


PAPER



## Effects of linseed processing method (ground versus extruded) and dietary crude protein content on performance, digestibility, ruminal fermentation pattern, and rumen protozoa population in growing lambs

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### ABSTRACT

Thirty-six male lambs (averaging BW = 24.4 kg) were assigned in a completely randomised design with the factorial arrangement (nine lambs per each treatment) to assess the effects of linseed processing method (ground vs. extruded) and dietary crude protein content (12% vs. 15%, DM basis) on performance, digestibility, ruminal fermentation, and protozoa population. Treatments were; (1) ground linseed with 12% CP (GLS-12CP), (2) ground linseed with 15% CP (GLS-15CP), (3) extruded linseed with 12% CP (ELS-12CP), and (4) extruded linseed with 15% CP (ELS-15CP). The study lasted 84 d, and the lambs had free access to experimental diets and water. Outcomes showed that dry matter intake, digestibility of organic matter, and crude protein were increased in lambs fed ground linseed compared to extruded linseed. Feeding extruded linseed decreased urinary allantoin concentration, total purine derivatives (PD), the ruminal proportion of propionate, and blood concentration of glucose and HDL-cholesterol compared to ground linseed diets. Regarding dietary protein content, results showed that dry matter intake, urinary concentration of allantoin and total PD were increased in 15CP diets compared to 12CP diets. Considering the interaction, results showed that average daily gain was improved in GLS-15CP treatment compared to other experimental treatments. In conclusion, results show that grinding the linseed is more beneficial compared to its extrusion in lamb nutrition. Moreover, 15CP diets had a beneficial effect when fed with either ground or extruded linseed and seem to compensate, to some extent, the negative effects of extruded linseed on the gain of growing lambs.

### HIGHLIGHTS

- Linseed processing methods (ground vs. extruded) and dietary protein content (12 vs. 15%, DM basis) were evaluated in growing lambs
- Ground linseed increased average daily gain, improved digestibility, and microbial protein yield compared to extruded linseed
- Ground linseed was more favourable than extruded linseed and 15% CP diet has additional advantages either with the ground or extruded linseed

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## Introduction

Lamb producers in intensive feeding systems, are interested to apply strategies to make the lamb production length shorter and hence have the opportunity to reduce the production costs. Fat supplementation in ruminant's nutrition was indicated to be a strategy to make the diet denser from the energy level (Kim et al. 2007) and also decrease methane emission to the environment (Machmüller

and Kreuzer 1999). In addition, literatures stated that carcass and adipose tissue fatty acid (FA) contents could be manipulated through fat sources incorporated in the diet of lambs that consequently linked to human health (Kim et al. 2007; SoltaniNezhad et al. 2016). On the other side, supplemental fat in lambs was shown to reduce feed efficiency (FE), reduce the digestibility of fibre that was due to the negative impacts of supplemental fat on protozoa population

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(Bhatt et al. 2011), and decreased bacterial protein synthesis (Ikwegbu and Sutton 1982). Oilseeds are the source of fat in ruminants' diets which were shown to have a lower detrimental effect on rumen fermentation and microbial activity compared to supplemental fat in the diet (Ghorbani et al. 2020). Hence, linseed which is rich in n-3 FA, a favourable FA to produce the healthier product for human consumption (Kim et al. 2007; Mohtashami et al. 2021), can be incorporated in ruminant's diet with a less detrimental effect on rumen fermentation and microbial activity rather than linseed oil (Ikwegbu and Sutton 1982).

Different methods of processing of oilseed such as rolling, roasting, pelleting, and extruding have been evaluated in ruminants (Giannico et al. 2009; Doreau et al. 2009). It seems that FA availability for ruminal microbes can be modulated through various oilseed processing methods. For instance, Doreau et al. (2009) stated that extruding could break down the plant cells, and cytosolic lipids would be more accessible to ruminal bacteria than un-processed whole seeds. However, contrary to Doreau et al. (2009), it was stated that the application of heat treatments can denature the protein matrix surrounded the fat droplet and therefore protects fat from ruminal biohydrogenation (Sterk et al. 2010). Moreover, it was shown that different availability of fat absorbed in the small intestine, can influence body energy status in ruminants and thus consequently have an impact on their growth performance (Ghorbani et al. 2020). Therefore, the first aim of the present study was to evaluate the ground versus extruded linseed processing method on ruminal fermentation and microbial activity in growing lambs.

Another strategy for having a shorter rearing period of lambs is to provide the diets with greater protein content to support faster growth and greater gain (Haddad et al. 2001). Extensive ranges of dietary protein content from 8.9% (Drouillard et al. 1991) to 18% (Haddad et al. 2001), (DM bases) were evaluated in lamb nutrition. Protein requirement might be influenced while the fat source is incorporated in the ruminant diet (Yousefinejad et al. 2021). This can be related to decrease available amino acids (AA) for ruminants via either decreased digestibility of protein (Hill et al. 2015; Ghorbani et al. 2020) or decreased microbial protein yield (Ikwegbu and Sutton 1982) due to high-fat content in diets. Therefore, it can be postulated that high-fat diets had the potential to reduce metabolisable protein (MP) reached in to the small intestine (Yousefinejad et al. 2021) and then have potential to negatively influence the albumin

and total protein (TP) concentrations in the blood (Ghorbani et al. 2020). MP is necessary for growing animals, from the other side, to support maximum growth (NRC 2001). Furthermore, adequate dietary MP granted sufficient blood albumin and TP concentrations, which are indicating the optimum growth rate from the protein nutrition perspectives in the growing lambs (Valizadeh et al. 2021). It has been indicated that feeding diets with high levels of AA had positive effects on blood glucose concentration in ruminants via gluconeogenesis pathway (Young 1977; Valizadeh et al. 2021). In contrast, higher blood urea nitrogen (BUN) concentration, from the other side, indicates the lower nitrogen efficiency in ruminants which was found in some high-fat diets (Ghorbani et al. 2020). Thus, we postulated that optimum dietary protein content might have supportive effects via providing greater MP required for maximum growth in diets with MP deficiency due to the negative effects of supplemented fat sources. Therefore, as the second aim of the study, it was hypothesised that greater protein content in the diet may reduce negative effects of fat that can be either found in the ground or in extruded linseed. Therefore, this study aimed to evaluate the different processing methods of linseed (ground vs. extruded) and dietary protein level (12% vs. 15%, DM basis) on performance, nutrient digestibility, ruminal fermentation profile, protozoa population, and blood metabolites in growing lambs.

