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EFFECT OF RECYCLED POULTRY BEDDING TREATED WITH PHENOLIC COMPOUNDS EXTRACTED FROM POMEGRANATE PEEL ON *IN VITRO* DIGESTION ACTIVITY OF RUMEN MICROBES

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ABSTRACT

This study was conducted to investigate the effect of treating recycled poultry bedding (RPB), with phenolic compounds extracted from pomegranate peel on *in vitro* digestion and fermentation activity of rumen bacteria and fungi. The eight treatments were included by adding pomegranate peel extract (PPE) to RPB at levels 0, 5, 10, 15, 20, 25, 30 and 35% of dry matter (DM). With increasing amount of PPE to 15 and 20%, respectively potential and volume of gas production of mixed rumen microorganisms were unchanged. Treatment RPB with PPE decreased rumen ammonia-N concentration ($P<0.05$), and increased partitioning factor (PF), microbial biomass (MB) production and efficiency of MB in compared to control treatment. Increasing the levels of PPE had no significant effect on DM digestibility by rumen bacteria in days 1 and 2. However, it was decreased 3 days after incubation by adding 25 and 30% PPE, but not 20% ($P<0.05$). The culture medium ammonia-N concentration in 6 days after incubation decreased, at levels 25 and 30% PPE ($P<0.05$). Results of present study showed that treatment RPB with PPE up to 25% of dry matter, had no negative effects on digestion and fermentation activity of mixed rumen microorganisms and fungi, and resulted in improve nitrogen metabolism in ruminants via decreasing rumen NH_3-N concentration.

Keywords: ammonia-N, microbial biomass production, non protein nitrogen, pomegranate peel extract, tannin, true protein.

INTRODUCTION

Dry climatic condition and lack of water resources in some countries, have led to decrease of the quantity and quality feedstuffs for ruminant feeding (Abarghuei *et al*., 2014). In order to compensate for this deficiency, proper utilization of wastes and animal or agricultural by-products, such as recycled poultry bedding (RPB) as ruminant feed, lead to improved conditions for animal production (Azizi-shotorkhoft *et al*., 2014).

Recycled poultry bedding (RPB) is a solid waste including poultry excreta, material of litter, feathers and particles of feed. The nutritional value of RPB as a feed is due to its crude protein and minerals. Crude protein (CP) content of RPB is in the range of 15-35% (Obeidat *et al*., 2011). The RPB have been used successfully in the diet of ruminants in most countries (Azizi-shotorkhoft *et al*., 2014). Recycled poultry bedding has a high rumen degradable protein (RDP). About half of crude protein (CP) content of RPB is in the form of non-protein nitrogen (NPN) which is quickly degraded in the rumen by microorganisms (Azizi-shotorkhoft *et al*., 2014). In ruminants, due to unfavorable ruminal protein degradation, a great part of nitrogen excreted through urine (Jolazadeh *et al*., 2015). Therefore, finding ways to reduce rumen degradability and urinary nitrogen excretion is essential.The phenolic compounds and tannins reduce protein degradation rate in rumen and facilitate passing of a portion of protein to the intestine (Makkar, 2003).

Pomegranate peel (PP) is a by-product of extracting the juice from pomegranates, with annually produce more than 120,000 tons in Iran (Abarghuei *et al*., 2014). The pomegranate fruit peel has a lot of phenolic and tannin that deposit the feed proteins and microorganisms building proteins (Abarghuei *et al*., 2014). Reducing the rate of protein degradation in animals fed with tannins, leads to decreases ammonia-N concentration, and it consequently reduce urinary nitrogen excretion and change nitrogen excretion via urine to feces. This causes more nitrogen retention and improve the efficiency of nitrogen utilization (Abarghuei *et al*., 2014; Jolazadeh *et al*., 2015).

Despite efforts to study the effect of tannin-rich plants on the microbial population in the rumen (Singh *et al*., 2011; Amira *et al*., 2014); scarce information is available on the effect of processing RPB with phenolic compounds on ruminants performance. Therefore, the aim of present study was to investigate the effect of processing RPB with different levels of tannins extracted from PP on digestion and fermentation parameters of mixed rumen microbial population or anaerobic bacteria and fungi isolated from Holstein cow.

MATERIALS AND METHODS

Preparation of recycled poultry bedding and pomegranate peel extract: Recycled poultry bedding (RPB) was prepared from the main factory in Sabzevar, Iran, in which RPB was humidified up to 23%, and then thermally processed by indirect vapor pressure 75-85 ^{0}C for 20 min.

