of dietary RPB with 25.00 or

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Table 5. Effects of recycled poultry bedding (RPB) treated with different levels of pomegranate peel extract (PPE) on total and some cellulolytic bacteria population in the rumen of male-Arabian lambs, (* indicates Fold change compared to control).

| Parameters                          | PPE supplement (%) | SEM  | p value |
|-------------------------------------|--------------------|------|---------|
|                                     | 0                  | 20   | 25      | 30      |
| pH                                  | 6.22               | 6.27 | 6.33    | 6.40    | 0.14  | 0.61  |
| Ammonia-nitrogen (mg dl-1)          | 17.60              | 16.10| 15.30   | 14.40   | 0.58  | 0.02  |
| Total VFA (mmol L-1)                | 99.20              | 97.70| 97.20   | 96.10   | 1.90  | 0.71  |
| Individual VFA (mmol L-1)           |                    |      |         |         |       |       |
| Acetate                             | 58.80              | 58.40| 57.50   | 56.30   | 1.36  | 0.50  |
| Propionate                          | 23.70              | 23.50| 23.40   | 22.70   | 1.15  | 0.66  |
| Butyrate                            | 11.80              | 11.40| 12.40   | 12.80   | 1.64  | 0.76  |
| Isovalerate                         | 2.51               | 2.16 | 2.09    | 2.32    | 0.43  | 0.45  |
| Valerate                            | 2.41               | 2.21 | 2.23    | 2.05    | 0.39  | 0.34  |
| Acetate: Propionate                 | 2.49               | 2.49 | 2.48    | 2.48    | 0.14  | 0.65  |

* Means with different letters are significantly different within rows (p < 0.05).
30.00% PPE compared to the control RPB. However, dietary treatments did not have effect on \( R. \ flavaefaciens \) population, ruminal pH, total VFA and individual VFA, while AN concentration in the diets containing RPB treated with PPE at levels 25.00 or 30.00% was decreased compared to that of the control treatment. More works especially on \textit{in vivo} animals are required to investigate change in other rumen microbes such as protozoa and proteolytic bacteria as well as other cellulosytic bacteria to evaluate the mechanism of tannin action in the rumen and interaction of these microorganisms together.

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**Conflict of interest**

The authors declare there is no conflicts of interest.

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