Effects of different carbohydrate sources on activity of rumen microbial enzymes and nitrogen retention in sheep fed diet containing recycled poultry bedding

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ABSTRACT
In a completely randomized design, 15 male Moghani sheep were fed to determine the influence of supplementing recycled poultry bedding (RPB) with different carbohydrate sources on the activities of rumen hydrolytic enzymes and nitrogen retention. In the first diet, corn and barley were used as the carbohydrate sources (M0). In the second and third diets, corn and barley were replaced by 50 (M50 diet) and 100 (M100 diet) g/kg of sugar beet molasses, respectively. The extracellular activity of carboxymethyl-cellulase (CMCase) was increased linearly by dietary inclusion of molasses [linear (L), \( P < .05 \)]. The extracellular and total activity of microcrystalline-cellulase in ruminal content was increased linearly (L, \( P < .05 \)), while its cellular activity remained unaffected (\( P > .05 \)) by dietary inclusion of molasses. Dietary inclusion of molasses in RPB-containing diets linearly increased filter paper degrading (FPD) activity in all fractions of ruminal content (L, \( P < .05 \)). Addition of molasses to the diets linearly decreased (L, \( P = .04 \)) total activity of \( \alpha \)-amylase, and cellular and total activity of proteases. Increasing dietary levels of molasses linearly increased retained N (L, \( P < .05 \)). In conclusion, dietary supplementation of RPB-containing diet with molasses improved activities of fibrolytic enzymes and nitrogen retention in sheep.

1. Introduction
Poultry bedding (PB) is a solid waste consisting of poultry excreta (urine and faeces), bedding material, feathers and spilled feed (Azizi-Shotorkhoft et al. 2012). In Iran, production of dry PB exceeds 1.3 million metric tonnes annually (Azizi-Shotorkhoft et al. 2012).

The proper use of inexpensive agro-industrial by-products such as PB is important to have a more profitable livestock production (Negesse et al. 2007). The commercial value of PB as a feedstuff is based on its crude protein content (CP) (150–350 g/kg dry matter (DM)) (Obeidat et al. 2011). However, most of its nitrogen (N) content is in the form of non-protein nitrogen (NPN), which can be easily and rapidly degraded in the rumen (Animut et al. 2002).

Rapid N degradation causes an imbalance between the supply of N and energy in the rumen for microbial protein synthesis (MPS). Excess ruminal protein is mainly absorbed as ammonia (\( \text{NH}_3 \)) by the rumen and is mainly lost by the animal (Broderick 1995; Yu et al. 2004). Excessive ruminal protein degradation also results in a smaller portion of dietary protein escaping to the lower digestive tract, a process which is required for optimum animal performance of high-producing ruminants (Dhiman & Satter 1993; Klopfenstein 1996). Besides, an imbalance between supply of rumen degradable N and rumen available energy may also impair rumen degradation of dietary fibres as fiber-degrading microorganisms require a synchronized supply of energy-yielding and N-containing substrates (Azizi-Shotorkhoft et al. 2012). A feeding strategy when an ingredient containing a high level of rumen degradable N (such as recycled poultry bedding, RPB) included in the diets is to provide the animal with a readily available source of rumen-fermentable carbohydrates. Molasses is one such an ingredient which is rich in water-soluble carbohydrates, and is an inexpensive source of energy in some regions compared to starchy feeds such as corn and barley (Oba 2011).

In the present study, we hypothesized that the activity of rumen microbes, measured as the activities of rumen hydrolytic enzymes, and nitrogen retention would improve when sheep ration containing RPB, as the main source of rumen degradable N, was supplemented with sugar beet molasses when compared to starchy cereals, namely barley and corn.

2. Materials and methods
2.1. Recycled poultry bedding
Hydrothermally processed broiler bedding (RPB; indirect pressurized vapour, 80°C for 20 min) with a geometrical mean diameter of 6 mm was purchased from a commercial company (Sabzevar, Khorasan, Iran). The RPB was a mixture of bird excreta (urine and faeces) and bedding material, including wood shaving, feathers and dropped feed.
2.2. Animal study