Pomegranate peel (PP) was obtained from the factory in Baghmalek city (Khuzestan province, Iran), which used similar varieties and processing methods. A two-step extraction process was performed according to procedure of Capparucci *et al*. (2011). In the first extraction step, sun dried pomegranate peel was ground through a 0.5 mm screen and soaked in water at a ratio of 1:10 (w/v) for 24 h. In the second step, the pomegranate peel extract was filtered and boiled to achieve pomegranate peel extract (PPE). The chemical composition of RPB and PPE are presented in Table 1.

Treatment of recycled poultry bedding (RPB): The experimental treatments were recycled poultry bedding that treated with levels of 0, 5, 10, 15, 20, 25, 30 and 35% of dry matter (DM) with phenolic compounds such as tannins of PPE. An aqueous solution of PPE was prepared by adding water to PPE (W/ 3V), and mixed with RPB in a bucket.

Table 1. Chemical composition of recycled poultry bedding (RPB) and pomegranate peel extract (PPE) (g/kg of DM or as stated).

Item	Recycled poultry bedding	Pomegranate peel extract
DM	900	875
OM	853	747
CP	224	203
Ash	147	253
NPN (g/kg CP)	402	-
TP (g/kg CP)	598	-
NDF	350	75.0
ADF	195	57.5
Total phenolic	-	151
Total tannin	-	111

DM- dry matter; OM- organic matter; CP- crude protein; NPN- non-protein nitrogen; TP- true protein; NDF- neutral detergent fiber; ADF- acid detergent fiber.

***In vitro* gas production parameters:** Rumen fluid collected before morning feeding from two fistulated Holstein steers (36 ± 3 months old), by plastic tube and vacuum pump. Animals were fed with diet containing 60% forage and 40% concentrate for two weeks. The diet was formulated to meet maintenance requirements according to NRC (2001) recommendations (Table 2). Rumen fluid immediately was filtered by four layers of cheesecloth, and then was placed into bottle within warm water insulated flask with a temperature of 39 °C, and transferred to the laboratory.

In vitro gas production of RPB samples processed with different levels of PPE was measured based on the method of Vercoe *et al*. (2010). Volume of gas production of experiment treatments (with particle size of 1 mm) was measured in 100 mL glass vials containing 500 mg of sample, 30 mL artificial saliva and 10 mL of rumen fluid. The amount of produced gas was determined by digital barometer device at times 0, 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 h of incubation. For determination of gas production parameters, following non-linear equation was used.

$P = b (1 - e^{-ct})$

Where, P is the gas produced at t time, b is gas production from the insoluble fraction (ml), c is the rate of gas production for b and t is the time of incubation.

Table 2. Feed ingredients and chemical composition (g/kg of DM or as stated) of experimental diet fed to Holstein cows.

Ingredients	Amount
Wheat straw	400
Corn silage	100
Alfalfa hay	100
Corn, ground	270
Wheat bran	110
Urea	9.0
Calcium carbonate	5.5
Minerals and vitamin[a]	2.5
Salt	2.5
Chemical composition	
DM	887
OM	926
CP	108
NDF	531
Calcium	6.0
Phosphorus	3.5
ME (MJ/kg DM)	8.78

DM- dry matter; OM- organic matter; CP- crude protein; NDF- neutral detergent fiber; ME- metabolizable energy.

[a] Contained (per kg): 99.2 mg Mn, 50.0 mg Fe, 84.7 mg Zn, 1.0 mg Cu, 1.0 mg I, 0.2 mg Se, 9000 IU vitamin A, 2000 IU vitamin D, and 18.0 IU vitamin E (Roshddaneh Co., Iran).

To determine the fermentation parameters (such as pH, partitioning factor (PF, expresses as ratio of truly degraded organic matter to gas produced during 24 hr. incubation periods.), microbial biomass (MB) production, efficiency of MB and truly degradable organic matter (TDOM), 8 replicates were considered per treatment. The amount of gas produced per vial was recorded during 120 h of incubation. Then pH of incubated samples was recorded by pH meter (model 744, Swiss Metrohm

companies). The content of each vial was centrifuged at 1500 rpm for 20 min and the residuals were collected and dried. Ruminal DM digestibility (DMD) was calculated by difference between weights of primary substrate from residual after incubation. To determination of ammonia-N, supernatant sample (5 ml) was mixed with one ml of HCl 0.2 N immediately, and stored at -20 °C. Partitioning factor, MBproduction, efficiency of MB and TDOM in samples were calculated according to Vercoe *et al*. (2010).

