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The effect of a bacterial inoculant, urea and molasses on chemical composition, *in vitro* gas production and energy content of ensiled pomegranate (*Punica granatum* L.) seeds and peel pulp

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Abstract

This study was carried out to determine the effect of a bacterial inoculant, urea and molasses on chemical composition and nutritive value of ensiled pomegranate seed pulp (PSP) and pomegranate peel pulp (PP). The treatments were 100% PSP (T1), 100% PP (T2), 75% PSP and 25% PP (T3), 25% PSP and75% PP (T4), 50% PSP and 50% PP (T5), 50% PSP and 50% PP with 5% urea (T6), 50% PSP and 50% PP with 5% molasses (T7), 50% PSP and 50% PP with bacterial inoculant (T8), 50% PSP, 50% PP with 5% urea and 5% molasses and 50% PSP (T9), 50% PP with 5% urea and 5% molasses and bacterial inoculant (T10). The bacterial inoculant (*Lactobacillus plantarum*) was applied at the recommended level of 1×10^5 CFU/g. The materials were ensiled for 30 days in plastic polyethylene bags. At the end of the ensiling period, all silages were subjected to chemical analysis and *in vitro* gas production. After fermentation, pH values in silage treated with inoculant decreased below 4.0 and the addition of urea increased pH to 5.47 and had highest crude protein (CP) content ($P<0.05$). Ensiling PP was superior to PSP with respect to higher ($P<0.05$) gas production, organic matter digestibility (OMD), digestible organic matter in dry matter (DOMD) and metabolizable energy (ME) values and T2 and T9 had higher ($P<0.05$) OMD, DOMD and ME values. Gas production was higher ($P<0.05$) for T2 but was intermediate for T9. Also, the results indicated that the application of urea and molasses resulted in silages with higher OMD, DOMD and ME values. In conclusion, ensiling pomegranate peel pulp with or without urea and molasses can provide a valuable feedstuff for ruminants.

Keywords: Urea; molasses; gas production; pomegranate seed and peel

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Introduction

The provision of adequate quantities of good-quality cheap forage is a major challenge in the development of livestock production system throughout the Middle East. Conventional feedstuffs for ruminants in Iran are often in short supply and expensive, so there is a need to search for non-conventional feedstuffs. Many by-products have a substantial potential value as animal feedstuffs (Bampidis and Robinson, 2006). Pomegranate by-product (the by-product of juice extraction) is one of such feedstuff available in Iran (Mirzaei-Aghsaghali et al., 2011). The by-product contains the seeds and the peel. Pomegranate by-product generated from extraction factories is estimated to be 120000 tons per year in Iran (Mirzaei-Aghsaghali et al., 2011). The pomegranate (*Punica granatum* L.) is one of the oldest edible fruits and is

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widely grown in many tropical and subtropical countries (Salaheddin and Kader, 1984). Iran, India and USA are three main pomegranate producer countries (FAO, 2005). It is an important commercial fruit in Iran with a total production of 800,000 tons (FAO, 2005). Edible parts of pomegranate fruit comprised of 78% juice and 22% seed (Kullkarni and Aradhya, 2005). Pomegranate seeds are rich in sugars, vitamins, polysaccharides, polyphenols and minerals. They have low oil content but are rich in polyunsaturated fatty acids (Miguel et al., 2004). The availability of pomegranate is seasonal and their use in animal feeding throughout the year requires preservation and storage. The main constrain to preserve pomegranate by-product is its high water content. Therefore, longterm storage of this by-product near the local factories may result in a considerable deterioration of the material and wastage of potential nutrients. Preservation of pomegranate by-products as silage and use of silage additives are likely to increase their consumption by improving their nutritive value. Microbial additives improved silage quality, nutrient digestibility and net energy for lactation, and reduced protein degradation (Filya, 2003; Rowghani et al., 2008a). It is shown that the inoculants of lactic acid bacteria (LAB) improved the fermentation quality, increased water soluble carbohydrates (WSC) and lactic acid contents, and decreased acetic acid, butyric acid, ammonia-N and also ensured a well-preservation of corn silage (Jatkauskas and Vrotniakiene, 2004). Addition of WSC during fermentation has been shown to improve silage quality of beet molasses (Rowghani et al., 2008b; Weinberg et al., 2008) and also results into an increase of CP content (urea) (Al Jassim et al., 1997; Rowghani et al., 2008b). Various approaches have been used to improve the nutritive value of pomegranate by-products, including addition of urea (Fernandez, 1998; Feyzi et al., 2010) and polyvinylpoly-pyrrolidon (Feyzi et al., 2010) as silage additives. There is little information about the nutritive value of pomegranate by-products or the effect of additives on its potentiality as feedstuff for ruminants in the literature.

