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The effects of dietary supplementation with red grape pomace treated with ozone gas on ruminal fermentation activities, nutrient digestibility, lactational performance, and blood metabolites in dairy ewes

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ABSTRACT

This study aimed to investigate the effect of red grape pomace (RGP) treated with ozone (O_3) gas as a substitute of low-quality alfalfa hay on the ruminal fermentation, nutrient digestibility, and milk production performance of fat-tailed, fed RGP substituted high fibrous alfalfa hay. Present study was performed in two experimental phases: 1) *In situ* and *in vitro* phase: Four Ghezel ewes in their third gestation (30 months old; weighing 51 ± 2 kg) with surgically implanted cannulas in their rumen were used to assess the in situ degradability and to obtain ruminal fluid to assay the *in vitro* gas production; the treatments were RGP not-processed (control; T0), and RGP processed with O_3 for 6 (T1), 12 h (T2), and 24 h (T3). Processing RGP with O_3 gas reduced ($P < 0.05$) neutral detergent fiber (NDF) and acid detergent fiber (ADF), although the processing time did not show differences. Ozone-treated RGP caused a reduction ($P < 0.02$) in total phenolics, total tannins, condensed tannins, protein-bound tannins, and fiber-bound tannins, compared to the control. Processing RGP with O_3 for 12 h improved the nutritional value of RGP because of the reduction of the concentration of phenolic compounds, the increase of the *in vitro* gas production and the dry matter (DM) degradability, and improved fermentation parameters and it was chosen for the second phase of the experiment. 2) *In vivo* phase: Forty-eight healthy multiparous fat-tailed Ghezel ewes received the control diet (0 % RGP), or diets that substituted 20, 40, and 60 % of the dietary alfalfa hay with RGP processed with O_3 for 12 h. Incremental dietary replacement of RGP with alfalfa hay did not influence the ruminal total volatile fatty acids but increased ($P < 0.03$) propionate. The digestibility coefficients of the DM and the NDF increased ($P < 0.001$) in diets containing RGP *versus* the control, but not for the crude protein and ether extract. The ruminal protozoa population exhibited a linear increase with the addition of O_3-treated RGP ($P < 0.001$). Plasma glucose and albumin concentration increased ($P < 0.04$), and milk production and milk fat, protein, and lactose contents also increased ($P < 0.05$) with the alfalfa substitution with O_3-processed RGP. In conclusion, replacing high-fibrous alfalfa hay with RGP treated with O_3 for 12 h improved the apparent digestibility of DM and NDF and maintained the performance of the ewes.

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1. Introduction

All over the world, agricultural and industrial waste and by-products are generated in increasing amounts [1], which can cause environmental pollution because they do not decompose quickly in the environment. Due to growing concerns about environmental pollution and global warming issues -particularly CH_4 formation from anaerobic digestion of waste biomass-effectively managing the utilization of agro-industrial by-products mainly as animal feedstuffs might reduce the environmental impact of agricultural and livestock production [2]. Most of the agro-industrial by-products have the potential to be used as animal feed and even some of them might reduce the carbon (C) footprint associated with farm animal production and help to achieve zero carbon production systems. Although by-products might be used for all farm animals, ruminants have an active and diverse microbial community in the rumen to meet their nutritional requirements from bulky and high fiber content agro-industrial by-products.

The grape has an important share of global fruit production, being among the five top-produced fruits in the world [3]. The world's annual grape production was estimated to be about 75 million metric tons, which accounts for 8–11 percent of total fruits produced in the world. Grapes are used mainly for juice and wine making, and the processing produces grape pomaces as the main by-product. Grape pomace constitutes 10–20 percent of grapes' weight. This waste material includes skins, seeds, and any other solid remaining materials after the grape juice extraction through pressing. It is estimated that the production of 6 L of wine generates one kg of grape pomace, which amounts to 10.5–13.1 million tons of grape pomace in the world annually [3]. Because of a low extraction during winemaking, grape pomace retains high levels of condensed tannins (CT), mainly proanthocyanidins composed of procyanidin and prodelphinidin units, anthocyanins, catechins, flavanols, alcohols, stilbenes, as well as benzoic and p-coumaric acids [4]. The content of CT and total polyphenols in RGP may reach a value of about 50 g/kg DM [5], which have both positive health effects as antioxidants [6], and negative digestibility consequences due to the formation of indigestible complexes with proteins and cell wall carbohydrates [7]. While low to moderate levels of CT offer health benefits, tannins have been observed to adversely affect palatability, feed intake, digestibility, and production efficiency in ruminants [8] and it is recommended to process the tanniferous feedstuffs in order to deactivate the CT. Hence, before utilizing RGP in animal feed, it is necessary to undergo a processing stage.

