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ORIGINAL ARTICLE

Effect of forage inclusion and particle size in diets of neonatal lambs on performance and rumen development

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Summary

A slaughter experiment was conducted to determine the effects of alfalfa particle size on rumen morphology and performance of lambs. Twenty-four Balouchi lambs aged 21 days (9.1 ± 1.1 kg) were randomly fed control (diet without alfalfa hay; CON) and mixed rations containing 15% finely ground (FINE; 2 mm) and 15% coarsely chopped alfalfa hay (LONG; 3 to 4 cm). After a 63 days feeding period, nine animals (three per treatment) were slaughtered to obtain ruminal tissue samples for morphological analyses. Alfalfa particle size did not affect ($p > 0.05$) papillae density, height, width, epithelium depth and surface area. Coarse alfalfa decreased the *stratum corneum* and increased ($p < 0.05$) muscle depth compared with fine and control diets. Neither DNA content and nor RNA concentration of rumen tissue was affected by feeding different diets. Forage particle size did not affect the blood concentration of glucose, urea nitrogen (BUN), beta-hydroxybutyric acid (BHBA) and non-esterified fatty acids (NEFA). Dry matter intake and feed conversion ratio were higher for control diet; however, there were no significant differences between treatments for average daily gain. These data suggest that coarse alfalfa significantly reduces the *stratum corneum* and increases muscularity of rumen wall and tended to better feed conversion ratio.

Keywords rumen development, particle size, alfalfa, lamb, performance

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Introduction

The gastrointestinal epithelium is responsible for many physiological functions, including absorption, transportation and metabolism of nutrients. Understanding the effect of the physical form and nutrient composition of the diet on the ruminal epithelium could lead to changes in dietary regimens that exploit beneficial tissue response (Baldwin, 1998). Previous studies have shown that increasing grain intake stimulated rumen mucosal development in growing ruminants (Zitnan et al., 2003; Odongo et al., 2006). This is related to increases in the supply of volatile fatty acids and rumen digestible starch (Lane and Jesse, 1997). However, excessive grain intake or low diet particle size decreases the rumen papillae development in growing ruminant (Wang et al., 2008; Norouzian et al., 2011). Ration particle size affects the ruminal environment, volatile fatty acid production, and papillae structure and function (Coverdale et al., 2004). There is limited information on forage particle size on gastrointestinal development in growing lambs. Therefore, the objective of this study was to determine the effects of alfalfa particle size on rumen morphology and performance of newborn lambs.

Materials and methods

Animals and treatments

The research proposal was reviewed and approved by the local animal care committee. Twenty-four 21-day old Balouchi lambs (9.1 ± 1.1 kg BW) were assigned to the following treatments: a ground starter (without alfalfa, control; CON; $n = 8$) and diets containing (on a dry-matter basis) 85% concentrate and either 15% coarsely chopped (3 to 4 cm) alfalfa hay (LONG; $n = 8$) and 15% finely ground (2 mm) alfalfa hay (FINE; $n = 8$, Tables 1 and 2).

The lambs were separated from their mothers at 3 weeks of age, housed in individual pens. Pens were equipped with feed boxes and plastic troughs for providing the constant supply of fresh water. The diets were offered *ad libitum,* and the intake was checked daily. The refusals were removed from the individual

Table 1 Composition of the experimental diets

Ingredients (%)	Diet*		
	CON	FINE	LONG
Corn	58	58	58
Soybean meal	21	21	21
Alfalfa hay	0	15	15
Wheat bran	15	0	0
Beet molasses	4	4	4
Vitamin–mineral pre-mix†	0.4	0.4	0.4
Salt (NaCl)	0.2	0.2	0.2
Limestone	1.4	1.4	1.4

*CON, the control diet (without alfalfa hay); FINE, diet containing 15% fine alfalfa hay; LONG, diet containing 15% coarse alfalfa hay.
†Each kg of vitamin–mineral pre-mix contained: vitamin A (50 000 IU), vitamin D3 (10 000 IU), vitamin E (0.1 g), calcium (196 g), phosphorus (96 g), sodium (71 g), magnesium (19 g), iron (3 g), copper (0.3 g), manganese (2 g), zinc (3 g), cobalt (0.1 g), iodine (0.1 g) and selenium (0.001 g).

