ORIGINAL ARTICLE

Veterinary Research Forum. 2019; 10 (2) 145 - 152
doi: 10.30466/vrf.2019.75050.2007
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

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

Afrooz Sharifi[1], Morteza Chaji[1]*, Alireza Vakili[2]

[1] Department of Animal Science, Faculty of Animal Science and Food Technology, Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Ahvaz, Iran; [2] Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.

Article Info

Article history:

Received: 11 November 2017
Accepted: 31 July 2018
Available online: 15 June 2019

Key words:

Cellulolytic bacteria
Pomegranate peel
Real-time PCR
Recycled poultry bedding

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*.

اثر کود مرغی عمل‌آوری شده با سطوح مختلف تانن مستخرج از پوست انار بر فراسنجه‌های تخمیر و جمعیت باکتری‌های سلولولایتیک شکمبه بره‌های عربی

چکیده

هدف از مطالعه حاضر بررسی اثر کود مرغی عمل‌آوری شده با سطوح مختلف تانن استخراج شده از پوست انار بر تغییر جمعیت کل باکتری‌ها، باکتری‌های سلولولایتیک و فراسنجه‌های تخمیر شکمبه‌ای در بره‌های پرواری عربی بود. آزمایش با استفاده از ۲۸ رأس بره نر نژاد عربی ۱۲ ± ۹۰ روزه با میانگین وزن زنده ۲/۴۵ ± ۱۹/۷۰ کیلوگرم در قالب طرح کاملاً تصادفی متعادل با چهار جیره (تیمار) و هفت بره (تکرار) در هر تیمار انجام شد. جیره‌های آزمایشی شامل کود مرغی عمل‌آوری نشده (شاهد)، و عمل‌آوری آن با سطوح ۲۰/۰۰، ۲۵/۰۰ و ۳۰/۰۰ درصد عصاره تانني استخراج شده از پوست انار بود. جمعیت باکتری‌ها توسط استخراج DNA از نمونه‌های مایع شکمبه با استفاده از تکنیک واکنش زنجیره ای پلیمراز در زمان واقعی صورت گرفت. فراسنجه‌های pH، غلظت اسیدهای چرب فرار و نیتروژن آمونیاکی نمونه‌های مایع شکمبه تعیین گردید. با افزایش سطح عصاره در جیره، جمعیت کل باکتری‌ها و باکتری‌های *رومینوکوکوس آلبوس* و *فیبروباکترسوکسینوژنز* کاهش یافت، اما تأثیری بر جمعیت باکتری *رومینوکوکوس فلاووفاسینز* نداشت. میزان pH شکمبه تحت تأثیر جیره‌های آزمایشی قرار نگرفت، اما با افزایش سطوح عصاره در جیره، غلظت آمونیاک شکمبه به طور معنی‌داری کاهش یافت. جیره‌های آزمایشی تأثیری بر غلظت اسیدهای چرب فرار شکمبه نداشتند. در کل، عمل‌آوری کود مرغی با تانن پوست انار سبب بهبود متابولیسم نیتروژن در بره‌های پرواری عربی گردید، هرچند، جمعیت باکتری‌های سلولولایتیک رومینوکوکوس فلاوسیانس شکمبه کاهش یافت.

واژه های کلیدی: باکتری‌های سلولولایتیک، عصاره پوست انار، کود مرغی، واکنش زنجیره ای پلیمراز در زمان واقعی

***Correspondence:**

Morteza Chaji. PhD
Department of Animal Science, Faculty of Animal Science and Food Technology, Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Ahvaz, Iran
E-mail: chaji@asnrukh.ac.ir

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.[1] 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.[2,3] 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.[2] 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[3]. Up to 96.00% of uric acid, the main component of NPN in RPB, is degraded in the rumen.[4]