Extensive research has been conducted and published on different processing techniques for various feed resources containing CT [9]. However, many of the currently recognized efficient processing methods rely on the use of chemicals or fossil fuels to deactivate or break down CT [10] and unfortunately, these approaches contribute to an amplified environmental impact and an increased carbon footprint in farm animal feeding.

Ozone (O_3) is a naturally occurring highly reactive molecule with a very short half-life gaseous molecule, generated immediately from freely available air and unlike other chemicals, the decomposition of ozone into oxygen does not leave toxic residues [11,12] and has been approved by the Food and Drug Administration (FDA). *'Ozonation'* is an emerging green technology that employs ozone for different purposes. Ozone has been proposed as an effective and environmentally friendly oxidative method for treating lignocellulosic materials while minimizing the impact on cellulose and hemicellulose [13,14]. Owing to its potent oxidizing ability, ozone seems to oxidize disulfide bonds in the feather meal and was found to improve its digestibility [15]. Accordingly, we postulate that the processing of RGP with O_3 gas leads to the inactivation or decomposition of a substantial portion of phenolic compounds, mainly CT and lignin, and boosts its nutritional value. Consequently, the present study was designed to examine the effect of O_3 gas on phenolic compounds, chemical composition, ruminal degradability, and digestibility of RGP and to test if it can replace the alfalfa hay in milking ewes. A second anticipated effect of the ozone is the inactivation of microbes, so that the storage time of RGP until feeding can be increased, adding safety.

2. Material and methods

2.1. In vitro and in situ experiments

2.1.1. Sample collection and chemical analysis

Samples of RGP were collected during four weekly visits to five industrial juice factories in Urmia city, located in the West Azerbaijan province of Iran. During each visit to the juice factories, 200 kg of RGP were collected, resulting in a total of 4000 kg of RGP. These samples were then dried in a large air-circulating oven at 40 °C. Once dried, the samples were thoroughly mixed and divided into twenty smaller samples, and a 10 kg subsample from each of the samples was used for different treatments, with a total of five replications for each treatment (processing time).

The DM, ash, ether extract, and crude protein content were determined according to Ref. [16]. Amylase-treated NDF and ADF contents were analyzed using the automated Ankom apparatus [17]. The RGP samples (200 mg) were ground and transferred into a 25 ml glass vessel. Next, 10 ml of acetone with a concentration of 70 % was introduced, and the container was then placed in an ultrasonic water bath at room temperature (Jencons, model 2800) for 20 min. Afterward, the materials underwent centrifugation at 3000*g* for 10 min at a temperature of 4 °C. The resulting supernatants were carefully collected, stored on ice, and utilized for assessing the levels of total phenolic and tannin compounds [18].

2.1.2. Reactor design, processing

A processing reactor was built of a stainless-steel tank with a double wall and a capacity of 10 kg (Fig. 1). To supply the necessary oxygen, an O_3 gas generator capable of producing 50 g of O_3 gas per hour was connected to the stainless-steel tank, along with an air pump. Additionally, a pre-inlet heater was utilized to raise the temperature of the O_3 gas inside the reactor to enhance the reaction rate [15]. The reactor was filled with dried RGP and O_3 gas was introduced while stirring for 6, 12, and 24 h at a pressure of 1.5 bar. To analyze the chemical composition, samples were ground to pass through a 1 mm screen using Wiley Mills.

2.1.3. In vitro gas production

Four Ghezel ewes in their third gestation (30 months old; weighing 51 ± 2 kg) with surgically implanted cannulas in their rumens were utilized in this research part of the experiment. The ewes were fed 10 % above maintenance level with 50 % alfalfa hay, 25 % corn silage, and 25 % barley. The Animal Care and Use Committee of Urmia University (IACUC Protocol #IR2018011) approved all animal procedures in advance [19]. The ewes were housed separately and subjected to proper care and sampling procedures as outlined by FASS, [20]. They were provided with two equally sized meals (one at 06:00 a.m. and another one at 06:00 p.m.) consisting of a balanced diet designed to meet energy requirements exceeding maintenance levels by 10 % according to National Research Council [21] guidelines.