The experiment was approved by the Animal Care and Ethics Committee of Tarbiat Modaress University (Tehran, Iran). Fifteen healthy male Moghani sheep (two years old: 62 ± 2.3 kg of live weight) were individually housed in the metabolic cages, where their faeces and urine were quantitatively collected. The animals were randomly assigned to the three experimental diets in a completely randomized design with five sheep in each diet-treatment. The three experimental diets (Table 2) consisted of 260 g alfalfa hay, 260 g wheat straw and 240 g RPB per kg of DM, and were supplemented with either 120 g corn and 120 g barley per kg of DM (control diet; M0), control diet in which 25 g of barley and 25 g of corn were replaced by 50 g of sugar beet molasses per kg of DM (M50 diet) and control diet in which 50 g of barley and 50 g of corn was replaced by 100 g of sugar beet molasses per kg of DM (M100 diet). All experimental diets were formulated to meet the nutrient requirements of animals based on National Research Council recommendations (2007). Sheep were individually fed twice daily at 08:00 and 16:00 h with equal aliquots. The animals had free access to experimental diets, fresh water and mineral-vitamin blocks. The experimental diets had no differences in type and proportion of ingredients except for main carbohydrate source (i.e. corn, barley or molasses) and their ratios in order to avoid the influences of ingredient characteristics and intake level on the activity of rumen hydrolytic enzymes (Seo et al. 2010).

2.3. Rumen liquor sampling

The experimental period lasted for 28 days with 21 days for adaptation period to metabolic cages and experimental diets, and 7 days for samples collection (Givens et al. 2000). On day 5 of collection period, rumen liquid (RL) samples (50–60 mL) were taken just before morning feeding, 3 and 6 h post feeding using a flexible polyvinyl chloride stomach tube. The rumen contents were immediately transferred to the laboratory in a preheated (39°C) insulated thermos flask.

2.4. Fractionation of RL and enzymes extraction

The microbial enzymes in different fractions of RL samples were extracted as described by Hristov et al. (1999). Approximately 50 mL of rumen contents were strained (strained rumen liquor (SRL)) through four layers of cheese cloth and divided into two fractions, extracellular (liquid portion) and cellular (cell suspended freely in the liquid portion of RL). About 10 mL of SRL was centrifuged at 450×g for 5 min at 37°C and the pellet was considered as protozoal fraction (PF). The supernatant was then centrifuged at 27000×g for 20 min at 4°C and the pellet was considered to be bacterial fraction (BF). After centrifuging, clear supernatant was used as the source for extracellular fraction. For the extraction of enzymes from cellular fraction, pellets containing microbial biomass (PF plus BF) were suspended in a volume of 0.1 M phosphate buffer (pH 6.8) equal to the extracellular liquid. Lysozyme solution (4 g/L) and carbon tetrachloride were separately added to the suspension at the rate of 5 mL/30 mL cell suspension. Lysozyme treatment was followed by sonication in ice bath for 6 min with 30 s pulse rate and power supply of 0.5. The suspension was then incubated for 3 h at 39°C and centrifuged at 27000×g for 30 min at 4°C. The supernatant was collected and used as enzyme source for cellular fraction.

2.5. Enzyme activity assay

Activity of microbial enzymes in different fractions of rumen content was determined as described in detail by Agarwal (2000). For the estimation of carboxymethyl-cellulase (CMCase) activity, the reaction mixture contained 1 mL phosphate buffer (0.1 M, pH 6.8), 0.5 mL strained RL (SRL), 0.5 mL carboxymethyl cellulose (carboxymethyl cellulose; 10 mg/mL for CMCase) and was incubated for 60 at 39°C. To determine the activity of microcrystalline-cellulase (MCCase), the reaction mixture, incubation temperature and time was similar to that of CMCase except that 1 mL of microcrystalline cellulose (10 mg/mL) was used as the reaction substrate. Reaction mixture to determine α-amylase activity consisted of 1 mL of 0.1 M phosphate buffer (pH 6.8), 0.5 mL of SRL and 0.5 mL of starch solution (1%, as the reaction substrate), which was incubated for 60 min at 39°C. For filter paper degrading (FPD) activity the assay mixture contained 1 mL phosphate buffer (0.1 M, pH 6.8), 0.5 g filter paper (Whatman No. 1) and 1 mL SRL, which was incubated for 60 min at 39°C. All reactions were stopped by the addition of dinitro-salicylic acid reagent. The glucose produced in all reactions was estimated according to the method described by Miller (1959) taking glucose as standard. The activities of CMCase, MCCase and α-amylase were then calculated considering that one unit of enzyme was able to produce 1 µmol glucose per hour per mL from degradation of respective substrates.

For estimation of the protease activity (µg hydrolysed protein/h/mL), the reaction mixture which contained 1 mL phosphate buffer (0.1 M, pH 6.8), 0.25 mL SRL and 0.25 mL casein (2.5 mg/mL) was incubated for 2 h at 39°C. The reaction was stopped by adding trichloroacetic acid (200 mL/L) and protein was determined according to the procedure of Lowry et al. (1951).