## Materials and methods

### Lambs and dietary treatments

This study was done at the Animal Research Station of Bahonar University of Kerman, Iran. All procedures related to animals were certified by the Animal Care and Use Committee of Bahonar University (IACUC Protocol #IR2018011) described by the Iranian Council of Animal Care (Iranian Council of Animal Care 1995). Thirty-six male Kermani lambs (averaging BW of  $24.4 \pm 1.3$  kg) were used in a complete randomised design with a  $2 \times 2$  factorial arrangement of dietary treatments as follow; (1) ground linseed with 12% CP (GLS-12CP), (2) ground linseed with 15% CP (GLS-15CP), (3) extruded linseed with 12% CP (ELS-12CP), and (4) extruded linseed with 15% CP (ELS-15CP).

A batch of the linseed (contained 21% and 37%, crude protein and ether extract based on DM, respectively) was divided into two identical portions, one portion was used as ground linseed and the other portion as an extruded form. The extrusion of linseed was done at  $148 \pm 2^\circ\text{C}$  with 25–32% moisture and

high pressure for 15 s. The extruder had a single screw (450 rpm speed, 10 cm diameter) and dual conditioner system (Amandus Kahl, Expander, GmbH and Co., KG, Germany) according to Berenti et al. (2021). Nutrient requirements except dietary protein contents were balanced according to NRC (2007). Furthermore, the degradable and undegradable protein (RDP and RUP) contents of experimental diets were calculated based on the NRC (2001). The experimental diets were fed to lambs as total mixed ration (TMR). Individual stalls with concrete surfaces were used to keep animals and were cleaned every 48 h during the study. The lambs were fed TMR in two identical meals at 0800 and 1600 h as *ad libitum* for at least 10% orts.

### Performance and digestibility trial

The experimental diets were fed *ad libitum* to permit at least 10% orts (i.e. the portion of the diet not consumed over a 24-h period). The dietary refusals were collected and recorded at 0730 hours to evaluate daily dry matter intake (DMI) and then fresh feed was fed at 0800 hours. Lamb's BW was measured at the beginning of the experiment and also at every 14 d until the end day of the experiment (days 14, 28, 42, 56, and 70). The weighting of lambs was performed prior to the morning feeding (0700) to exclude the impacts of empty/full condition of the digestive tract on BW. The ratio of feed conversion was calculated by division of intake to gain of animals. The TMR was sampled weekly and dry matter was measured at 60 °C for 48 h (AOAC 2002). After that, feeds and orts samples were mixed completely and ground (1-mm screen, Ogawa Seiki CO., Ltd., Tokyo, Japan) for analyses based on AOAC (2002) for ether extract (method 920.39) CP (method 988.05), acid detergent fibre (method 973.18), and neutral detergent fibre (Van Soest et al. 1991). Diets' non-fibrous carbohydrates (NFC) was calculated as  $100 - (\text{CP} + \text{NDF} + \text{EE} + \text{ash})$  according to NRC (2001). Dietary FA profile was evaluated using gas-liquid chromatography based on methyl esters assay approved by Ichihara and Fukubayashi (2010). Hexane was utilised as a solvent for extraction, and nonadecanoic acid was utilised as an internal standard.

During the last 5 days of the experiment (d 66 to d 70), faecal grab samples (three samples from each animal) were collected daily at 6, 12, and 18 h post morning feeding. All the chemical analysis of the faecal samples was done similar to methods before explained for TMR samples (Table 1). An internal marker (acid insoluble ash) was used for measuring apparent digestibility coefficients of DM, OM, CP, EE,

NDF, and ADF through total digestive (Van Keulen and Young 1977) according to the following equation:  $\text{AD} (\%) = 100 - 100 \times (\text{MD}/\text{MF}) \times (\text{NF}/\text{ND})$ , where AD is the apparent digestibility (%), MD is the marker in the diet (%), MF is the marker in the faeces (%), NF is the nutrient in the faeces (%), and ND is the nutrient in the diet (%).

### Rumen fermentation characteristics and protozoa population

Samples of the rumen (15 mL) were collected from all animals through an oesophageal tube at 4 h post morning feeding on days 35 and 70 of the trial. To prevent contamination of rumen samples with saliva, the first 5 ml was discarded, and then rumen pH was determined instantly by a portable pH metre (model

**Table 1.** The ingredients and chemical analysis of experimental diets (% of DM, unless differently shown).

|  | Treatments <sup>a</sup> |       |       |       |
|--|-------------------------|-------|-------|-------|
|  | GLS                     |       | ELS   |       |
|  | 12CP                    | 15CP  | 12CP  | 15CP  |
| <b>Ingredients</b>                                       |                         |       |       |       |
| Alfalfa hay, chopped                                     | 30                      | 30    | 30    | 30    |
| Wheat straw, chopped                                     | 10                      | 10    | 10    | 10    |
| Barley grain, grounded                                   | 25                      | 25    | 25    | 25    |
| Corn grain, grounded                                     | 15                      | 13    | 15    | 13    |
| Wheat bran   | 7                       | 4     | 7     | 4     |
| Soybean meal   | 1                       | 6     | 1     | 6     |
| Grounded linseed   | 10                      | 10    | 0.00  | 0.00  |
| Extruded linseed   | 0.00                    | 0.00  | 10    | 10    |
| Mineral-vitamin mix <sup>b</sup>                         | 1.10                    | 1.10  | 1.10  | 1.10  |
| Salt   | 0.40                    | 0.40  | 0.40  | 0.40  |
| Calcium carbonate  | 0.50                    | 0.50  | 0.50  | 0.50  |
| <b>Chemical composition based on %, otherwise stated</b> |                         |       |       |       |
| Dry matter   | 90                      | 90    | 90    | 90    |
| Crude protein  | 12.00                   | 15.00 | 12.00 | 15.00 |
| Rumen degradable protein                                 | 7.95                    | 9.93  | 8.20  | 10.18 |
| Rumen undegradable protein                               | 4.05                    | 5.07  | 3.80  | 4.82  |
| Organic matter   | 92.60                   | 92.40 | 92.80 | 92.80 |
| Ether extract  | 5.91                    | 5.73  | 5.89  | 5.63  |
| Neutral detergent fibre                                  | 36.20                   | 36.50 | 36.10 | 36.40 |
| Non-fibre carbohydrate <sup>c</sup>                      | 38.70                   | 35.90 | 39.00 | 36.60 |
| Metabolisable energy <sup>c</sup> , MJ/kg                | 10.80                   | 10.83 | 10.78 | 10.71 |
| <b>Fatty acid profile, %</b>                             |                         |       |       |       |
| C14:0  | 0.53                    | 0.59  | 0.50  | 0.55  |
| C16:0  | 15.77                   | 15.83 | 15.69 | 15.72 |
| C16:1  | 0.84                    | 0.71  | 0.78  | 0.69  |
| C18:0  | 3.41                    | 3.48  | 3.37  | 3.33  |
| C18:1; n9  | 14.16                   | 13.87 | 14.00 | 13.96 |
| C18:2; n6  | 43.73                   | 43.58 | 43.86 | 43.97 |
| C18:3; n3  | 20.01                   | 20.26 | 20.19 | 20.31 |
| Other fatty acids  | 1.55                    | 1.68  | 1.61  | 1.47  |