To assess the effects of sample treatments on *in vitro* gas production (GP), which was performed according to Menke and Steingass [22] using a digital barometer [23] in an automated gas determination system with gas venting and continuous shaking at 39 °C. Rumen contents (liquid and solid phases) were obtained from four fistulated ewes before their morning meal, mixed and filtered through four layers of cheesecloth, and transported to the laboratory in insulated flasks at a temperature of 39 °C [22].

Dried RGP samples were ground to pass through a one mm screen, and 500 mg of each sample were weighed into incubation flasks. Anaerobically, 50 mL of buffered rumen fluid (a mix of rumen fluid and buffer at a ratio of 1:2 v/v) were added to 120 mL screw cap flasks. The

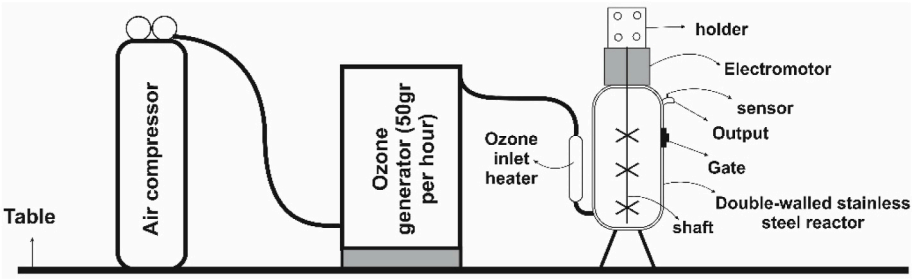

Fig. 1. Schematic of O_3 reactor for processing red grape pomace (RGP).

cumulative GP volume was measured in two separate runs with three replicates in each run for each experimental replication (n = 5) at different time intervals during the incubation (2, 4, 6, 8, 10, 12, 24, 48, 72, 96, and 120 h). The data were fitted using the Mitscherlich model proposed by France et al. [24] with the NLIN PROC of SAS according to Eq. (1).

$$GP = A\ (1 - e^{-c\ (T - L)}) \quad \text{Equation (1)}$$

Where: GP, total gas production at a specific time (measured in mL); A, potential cumulative GP (measured in mL); C, the rate at which gas is being produced (expressed as h^{-1}); T, the duration of fermentation (measured in hours); and L, time delay before GP begins (Lag phase; measured in hours).

Methane production and fermentation parameters were assessed using separate incubation flasks following a 24-h incubation period. To measure CH_4 production, the gas collected from three bottles in each trial was analyzed after the same 24-h incubation period. Gas samples from the headspace were obtained using gas-tight syringes (Hamilton, Reno, NV, USA).

Subsequently, 30 μL of each gas sample were injected into a gas chromatography system (Agilent 6820 series; Agilent Technologies Inc., Santa Clara, CA) equipped with a thermal conductivity detector and an HP-PLOT Q capillary column (Agilent Technologies Inc.) [25]. The ratio of CH_4 production to total gas production (mL of CH_4/mL total gas after 24 h) was then calculated by the method of Dehority, [26]. Following pH measurement, the contents of the vials were immediately placed in cold water at 4 °C and subsequently transferred into centrifuge tubes.

The vials were then centrifuged (15000 g, 20 min, 4 °C), and the supernatants were acidified (using metaphosphoric acid) and kept frozen until further analysis. Additionally, the amounts of *in vitro* degradability organic matter (DOM_{24}) and metabolizable energy (ME_{24}) were calculated using the models proposed by Menke and Steingass [22] according to Eqs. (2) and (3).

$$ME_{24}\ (MJ/kg\ DM) = 1.06 + (0.157 \times GP_{24}) + (0.084 \times CP) + (0.22 \times CF) - 0.081 \times Ash \quad \text{Equation (2)}$$

$$DOM_{24}\ (\%) = 0.9991 \times GP_{24} + 0.0595 \times CP + 0.0181 \times Ash + 9 \quad \text{Equation (3)}$$