Protection of protein from ruminal microbial degradation enhances amino acids flow to the duodenum,[5] thus, the efficiency of microbial protein production and performance of the animal would be improved. Pomegranate peel (PP) is a by-product of extracting the juice from pomegranates, with annual production of more than 120,000 tons in Iran.[6] The PP also have some anti-microbial factors such as saponin, polyphenolic compounds, primarily punicalagin and ellaggi tannins.[7] Tannins are phenolic secondary compounds of plants that are usually classified as hydrolysable and condensed.[8] Hydrolysable tannins contain a carbohydrate core, often glucose, esterified with gallic and/or ellagic acid. Condensed tannins (CT) are oligomers or polymers of flavonoid units linked by carbon–carbon bonds.[9] Condensed tannin can complex with proteins[10] through the multiple phenolic hydroxyl groups of CT[8] and have both adverse and beneficial nutritional effects on herbivores depending on their chemical structure and dietary concentration.[11] The beneficial effects of CT are associated with their capacity to increase digestive utilization of dietary protein for ruminants,[8] largely by binding 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.[12] 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.[13]

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.[14] The fibrolytic bacteria *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* are generally considered as the primary organisms responsible for ruminal plant cell walls degradation.[14] 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.[5,6,15] 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.[10] It has been suggested that phenolic monomers decrease cellulose and xylan digestion by inhibiting the attachment of ruminal cellulolytic bacteria such as *F. succinogenesto* to fiber particles.[16] Reduction of cellulolytic bacteria population by dietary supplementation with tannins has been shown *in vivo* and *in vitro*. [17,18] Recently the dynamics of cellulolytic 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.[17,19] Thus, the objective of this study was to investigate the effect of RPB treated with tannins extracted from PP (PPE) on changing total and cellulolytic 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.[20] 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.[21] 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 12.00 mL per lamb; Darou-Pakhsh Co., Tehran, Iran) parasites and vaccinated against enterotoxaemia (3.00 mL per lamb; Razi Vaccine and Serum Research Institute, Karaj, Iran).

Four iso-caloric and iso-nitrogenous diets (Table 2) containing RPB (18.00% of DM) treated with different levels of PPE (0.00, 20.00, 25.00 or 30.00% of DM) were formulated to meet the nutrient requirements of growing lambs.[23] Diets as total mixed ration (TMR) were offered to the lambs two times a day at 8:00 and 17:00 hr *ad libitum*. Animals had free access to fresh water.

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).

Parameters	PPE supplement (%)			
	0	20	25	30
Ingredients (g)				
Alfalfa hay	150.00	150.00	150.00	150.00
Wheat straw	50.00	50.00	50.00	50.00
Corn silage	50.00	50.00	50.00	50.00
Treated RPB	180.00	180.00	180.00	180.00
Soybean meal	40.00	40.00	40.00	40.00
Corn grain, ground	169.00	169.00	169.00	169.00
Barley, ground	250.00	250.00	250.00	250.00
Wheat bran	100.00	100.00	100.00	100.00
Urea	2.00	2.00	2.00	2.00
Salt	3.00	3.00	3.00	3.00
Magnesium oxide	1.00	1.00	1.00	1.00
Sodium bicarbonate	2.00	2.00	2.00	2.00
Minerals and vitamins	3.00	3.00	3.00	3.00
Chemical composition (g kg^{-1} DM)				
Dry matter	878.00	877.00	875.00	872.00
Organic matter	813.00	812.00	810.00	808.00
Crude protein	157.00	153.00	151.00	149.00
Neutral detergent fiber	304.00	300.00	293.00	290.00
Acid detergent fiber	167.00	164.00	162.00	160.00
Lignin (surface area)	29.60	27.00	26.90	26.70
Metabolizable energy*	2.51	2.49	2.48	2.47

* 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.

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	Level of treated RPB*			
		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.
Non-protein nitrogen (NPN) content of RPB was 402 g kg^{-1} total nitrogen.