<sup>a</sup>Treatments were: 1) Ground linseed (GLS) with 12% dietary protein content (GLS-12CP); 2) Ground linseed with 15% dietary protein content (GLS-15CP); 3) Extruded linseed (ELS) with 12% dietary protein content (ELS-12CP); 4) Extruded linseed with 15% dietary protein content (ELS-15CP).

<sup>b</sup>Each kg of the supplement contained vitamin D (100,000 IU), vitamin A (600,000 IU), vitamin E (3000 IU), calcium (185 g), phosphorus (35 g), magnesium (21 g), sodium (24 g), manganese (3 g), copper (0.8 g), iron (2 g), zinc (4 g), iodine (50 mg), selenium (14 mg), cobalt (29 mg).

<sup>c</sup>Calculated based on NRC (2001) equations [non-fibrous carbohydrate (NFC) was calculate as  $100 - (\text{CP} + \text{NDF} + \text{EE} + \text{ash})$ ].

8601, AZ-Complete, Taiwan). After that, the rumen samples were passed through cheesecloth with four layers. Rumen fluid samples (5 ml each) were blended with 0.1 ml sulphuric acid 50% and kept at  $-20^{\circ}\text{C}$  for later ammonia N analysis. Just prior to analysis, these samples were defrosted, centrifuged at  $12,000 \times g$  ( $4^{\circ}\text{C}$ , 18 min) and the clear supernatant was analysed for ammonia N based on a method described by Broderick and Kang (1980). Rumen liquid (5 ml) was blended with 25% (wt/vol) metaphosphoric acid (1 ml) and then analysed for short-chain fatty acids (SCFA) using a gas chromatograph (model 5890, Hewlett-Packard, Avondale, PA, USA) as reported in our previous study (Khezri et al. 2017). Rumen protozoa were counted by use of a Neubauer counting chamber (0.1-mm depth; Hausser Scientific Co., Horsham, USA) from aliquot of rumen contents conserved in the solution of methyl green-formalin-saline according to Dehority (1993). The genera were grouped as *Entodinium* sp., *Holotrichs* (*Dasytricha* sp. and *Isotricha*), and cellulolytic protozoa (*Entoploplastron* sp., *Diplodinium* and *Polyplastron*).

### Purine derivatives in urine

Daily volume of urine was estimated by spot sampling method based on urine creatinine concentration as described in growing lambs (Valizadeh et al. 2021). On days 32 and 64 of the experiment, urine samples were taken from lambs during the morning (between 0900 and 1100 h), when animal urinated spontaneously. Urine subsamples (5 ml) were mixed instantly with 0.036 N sulphuric acid (45 ml) and kept at  $-20^{\circ}\text{C}$  for next measurements. The concentrations of the creatinine, allantoin, and uric acid in the urine samples were determined based on procedures explained in our earlier work (Khezri et al. 2017). The concentrations of xanthine and hypoxanthine in the urine samples were detected based on their conversion to uric acid with xanthine oxidase (Sigma; Catalog No. X-1875, 5 Units, Germany), with following optical density at 293 nm (Rajabi et al. 2017). To estimate total urine volume, an average creatinine excretion of 9.79 mg/kg BW was applied (David et al. 2015). The ruminal microbial nitrogen supply was calculated from excretion of purine derivatives (PD) in urine according to Chen and Gomes (1992).

### Metabolites and liver enzymes of blood

Blood samples were collected into heparinised tubes (10 ml) from jugular vein of lambs at 3 to 4 h post

morning feeding (days 34 and 68 of the experiment) and centrifuged at  $5000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . Plasma samples were kept at  $-20^{\circ}\text{C}$  until analysis for glucose, urea N (BUN), cholesterol, triglyceride, total protein (Kit NOs. 93008, 93013, 110500, 132500, 9304, respectively), according to manufacturer's guidelines (Pars Azmoon Co., Tehran, Iran). Enzymes related to function of liver including alanine aminotransferase and aspartate aminotransferase (Kit Nos. 92004 and 92005, respectively. Pars Azmoon Co., Tehran, Iran) were determined by use of ELISA (Auto Analyzer Hitachi 717, Japan).