The objective of this work was to study the effect of adding a microbial inoculant (*Lactobacillus plantarum*), urea and molasses on chemical composition, gas production and energy content of ensiled pomegranate seed pulp (PSP) and pomegranate peel pulp (PP).

Material and Methods

Randomly fresh PSP (after extracting juice) and PP samples were collected from the "Noshin Shahd" factory in Ferdows , Iran. The treatments were 100% PSP (T1), 100% PP (T2), 75% PSP and 25% PP (T3), 25% PSP seed and 75% PP (T4), 50 % PSP and 50% PP (T5), 50% PSP and 50% PP with 5% urea (T6), 50% PSP and 50% PP with 5% molasses (T7), 50% PSP and 50% PP with bacterial inoculant (T8), 50% PSP, 50% PP with 5% urea and 5% molasses and 50% PSP (T9), 50% PP with 5% urea and 5% molasses and bacterial inoculant (T10). All additives were added based on DM basis. The bacterial inoculant was applied at the recommended level of 1×10^5 CFU/g. The same volume of water used to dissolve the additives was added to the control (untreated treatments) to maintain equal moisture. Dark polyethylene bags were packed with 5 kg of treated materials, kept indoors (22°C) and opened after 30 days of ensiling. The samples were air-dried and ground (1 mm and 5 mm screen) for chemical analysis and *in vitro* gas production at the Animal Science Research Institute Laboratories in Birjand, Iran.

Chemical analysis

The dry matter (DM), ether extract (EE), organic matter (OM), ash, calcium (Ca), phosphorus (P) and crude protein (CP) contents of silage samples were determined following the procedures of AOAC (2000). Neutral detergent fibre (NDF) and acid detergent fiber (ADF) were measured according to the method of Goering and Van Soest (1970). The pH of each sample was determined using 25 g of wet material added to 100 ml of distilled water. After homogenizing for 10 min in a blender, the pH was determined using a digital pH meter (Pye Unicam, Phillips) (Polan et al., 1998). All analyses were carried out in triplicates.

***In vitro* gas production (GP), *in vitro* Organic matter digestibility (IVOMD), digestible organic matter in dry matter (DOMD), and metabolizable energy (ME) estimation**

Fermentation of PSP and PP samples were carried out with rumen fluid obtained from three mature canulated steers (450 kg BW) fed twice daily a diet consisting of 60% of Lucerne hay and 40% concentrate mixture (as fed basis) in equal portions every 12 h to maintain a relatively stable ruminal environment, following the method described by Menke and Steingass (1988). Rumen fluid was pumped using manually operated vaccum pump from the rumen into pre-warmed (39°C) thermos flasks. The rumen fluid from steers were mixed and filtered through four layers of cheese cloth and flushed with CO_2. The thoroughly mixed and CO_2 flushed rumen fluid was added to the buffered mineral solution (1:2 v/v), which was maintained in a water bath at 39°C and mixed. Buffered rumen fluid (30 ml) was pipetted into a syringe. The syringe was immediately placed in a water bath maintained at 39°C. Approximately, 200 mg of each sample was weighed into the graduated glass syringes of 100 ml. The glass syringes containing samples and rumen fluid-buffer mixture were incubated at 39°C. The syringes were gently shaken 30 min after the start of incubation. The gas production was determined at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h of incubation. To estimate the IVOMD and ME, triplicates of each sample were used

and the GP was corrected for the GP of buffered rumen fluid with no sample. Estimated ME (MJ/kg DM) concentration and IVOMD (g/kg) of the samples were calculated as described by Makkar (2004). DOMD (g/kg) was calculated as IVOMD × %OM.

Statistical Analysis

The data were subjected to analysis of variance using General Linear Model procedure of SAS, 1996. Mean separation was performed by the Duncan's multiple range tests, and the level of significance was set at 5%.