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[25] 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.[26] 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.[27] After thawing, RL was analyzed for ammonia-nitrogen (AN) using the phenol-hypochlorite method according to Broderick and Kang.[28] Ruminal VFA concentrations were determined by gas chromatography.[29] For this purpose, the VFAs were 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*.[30] The 16S rRNA gene-targeted primer sets used in the present study are described in Table 3.

Templates (3.00 µL; 28 sample in two replicates) were added to amplification reactions (15.00 µL) containing 0.50 µL of primer mixture containing 10.00 pM of each primer, 7.50 µL of SYBR Green I qPCR Master Mix (K0221; Syntol, Moscow, Russia) and 4.00 µL of deionized water. Master Mix contained KCl, Tris-HCL (pH 8.80), 6.25 mM $MgCl_2$, dNTP, Taq DNA polymerase, Tween, and SYBR Green I. A non-template (sterile distilled water) negative control was loaded on each plate run to screen for contamination and dimmer formation and to set the background fluorescence for plate normalization. Amplification and detection were performed using an ABI 7300 (Applied Biosystems; Massachusetts, USA) sequence detection system under the following conditions: initial denaturation at 95 ˚C for 5 min was followed by 45 cycles of denaturation at 95 ˚C for 30 sec, annealing at 61 ˚C for 30 sec, extension at 72 ˚C for 30 sec, and then by the melting curve program (60-95 ˚C with a heating rate of 0.10 ˚C per sec and a continuous fluorescence measurement). At the end of each experiment, melting curve was analyzed for non-specific PCR products in each sample.

Simultaneously, DNA extracted from RL of each animal was subjected to real-time qPCR for all of the bacteria (total and three cellulolytics). A bacterial rDNA standard curve was generated from DNA extracted from a mix (equal volumes) of 24 cultures of the following rumen bacterial strains grown on Hobson's medium 2:[31] *Prevotella ruminicula* 23, *Butyrivibrio fibrisolvns* SH13, *Ruminococcus albus* SY23, *Prevotella albensis* M384, *Clostridium sticklandii* 12 662, *Peptostreptococcus anaerobius* 27 337, *Ruminococcus falvefaciens* Fd1, *Mitsuokella multiacidus* D15d, *Veillonela parvula* L59, *Prevotella bryantii* B14, *Prevotella brevis* GA33, *Lactobacillus casei* LB17, *Clostridium aminophilum* 49 906, *Streptococcus bovis* ES1 and *Megasphera elsdenii* J1 were obtained from the Rowett Research Institute (Aberdeen, UK) culture collection.

For total bacteria, the threshold cycle of each standard dilution was determined during the exponential phase of amplification and regressed against the logarithm of known total bacterial DNA standards that had been prepared for each animal. Total bacterial population size is reported as nano gram (ng) per µL of extracted DNA.

Shift in the population of cellulolytic bacteria species of lambs fed with experimental diets was compared to the animals fed with control diet in the same period using the methods of relative quantification as:

$$\text{Relative fold change in genomic DNA} = 2^{-\Delta Ct}$$

Table 3. The PCR primers used for amplifying the target bacteria.

Target species	Primer sequence	Annealing temperature (˚C)	PCR product size (bp)
Total bacteria	F: GTGSTGCAYGGYTGTCGTCA	61	120
	R: ACGTCRTCCMCACCTTCCTC	61	
F. succinogenes	F: GTTCGGAATTACTGGGCGTAAA	61	175
	R: CGCCTGCCCCTGAACTATC	61	
R. flavefaciens	F: CGAACGGAGATAATTTGAGTTTACTTAGG	61	155
	R: CGGTCTCTGTATGTTATGAGGTATTA	61	
R. albus	F: CCCTAAAAGCAGTCTTAGTTCG	61	122
	R: CCTCCTTGCGGTTAGAACA	61	