### Statistical analysis

Statistical analyses were done using PROC MIXED of SAS (version 9.1; SAS Inst. Inc., Cary, NC). The adopted model was as:  $Y_{ijklm} = \mu + \text{Lamb}_i + \text{LS}_j + \text{CP}_k + \text{P}_l + (\text{LS} \times \text{P})_{il} + (\text{CP} \times \text{P})_{kl} + (\text{LS} \times \text{CP})_{jk} + (\text{LS} \times \text{CP} \times \text{P})_{jkl} + \beta(\text{Xi} - \bar{\text{X}}) + \varepsilon_{ijklm}$ ; where  $Y_{ijklm}$  is the dependent variable;  $\mu$  is the overall mean;  $\text{LS}_j$  is the linseed processing method ( $j$ =ground linseed vs. extruded linseed);  $\text{CP}_k$  is the impact of dietary protein level ( $k$ =12% vs. 15%, DM basis);  $\text{P}_l$  is the impact of sampling period;  $(\text{LS} \times \text{P})_{il}$  is the interaction between linseed processing method and period;  $(\text{CP} \times \text{P})_{kl}$  is the interaction between dietary protein content and period;  $(\text{LS} \times \text{AH})_{jk}$  is the interaction between linseed processing method and dietary protein content;  $(\text{LS} \times \text{CP} \times \text{P})_{jkl}$  is the tripartite effect of linseed processing method, dietary protein content and period;  $\beta(\text{Xi} - \bar{\text{X}})$  is the covariate variable and  $\varepsilon_{ijklm}$  is the residual error. Before analyses, the normality of all data were checked by the UNIVARIATE procedure of SAS. The model included lamb within treatment as a random effect and the first-order autoregressive covariance structure (AR1) was choosed as the most suitable covariance structure for all repeated statements according to the Bayesian information criterion (BIC) and Akaike's information criterion (AIC). The BW of animals in the first day of the trial was employed as a covariate for final calculation of associated parameter. Effects were declared as significant when  $p \leq .05$ , and tendency was shown when  $0.05 < p \leq .10$ . Data of the tables were outlined as least squares means.

## Results

### Intake, performance and digestibility

Dry matter intake was increased when lambs fed ground linseed compared to lambs fed extruded linseed ( $p=.02$ ; Table 2). The final BW was greater in



**Table 2.** Least square means for performance and nutrient digestibility in lambs fed diets with different linseed processing method (ground vs. extruded) and dietary protein content (12% vs. 15%, DM basis).

| Item                              | Treatments <sup>a</sup> |                  |                  |                  | SEM   | <i>p</i> -value <sup>b</sup> |     |         |
|-----------------------------------|-------------------------|------------------|------------------|------------------|-------|------------------------------|-----|---------|
|                                   | GLS                     |                  | ELS              |                  |       | LS                           | CP  | LS × CP |
|                                   | 12CP                    | 15CP             | 12CP             | 15CP             |       |                              |     |         |
| Performance                       |                         |                  |                  |                  |       |                              |     |         |
| Initial body weight, kg           | 24.20                   | 24.40            | 24.60            | 24.70            | 0.67  | .58                          | .79 | .84     |
| Final body weight, kg             | 39.80                   | 41.10            | 37.20            | 40.10            | 0.76  | .04                          | .01 | .27     |
| Dry matter intake, g/d            | 1365                    | 1401             | 1286             | 1361             | 25.30 | .02                          | .03 | .46     |
| Metabolisable energy intake, MJ/d | 14.72                   | 15.16            | 13.85            | 14.55            | 1.34  | .03                          | .05 | .34     |
| Protein intake, g/d               | 162.30                  | 210.00           | 155.20           | 204.30           | 9.12  | .02                          | .04 | .38     |
| Average daily gain, g/d           | 222 <sup>a</sup>        | 237 <sup>a</sup> | 180 <sup>b</sup> | 221 <sup>a</sup> | 8.57  | .01                          | .02 | .05     |
| Feed conversion ratio             | 6.44                    | 6.23             | 7.68             | 6.48             | 0.38  | .03                          | .04 | .06     |
| Nutrient digestibility,%          |                         |                  |                  |                  |       |                              |     |         |
| Dry matter                        | 73.78                   | 75.21            | 71.66            | 72.98            | 1.03  | .04                          | .18 | .95     |
| Organic matter                    | 77.45                   | 78.03            | 71.89            | 73.47            | 1.69  | .03                          | .49 | .78     |
| Crude Protein                     | 67.66                   | 68.97            | 61.73            | 65.22            | 1.51  | .01                          | .15 | .72     |
| Neutral detergent fibre           | 52.81                   | 53.64            | 51.28            | 51.92            | 2.78  | .37                          | .69 | .95     |
| Acid detergent fibre              | 43.68                   | 44.54            | 42.10            | 42.51            | 2.14  | .09                          | .54 | .82     |
| Ether extract                     | 81.35                   | 81.19            | 78.12            | 78.86            | 1.77  | .08                          | .84 | .76     |

<sup>a</sup>Treatments were: 1) Ground linseed (GLS) with 12% dietary protein content (GLS-12CP); 2) Ground linseed with 15% dietary protein content (GLS-15CP); 3) Extruded linseed (ELS) with 12% dietary protein content (ELS-12CP); 4) Extruded linseed with 15% dietary protein content (ELS-15CP).

SEM = Standard error of means; LS = Linseed; CP = Crude protein.

<sup>b</sup>Statistical comparisons: LS = linseed processing method (ground linseed vs. extruded linseed); CP; 12 vs.15% dietary protein content based on the dry matter; LS × CP = interaction of linseed processing method and dietary protein content.

lambs fed GLS in comparison with lambs fed ELS diets ( $p = .04$ ). The ratio of feed conversion was improved in GLS compared to ELS diets ( $p = .03$ ). DMI ( $p = .03$ ) and final BW ( $p = .01$ ) were greater and FCR ( $p = .04$ ) was improved in 15CP diets compared to 12 CP diets. Considering the interaction between linseed processing method and dietary protein content, results indicated that the greatest ADG was observed in lambs fed GLS-15CP diet compared to the other experimental treatments ( $p = .05$ ). The FCR also was improved (tendency,  $p = .06$ ) when lambs received GLS-15CP diets among different dietary treatments.

The digestibility of nutrients including DM ( $p = .04$ ), OM ( $p = .03$ ), CP ( $p = .01$ ), EE (tendency;  $p = .08$ ), and ADF (tendency;  $p = .09$ ) were improved when GLS was fed to lambs compared to ELS diets (Table 2). The dietary protein content did not have impact on nutrient digestibility. No interaction was found regarding the linseed processing method and dietary protein content with respect the nutrient digestibility ( $p > .05$ ).