Two-step digestibility: *In vitro* nutrients digestibility were determined based on two-step digestion as described by Tilley and Terry (1963). The 100 ml test tubes containing 0.5 g sample, 40 ml artificial saliva and 10 ml rumen fluid (ratio 4:1) were used. Artificial saliva was prepared according to McDougall (1948). Finally, the content of the tubes was filtered and digestibility of DM, CP, neutral detergent fiber (NDF) and acid detergent fiber (ADF) was calculated, by subtracting the amount of the nutrients in the initial sample from the remaining after incubation and drying in the oven.

Incubation of treatment RPB in culture medium of rumen isolated bacteria and fungi: After analysis of previous results, some treatments (including treatments containing 15, 20 and 25% of PPE) were selected and their effect on growth of ruminal bacteria and fungi of Holstein cow in culture medium were tested according to following methods.

Digestion in culture medium of rumen bacteria: This experiment was conducted in culture medium of rumen bacteria according to method of Mohammadabadi and Chaji (2010). First for isolation of bacteria, culture glasses containing 1 g experimental sample were autoclaved at 120 °C for 15 minutes. Rumen fluid was centrifuged (at 1000 rpm for 10 min) and supernatant was added to culture medium of bacteria that contained cellobiose, sodium sulfide, sodium carbonate, fungicides (benomyl and metalaxyl), cysteine-HCl, peptone, Trypticase and yeast extract mixture, under anaerobic condition. The amount of 36 ml of this solution as culture medium and 4 ml rumen fluid were inoculated into each culture glass. Then the samples were cultured in incubator at 39 °C for 24, 36 and 48 h. At the end of each of the mentioned times, 3 replicates per treatment were considered to determine the DMD and ammonia-N.

Digestion in culture medium of rumen fungi: Experimental treatments were cultured (with particles size of one millimeter, 9 replicates per treatment) in culture medium of rumen fungi at 39 °C for 1, 3 and 6 days. At the end of each from the times, to determine the DMD and ammonia-N concentration were considered of three replicates per treatment. The fungi isolation and culture was prepared according to Rezaeian *et al*. (2005). The rumen fungi culture medium contained salt solution 1 (phosphate hydrogen dipotassium per liter of sterile water), salt solution 2 (phosphate hydrogen potassium, sulfate ammonium, sodium chloride and calcium chloride per liter of sterile water), rumen fluid (centrifuged), yeast extract, peptone, Trypticase, glucose, cellobiose, sodium bicarbonate, cysteine-HCl and 1.0% resazurinper liter of culture medium. Culture medium was transferred under anaerobic conditions into medium glasses and then autoclaved for 15 minutes at 120 ° C. Thus, culture medium of fungi was prepared. Isolated rumen fungi prepared as inoculants (wheat straw was incubated in the rumen of fistulated animal, and used as the carbon source for growth of the rumen anaerobic fungi, Rezaeian *et al*., 2005). The isolated rumen fungi was cultured in glass serum bottle containing rumen anaerobic fungi culture, experimental samples and antibiotics (penicillin, streptomycin and chloramphenicol, each of 0.1 mg/L), for purifying the cultures three subculture was done. At days 1, 3 and 6 of growth ruminal fungi, disappearance of dry matter and samples nitrogen by fungi were calculated.

Chemical analysis of experimental samples: Recycled poultry bedding, PPE and experimental treatments were analyzed for DM (method 930.15), Ash (method 924.05) and N (method 984.13) of AOAC (1990). Acid detergent fiber (method 973.18) was determined and expressed inclusive of residual ash (AOAC, 1990). Neutral detergent fiber was determined without the use of sodium sulphite or α-amylase according to Van Soest *et al*. (1991). Tungstic acid was used to determine true protein (TP) content of RPB as a precipitating agent. Amount of protein deposited was measured as TP (Licitra *et al*., 1996). The NPN content of RPB was calculated by subtraction of crude protein from TP.

The measurement of the total phenolic and total tannins in PPE was conducted as described by Makkar (2000). Total phenol was determined using Folin–Ciocalteu's reagents, and the concentration was measured as tannic acid equivalent using tannic acid (Merck, Germany) as a standard. Total tannins were measured as described by Makkar (2000).