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 µL 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, μ 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 (*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.[32] Moreover, previous report suggested that tannins may form strong complexes with substrates and reduce adhesion of microbes.[33] Tannins also are known for their antimicrobial activity either on cellulolytic or proteolytic bacteria,[34] as reduction of microbial attachment caused by tannin is supported by the reduction ruminal microbial population.[32] 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 interfere of tannin with the adhesion process.[35] 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.[36] Similar to results obtained in the present study, tannins have reduced cellulolytic bacteria population *in vivo* [37] and *in vitro* by inhibiting microbial growth.[18] Bento *et al.* observed reduction of microbial attachment when supplementing cellulose with mimosa tannin compared to cellulose alone.[38] 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).[35] 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,[39] and it was concluded that CT reduce fiber digestion by inhibiting fibrolytic 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^{ab}	392.00^{bc}	365.00^{c}	26.10	0.01
F. succinogenes*	1.03^{a}	0.72^{ab}	0.81^{ab}	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

^{abc} Means with different letters are significantly different within rows ($p < 0.05$).

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^{a}	16.10^{ab}	15.30^{b}	14.40^{b}	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

^{ab} 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.[40] 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.[38] 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. flavefaaciens* 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 NH_3–N.[36] 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 RPB 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

30.00% PPE compared to the control RPB. However, dietary treatments did not have effect on *R. flavefaciens* 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 *in vivo* animals are required to investigate change in other rumen microbes such as protozoa and proteolytic bacteria as well as other cellulolytic bacteria to evaluate the mechanism of tannin action in the rumen and interaction of these microorganisms together.

Acknowledgments

The authors appreciate the honorable officials of the Agricultural Sciences and Natural Resources University of Khuzestan and Ferdowsi University of Mashhad to support and provide the facilities of research. The authors thank Denis Meehan, PhD student in Animal Nutrition Department, University of Porto for some helps.

Conflict of interest

The authors declare there is no conflicts of interest.

References

1. Daniel J, Olson KC. Feeding poultry litter to beef cattle. MU Guide. MU Extension, University of Missouri-Columbia, 2005: G2077.
2. Rankins DL, Poore MH, Capucille DJ, et al. Recycled poultry bedding as cattle feed. Vet Clin North Am Food Anim Prac 2002; 18(2): 253-266.
3. Azizi-Shotorkhoft A, Rouzbehan Y, Fazaeli H. The influence of the different carbohydrate sources on utilization efficiency of processed broiler litter in sheep. Livest Sci 2012; 148(3): 249-254.