2.6. Nitrogen retention

To measure nitrogen balance, the animals in each treatment group were individually housed in metabolic cages. After 21-day adjustment period, faeces were collected during 7 days and daily aliquot (150 g/kg) was stored at −4°C. Faecal samples were dried to a constant weight in a forced air oven at 60°C and stored before grinding for chemical analyses. During the collection period, urine was collected in buckets containing 100 mL of sulphuric acid (H2SO4; 10% v/v). Urine samples (100 mL) were stored at −4°C. At the end of the collection period, an aliquot of the composted urine from each sheep was sampled and analysed for total urinary nitrogen (UN).

2.7. Laboratory analysis

The samples of RPB, molasses and experimental diets and faeces were oven-dried at 55°C for 72 h and ground to pass
through a 1 mm sieve (Wiley mill, Swedesboro, USA) and were analysed for DM (AOAC, 930.15), ash (AOAC, 942.05), N (AOAC, 984.13) and ether extract (EE, AOAC, 954.02) according to AOAC (1990). The ADFom and anNDFom (without sodium sulphite) were determined according to the procedures of Van Soest et al. (1991). Lignin(sa) was determined by solubilization of cellulose with 720 g/kg sulphuric acid, according to the procedure of Robertson and Van Soest (1981). NPN was analysed according to Licitra et al. (1996). Metabolizable energy (ME) of RPB was calculated as described elsewhere (Azizi-Shotorkhoft et al. 2012). Starch content of the RPB and experimental diets were analysed according to AOAC (996.11; 1990).

2.8. Statistical analysis

Statistical analyses were performed using the GLM procedure of SAS (Version 9.1, SAS Institute, Cary, NC, USA) using the following model:

\[ Y_{ij} = \mu + T_i + e_{ij} \]

where \( Y_{ij} \) is an observation of dependent variable, \( \mu \) is the population mean for the variable, \( T_i \) is the effect of treatment (\( i = 3 \); M0, M50 and M100) and \( e_{ij} \) is the random error associated with the observation \( ij \). For all analyses, specific orthogonal contrasts were used to test linear (L) and quadratic (Q) effects of treatments. Significance was declared at \( p \leq 0.05 \) and trends at \( p \leq 0.10 \), unless otherwise stated.

3. Results

Chemical composition and ME of RPB and liquid molasses are presented in Table 1.

### 3.1. Enzyme activity

The activity of CMCase in the extracellular fraction of ruminal content was increased linearly (L, \( P < 0.05 \)), while the cellular and total (extracellular plus cellular fraction) activity of this enzyme remained unaffected as the inclusion level of molasses increased in the diets (Table 3). The extracellular and total activity of MCCase in ruminal content were increased linearly (L, \( P < 0.05 \)), while its cellular activity remained unchanged (\( P > 0.05 \)) as the dietary levels of molasses increased (Table 3). The activity of FPD showed a linear increase in all fractions of ruminal content with increasing the inclusion level of molasses was higher (L, \( P < 0.05 \); Table 3).

Addition of molasses to the diets linearly decreased (L, \( P = 0.04 \)) total activity of \( \alpha \)-amylase, and cellular and total activity of proteases. However, their activities were similar in the other fractions of ruminal content (Table 3).

### 3.2. N retention

The N intake, N excreted via faeces and urine were not affected (\( P > 0.05 \)) by dietary inclusion of molasses (Table 4). Increasing feeding levels of molasses, linearly decreased total N excreted (L, \( P = 0.04 \)). Consequently, retained N increased linearly (L, \( P < 0.05 \)) when sheep were fed with molasses-containing diets (Table 4).

4. Discussion

4.1. Enzyme activity

Studies carried out on the effects of synchronized supply of N and energy sources in ruminants have mostly focused on productive performance of animals. The present study was among the few studies in which the effects of synchronized supply of N and energy sources on the activity of rumen microbial enzymes were assessed.

In agreement with published results (Agarwal 2000), the activities of microbial enzymes (CMCase, MCCase, \( \alpha \)-amylase, FPD activity and proteases) were higher in the cellular fraction of rumen content compared with the other fractions. This may be attributed to the higher numbers of particle-associated bacteria, which comprise 50–70% of rumen microbial population (Minato et al. 1966) and are mostly responsible for fibrolytic enzyme activities and mainly involved in fibre digestion (Cheng & McAllister 1997; Raghuvansi et al. 2007).