Ammonia-N concentration of samples was determined according to method of Broderick and Kang (1980).

Statistical analysis: A completely randomized design was used to determine the effect of processing RPB with phenolic compounds extracted from pomegranate peel on various parameters. All data were analyzed using the GLM procedure in SAS 9.2, based on the statistical model:

$Y_{ij}= \mu+T_i+e_{ij}$

Where Y_{ij} is the observation (*in vitro* rumen fermentation parameters, nutrient digestibility and growth of ruminal bacteria and fungi), μ is the general mean, T_i is the effect of processing broiler litter with phenolic compounds extracted from pomegranate peel and e_{ij} the

standard error of term. Differences among means (P≤0.05) were compared using Duncan's multiple range test method (Duncan, 1955).

RESULTS

Chemical composition of RPB, PPE and experimental diets: The chemical composition of RPB and PPE are presented in Table 1.Table 3 shows the results of chemical composition of treatment RPB with different levels of tannin extract from PP. The DM, OM and ash were not affected by treatments.The treatments had significant effect on the CP, NDF and ADF content of RBP, and these components decreased with increased level of PPE supplementation.

***In vitro* rumen fermentation parameters:** *In vitro* rumen fermentation parameters of RPB processed with different levels of PPE is presented in Table 4. With increasing PPE level up to 20 and 15%, the volume and potential of gas production were similar among treatments, but they decreased in treatments containing higher levels of PPE as compared to the control treatment (P<0.05). In addition, the rate of gas production was similar among treatments, except for treatment containing 35% of PPE which decreased significantly (P<0.05). After 120 h of incubation, the highest and lowest volume of gas production was obtained for the control and treatment containing the highest amount of PPE, respectively (P<0.05).

With increasing the amount of PPE up to 25%, pH was similar among experimental treatments (Table 4), but it decreased significantly in treatments containing 30 and 35% PPE (P<0.05). With increasing levels of PPE in the treatment, ammonia-N concentration reduced (P<0.05).

Increasing the level of PPE up to 25% in the treatment, ruminal DMD and TDOM were not affected (Table 4), but they decreased significantly in treatments containing 30 and 35% PPE compared to control (P<0.05). Partitioning factor, MB production and efficiency of MB increased by increasing the amount of PPE in treatment.

Digestibility by rumen isolated bacteria: As shown in Table 6, DMD after 1 and 2 days of incubation in culture medium of mixed rumen bacteria was not affected by treatments. However, 3 days after incubation, DMD in treatments containing 25 and 30 % of PPE decreased as compared to the control treatment (P<0.05).

One day after incubation by mixed rumen bacteria, ammonia-N concentration was not changed among the experimental treatments while, 2 day after incubation, its concentration decreased (P<0.05) significantly with increasing the level of extract in the treatment (Table 6). About the day 3th after incubation, treatment containing 20 and 25% of PPE had no effect on NH_3-N concentration, and then it was significantly decreased (Table 6).

Regardless of incubation time (Table 6), level of PPE affected DMD and ammonia-N in rumen bacteria culture medium. For both parameters, the highest and lowest amounts were observed in the control treatment and treatment containing 30% extract, respectively (P<0.05).

Digestibility by rumen isolated fungi: Digestibility of DM by mixed rumen fungi 1, 3 and 6 days afterincubation times was not affected by experimental treatments (Table 7).

Two-steps digestibility: Two-step digestibility of nutrients in RPB treated with different levels of PPE is presented in Table 5. Adding PPE to treatment up to 25% had no effect on digestibility of DM, CP, NDF and ADF, but they decreased significantly in treatments containing 30 and 35% extract (P<0.05). However, highest CP digestibility was obtained in treatment with 15% extract (P<0.05).

The experimental treatments had no effect on the ammonia-N concentration in 1 and 3 days after incubation (Table 7). However, in day sixth significantly decreased in treatments contain 25, 30% PPE in compared with control (P<0.05).

Table 3. Effect of treatment of recycled poultry bedding (RPB) with different levels of pomegranate peel extract (PPE) on its chemical composition.