Table 2 Chemical composition and physical characteristics of the experimental diets

Measurement	Diet*		
	CON	FINE	LONG
Chemical composition			
Dry matter (%)	90.2	91.1	90.3
Crude protein (%)	18.3	18.2	18.2
Ether extract (%)	2.35	2.31	2.30
Neutral detergent fibre (%)	15	15.3	15.3
Acid detergent fibre (%)	6.4	8.4	8.4
Ash (%)	3.1	4.1	4.2
Physical characteristics			
Bulk density (g/cm^3)	0.71^a	0.61^b	0.57^c
Water-holding capacity (g/g DM)	2.57^c	2.93^b	2.97^a
Particle size distribution (% of DM)			
19 mm	0	0	0
8 mm	0^b	0^b	2^a
1.18 mm	58.0^b	57.0^b	63.0^a
Pan	42.0^a	43.5^a	35.0^b
Physical effective factor (pef$_{>1.18}$)†	60.0^b	57.0^b	70.0^a
Physical effective NDF (peNDF)‡	8.84^b	8.60^b	10.57^a
Geometric mean of particle size (mm)	2.02^b	1.94^b	2.43^a
Abrasive value (g)	5.5^c	9.0^b	15.5^a

Means with different superscripts within a row differ significantly ($p < 0.05$).
*CON, the control diet (without alfalfa hay); FINE, diet containing 15% fine alfalfa hay; and LONG, diet containing 15% coarse alfalfa hay.
†pef was determined based on proportion of DM retained on the 1.18-mm sieve.
‡peNDF was calculated by multiplying NDF content of the feed by the pef.

feeders daily and weighed. The lambs were weighed at week 0 and then once a week (up to 9 week) before morning feeding.

Physicochemical analysis

The diets were sampled weekly and stored at −20 °C prior to analysis. Bulk density and water-holding capacity (WHC) of the samples were measured according to Giger-Reverdin (2000). Dry matter (DM), crude protein (CP), ether extract (EE), ash (AOAC, 2002) and NDF and ADF (Van Soest et al., 1991) were determined. Feed particle size was determined by dry sieving using the new Penn state particle separator (PSPS; Kononoff, 2002). The NDF content of all materials retained on PSPS sieves was measured (Van Soest et al., 1991). According to Mertens (1997), the physical effective factor (pef) was determined based on proportion of DM retained on the 1.18-mm sieve (pef$_{>1.18}$). The peNDF was calculated by multiplying NDF content of the feed by the pef$_{>1.18}$. Abrasive value of diets was measured according to Greenwood et al. (1997). In this method, a mixer hook was evenly coated with paraffin and used to mix moistened feedstuffs. Abrasive value was measured according to the amount (g) of paraffin that was abraded during the testing.

Collection of blood samples

Blood samples were obtained approximately 3 h after the morning meal from the jugular vein at the beginning of the experiment and the end of each week. The serum was separated by centrifugation at 1800 ***g*** for 10 min and stored at −18 °C until analysis. Samples were then transported to the veterinary medicine clinical pathology laboratory. Serum samples were allowed to thaw and analysed for beta-hydroxybutyric acid (BHBA; Ranbut, Randox, Crumlin, UK), non-esterified fatty acids (NEFA; Randox), glucose and blood urea nitrogen (BUN; Zist Shimi, Tehran, Iran) by commercial kits using a spectrophotometer (Biotechica Instruments, TARGA 3000, Rome, Italy).

Tissue sample collection and handling

Nine lambs (three per treatment) were slaughtered at 63 days of age. The entire digestive tract was removed from the carcass, and the ruminal compartment was separated (the reticulo-rumen remained together), emptied, washed clean, drained of excess water and weighed. Samples (approximately 1 cm^2) were collected from the dorsal, ventral, caudal dorsal, caudal ventral blind sacs, left pillar and atrium area.

Tissue samples were obtained within 30 min after slaughter, placed in individual containers and fixed immediately in a 10% formaldehyde solution for

subsequent measurements. In the laboratory, tissue samples were dehydrated in a series of ethanol solutions from 70% to 100%. The materials were sectioned with an automatic microtome, at 6 μm thickness, stained with haematoxylin mixture and eosin. The materials were observed under a light microscope (Olympus BX-51, Olympus, USA) at 20 and 40×. Digital images of stained sections were taken using an Olympus BX-51 camera (DP 11), and measurements were made using image analysis computer software (DP2-BSW Version 1.3, Olympus, USA). An example of representative rumen morphology from each feeding treatment is shown in Figs 1 and 2.