### Ruminal fermentation characteristics and protozoa population

Ruminal pH was influenced neither with linseed processing method nor with dietary protein content ( $p > .05$ ; Table 3). Total concentrations for ruminal SCFA (tendency;  $p = .09$ ) was increased when lambs fed GLS compared to ELS diets. In addition, ruminal proportion of propionate was increased ( $p = .03$ ) in lambs received GLS compared to ELS diets. However, ruminal proportions of butyrate ( $p = .01$ ) and BCVFA

( $p = .05$ ), as well as ruminal concentration of  $\text{NH}_3\text{-N}$  ( $p = .02$ ) were increased in ELS diets compared with GLS diets. Regarding the dietary protein content, the 15CP diets increased ruminal  $\text{NH}_3\text{-N}$  concentration compared to 12CP diets (tendency;  $p = .09$ ). Besides, feeding 15CP diet reduced butyrate proportion compared to 12CP diets ( $p = .02$ ) and no other changes were found between 15CP diets and 12CP diets regarding the ruminal fermentation pattern.

There was greater number of cellulolytic protozoa in ruminal fluid of lambs received GLS compared to lambs fed ELS ( $p = .03$ ; Table 3). No difference was found regarding the entodinium and holotrichs protozoa when lambs fed different experimental diets.

### Purine derivatives in urine and microbial protein yield

The results for excretion of urinary purine derivatives and microbial protein production are indicated in Table 4. The allantoin excreted through urine ( $p = .02$ ) and total PD excretion ( $p = .01$ ) are increased in lambs fed GLS compared to lambs received ELS diets. Accordingly, microbial protein yield (MPY) was greater in lambs fed GLS diets compared to ELS diets ( $p = .01$ ). Regarding the dietary protein content, the 15CP diets increased allantoin ( $p = .05$ ) and total PD ( $p = .04$ ) excretions through urine compared to 12CP diets and then MPY increased in lambs fed 15CP diet compared to 12CP diets ( $p = .04$ ). No interaction was found regarding the linseed processing method and dietary

**Table 3.** Least square means for rumen fermentation profile and protozoa population in lambs fed diets with different linseed processing methods (ground vs. extruded) and dietary protein content (12% vs. 15%, DM basis).

| Item   | Treatments <sup>a</sup> |        |        |        | SEM   | <i>p</i> -value <sup>b</sup> |     |         |
|--|-------------------------|--------|--------|--------|-------|------------------------------|-----|---------|
|  | GLS                     |        | ELS    |        |       | LS                           | CP  | LS × CP |
|  | 12CP                    | 15CP   | 12CP   | 15CP   |       |                              |     |         |
| Rumen fermentation profile                               |                         |        |        |        |       |                              |     |         |
| pH   | 6.36                    | 6.38   | 6.43   | 6.48   | 0.06  | .14                          | .56 | .82     |
| Short-chain fatty acids, mmol/L                          | 102.70                  | 103.40 | 99.20  | 101.10 | 1.73  | .09                          | .43 | .71     |
| Acetate, mM/100 mM                                       | 60.80                   | 61.20  | 59.70  | 59.90  | 0.62  | .12                          | .70 | .83     |
| Propionate, mM/100 mM                                    | 25.70                   | 25.90  | 23.80  | 24.60  | 0.59  | .03                          | .29 | .47     |
| Butyrate, mM/100 mM                                      | 10.80                   | 9.50   | 12.90  | 11.70  | 0.47  | .01                          | .02 | .85     |
| Acetate: Propionate                                      | 2.30                    | 2.30   | 2.50   | 2.40   | 0.08  | .13                          | .57 | .48     |
| Branched-chain volatile fatty acids, mM/100 mM           | 2.70                    | 3.40   | 3.60   | 3.80   | 0.26  | .05                          | .21 | .26     |
| NH <sub>3</sub> -N, mg/dL                                | 13.40                   | 14.70  | 15.50  | 16.00  | 0.58  | .02                          | .09 | .71     |
| Protozoa population (×10 <sup>3</sup> mL <sup>-1</sup> ) |                         |        |        |        |       |                              |     |         |
| Entodinium sp  | 605.60                  | 617.80 | 511.30 | 567.70 | 47.16 | .13                          | .45 | .64     |
| Holotrichs   | 19.20                   | 21.10  | 15.90  | 17.80  | 2.47  | .19                          | .34 | .99     |
| Cellulolytic <sup>c</sup>                                | 4.10                    | 4.90   | 3.30   | 3.00   | 0.65  | .03                          | .68 | .36     |
| Total  | 628.90                  | 643.80 | 530.50 | 588.40 | 47.43 | .11                          | .44 | .65     |

<sup>a</sup>Treatments were: 1) Ground linseed (GLS) with 12% dietary protein content (GLS-12CP); 2) Ground linseed with 15% dietary protein content (GLS-15CP); 3) Extruded linseed (ELS) with 12% dietary protein content (ELS-12CP); 4) Extruded linseed with 15% dietary protein content (ELS-15CP).

SEM = Standard error of means; LS = Linseed; CP = Crude protein.

<sup>b</sup>Statistical comparisons: LS = linseed processing method (ground linseed vs. extruded linseed); CP; 12 vs.15% dietary protein content based on dry matter; LS × CP = interaction of linseed processing method and dietary protein content.

<sup>c</sup>The cellulolytic protozoa family was Epidinium + Eudiplodinium + Polyplastron.

**Table 4.** Least square means for urinary purine derivatives, microbial protein synthesis, and blood metabolites in lambs fed diets with different linseed processing methods (ground vs. extruded) and dietary protein content (12% vs. 15%, DM basis).