Item	Level of pomegranate peel extract (% of DM)								SEM	P value
	Control	5	10	15	20	25	30	35		
DM	900	899	897	894	885	884	879	878	9.0	0.46
OM	853	847	847	846	836	836	831	826	36.0	1.00
Ash	147	153	153	154	164	164	169	174	6.7	0.08
CP	224^{a}	213^{ab}	212^{ab}	211^{ab}	209^{ab}	208^{ab}	202^{bc}	199^{c}	4.8	0.02
NDF	350^{a}	341^{a}	333^{b}	329^{b}	291^{c}	291^{c}	288^{c}	274^{d}	4.0	<0.01
ADF	195^{a}	186^{b}	182^{bc}	179^{bc}	175^{cd}	171^{d}	170^{d}	168^{d}	2.2	<0.01

DM- dry matter; OM- organic matter; CP- crude protein; NDF- neutral detergent fiber; ADF- acid detergent fiber.
a, b, c- values in rows with different letters differ significantly (P<0.05).

Table 4. Effect of treating recycled poultry bedding (RPB) with different levels of pomegranate peel extract (PPE) on rumen fermentation parameters by *in vitro* gas production technique.

Item	Level of pomegranate peel extract (% of DM)								SEM	P-value
	Control	5	10	15	20	25	30	35		
IVGP (ml)	77.9^{a}	72.9^{ab}	76.1^{a}	75.8^{ab}	69.9^{ab}	68.9^{b}	61.2^{c}	58.4^{c}	2.29	<0.01
b (ml)	85.8^{a}	83.5^{a}	84.5^{a}	82.8^{ab}	78.8^{c}	79.9^{bc}	67.4^{c}	63.2^{d}	1.09	<0.01
c (ml/h)	0.011^{ab}	0.009^{ab}	0.011^{ab}	0.012^{a}	0.009^{b}	0.012^{ab}	0.009^{ab}	0.006^{c}	0.001	<0.01
pH	6.78^{a}	6.76^{a}	6.76^{a}	6.75^{a}	6.74^{a}	6.70^{ab}	6.64^{b}	6.44^{c}	0.027	<0.01
Ammonia Nitrogen (mg/100 ml)	17.1^{a}	15.7^{b}	15.4^{b}	13.4^{c}	12.2^{d}	12.1^{d}	12.^{2d}	11.6^{d}	0.26	<0.01
DMD (g/kg)	612^{a}	610^{a}	617^{a}	591^{a}	581^{ab}	589^{a}	518^{b}	497^{b}	18.7	<0.012
TDOM (mg)	251^{a}	248^{a}	252^{a}	250^{a}	248^{a}	246^{a}	238^{b}	236^{b}	3.11	0.017
PF (mg/ml)	3.31^{c}	3.39^{bc}	3.50^{b}	3.52^{c}	3.55^{b}	3.58^{b}	3.89^{a}	4.01^{a}	0.12	0.015
MB (mg)	79.6^{d}	87.6^{c}	84.6^{c}	83.2^{c}	94.2^{b}	94.4^{b}	103.4^{a}	107.5^{a}	2.09	<0.01
MBE (%)	31.7^{c}	35.3^{bc}	33.6^{c}	33.3^{c}	38.0^{b}	38.4^{b}	43.4^{a}	45.5^{a}	2.02	<0.01

IVGP- *in vitro* gas production for 24 h (mL/200 mg DM); *b*- the gas production from the insoluble but fermentable fractions for 120 h (mL); *c*- rate constant of gas production during incubation (ml/h); DMD- dry matter digestibility; TDOM- truly degradable organic matter ; PF- partitioning factor; MB- microbial biomass; MBE- microbial biomass efficiency.
a, b, c, d- values in rows with different letters differ significantly (P<0.05).
SEM- Standard error of means

Table 5: Effect of treatment recycled poultry bedding (RPB) with different levels of pomegranate peel extract (PPE) on two-step nutrient digestibility (g/kg of DM).

Item	Level of pomegranate peel extract (% of DM)								SEM	P-value
	Control	5	10	15	20	25	30	35		
DM	581^{a}	581^{a}	587^{a}	592^{a}	564^{a}	540^{a}	471^{b}	390^{b}	25.3	<0.01
CP	651^{ab}	650^{ab}	657^{a}	695^{a}	668^{a}	659^{a}	593^{c}	550^{d}	19.8	<0.01
NDF	476^{a}	466^{a}	448^{ab}	456^{a}	441^{a}	411^{b}	339^{b}	301^{c}	28.6	<0.01
ADF	433^{a}	443^{a}	419^{ab}	425^{a}	428^{a}	395^{ab}	319^{bc}	290^{c}	31.4	<0.01

DM- dry matter; CP- crude protein; NDF- neutral detergent fiber; ADF- acid detergent fiber.
a, b, c, d- values in rows with different letters differ significantly (P<0.05).
SEM- Standard error of means.