Papillary height was defined as the distance from the tip to the base of the papillae, and papillary width was defined as the average width of the base, middle and tip of the papillae. Papillae length and width were used to estimate surface area per cm^2 of each ruminal section. Density of papillae was determined using digital images, from scanning electron microscopy (SEM, Tescan Vega, TS 5130MM, Czech Republic). Surface area of papillae per surface area of each ruminal section was presented as the surface area ratio (SAR) based on Hill et al. (2005). Papillae were considered to be cylindrical in shape with one closed end. Therefore, equation 1 was used to calculate lateral area of papillae, based on the surface of a cylinder plus the area of a circle. Equation 2 was used to calculate the average SAR of each section of the rumen by multiplying the average surface area of the papillae in each section by the average density or number of papillae per unit area in that section:

$$\text{Surface Area of Papillae}\,(cm^2) = 2 \times r \times \pi \times L + \pi \times r^2 \quad (1)$$

where r = radius in cm and L = length in cm.

$$SAR = (\text{average surface area of papillae in section A}) \times (\text{average papillae density in section A}) \quad (2)$$

where A can be caudal, ventral, dorsal, pillar or atrium.

The keratin layer (*stratum corneum*) and tunica muscularis (muscular layer) were measured on each slide. A minimum of 10 measurements was recorded for each stratum for statistical analysis.

Epithelium colour score measured according to the 1–3 scale: 1 = yellow, 2 = light brown, 3 = dark brown grey (Álvarez-Rodríguez et al., 2012; Fig. 3).

Ribosomal capacity (the capacity for protein synthesis) and cell size were calculated as the ratio of RNA to protein and protein to DNA respectively (Tesseraud et al., 1996) . For extract of total RNA and DNA, the Trizol RNA Prep 100 kit and Accuprep Genomic DNA Extraction Kit; Cat No: K-3032 were used respectively. Quality and quantity of RNA and DNA were assessed by absorbance measurement using a NanoDrop (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

The Proc. mixed program of SAS (Version 9.1) was used to analyse the measurements. Because blood parameters and rumen morphological characteristics were measured over the time and area, a repeated measures approach using ANOVA with mixed linear models in SAS 9.1 was used. The means were compared by the Duncan test.

Results and discussion

Physicochemical properties

Reduction in particle size decreased WHC ($p < 0.05$) but increased bulk density ($p < 0.01$) of diets (Table 2). The LONG diet had the lowest, but the CON diet had the highest bulk densities (Table 2). Teimouri Yansari et al. (2004) and Teimouri Yansari and Primohammadi (2009) found that reduction in particle size increased bulk density and decreased WHC of alfalfa.

Using the new PSPS, the proportion of material retained on upper sieves of 1.18 mm was higher for diets containing coarse alfalfa hay ($p < 0.01$), but the proportion of material retained on pan was higher in CON and FINE compare with CA diets ($p < 0.01$).

Reduction in alfalfa particle size decreased $pef_{>1.18}$ and peNDF values (Table 2). The geometric mean of diet particles was significantly influenced by

Fig. 1 Ruminal epithelium colour in lambs fed control (a), fine (b) and coarse chopped alfalfa hay containing (c) diets.

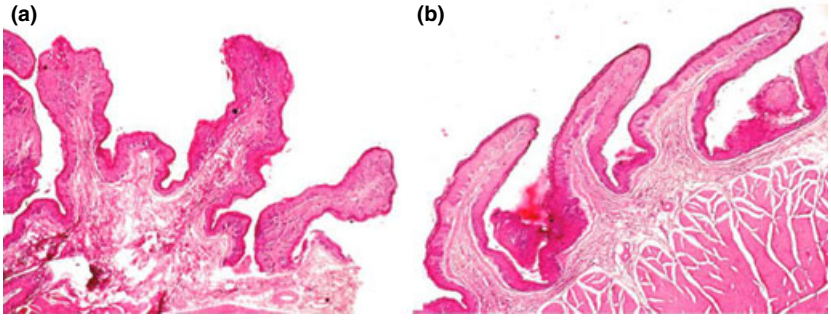

Fig. 2 Morphology of ruminal dorsal sac papilla (320 ×) in lambs fed control (a) and coarse chopped alfalfa hay containing (b) diets.

treatments and increased with increasing of alfalfa hay particle size ($p < 0.01$). These results are similar to previous studies (Kononoff, 2002; Teimouri Yansari et al., 2004; Teimouri Yansari and Primohammadi, 2009). Abrasive value increased significantly ($p < 0.01$) when coarse alfalfa hay was used (Table 2). Greenwood et al. (1997) in a study with different particle size of forage reported that diet abrasive value increased as particle size increased. They suggested that abrasive value of forages produced a sigmoid curve as particle size increased.