| Item   | Treatments <sup>a</sup> |        |        |        | SEM  | <i>p</i> -value <sup>b</sup> |     |         |
|--|-------------------------|--------|--------|--------|------|------------------------------|-----|---------|
|  | GLS                     |        | ELS    |        |      | LS                           | CP  | LS × CP |
|  | 12CP                    | 15CP   | 12CP   | 15CP   |      |                              |     |         |
| Urinary purine derivatives and microbial protein synthesis |                         |        |        |        |      |                              |     |         |
| Allantoin, mmol/d  | 8.34                    | 8.96   | 6.14   | 8.03   | 0.65 | .02                          | .05 | .33     |
| Uric acid, mmol/d  | 2.67                    | 2.71   | 2.46   | 2.41   | 0.19 | .21                          | .99 | .83     |
| Xanthine + hypoxanthine, mmol/d                            | 1.21                    | 1.28   | 1.03   | 1.12   | 0.12 | .11                          | .43 | .95     |
| Total PD <sup>c</sup> , mmol/d                             | 12.21                   | 12.95  | 9.64   | 11.57  | 0.84 | .01                          | .04 | .36     |
| Microbial protein synthesis, g/d                           | 65.70                   | 69.73  | 51.79  | 62.13  | 3.59 | .01                          | .04 | .36     |
| Blood metabolites and liver enzymes                        |                         |        |        |        |      |                              |     |         |
| Glucose, mg/dl   | 66.40                   | 67.60  | 62.40  | 65.10  | 1.53 | .03                          | .09 | .56     |
| Blood urea nitrogen, mg/dl                                 | 18.80                   | 20.90  | 20.60  | 21.30  | 0.70 | .04                          | .08 | .29     |
| Cholesterol, mg/dl   | 60.30                   | 62.20  | 56.50  | 57.20  | 1.82 | .02                          | .47 | .73     |
| HDL-cholesterol, mg/dl                                     | 37.90                   | 39.30  | 33.80  | 35.30  | 1.64 | .04                          | .41 | .99     |
| LDL-cholesterol, mg/dl                                     | 22.40                   | 22.90  | 22.70  | 21.90  | 1.16 | .74                          | .95 | .60     |
| Triglyceride, mg/dL  | 25.50                   | 24.70  | 27.10  | 26.40  | 1.01 | .09                          | .55 | .84     |
| Total protein, g/dl  | 5.60                    | 6.00   | 5.30   | 5.90   | 0.34 | .23                          | .06 | .81     |
| Aspartate aminotransferase, U/L                            | 104.90                  | 103.50 | 106.70 | 111.20 | 3.46 | .21                          | .65 | .34     |
| Alanine aminotransferase, U/L                              | 19.30                   | 20.20  | 18.10  | 18.60  | 1.21 | .26                          | .42 | .84     |

<sup>a</sup>Treatments were: 1) Ground linseed (GLS) with 12% dietary protein content (GLS-12CP); 2) Ground linseed with 15% dietary protein content (GLS-15CP); 3) Extruded linseed (ELS) with 12% dietary protein content (ELS-12CP); 4) Extruded linseed with 15% dietary protein content (ELS-15CP).

SEM = Standard error of means; LS = Linseed; CP = Crude protein; PD = purine derivatives; HDL = High density lipoprotein; LDL = Low density lipoprotein; DM = Dry matter.

<sup>b</sup>Statistical comparisons: LS = linseed processing method (ground linseed vs. extruded linseed); CP; 12 vs.15% dietary protein content based on dry matter; LS × CP = interaction of linseed processing method and dietary protein content.

<sup>c</sup>Total PD (mmol/d) = allantoin + uric acid + xanthine + hypoxanthine.

protein content with respect to the urinary PD excretion.

### Metabolites and liver enzymes of blood

The results indicated that feeding diets contained GLS to lambs increased the blood concentrations for glucose ( $p = .03$ ), total cholesterol ( $p = .02$ ), and HDL-cholesterol ( $p = .04$ ) compared to feeding ELS diets (Table

4). However, feeding ELS diets increased the concentrations of BUN ( $p = .04$ ) and triglyceride (tendency;  $p = .09$ ) in the blood of lambs compared to feeding GLS diets. The 15CP diets increased the concentrations of glucose (tendency;  $p = .09$ ), BUN (tendency;  $p = .08$ ) and total protein (tendency;  $p = .06$ ) in the blood of lambs compared to 12CP diets. No effect of experimental treatments was found on liver enzymes activity when lambs fed different processed linseeds or

different dietary protein content ( $p > .05$ ). There was no interaction between linseed processing method and the level of dietary protein for blood metabolites and liver enzymes.

## Discussion

### *Linseed processing method*

DMI was greater in lambs received GLS compared to ELS diets (1383 vs. 1323 g/d for GLS and ELS, respectively) probably because of greater ruminal availability of FA in extruded linseed can contribute to appetite control. As Doreau et al. (2009) stated before, the extrusion process of oilseeds can rupture the plant cells, and subsequently enhance the accessibility of cytosolic lipids to the animal compared with feeding the whole seed. Thus, the processed oilseeds have the potential to modify the intake, ruminal fermentation pattern, nutrient digestibility, and microbial activity compared to intact oilseeds via the modulation the fat availability due to the effect of the processing method. In general, the impact of fat supplementation on feed intake in ruminants was ascribed to factors such as dietary energy level, forage to concentrate ratio, level and source of supplemented fat, animal housing and management, and environmental condition (Allen et al. 2009; Hill et al. 2015; Kandi et al. 2020). In the current study, the dietary energy content was constant among experimental diets; therefore, it can be postulated that probably modification of the ruminal fermentation of the linseed processing method may contribute to lower intake in lambs fed extruded linseed in comparison with lambs fed ground linseed.

Evaluating the digestibility results suggests that lower digestibility of DM, CP, and OM found in ELS diet compared to GLS diet may partly involve intake reduction when lambs fed extruded linseed. Previous studies clarified that lower nutrient digestibility was observed when ruminally unprotected FAs are included in lamb's diet (Bhatt et al. 2011). The lower nutrient digestibility then can lead to a lower intake of ruminants (Ghorbani et al. 2020). The related mechanism has been discussed to be the adverse influences of FAs on the ruminal microbes that can enhance the rate of passage in digestive tract, causing looser faeces and hence lower intake and digestibility (Ghorbani et al. 2020). The bacterial population was not measured in our study; however, reduced cellulolytic protozoa population in ELS diets compared to GLS diets may indicate the negative effect of extrusion on fibre digestion. Hence, ADF digestibility was negatively

influenced by feeding extruded linseed. Previous studies indicated negative effects of FA on ruminal protozoa population and hence reduced fibre digestion in ruminants (Enjalbert et al. 2017). Because bacteria play a pivotal role in fibre digestion in the rumen, more research are necessary to evaluate the impact of oilseed processing methods on the rumen bacteria population.