Table 6. Effect of processing recycled poultry bedding (RPB) with different levels of pomegranate peel extract (PPE) on dry matter digestibility and ammonia nitrogen concentration by mixed rumen bacteria.

Incubation time	Level of pomegranate peel extract (% of DM)				SEM	P-value
	Control	20	25	30		
Dry matter digestibility (g/kg)						
1 day after incubation	292	290	289	285	6.1	0.55
2 days after incubation	363	376	362	348	17.5	0.33
3 days after incubation	463^{a}	413^{ab}	391^{bc}	362^{bc}	31.0	<0.01
Ammonia nitrogen(mg/100 ml)						
1 day after incubation	14.3	14.2	13.7	13.7	0.62	0.52
2 days after incubation	15.7^{a}	14.9^{b}	14.7^{b}	14.5^{b}	0.30	0.04
3 days after incubation	17.4^{a}	17.3^{a}	16.9^{a}	16.0^{b}	0.31	0.01
Regardless of time						
DMD (g/kg)	373^{a}	360^{a}	347^{b}	332^{c}	23.2	<0.01
Ammonia nitrogen(mg/100 ml)	15.8^{a}	15.4^{b}	15.1^{b}	14.7^{c}	0.15	<0.01

DMD- dry matter digestibility.
a, b, c- values in rows with different letters differ significantly (P<0.05).
SEM- Standard error of means.

Table 7. Effect of processing recycled poultry bedding (RPB) with different levels of pomegranate peel extract (PPE) on dry matter digestibility and ammonia nitrogen concentration by mixed rumen fungi.

Incubation time	Level of pomegranate peel extract (% of DM)				SEM	P-value
	Control	20	25	30		
	Digestibility of dry matter (g/kg)					
1 day after incubation	201	191	214	200	15.0	0.32
3 days after incubation	252	236	266	232	16.3	0.23
6 days after incubation	402	402	382	372	18.9	0.25
	Ammonia nitrogen (mg/100 ml)					
1 day after incubation	12.1	11.7	11.5	11.1	0.55	0.20
3 days after incubation	13.4	12.9	12.8	12.3	0.54	0.19
6 days after incubation	14.2[a]	13.3[ab]	12.5[b]	12.6[b]	0.46	0.023
	Regardless of time					
DMD (g/kg)	285	276	287	268	13.1	0.18
Ammonia nitrogen (mg/100 ml)	13.5[a]	12.6[a]	12.3[ab]	12.0[b]	0.21	0.02

DMD- dry matter digestibility.
a, b- values in rows with different letters differ significantly (P<0.05).
SEM- Standard error of means.

Regardless of the incubation time (Table 7), the experimental treatments had no effect on DMD by mixed rumen fungi. The concentration of ammonia-N was same as control until treatment contained 25% PPE, then it was significantly decreased (P<0.05).

DISCUSSION

Chemical composition of RPB, PPE and experimental treatments: Recycled poultry bedding used in this experiment was containing the considerable amount of CP (Table 1) that is comparable with results reported by Azizi-shotorkhoft *et al*. (2014) and Obeidat *et al*. (2011). More than half of the nitrogen content of RPB was with origin of TP that agreed with the results of Azizi-shotorkhoft *et al*. (2014). Recycled poultry bedding used in the present experiment had a good quality as ruminant feed because of its appropriate CP content (Obeidat *et al*., 2011).

Total phenolic compounds and total tannin of PPE used in the present study (Table 1) were more than PPE obtained by Abarghuei *et al*. (2014). This may be due to more boiling and consequently more extract concentration in our study. Jolazadeh *et al*. (2015) extracted phenolic compounds from pistachio hulls by the same method, and their results were comparable with results of present experiment.

Reduction of DM, OM, CP, NDF and ADF contents of the treatments (Table 3) with increasing the level of extract was probably due to the lower concentration these compounds in the extract than RPB (Table 1).