Rumen morphological and molecular characteristics

Rumen morphological characteristics are summarized in Table 3. There were no significant differences between treatments for papillae height, width, epithelium depth, papilla density and surface area ratio (SAR). Keratinized layer (*Stratum corneum*) was thickest ($p = 0.02$), and muscular layer was thinnest ($p = 0.04$) in lambs fed control diet (Fig. 2). Forage particle size affected ruminal epithelium colour score ($p = 0.03$), which was greater in LONG than in FINE and control lambs. Coarse alfalfa feeding induced darker brown epithelium colour than control and lambs fed diet containing finely ground alfalfa hay, which turned lighter (Table 3, Fig. 3). The rumen tissue alterations have been shown to be influenced by physical texture of diet (Coverdale et al., 2004) and particle size (Greenwood et al., 1997). McGavin and Morrill (1976) reported that calves fed finely ground diets developed extensively keratinized papillae with feed and hair particles adhered between them; however, calves fed a coarse diet did not. This is also supported by Norouzian et al. (2011) who reported that low particle size decreases abrasive ability. In the current experiment, changes in physical structure may result in the decrease in keratinization of the inner surface of rumen (*stratum corneum*) and increase in rumen wall muscularity. As ruminal contractions mix digesta, fine particles adhere to the ruminal wall between papillae, and larger particles slide across the surface and cause abrasion (Greenwood et al., 1997).

Feed physical structure likely has the greatest influence on the development of rumen muscularization and volume. Stimulation of rumen motility is governed by the same factors, particle size and effective fibre, in the neonatal ruminant similar to adults (Van Soest, 1994). In contrast to concentrate's advantages for epithelial development (Nocek et al., 1984), fibre characteristics such as particle size appear to be the primary stimulators of rumen muscularization development and increased rumen volume (Zitnan et al., 1998). Large particle size, high content of effective fibre and increased bulk of forages or high fibre sources increase rumen wall stimulation physically, subsequently rumen motility, muscularization, and volume is increased (Vazquez-Anon et al., 1993).

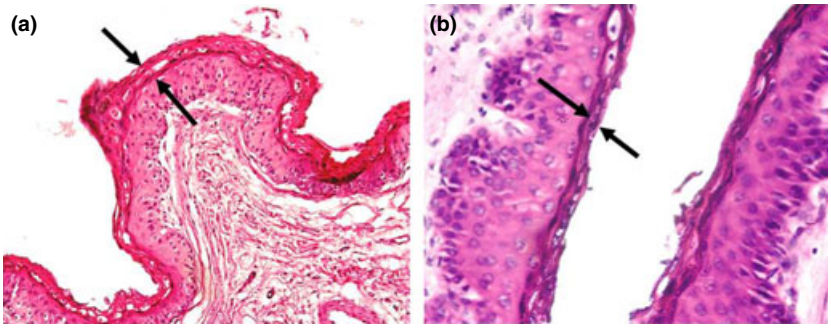

Fig. 3 Keratinization of ruminal papillae (640 ×) in lambs fed control (a) and coarse chopped alfalfa hay containing (b) diets.

Table 3 Effect of dietary treatments on rumen morphological characteristics

	Diet*				
Measurement	CON	FINE	LONG	SEM	p value
Papillae height (μm)	1630.0	1482.9	1679.9	113.9	0.47
Papillae width (μm)	288.8	303.5	306.6	23.2	0.40
Epithelium (μm)	59.7	62.7	69.4	2.90	0.13
Stratum corneum (μm)	13.96^{a}	12.68^{ab}	8.35^{b}	0.85	0.02
Muscular layer (μm)	778.3^{b}	993.2^{ab}	1072.3^{a}	90.0	0.04
Papilla density (No/cm^2)	108.5	107.6	107.0	0.60	0.30
Surface area ratio (cm^2)	1.09	1.06	1.06	0.01	0.11
Epithelium colour score	2.00^{a}	2.21^{a}	2.87^{b}	0.18	0.03

Means with different superscripts within a row differ significantly ($p < 0.05$).
*CON, the control diet (without alfalfa hay); FINE, diet containing 15% fine alfalfa hay; and LONG, diet containing 15% coarse alfalfa hay.

Molecular characteristics of ruminal tissue samples are shown in Table 4. Treatment had no significant effect on molecular indices of ruminal tissue. Neither DNA content ($p = 0.92$) nor RNA ($p = 0.84$) concentration was affected by reduction in alfalfa particle size. Although no data were found in molecular approaches of the effect of forage particle size on epithelium cells proliferation, data concerning the dietary-energy-dependent enhanced transport capacities of the rumen epithelium cells are supported by recent findings that the abundance of DNA and RNA of rumen epithelial cells increases with greater intake of metabolizable energy (Shen et al., 2004). Our data showed that all experimental treatments were able to provide the minimum energy required for proliferation of epithelium cells.