2.1.4. *In situ nutrient degradability*

An in situ experiment was carried out to examine the effects of RGP processing on DM and NDF degradability parameters according to Vanzant et al. [27]). The samples were finely ground until they passed through a 2 mm screen [28], and particles smaller than 50 μm were removed by sieving. Nylon (PA) bags measuring 80 × 160 mm with a pore size of 50 μm were utilized to hold 4 g of the samples, achieving a sample size to surface area ratio of 100 mg/cm^2. Bags were then placed in the ventral rumen just before the morning meal for incubation periods of 2, 4, 8, 12, 24, 48, 72, and 96 h during two separate runs in triplicate. Portions of the dried bag residues were utilized for DM and NDF analysis. To calculate the effective degradability (ED) for DM and CP variables and degradation parameters were computed by fitting the data to the nonlinear model presented by Ørskov and McDonald [29] (Eq. (4)) utilizing the NLIN PROC of SAS Institute [30].

$$P = a + b\ (1 - e^{-ct}) \quad \text{Equation (4)}$$

Where: P, proportion degraded at a specific time point t; a, proportion that can dissolve in water; b, portion that is not water-soluble but can potentially be degraded in the rumen; c, degradation rate of the b fraction; t, duration of incubation; and e = exponential constant.

The sum of a and b is equated to the potentially degradable fraction. To determine the ED, assumed outflow rates of 0.02, 0.05, and 0.08 per h were utilized, following the methodology described by Ørskov and McDonald [29], as Eq. (5).

$$ED = a + (bc/c + k) \quad \text{Equation (5)}$$

Where: ED, effective degradability; a, b, and c, constants as stated in Eq. (4); and k, is the passage rate.

2.2. *In vivo evaluation study*

2.2.1. *Animals and diets*

A total of 48 early lactating Ghezel ewes in their third gestation (30 ± 2 months old; weighing 56 ± 3 kg) were used. The experiment spanned from 7 days post-partum to 45 days in milk. The ewes were randomly assigned into four groups, each consisting of 12 ewes. The allocation of animals in four groups was random. The feeding was individually based. Diet in terms of protein and energy did not change during the experimental period. Considering that the highest degradability and gas production was obtained in 12 h of processing with O_3, therefore, 12 h of processing was used in this part of the experiment.

The different treatments given to the ewes were as follows: 1) control diet with no processed RGP, 2) control diet with O_3-processed RGP for 12 h, replacing 20 % of alfalfa hay, 3) control diet with O_3-processed RGP for 12 h, replacing 40 % of alfalfa hay, and 4) control diet with O_3-processed RGP for 12 h, replacing 60 % of alfalfa hay. The ewes had free access to the total mixed ration diets that are presented in Table 1. Their energy and protein requirements, as specified by the National Research Council [21], were met using SRNS software (Version September 1, 6069). The ewes were individually fed twice daily at 6:00 and 18:00 h, and they had ad libitum to drinking water.

2.2.2. *Dry matter intake, weight changes, and nutrient digestibility*

The daily monitoring of dry matter intake (DMI) involved subtracting the weight of the remnants from the initial feed provided individually to each one. Additionally, the determination of DM content in the TMR and refusals was conducted every week through sampling. Weighing of all animals took place weekly, utilizing an on-farm digital scale. Fecal samples were collected directly from the rectum for five consecutive days in the last week of the experiment. The fecal collection was done two times a day, and samples for each animal were mixed after oven drying. Feed and ort sampling were also made during the fecal

Table 1
Feed composition of experimental diets.

Ingredient, g/kg DM	Ozone-12-h treated RGP, %[a]			
	0	20	40	60
Alfalfa hay	456.6	378.2	289.8	199.0
Wheat straw	91.4	93.9	96.6	99.5
Barley grain	146.1	122.1	96.6	69.7
Corn grain	137.0	140.8	144.9	149.3
Soybean meal	114.2	114.7	120.8	124.2
Red grape pomace (RGP)[b]	0.0	93.9	193.2	298.5
Mineral and vitamin premix[c]	22.8	23.5	24.2	24.9
Calcium Carbonate	22.8	23.5	24.2	24.9
Dicalcium Phosphate	9.1	9.4	9.7	10.0
Calculated Chemical analysis[d]				
OM, g/kg DM	119.7	116.3	112.8	109
CP, g/kg DM	152.3	152.7	153	153.4
EE, g/kg DM	20.3	23.2	26.3	29.6
NDF, g/kg DM	405.3	403.2	401	398.6
Lignin, g/kg DM	165.3	146.7	126.8	105.4
NEL, Mcal/day	1.34	1.38	1.42	1.46
MP, g/kg DMI	99.80	100.90	101.85	102.91