Fermentation parameters: Reduction potential of gas production (Table 4) in treatments containing PPE compared to the control treatment represents formation of tannin complex with nutrients such as carbohydrates, proteins, polysaccharides, bacterial cell membrane and enzymes digesting protein and carbohydrates and is likely to access lower rumen microorganisms to degradation of them (Chung *et al*., 1998; Patra, 2012). Consistent with results of present study, Alipour and Rouzbehan (2010) with treatmentof soybean meal with 0, 15, 30, 40 and 60 g per kg of tannin extracted from grape pomace, reported that the treatment resulted in 4-11.5% reduction in gas production. Tan *et al*. (2011) investigated the effect of 0, 10, 15, 20, 25 and 30 mg condensed tannin (CT) extracted from *Leucaena* plant on gas production and ruminal fermentation parameters of diet containing forage *Panicum maximum* (500 mg). After 24 h of incubation, the gas production volume decreased by condensed tannins.

The DMD and ammonia-N concentration decreased with increasing the levels of PPE (Table 4). Sharifi *et al*. (2013) evaluated the effect of treating soybean and canola meal with tannin extracted from the pistachio by-product on *in vitro* gas production, the *b* and *c* fractions decreased numerically in compared with untreated meals. Tannins reduced cumulative gas production by formation of tannin-macromolecule complexes with inhibiting the activity of microbial enzymes (Sharifi *et al*., 2013). Tabacco *et al*. (2006) showed that high tannin concentration in diet might be a cause for reduction in microbial enzyme activities like cellulase. Mohammadabadi *et al*. (2010) reported that adding tannic acid (hydrolysable tannin) to soybean meal decreased cumulative gas production, *b*, and *c* fractions and concluded that processing soybean meal with tannic acid protected protein from degradation in the rumen.

Reduction of the ammonia-N concentration by increasing the levels of PPE was consistent to other studies (Yanez Ruiz *et al*., 2004; Sharifi *et al*., 2013) Mohammadabadi and Chaji (2012) demonstrated reduction of ammonia-N with supplementation of soybean meal with tannins extracted from oak and pistachio hull. Jolazadeh *et al*. (2015) also reported that ammonia-N concentration of Holstein calves was significantly affected by soybean meal processed with different levels of tannins extracted from the pistachio hull. Lower crude protein content of experimental treatments (Table 3) with increasing PPE, may be also another reason for reduction of ammonia-N concentration.

Higher PF in treatments with levels of 20, 25, 30 and 35% was probably due to lower gas production in these treatments, which is the reason for increasing the amount of PF compared to treatments with lower content of PPE (Table 4).

Nutrient digestibility: Digestibility of nutrients (*i.e.,* DM, NDF and ADF) in experimental treatments containing PPE up to 25% was comparable with control (Table 5). That indicates a lack of negative effect of tannin and phenolic compounds extract on rumen fermentation conditions. In treatments containing high levels of the PPE, digestibility of nutrients decreased, probably due to the negative effect of tannin on rumen microorganisms due to the higher concentration of tannin in them. A study have shown that the tannins with concentration higher than rumen capacity selectively inhibit the growth of gastrointestinal tract microorganisms (Patra, 2012).The antimicrobial effects of phenolic compounds are probably related to the inhibition of bacterial enzymes, alterations in cell wall permeability, an increase in the hydrogen ion activity of the microbial environment, chelation of essential minerals, particularly iron with a concomitant impairment of the microbial oxidative metabolic system (Chung *et al*., 1998; Patra, 2012). Similar to the results of present study, Abarghuei and Ruzbehan (2015) reported that grape pomace tannin decreased ruminal DMD of common dairy cows diet, where the treatments containing 36, 48 and 60 µl of the extract. In other studies, using secondary metabolites, especially condensed tannins, reduced ruminal DMD (Frotus *et al*., 2004; Cortés *et al*., 2009).

The highest CP digestibility of treatment containing 15% PPE was probably because of protective effects of tannins on hydrolysis and deamination of protein. Thus, the availability of feed proteins increased far more digestion and absorption post rumen (Abarghuei and Ruzbehan, 2015). Mezzomo *et al*. (2011) reported that supplementation of soybean meal as a major source of protein with condensed tannins of *Quebracho* plant in calf diet, decreased ruminal CP digestibility and subsequently increased by-pass metabolizable protein to the duodenum.