Blood metabolites

Forage particle size did not affect the blood concentration of glucose, BUN, BHBA and NEFA (Table 5).

Table 4 Effect of dietary treatments on molecular indices of ruminal tissue

	Diet*				
Measurement	CON	FINE	LONG	SEM	p value
RNA (μg/g)	2977.6	3163.0	3206.5	486.4	0.84
DNA (μg/g)	144.9	141.6	132.6	35.5	0.92
Pr (mg/g)	400.0	398.7	406.8	18.6	0.72
Cs ($\times 10^{-3}$)	7.45	7.92	7.50	1.17	0.86
Cell Size ($\times 10^{3}$)	2.79	3.05	3.07	0.59	0.92

Means with different superscripts within a row differ significantly ($p < 0.05$).
*CON, the control diet (without alfalfa hay); FINE, diet containing 15% fine alfalfa hay; and LONG, diet containing 15% coarse alfalfa hay.

Table 5 Effect of dietary treatments on blood metabolites of experimental lambs

	Diet*				Effect	
Measurement	CON	FINE	LONG	SEM	Diet	Time
Glucose (mg/dl)	104.7	91.25	96.1	12.26	0.62	0.08
BUN (mg/dl)	5.09	5.13	5.97	0.07	0.43	0.003
Beta-hydroxybutiric acid (mM)	0.56	0.47	0.42	0.04	0.12	<0.001
Non-esterified fatty acids (mM)	0.20	0.21	0.18	0.03	0.45	0.11

*CON, the control diet (without alfalfa hay); FINE, diet containing 15% fine alfalfa hay; and LONG, diet containing 15% coarse alfalfa hay.

Plasma BHBA concentrations increased with age of the lambs ($p < 0.01$). Similar results have been reported by other researchers; they have emphasized that such increment may have been resulted from increased consumption of fermentable carbohydrates (Quigley and Bernard, 1992; Greenwood et al., 1997). Glucose concentration tended to decrease with increasing age of lambs ($p = 0.08$). It has been suggested that this decline could be related to both age of animals and their diet. Solid feed intake and rumen development causes the decline in blood glucose concentrations due to the production of VFAs (Baldwin et al., 2004).

Animal performance

DMI was higher ($p < 0.001$) in lambs fed control diet compared with alfalfa-containing diets (Table 6). Reduction in alfalfa particle size increased DMI, as lambs fed diet containing fine alfalfa hay had higher DMI than LONG lambs. Teimouri Yansari et al. (2004) and Teimouri Yansari and Primohammadi (2009) reported that reduction in particle size had a positive

Table 6 Effect of dietary treatments on performance of experimental lambs

	Diet*				
Measurement	CON	FINE	LONG	SEM	p value
Initial body weight (kg)	9.2	9.4	9.3	1.1	0.54
Final body weight (kg)	18.8	19.6	19.3	1.8	0.35
Dry matter intake (g/d)	533.5^{a}	486.4^{b}	464.9^{c}	6.58	<0.001
Average daily gain (g/d)	150.39	169.7	160.0	9.79	0.31
Feed conversion ratio	4.11^{b}	3.17^{a}	3.26^{a}	0.34	0.01

Means with different superscripts within a row differ significantly ($p < 0.05$).
*CON, the control diet (without alfalfa hay); FINE, diet containing 15% fine alfalfa hay; and LONG, diet containing 15% coarse alfalfa hay.

effect on DMI. Feeds with longer particle size usually led to greater fill because of their slower passage rate, which limit DMI mainly because of distension effect (Teimouri Yansari et al., 2004). Therefore under this theory, particle size reduction could positively affect DMI because of density of particles increases (Allen, 2000). Voluntary intake and nutrient supply can be constrained by rumen fill and clearance of digesta from the rumen. Hence, forages that occupy lower bulk density should have a greater ruminal fill factor than more dense forages (Wattiaux, 1990).

There were no differences in average daily gain (ADG) of lambs throughout the entire trial (Table 6), but feed conversion ratio (FCR) affected by the treatments and was worst for control diet. This is supported by the observation that DMI was higher in CON, while the ADG was not different between treatments. Coverdale et al. (2004) reported greater body weight in calves fed diets containing 15% coarse bromegrass hay. In their study, calves fed by these diets were heavier, had greater ADG, and greater gain to feed than calves fed commercial starter.

In conclusion, our results suggest that including 15% coarse alfalfa hay in the starter diet could decrease thickness of the keratinization layer and increase muscularity of rumen wall and tended to improve FCR overall.

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