[a] Dietary level of the ozone-12-h treated red grape pomace (RGP) replaced by alfalfa hay at 0, 20, 40, and 60 % of RGP in the total mixed ration.
[b] Processed with O3 gas for 12 h.
[c] Mineral and vitamin premix Composition (in kg): Vitamin A, 600,000 International Units, Vitamin D3, 100,000 International Units; Vitamin E 300 International Units, Iron, 2000 mg, Copper, 200 mg, Manganese, 2000 mg, zinc, 3000 mg, cobalt, 100 mg, iodine, 100 mg, selenium, 1 mg, antioxidant 500 mg, magnesium, 18000 mg, Phosphorus, 90,000 mg, calcium, 160,000 mg, sodium, 50,000 mg.
[d] OM, Organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; MP, metabolizable protein; NEL, Lactation net energy.

collection period.

To calculate nutrient digestibility coefficients, acid-insoluble ash was employed as an internal marker, following the method described by Van Keulen and Young, [31].

2.2.3. Rumen fermentation parameters

On day 33 after regimen application, a rumen fluid sample was collected to assess the impact of dietary treatments on rumen parameters. The collection is performed through an esophageal tube and a vacuum pressure pump. To ensure rumen fluid without saliva, the initial samples were removed after entering the esophageal probe into the rumen, and then the main sampling was done. About 4 h after the morning meal, approximately 200 mL of saliva-free rumen fluid were obtained.

The pH of the rumen fluid was immediately determined using a calibrated pH meter (Titroline Easy 5000, Schott Titrator, SCHOTT AG, Mainz, Germany). After that, the rumen fluid was subjected to centrifugation at 15,000g for 20 min at 4 °C. Subsequently, 4 mL portions of the supernatant were combined with 1 mL of 25 % metaphosphoric acid and then stored at −20 °C until analysis for volatile fatty acids.

Analysis of VFA (volatile fatty acids) was conducted using GC (6820 Gas Chromatograph, Agilent Technologies, Santa Clara, CA, USA) equipped with HP-FFAP columns (J&W HP-FFAP GC Column, 30 m, 0.25 mm, 0.25 mm, 7-inch cage, Agilent Technology, USA). A previously described thermal programming of the GC column was utilized [32]. To count the number of protozoa, 50 % formalin solution was added to the rumen fluid at a ratio of 1:1 and kept at room temperature. The number of protozoa was counted under a light microscope [26].

2.2.4. Blood sampling and measurement of plasma metabolites

During the final two days of the experiment, blood samples were obtained from all ewes via the left external jugular vein and collected into 10 mL pre-evacuated tubes. These tubes contained heparinized fluid along with sodium fluoride and potassium oxalate. The collected blood samples were immediately subjected to centrifugation at a speed of 2500 g and a temperature of 25 °C for 15 min to separate the plasma. Subsequently, the plasma samples were stored at a temperature of −20 °C until they were ready for further analysis to determine the glucose (intra and inter-assay CV of 1.07 and 1.86, respectively), triglyceride (intra and inter-assay CV of 1.19 and 1.56, respectively), cholesterol (intra and inter-assay CV of 0.94 and 1.42, respectively), albumin (intra and inter-assay CV of 0.78 and 1.11, respectively), total protein (intra and inter-assay CV of 1.45 and 1.88, respectively) and non-esterified fatty acids (NEFA) (intra and inter-assay CV of 2.83 and 3.47, respectively) using commercially available diagnostic kits (Biorex-Fars, Shiraz, Iran) using a calibrated auto analyzer (BT-1500, Biotecnica Instruments, Rome, Italy).

2.2.5. Milk yield and composition

Milk production was measured at three-day intervals using the lamb weighing technique. To determine the amount of milk production, the lambs were kept apart from the mother for 12 h, then the lambs were weighed with a digital scale (with high accuracy), and at the same moment they ate the mother's milk for 10 min. The lambs were reweighed immediately after weaning and the difference in weight before and after milk consumption was recorded. Also, after separating the lambs from the mother, the remaining milk from the ewes' breasts was milked after an intramuscular injection of 3 cc of oxytocin and its amount was recorded. Finally, the total amount of milk produced in 12 h was multiplied by two to become 24 h [33].