Results

The chemical composition of pomegranate by-products is presented in Table 1. DM content varied significantly among different treatments (P<0.05) except between T2 and T9 and between treatments T8 and T10. Treatment 3 had the highest DM content while T 2 and T9 had lowest DM contents (P<0.05). Ash content of T2 and T9 were highests and significantly differed from other treatments while T1 had lower (P<0.05) ash content than other treatments. Treatments 1, 3, 4, 5, 6 had significantly higher OM than other treatments while T2 and T9 had the lowest OM content (P<0.05). Crude protein was affected by treatments and was higher for T6 and was lower for T2 and T9 (P<0.05). NDF content was significantly higher in T3 and T5 while T4 had the lowest NDF content. The ADF content of silages was affected (P<0.05) by treatments. Treatments 2, 5 and 9 had significantly higher ADF and it was lowest in treatment 4. Treatments 1, 2 and 5 had higher (P<0.05) EE content compared to other treatments. Treatments 1, 2, 3, 9 and 10 had higher (P<0.05) Ca content compared to other treatments. Treatments 1, 3, 4, 5, 6, 7 and 10 had higher P content (P<0.05) and treatments 2 and 9 had the lower P content. As expected, treatments 6, 9 and 10 had higher pH values than other treatments and T8 had the lowest (P<0.05) pH value (3.80).

Table 2 presents *in vitro* gas production (GP), estimated ME, DOMD and IVOMD values of pomegranate by-product silages. Up to 8 h of incubation, GP values were highest in T8 (P<0.05) and from 12 to 96 h of incubation period, GP values were found highest in treatment 2 (100% PP). Treatments 2 and 9 had higher IVOMD and ME values and T9 had higher DOMD value compared to other treatments (P<0.05). Treatment 1 had lower (P<0.05) IVOMD, DOMD and ME values compared with other treatments.

Discussion

In semi-arid areas which have a scarcity of natural pastures and conventional animal foods, using pomegranate by-products for ruminant feeding could contribute to the development of livestock production system. Lower DM content of silages treated with molasses or molasses + urea compared with treatment 5 may have resulted from higher extent of fermentation by providing soluble substrates from molasses and the effect of urea on cell wall content of material (McDonald et al., 1991). The higher DM content of silages treated with inoculant (treatment 8) and molasses + urea + inoculant (treatment 10), might be due to the lower pH and lower DM loss of treatment 8 and high DM content of molasses (Hinds et al., 1985) and less seepage from the molasses-treated silages, respectively (McDonald et al., 1991). The increased CP content of urea-treated silages was a consequence of urea addition (Khorasani et al., 2010). The lower CP content of treatment 9 compared with the treatment 5, might be due to the volatilization of NH_3 or sampling error. Addition of molasses increased (P<0.05) CP content (treatment 7) compared with treatment 5 which is in agreement with the findings of Lattemae et al. (1985) and Kennedy (1990). There was some reduction in NDF content in treatments 6, 7, 8, 9 and 10 compared with treatment 5 which might be due to the acid hydrolysis of fibre, the effect of urea on cell-wall or higher extent of fermentation by providing soluble substrates from molasses (McDonald, 1981; Deinum and Massen, 1994). The lower ADF content of treatments 6, 7, 8, 9 and 10 compared with treatment 5 is an indication of cell wall digestion during fermentation (McDonald, 1981; Bolsen et al., 1996). All silages (except urea-treated silages) had pH values less (P<0.05) than 4.41, indicating successful preservation and fermentation. The lowest pH value was in inoculant-treated silage (3.80), which can minimize the growth of clostridia due to lower concentration of butyric acid (McDonald, 1981). The pH values in urea-treated silages was highest (P<0.05) due to urea addition, which can be explained by the conversion of urea to ammonia (Rowghani et al., 2008b).