Lower final BW observed in lambs received ELS compared to lambs fed GLS (40.4 vs. 38.6 kg for GLS and ELS, respectively) can be mostly related to the lower intake and nutrient digestibility which can influence animal performance. Greater intake in lambs fed GLS provides more metabolisable energy (14.94 vs. 14.20 MJ/d for GLS and ELS, respectively) which has been expended towards greater gain. In addition to the greater intake and energy supplied in GLS compared to ELS, the more favourable rumen fermentation profile and nutrient digestibility, and also higher microbial activity seem to be the influencers on achieving better gain in GLS diets compared to ELS diets. In fact, the higher availability rate of FA in for ruminal microbes in GLS diets than ELS diets had a negative effect on the fermentation rate in the rumen. Although the duodenal flow of n-3FA in lambs fed with different experimental treatments was not measured in our study; however, Doreau et al. (2009) revealed that the duodenal flow of n-3FA was equal to 7.8, 5.9, and 2.1 g/d (based on total FA flow into duodenum) in extruded, rolled and unprocessed linseeds, respectively. As it was well documented, linseed is rich in UFA (i.e. C18:3) which has the potential to have negative effects on ruminal microbial activity (Ikwegbu and Sutton 1982) and reduce nutrient digestibility in lambs (Lyons et al. 2017). Changes in proportions of propionate and butyrate are in agreement with previous works which resulted in a shift in fermentation pattern and hence differed in individual SCFA production in the rumen due to the various availability to UFA for ruminal microbes (Martin et al. 2016), which was caused by different processing methods. The total SCFA concentration in the ruminal fluid is reduced (tendency) when lambs received ELS diets. Besides, propionate proportion was also reduced in lambs fed ELS diets compared to GLS diets. The high amount of the energy requirement in ruminants (nearly 70%) is provided via SCFA production in the rumen (Bergman 1990). Moreover, propionate is a key SCFA which contributes to the energy status of ruminants due to its role in glucose homeostasis in ruminants (Bergman 1990). The greater total SCFA concentration and the propionate proportion found in

the rumen of lambs fed GLS are possibly because of higher digestibility of OM found in GLS diets (Clark et al. 1992).

Greater PD excretion via urine and higher MPY in GLS diets partly contributed better gain than ELS diets. The greater urinary concentration of allantoin and subsequently total PD observed in GLS diets (total PD was 12.58 vs. 10.60 mmol/d for GLS and ELS, respectively) indicated the greater ruminal microbe's activity when lambs fed ground linseed compared to feeding extruded linseed. Higher fat availability for ruminal microbes interferes with their activity and reduces produced microbial mass (Ikwegbu and Sutton 1982). The negative effects of FA on rumen microbes in ruminants is discussed to be related to the oily nature of FA that interferes with the ruminal microorganism ecosystem (Palmquist and Jenkins 1980). In this regard, Palmquist and Jenkins (1980) explained how the structure of fats might influence rumen microorganisms' activity. For instance, the possible adsorption of FA on to the bacterial membrane surfaces can lower the microbial activity. This can reduce the provision of some minerals, such as magnesium and calcium, needed for the optimum ruminal fermentation by forming soaps of magnesium and calcium. Furthermore, some antimicrobial impacts of polyunsaturated fatty acids (PUFAs) can be related to the potential toxicity on cellulolytic bacteria or coat the fibre in the rumen fluid and prevent optimum fibre digestion. Indeed, different availability to PUFAs in the rumen which can be modified via different processing methods, have the potential to produce numerous intermediates during the rumen bio-hydrogenation which may have negative effects during fermentation process. The higher ruminal  $\text{NH}_3\text{-N}$  concentration along with lower MPY estimated in ELS indicates the lower nitrogen efficiency in this diet. In fact, accumulated  $\text{NH}_3\text{-N}$  concentration in the rumen fluid was not used efficiently towards microbial protein synthesis. Some discrepancies are present regarding the effect of extruding of linseed on its ruminal protein degradation rate. For instance, some studies reported which extrusion reduced ruminal degradability of oilseeds and legume seeds (Bayourthe et al. 1998; Orias et al. 2002). However, in contrast, some others believe that high fat content of oilseeds may limit thermal and mechanical effects by reducing the shearing force and heat elevation inside the extruder (Melcion et al. 1988). Besides, extrusion of oilseeds may release some of the oil, and disrupt the protein matrix, and even increase ruminal degradability (Reddy et al. 1994). However, the effect of extrusion

on ruminal nutrient degradability may vary from one type of feed to another (Mustafa et al. 2003). Higher ruminal  $\text{NH}_3\text{-N}$  concentration shows that the extrusion of linseed cannot protect protein via microbial degradation or even increased protein degradation which was reported in previous work (Mustafa et al. 2003). Because oilseeds contain a high amount of fat and protein, more work is granted to evaluate the interactive effects of various processing methods on fat and protein degradation in the rumen.

The higher BUN concentration in lambs received ELS diets is in line with higher ruminal  $\text{NH}_3\text{-N}$  concentration which indicated lower nitrogen efficiency (Makizadeh et al. 2020). Blood glucose concentration is reduced in ELS diets because of lower intake compared to GLS diets. Veterinary works on growing calves were indicated that the HDL-cholesterol status can be an indicator for diarrhoea and illness in growing animals (Kazemi-Bonchenari et al. 2020). Discussing cautiously, higher HDL-cholesterol in GLS may be an indicator for the healthier condition of lambs fed this diet compared to ELS diet. Gathering all results together for discussed items, it can be concluded that extruding the linseed is not an appropriate processing method for ruminal fermentation, microbial activity, and health of lambs compared to grinding type.

### **Dietary protein content**

DMI was improved when lambs fed 15CP diets compared to feeding 12CP diets probably because positive effects of higher dietary content on appetite which was stated in lambs (Haddad et al. 2001). Based on a vast protein difference from 8.5% to 14.5% in growing lambs, Drouillard et al. (1991) reported a 7% higher DMI when a 14.5% CP diet were fed to lambs. A continual rise in DMI was reported as the level of dietary protein increased being highest for the 16% diet compared with 10, 12 and 14% dietary protein levels (Haddad et al. 2001). Because the metabolisable energy content of the dietary treatments was relatively similar in the current study, the change in DMI is mainly due to the dietary protein content. In contrast, some researchers did not report any effect of dietary CP level on DMI in lambs (Beauchemin et al. 1995). Discrepancies among studies may be linked to factors including the ratio of concentrate to forage, the stage of animal growth, and the range of dietary protein evaluated (Beauchemin et al. 1995; Haddad et al. 2001).