The rumen microbial enzyme activities are a qualitative reflection of rumen microbes involved in the digestion of feed (Raghuvansi et al. 2007; Kamra et al. 2010). In the present study, the activity of fibrolytic enzymes (CMCase, MCCase and FPD) linearly improved when sheep ration containing RPB, as the source of rumen degradable N, was supplemented with increasing levels of molasses rather than with starchy feeds, namely barley and corn. This would show a more synchronized supply of nitrogen and energy to rumen microbes, which might have led to an improved fibre digestion in the rumen. Indeed, Azizi-Shotorkhoft et al. (2012) observed that supplementing RPB with molasses compared with barley and corn improved total tract digestibility of NDF. Rumen ammonia concentration is an index for investigating the effect of nutrient synchrony on rumen performance. In our previous work, supplementing RPB with increasing levels of molasses decreased rumen ammonia concentration and increased microbial growth and N efficiency compared with starchy diets in sheep (Azizi-Shotorkhoft et al., 2013). Additionally, reduction in ruminal ammonia concentration and improving MPS by replacing wheat starch with sucrose in grass-silage-based diets fed to mature sheep has been already reported (Chamberlain et al., 1993).

### Table 1. Chemical composition and ME of RPB and liquid molasses (g/kg DM or as stated).

| Item           | RPB  | Liquid molasses |
|----------------|------|-----------------|
| DM (g/kg fresh weight) | 930  | 870             |
| CP             | 238  | 81.0            |
| NPN (g/kg total N) | 451  | 763             |
| EE             | 22.3 | 2.00            |
| NDFom          | 353  | 0.0             |
| ADFormat       | 185  | 0.0             |
| Lignin(sa)     | 75.0 | 0.0             |
| Ash            | 184  | 112             |
| ME (MJ/kg DM)  | 9.3  | 11.7            |

Note: DM, dry matter; CP, crude protein; NPN, non-protein nitrogen; EE, ether extract; NDFom, ash-free neutral detergent fiber; ADFormat, ash-free acid detergent fiber; ME, metabolizable energy.
The α-amylase enzyme plays an important role in the rumen because it hydrolyses dietary starch (Engvall 1980). The lower α-amylase activity observed by molasses supplementation in the present study could be due to a less available starch for α-amylase activity observed by molasses supplementation in the present study could be due to a less available starch for amylo-lytic microbes (Table 2). Nasr (1950) also showed a positive correlation between rumen α-amylase activity and the amount of starch offered to sheep. However, Azizi-Shotorkhoft et al. (2014) recently found that sheep fed molasses-containing diet had a higher rumen α-amylase activity than those fed with corn- or barley-containing diets. The reason for this inconsistency is not clear.

### Table 2. Ingredients and chemical composition (g/kg DM or as stated) of the experimental diets containing RPB.

| Ingredients   | M0   | M50  | M100  |
|---------------|------|------|-------|
| Alfalfa hay   | 260  | 260  | 260   |
| Wheat straw   | 260  | 260  | 260   |
| Ground corn   | 120  | 95.0 | 70.0  |
| Ground barley | 120  | 95.0 | 70.0  |
| Liquid molasses | 0.0  | 50.0 | 100   |

Note: DM, dry matter; CP, crude protein; NDFom, ash-free neutral detergent fiber; ADFom, ash-free acid detergent fiber; CP, metabolizable energy. Chemical compositions of the diets were calculated from each feed composition.

### Table 3. Effect of replacing corn/Barley with molasses on rumen microbial enzymes activities (µmol/h per mL or as stated) in sheep fed experimental diets containing RPB.

| Enzymes activity | Experimental diets<sup>a</sup> | M0 | M50  | M100  | SEM | L | Q |
|------------------|-------------------------------|----|------|-------|-----|---|---|
| Carboxymethyl-cellulase (CMCase) | Extracellular | 70.2 | 74.3 | 84.3  | 0.99 | <0.01 | 0.10 |
|                   | Extracellular | 103 | 110  | 99.1  | 5.94 | 0.66 | 0.24 |
|                   | Total         | 173 | 185  | 183   | 6.59 | 0.31 | 0.45 |
| Microcystalline-cellulase (FPG) | Extracellular | 13.7 | 17.9 | 25.7  | 0.78 | <0.01 | 0.11 |
|                   | Extracellular | 46.1 | 42.3 | 53.5  | 3.76 | 0.21 | 0.16 |
|                   | Total         | 59.7 | 60.2 | 79.2  | 4.20 | 0.02 | 0.12 |
| α-amylase activity | Extracellular | 154 | 140  | 136   | 8.21 | 0.17 | 0.64 |
|                   | Extracellular | 312 | 319  | 288   | 10.9 | 0.18 | 0.20 |
|                   | Total         | 465 | 459  | 424   | 12.4 | 0.04 | 0.38 |
| Protease activity (µg/h per mL) | Extracellular | 79.3 | 71.2 | 67.2  | 5.37 | 0.16 | 0.76 |
|                   | Extracellular | 156 | 151  | 144   | 3.24 | 0.04 | 0.75 |
|                   | Total         | 235 | 222  | 211   | 6.66 | 0.04 | 0.93 |

Note: L; linear; Q; quadratic; CMCase; carboxymethyl-cellulase, MCCase; microcrys-talline-cellulase, FPD; filter paper degrading. Values of enzyme activity are means across sampling times of 0, 3 and 6 h after morning feeding.