Up to 8 h of incubation, the GP was highest (P<0.05) for treatment 8 than others and from 12 up to 96 h of incubation treatment 8 was intermediate in GP. GP has negative relation with NDF content and a positive relation with starch content of material (De Boever et al., 2005). In addition, it has been reported that GP in phase 1 (fermentation of soluble fraction) of incubation was affected by soluble fraction "a" for corn silage (Cone et al., 1997). Although fraction "a" was not measured in present experiment, the high GP in inoculant-treated silage might be due to the effect of microbial inoculation in degrading the lignocellulose fraction of the cell wall and providing more soluble materials (Bolsen et al., 1996; Adesogan and Salawu, 2004). Besides, treatment 8 had intermediate NDF content. From 12 to 96 h of incubation, treatment 2 had the highest (P<0.05) GP which might be due to low NDF content. Lowest GP was seen in urea, urea + molasses and urea +molasses + inoculant treated silages. Fernandez (1998) reported that silages with high CP content had lower GP due to the fact that in the high

Table 1: The chemical composition (DM basis) and pH of pomegranate seed pulp and pomegranate peel after 30 days of ensiling

	Treatments										
Parameters	1	2	3	4	5	6	7	8	9	10	SEM
DM	38.4^{b}	24.9^{h}	39.5^{a}	28.70^{f}	31.8^{e}	34.40^{d}	27.70^{g}	36.40^{c}	24.90^{h}	36.40^{c}	0.23
OM	96.85^{a}	94.08^{c}	96.03^{a}	96.33^{a}	96.20^{a}	96.36^{a}	95.89^{ab}	95.17^{b}	94.08^{c}	95.98^{a}	1.87
Ash	3.15^{d}	5.92^{a}	3.97^{bcd}	3.67^{dc}	3.80^{dc}	3.68^{c}	4.11^{bc}	4.83^{b}	5.92^{a}	4.02^{bcd}	0.13
CP	10.36^{c}	3.7^{g}	9.22^{d}	8.43^{e}	8.05^{e}	39.32^{a}	10.04^{c}	7.75^{f}	3.70^{g}	30.22^{b}	2.19
NDF	39.19^{d}	39.80^{d}	42.29^{a}	37.93^{e}	42.66^{a}	41.82^{ab}	41.98^{ab}	41.7^{ab}	39.80^{d}	40.31^{c}	1.06
ADF	29.71^{c}	31.11^{a}	30.49^{ab}	28.58^{d}	31.52^{a}	30.74^{ab}	30.68^{ab}	29.11^{c}	31.11^{a}	28.66^{d}	2.21
EE	11.37^{a}	10.80^{a}	10.44^{b}	10.11^{b}	11.03^{a}	10.00^{b}	10.32^{b}	10.16^{b}	10.80^{b}	10.25^{b}	0.47
Ca	0.435^{a}	0.420^{a}	0.415^{a}	0.170^{b}	0.175^{b}	0.190^{b}	0.190^{b}	0.180^{b}	0.420^{a}	0.430^{a}	0.05
P	0.039^{a}	0.014^{c}	0.031^{a}	0.037^{a}	0.032^{a}	0.03^{a}	0.037^{a}	0.028^{b}	0.014^{c}	0.035^{a}	0.03
pH	4.19^{b}	4.41^{b}	4.26^{b}	4.00^{b}	3.91^{b}	5.47^{a}	4.00^{b}	3.80^{b}	5.15^{a}	5.31^{a}	0.07

Means within a row with similar superscript(s) are not significantly different (Duncan's test, P>0.05). *Treatments 1) %100 SPP , (2) %100 PP, (3) % 75 SPP and %25 PP, (4) %25 SPP and %75 PP, (5) %50 PSP and % 50PP , (6) %50 PSP and % 50 PP with %5 urea, (7) %50 PSP and % 50 PP with %5 molasses, (8) %50 PSP and % 50PP with bacterial inoculant (1×10^{5} CFU/g) , (9) %50 PSP,% 50 PP with %5 urea and %5 molasses and (10)) %50 PSP and % 50 PP with %5 urea and %5 molasses and bacterial inoculant (1×10^{5} CFU/g). SEM= standard error of the mean.