ADG and the final BW were greater in lambs received 15CP diets compared to feeding 12CP diets, mainly due to the greater intake in this diet. Increased intake amount in 15CP diets increased metabolisable energy supplementation to the animals (14.28 vs. 14.85 MJ/d for 12 and 15CP diets, respectively) that granted better growth and gain.

Different dietary protein contents did not have an impact on total SCFA production in the rumen fluid of lambs that can be related to similar nutrient digestibility observed for 12CP and 15 CP diets. However, the ruminal butyrate proportion was increased in 12CP diets compared to 15CP diets that can be due to the higher NFC content in 12CP diets compared to 15CP diets which were related to the different amounts of corn grain and wheat bran incorporated in experimental diets. Indeed, various dietary carbohydrate source and level provided different fermentation substrates for ruminal microbes that subsequently supposed to affect the ruminal fermentation pattern, which resulted in different total SCFA concentration and proportions of individual SCFAs (Jiriaei et al. 2020; Makizadeh et al. 2020).

Regardless of the difference found for intake and the amount of energy supplied across experimental treatments for different dietary protein contents in the current study, increased microbial protein yield in 15CP diets also can contribute to improved gain compared to lambs fed 12CP diets. The higher dietary protein content provided adequate nitrogen requirement towards microbial protein synthesis. As indicated in previous works, in addition to the ruminal  $\text{NH}_3\text{-N}$  concentration, free AA and peptide sources can positively impact microbial activity in ruminants (Griswold et al. 1996). The 15CP diets had greater soybean meal content compared to 12CP diets that can enhance the production of free AA and peptide in rumen after degradation (Khezri et al. 2009; Makizadeh et al. 2020). Therefore, expected higher ruminal AA and peptide concentration in 15CP diets can have a positive impact on microbial protein synthesis (Griswold et al. 1996). Our results suggest that although higher dietary protein content increased ruminal  $\text{NH}_3\text{-N}$  concentration which can be an indicator of lower nitrogen efficiency; but, increased MPY seems to provide higher supply of metabolisable protein (MP) and subsequently higher AA for absorption in the small intestine (Kazemi-Bonchenari et al. 2018).

The higher blood level of glucose (tendency) in lambs fed 15CP diets is partly related to increased DMI intake found in this diet. Greater intake can provide more energy supplied for animal; hence, glucose

as an energy indicator can increase with higher DMI. Moreover, as another mechanism contributed to higher blood glucose concentration in 15CP diets compared with 12CP diets, it can be postulated that more MP entered into the small intestine via improved MPY may contribute in gluconeogenesis and positively impact the blood glucose concentration (Young 1977). The greater blood TP (tendency) concentration observed in 15CP diets can result from more AA provided for protein synthesis in the liver (Makizadeh et al. 2020; Yousefinejad et al. 2021). Evaluating the diets with 15CP diets compared to 12CP in growing lambs indicate that the performance would be more favourable when lambs fed 15CP diets which are supposed to be achieved mainly via increased microbial protein synthesis and higher availability of MP into small intestine.

### ***Interaction between linseed processing and level of dietary protein***

Looking into the interaction results between linseed processing method and dietary protein level shows that ADG was increased and FCR was improved when lambs fed ground linseed with 15% protein content in the diet. Regardless of the processing method, feeding oilseeds can negatively affect the performance of growing ruminants (Ghorbani et al. 2020). However, comparing the ADG found in ELS diets and different dietary protein content (180 and 221 g/d for ELS-12CP and ELS-15CP, respectively) show that higher dietary protein content can partly compensate negative influences of feeding extruded linseed diet in lamb. Focussing on dietary protein content, it could be declared that higher dietary protein content can provide higher rumen undegradable protein (RUP) in experimental diets. Besides, different processing methods on oilseeds can influence the nitrogen degradability in the rumen. Thus, different dietary RUP contents are expected to be provided by experimental diets that can partly have an impact on animal performance via the alteration of AA available in the small intestine. Although we did not measure RUP content in the experimental treatments in the current study; however, it should be considered that the greater RUP content can increase MP reaching in to the small intestine and then provide a greater AA level towards animal growth performance improvement (Makizadeh et al. 2020).

Previous studies in ruminant nutrition revealed that supplemental fat reduced intake, protein digestibility, and microbial protein yield (Bhatt et al. 2011) that all

have potential to contribute to achieve the lower MP reached small intestine, which is critical for obtaining optimum growth in growing animals (NRC 2007). The results of current study confirm previous studies which was proposed that higher dietary protein content may compensate the negative effects of dietary fat on lower MP (Yousefinejad et al. 2021). Furthermore, our results suggest that although the extrusion of the linseed reduced MPY and negatively influenced the growth rate of lambs; however, higher dietary protein content can improve daily gain (41 g more ADG) as observed in lambs fed ELS-15CP diet compared to ELS-12CP diet. It can be postulated that in addition to valuable effect of higher dietary protein content on growth performance in growing animals, this can be a strategy for reducing the negative effects of processed oilseeds to improve ruminal fermentation and microbial activity in ruminant nutrition which needs to be more evaluated in future works.

## Conclusions

Feeding extruded linseed in growing lambs reduced intake, gain, digestibility of OM and CP compared with the diets contained ground linseed. Moreover, negatives effects of extruded linseed were found on ruminal fermentation pattern and microbial protein yield when compared with feeding the ground linseed. Regarding the dietary protein content, intake, gain, and microbial protein yield was improved when growing lambs received 15CP diets compared to 12CP diets. In summary, it was found that ground linseed resulted in more favourable performance, ruminal fermentation profile, and nitrogen metabolism in growing lambs than the extruded linseed. Moreover, greater dietary protein content has additional value when lambs received either ground or extruded linseed diets.

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## Ethical approval

All procedures related to animals were certified by the Animal Care and Use Committee of Bahonar University (IACUC Protocol #IR2018011) described by the Iranian Council of Animal Care (Iranian Council of Animal Care 1995).

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Data availability statement

Data available on request due to privacy/ethical restrictions.

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