4. Zinn RA, Barajas R, Montan M, et al. Protein and energy value of dehydrated poultry excreta in diets for feedlot cattle. J Anim Sci 1996; 74(10): 2331-2335.
5. Jolazadeh AR, Dehghan-banadaky M, Rezayazdi K. Effects of soybean meal treated with tannins extracted from pistachio hulls on performance, ruminal fermentation, blood metabolites and nutrient digestion of Holstein bulls. Anim Feed Sci Technol 2015; 203: 33-40.
6. Abarghuei MJ, Rouzbehan Y, Salem AZM, et al. Nutrient digestion, ruminal fermentation and performance of dairy cows fed pomegranate peel extract. Livest Sci 2013; 157(2-3): 452-461.
7. El-Waziry AM, Nasser ME, Sallam SMA. Processing methods of soybean meal. 1- Effect of roasting and tannic acid treated soybean meal on gas production and rumen fermentation *in vitro*. J Appl Sci Res 2005; 1(3): 313-320.
8. Makkar HPS. Effects and fate tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin Res 2003; 49(3): 241-256.
9. Hagerman AE and Butler LG. The specificity of proanthocyanidin- protein interactions. J Biol Chem 1981; 256(9): 4494-4497.
10. McSweeney CS, Palmer B, Bunch R, et al. Effect of the tropical forage Calliandra on microbial protein synthesis and ecology in the rumen. J Appl Microbiol 2001; 90(1): 78-88.
11. Min B, Barry T, Attwood G, et al. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim Feed Sci Technol 2003; 106(1-4): 3-19.
12. Barry TN, Manley TR, Duncan SJ. The role of condensed tannins in the nutritional value of Lotus *pedunculatus* for sheep. 4. Sites of carbohydrate and protein digestion as influenced by dietary reactive tannin concentration. Br J Nutr 1986; 55(1): 123-137.
13. Adamczyk B, Simon J, Kitunen V, et al. Tannins and their complex interaction with different organic nitrogen compounds and enzymes. Chem Open 2017; 6(5): 610-614.
14. Nagpal R, Puniya AK, Griffith GW, et al. Anaerobic rumen fungi: Potential and applications. In: Khachatourians GG, Arora DK, Rajendran TP, et al. (Eds). Agriculturally important micro-organisms. Kamal, India: Academic World International 2009: 375-393.
15. Patra Ak. Dietary tannins on microbial ecology of the gastrointestinal tract in ruminants. In: Patra Ak (Eds). Dietary phytochemicals and microbes. Dordrecht, Netherlands: Springer Science & Business Medi: 2012; 237-256.
16. Akin DE, Rigsby LL, Theodorou MK, et al. Population changes of fibrolytic rumen bacteria in the presence of phenolic acids and plant extracts. Anim Feed Sci Technol 1988; 19(3): 261-275.
17. Ghasemi S, Naserian AA, Valizadeh R, et al. Inclusion of pistachio hulls as a replacement for alfalfa hay in the diet of sheep causes a shift in the rumen cellulolytic bacterial population. Small Rumin Res 2012; 104(1-3): 94-98.
18. Bhatta R, Uyeno Y, Tajima K, et al. Difference in the nature of tannins on *in vitro* ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J Dairy Sci 2009; 92(11): 5512-5522.
19. Wanapat M, Cherdthong A. Use of real-time PCR technique in studying rumen cellulolytic bacteria population as affected by level of roughage in swamp buffalo. Curr Microbiol 2009; 58(4): 294-299.
20. Capparucci C, Gironi F, Piemonte V. Tannins extraction from walnuts residues. Chem Eng Trans 2011; 24: 469-474.