<sup>a</sup>Diets were: molasses at the levels of 0 (M0), 50 (M50) or 100 (M100) g/kg DM in the diets containing RPB.

### Table 4. Effect of replacing corn/Barley with molasses on N retention (g/day or as stated) in sheep fed experimental diets containing RPB.

| Experimental diets<sup>a</sup> | M0 | M50  | M100  | SEM | L | Q |
|------------------------------|----|------|-------|-----|---|---|
| N intake                     | 24.2 | 24.7 | 25.2   | 0.78 | 0.40 | 0.97 |
| Faecal N excreted            | 9.14 | 9.03 | 8.73   | 0.176 | 0.16 | 0.68 |
| Urinary N excreted           | 11.1 | 10.9 | 10.6   | 0.171 | 0.10 | 0.98 |
| Total N excreted             | 20.3 | 19.9 | 19.4   | 0.26 | 0.04 | 0.76 |
| Retained N                   | 3.94 | 4.75 | 5.82   | 0.57 | 0.04 | 0.85 |
| Retained N (g/kg BW)         | 0.067 | 0.075 | 0.099   | 0.004 | <0.01 | 0.18 |
| Retained N (g/ BW<sup>b</sup>) | 0.18 | 0.22 | 0.28   | 0.003 | <0.01 | 0.11 |

Note: L; linear; Q; quadratic.

<sup>a</sup>Molasses at the levels of 0 (M0), 50 (M50) or 100 (M100) g/kg DM in the diets containing RPB.

Poultry litter is a good source of CP for ruminants. However, most of its protein content is in the form of NPN, which can be easily and quickly degraded by rumen microorganisms, leading to surplus ammonia production in the rumen (Van Ryssen 2001; Azizi-Shotorkhoft et al. 2014). It has been known that ruminal degradation of proteins should be restricted to an extent that is necessary for maintaining efficient microbial activity and growth, provided that the rumen undegraded fraction is digested in the small intestine (Goelena 1999). It has been demonstrated that up to 70% of dietary protein is degraded by the combined actions of microbial proteases and peptidases (Selinger et al. 1996). The linear reduction of proteolytic activity in sheep fed RPB-containing diets supplemented with molasses compared to those fed M0 diet might be a reflection of less protein being degraded in the rumen. This might show that when sheep are fed diets containing an ingredient rich in NPN (like RPB), providing a readily available source of soluble sugars (such as molasses) could decrease unnecessary rumen degradation of dietary proteins.

### 4.2. N retention

The improvement of N retention in sheep fed molasses-containing diets (M50 and M100) than those fed with the M0 may be attributed to a synchronized supply of more readily fermentable energy source (i.e. sucrose in molasses) with soluble N of RPB, which led to a higher production of microbial protein in the rumen (Sinclair et al. 1993; Van Soest 1994; Richardson et al. 2003). Indeed, it is reported that supplementing RPBC-containing diets with molasses compared to barley and corn improved microbial protein synthesized in the rumen of sheep (Azizi-Shotorkhoft et al. 2012).

Moreover, lower rumen protease activity, as the indication of less protein being degraded in the rumen, might be another plausible explanation for the higher retained N observed in M50- and M100-fed sheep (Table 3). In agreement with our results, N retention improved when corn was replaced by molasses in the ration of feedlot calves (Hatch & Beeson 1972). Adding sucrose to a basal diet of grass silage as energy source compared with starch increased MP synthesized in the rumen and efficiency of N utilization in mature wethers (Chamberlain et al. 1993). In contrast, Broderick and Radloff (2004) showed that the efficiency of N utilization in dairy cows was linearly decreased when corn was replaced by sucre.
5. Conclusion

Incorporation of molasses in RPB-containing diet improved activities of fibrolytic enzymes in sheep, while the activities of α-amylase and protease were decreased. Additionally, supplementing RPB-containing diet with increasing levels of molasses increased N retention due to a less N exerted via urine. This could have implications on improved feed efficiency and reduced N loss to the environment.

Disclosure statement

No potential conflict of interest was reported by the authors.

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