Table 2: In *vitro* gas production (ml/200 mg DM) and estimated parameters of pomegranate by-product at different incubation time

Gas production	Treatments										
after (h)	1	2	3	4	5	6	7	8	9	10	SEM
2	5.95^{c}	7.04^{ab}	6.89^{ab}	6.92^{ab}	6.68^{b}	4.96^{d}	7.26^{a}	7.30^{a}	5.28^{d}	4.93^{d}	0.087
4	9.43^{c}	11.57^{b}	11.65^{b}	11.55^{b}	11.39^{b}	7.97^{d}	12.04^{ab}	12.61^{a}	8.57^{d}	8.22^{d}	0.191
6	12.08^{d}	15.77^{ab}	14.94^{c}	14.68^{c}	15.13^{cd}	9.47^{f}	16.00^{a}	16.43^{a}	10.39^{e}	9.07^{ef}	0.182
8	15.39^{d}	20.30^{ab}	18.72^{c}	18.15^{c}	19.69^{b}	11.80^{f}	19.96^{b}	20.07^{a}	13.19^{e}	12.66^{ef}	0.314
12	19.20^{e}	27.52^{a}	22.50^{d}	21.95^{d}	26.04^{b}	15.04^{g}	24.09^{c}	24.89^{c}	17.49^{f}	16.77^{f}	0.421
24	21.68^{g}	38.92^{a}	29.23^{d}	25.24^{e}	36.29^{b}	19.36^{h}	28.54^{d}	32.19^{c}	23.26^{f}	20.72g	0.503
48	21.82^{f}	44.63^{a}	32.22^{d}	26.90^{ef}	41.01^{b}	24.02^{g}	33.16^{d}	35.52^{c}	27.60^{e}	23.52g	0.477
72	26.39^{g}	46.14^{a}	31.17^{d}	28.30^{f}	42.15^{b}	26.18^{g}	34.32^{d}	36.84^{c}	30.36^{e}	25.32^{g}	0.467
96	26.81^{g}	47.31^{a}	34.15^{d}	28.87^{f}	42.96^{b}	27.01^{g}	34.81^{d}	37.50^{c}	30.85^{e}	26.47g	0.470
IVOMD(g/kg)	40.85^{g}	55.01^{a}	47.95^{e}	43.50^{f}	53.27^{b}	52.11^{c}	47.43^{e}	50.13^{d}	54.71^{a}	49.46^{d}	0.421
DOMD(g/kg)	39.56^{f}	51.75^{a}	45.70^{d}	41.90^{e}	51.22^{a}	50.19^{b}	45.48^{d}	47.71^{c}	51.47^{a}	47.47^{c}	0.378
ME(MJ/kg DM)	5.73^{c}	7.69^{a}	6.69^{b}	6.10^{b}	6.64^{b}	7.08^{ab}	6.64^{b}	7.01^{ab}	7.46^{a}	6.74b	0.321

DOMD was calculated as IVOMD× % OM

concentrations of ruminal $NH4^{+}$, $NH4^{+}$ will react with fatty acids which in turn will result in lower CO_2 production. The same pattern was found in the present work for urea-treated silages which is in agreement with the findings of Feyzi et al. (2010). Treatments 2 and 9 had higher (P<0.05) IVOMD, DOMD and ME values which might be due to their lower NDF content. The results for treatment 2 is in agreement with the findings of Feyzi et al. (2010) who reported higher IVOMD and ME in silages without urea. GP was positively correlated to IVOMD, DOMD and ME for treatment 2 which is in the line with the findings of Al-Masri (2003). The IVOMD and ME values for treatments 2 and 9 are very similar to the values reported by Feyzi et al. (2010) for pomegranate peel. Van Soest and Robertson, (1985) showed a highly significant and positive relation between CP and *in vitro* apparent and true digestibility. The volume of GP has a close association with level of intake (Blummel and Becker, 1997) and cow growth rate (Blummel and Ørskov, 1993). Tannin content of PSP and PP was not measured in the current experiment but any discrepancies in the results of GP might be due to the high content of tannin in pomegranate by-product and also the fact that different sources of tannins have different natures and different biological responses (Khazaal et al., 1994; Antenello, 1997; Makkar, 2003). There was no significant difference between PSP and PP silages without additive (T1 and T2) for EE. This result is in close agreement with the findings of Mirzaei-Aghsaghali et al. (2011).

Conclusion

The results of the current study confirmed the findings of other researchers that pomegranate by-product can be preserved well by ensiling. The application of molasses and urea at ensiling can improve its chemical composition and nutritive value. This by-product provides a cheap energy source and fibre for the ruminant animal which reduces the costs related to waste management of this potential pollutant. Additional farm trials, however, are needed to confirm the findings of the present study.

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