21. Sharifi A, Chaji M, Vakili A. Effect of treatment recycled poultry bedding with pomegranate peel tannins extract on *in vitro* digestion and fermentation activity of cow rumen microorganisms [Persian]. J Anim Prod 2017; 19(2):321-336.
22. International Atomic Energy Agency website (IAEA). Quantification of Tannins in Tree Foliage. Available at: http://www.naweb.iaea.org/nafa/aph/public/pubd31022manual-tannin.pdf. Accessed Nov 11, 2017.
23. National Research Council. National research council nutrient requirements of small ruminants, sheep, goats, Cervids and new world Camelids. Washington, USA: National Academy of Science 2007; 244-270.
24. Jasmin BH, Boston RC, Modesto RB, et al. Perioperative ruminal pH changes in domestic sheep (Ovis aries) housed in a biomedical research setting. J Am Assoc Lab Anim Sci 2011; 50(1): 27-32.
25. AOAC. Official methods of analysis of the association of official analytical chemists. 15th ed. Arlington, USA: Association of Official Analytical Chemists 1990; 69-88.
26. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci 1991; 74(10): 3583-3597.
27. Robertson JB, Van Soest PJ. The detergent system of analysis and its application to human foods. In: Sames WPT, Theander O (Eds). The analysis of dietary fibre in food. New York, USA: Marcel Dekker 1981; 123-158.
28. Broderick G A, Kang JH. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. J Dairy Sci 1980; 63(1): 64-75.
29. Stewart CS, Duncan SH. The effect of avoparcin on cellulolytic bacteria of the ovine rumen. J Gen Microbiol 1985; 131: 427-435.
30. Valizadeh R, Behgar M, Mirzaee M, et al. The effect of physically effective fiber and soy hull on the ruminal cellulolytic bacteria population and milk production of dairy cows. Asian-Aust J Anim Sci 2010; 23(10): 1325-1332.
31. Stewart CS, Flint HJ, Bryant MP. The rumen bacteria. In: Hobson PN, Stewart CS (Eds). The rumen microbial ecology. Dordrecht, Netherlands: Springer 1997; 10-54.
32. Ghasemi S, Naserian AA, Valizadeh R, et al. Partial and total substitution of alfalfa hay by pistachio byproduct modulated the counts of selected cellulolytic ruminal bacteria attached to alfalfa hay in sheep. Livest Sci 2012, 150(1-3): 342-348.
33. Makkar HPS, Blummel M, Becker K. *In vitro* effects of and interactions between tannins and saponins and fate of tannins in the rumen. J Sci Food Agric 1995; 69: 481-493.
34. Goel G, Puniya AK, Singh K. Xylanolytic activity of ruminal Streptococcus bovis in presence of tannin acid. Annu Microbiol 2005; 55(4): 295-297.
35. Bae HD, McAllister TA, Yanke J, et al. Effects of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogenes* S85. Appl Environ Microbiol 1993; 59(7): 2132-2138.
36. Molan AL, Attwood GT, Min BR, et al. The effect of condensed tannins from lotus pedunculatus and lotus corniculatus on the growth of proteolytic rumen bacteria *in vitro* and their possible mode of action. Can J Microbiol 2001; 47(7): 626-633.
37. Newbold CJ, Elhassan SM, Wang J, et al. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. B J Nutr 1997; 78(2): 237-249.
38. Bento MHL, Acamovic T, Makkar HPS. The influence of tannin, pectin and polyethylene glycol on attachment of 15N-labelled rumen microorganisms to cellulose. Anim Feed Sci Technol 2005; 122(1-2): 41-57.
39. McAllister TA, Bae HD, Jones GA, et al. Microbial attachment and feed digestion in the rumen. J Anim Sci 1994; 72(11): 3004-3018.
40. Min BR, Attwood GT, McNabb WC et al. The effect of condensed tannins from Lotus corniculatus on the proteolytic activities and growth of rumen bacteria. Anim Feed Sci Technol 2005; 121(1-2): 45-58.
41. Cheng K J, Costerton JW. Microbial adhesion and colonization within the digestion tract. Soc Appl Bacteriol Symp Ser 1986;13: 239-261.
42. Singh B, Chaudhary LC, Agarwal N, et al. Effect of feeding *Ficus infectoria* leaves on rumen microbial profile and nutrient utilization in goats. Asian-Aust. J Anim Sci 2011; 24 (6): 810-817.
43. Van Soest PJ. Nutritional ecology of the ruminant. Ithaca, USA: Cornell University Press 1994; 230-280.
44. Yildiz S, Kaya I, Unal Y, et al. Digestion and body weight change in Tuj lambs receiving oak (*Quercus hartwissiana*) leaves with and without PEG. Anim Feed Sci Technol 2005; 122(1-2): 159-172.
45. Min BR, Pinchak WE, Fulford JD, et al. Wheat pasture bloat dynamics, *in vitro* ruminal gas production, and potential bloat mitigation with condensed tannin. J Anim Sci 2005; 83(6): 1322-1331.
46. Krueger WK, Gutierrez-Banuelos H, Carstens GE, et al. Effects of dietary tannin source on performance, feed efficiency, ruminal fermentation, and carcass and non-carcass traits in steers fed a high-grain diet. Anim Feed Sci Technol 2010; 159(1-2): 1-9.
47. Theodoridou K, Aufrere J, Niderkorn V, et al. *In vitro* study of the effects of condensed tannins in sanfoins on the digestive process in the rumen at two vegetation cycles. Anim Feed Sci Technol 2011; 170(3-4): 147-159.
48. Tan H, Sieo C, Abdullah N, et al. Effects of condensed tannins from Leucaenaon methane production, rumen fermentation and populations of methanogens and protozoa *in vitro*. Anim Feed Sci Technol 2011; 169(3-4): 185-193.