Digestibility by isolated rumen bacteria: Reducing the concentration of ammonia-N in treatments containing PPE (Table 6) probably was due to the inhibitory effect of phenolic components in the extract on rumen proteolytic activity (Frotus *et al*., 2004). Decrease of proteolysis may be due to direct effects of condensed tannins on the activity of microbial proteolytic enzymes, or indirect effects on the metabolite concentration in the rumen, which can be attributed to regulate the activities of some proteolytic bacteria (Waghorn *et al*., 1994). Singh *et al*. (2011) reported that the concentration of ammonia-N was significantly reduced in diets contain pakar leaves fed to goats, which might be due to reduced proteolytic activity in the rumen of these animals. Min *et al*. (2002) also reported similar results by tannin of *Lotus corniculatus,* a CT tannin rich plant, where rumen proteolytic activity and rumen ammonia concentration were markedly reduced in sheep.

Regardless of incubation time, level of PPE affected DMD and ammonia-N, so that the highest and lowest values were observed in the control and treatment containing 30% PPE, respectively. Reduction of DMD by enhanced PPE in treatment may have been due to negative effect on decline in population of the rumen bacteria, especially cellulolytic bacteria. Because, it has been reported that effect of tannins on rumen bacteria depends on the type of bacterial species. Use of Calliandra plant tannins in the diet (2-3 % of tannin) decreased fiber degrading bacteria population (McSweeny *et al*., 2001). In a study of microbial pure culture, used 400 mg per liter of condensed tannins from *Cynodon dactylon* clover lead to the inhibition the growth of *Fibrobacter suucsinogenes* bacteria, but the concentrations under 400 mg per liter, had no significant inhibitory effect on the growth of bacteria (Bae *et al*., 1993). Salawu *et al*. (1999) showed that quebracho tannins (5% of DM) reduced cellulase and xylanase activities of the rumen microbes and total rumen protozoa in sheep fed a grass-barley diet. Singh *et al*. (2011) also investigated effect of feeding *Ficus infectoria* leaves (Pakar is as rich source of tannin) on rumen microbial profile and nutrient utilization in goats, and showed that digestibility of DM were reduced in experimental as compared with the control group. The rumen microbial profile as obtained by MPN technique showed no change in total bacterial population but cellulolytic bacteria were reduced ($P<0.05$).

Digestibility by isolated rumen fungi: The ammonia-N concentration (Table 7) after 6 days of incubation in treatments containing PPE, decreased significantly compared to the control treatment and the significant difference was observed in treatment with 25 and 30% PPE. Lower concentrations of ammonia-N mainly are due

to a decrease in degradation of amino acids in the rumen (Frotus *et al*., 2004). It was reported that tannins can protect dietary protein from ruminal degradation and thus increased by-pass protein and the performance of ruminants (Makkar, 2003). According to the results of this research, by increasing the amount of tannin level, protein degradation and ammonia concentration was decreased.

Regardless of incubation time, the experimental treatments had no significant effect on dry matter digestibility of treatment, but the concentration of ammonia-N in medium was significantly decreased with increasing the amount of extract.Unchanged DMD among experimental treatments was probably due to less sensitivity of rumen fungi to tannins and phenolic compounds than other ruminal microorganisms. Additionally, resistance of fungal species against different types of tannins could be due to their morphological structure, because fungi have thicker cell walls and contain higher percentage of chitin (Madigan and Martinko, 2006). Similar to the present results, Muhammed *et al*. (1995) investigated the effects of tannic acid, ellagic acid, gallic acid and catechin on rumen fungus *Neocallimastix frontalis* strain RE1. All these compounds had little effect on the inhibition of zoospore attachment. According to Paul *et al*. (2003), fungus could grow at tannic acid concentration up to 20 g/l and the growth was not appreciably affected up to 10 g/l concentration acid. However, fiber-degrading ability of rumen fungi may be less sensitive to the inhibitory effects of CT compared to cellulolytic bacteria. McSweeny *et al*. (2001) investigated the effects of Calliandra tropical forage (with and without using PEG) on rumen microbial population and microbial protein synthesis. They reported that the fungi population was less affected. Lim *et al*. (2006) also investigated antimicrobial activity of tannin extracted from the *Rhizophora apiculata* barks. Their results also showed that all three types of tannins (total tannins, hydrolysable tannin and condensed tannin) had no antifungal activity.

Results of the present study showed that treatment of recycled poultry bedding as an inexpensive protein resource with pomegranate peel extract up to 25% of dry matter, without negative effects on nutrients digestibility and the growth of rumen bacteria and fungi, may improve nitrogen metabolism in gastrointestinal tract via decreasing rumen ammonia-N concentration. However, further studies in this field of ruminant feeding are necessary.

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