To determine the composition of the milk, samples were collected from individual ewes over two consecutive days every week. Both mammary glands were milked, and a representative sample was obtained through thorough mixing. Potassium dichromate samples were utilized to analyze the concentrations of total solids, solids non-fat, fat, protein, and lactose using the MilkoScan FT6000 Spectrometer manufactured by Foss (Hillerød, Denmark).

The milk analysis was conducted by the Rajzhan Ltd. milk laboratory located in Karaj, Iran, adhering to the ISO 9622:2017 standard.

2.3. Statistical analysis

2.3.1. In vitro and in situ experiment

The RGP processing was conducted in five repetitions. In terms of chemical composition analysis, the average of laboratory replications (n = 5) was employed for statistical analysis. For the assessment of *in vitro* GP and fermentation parameters, the average of intra- and inter-run replications within each experimental replication (n = 5) was used for statistical comparison. The main effect was the processing time. In the case of *in vitro* gas production and ruminal nutrient degradability kinetics, incubation time and the interaction between time and treatment were also included in the model. The data were analyzed using a mixed model procedure, with a first-order variance-covariance structure selected based on the smallest Schwarz's Bayesian information criterion SAS [30]. Tuckey corrected least square means were compared using the PDIFF option.

A regression analysis was also carried out using Proc Reg to determine linear or quadratic effects of different processing times on chemical composition, phenolic compounds, nutrient degradability, GP coefficients, and rumen parameters.

2.3.2. In vivo experiment

The data was examined in a randomized design that had four different treatments. The statistical model employed for the measured parameters encompassed both random animal effects and fixed effects of experimental diets. Variables that had multiple measurements such as DMI, milk yield, and milk composition within the same ewe were examined using the fixed effects of treatment, time of measurement (in days), the interaction between treatment and time, and the random effect of ewe nested within a given treatment. The data was analyzed using the MIXED procedure of SAS 9.4 software. For the repeated measures,

the most suitable covariance structure (autoregressive order 1) was chosen. Data correction was carried out utilizing the Tukey test, while means were compared using the PDIFF option at a statistical probability level of 0.05. The data was reported as the least mean square along with the corresponding standard error of means.

Additionally, a regression analysis was conducted to ascertain the linear or quadratic effects of varying levels of RGP processed with O_3 gas on DMI, milk yield and composition, weight changes, apparent nutrient digestibility, plasma metabolites, and rumen fermentation parameters.

3. Results

3.1. In vitro and in situ experiment

3.1.1. Chemical composition

The processing of RGP with O_3 resulted in a linear increase ($P < 0.005$) in DM and CP contents, while the highest levels of DM and CP values were observed after 24 h of processing. Conversely, the content of NDF, ADF, and EE decreased ($P < 0.05$) after O_3 processing. Regression analyses revealed a linear increase ($P < 0.003$) in DM and CP contents with increasing treatment time, while NDF and ADF content showed a linear decrease (Table 2).

Various processing times of RGP ozonation linearly decreased ($P < 0.001$) in total phenolic compounds, total tannins, condensed tannins, protein-bond tannins, and fiber-bond tannins *versus* control samples (Table 3). However, total tannins and condensed tannins showed quadratic effects ($P < 0.007$) suggesting that best O_3-pretreatment results on RGP could be obtained after 12 h.

3.1.2. Gas production and fermentation parameters

The cumulative GP was not affected by the treatment of O_3 gas until 4h of incubation. However, after that point, there was an increase ($P < 0.05$) in GP for the samples treated with O_3 (Fig. 2).

Table 4 demonstrates that the gas produced from the potentially fermentable fraction (B) showed a notable increase and the rate of GP (C) decreased ($P < 0.001$) at 6 h in the samples with O_3-processed RGP for 6 and 12 h. In contrast, while DOM_{24} showed an increase ($P < 0.03$) in the O_3-processed RGP for 12 h, there were no differences observed in ME_{24} content and CH_4 production among the experimental treatments indicating that they were not influenced by the processing.

3.1.3. Rumen degradability of DM and NDF

The degradability of DM demonstrated a noteworthy rise in RGP that underwent processing for 6 and 12 h (Fig. 3, $P < 0.05$). The NDF degradability kinetics exhibited a significant increase ($P < 0.05$) in O_3-processed samples regardless of the duration of processing (Fig. 4).

The soluble fraction (A) and degradation rate of the potentially degradable fraction (C), for both DM and NDF, were not influenced by the O_3 processing (Table 5). However, the degradable fraction (B) and effective degradability of DM were increased ($P < 0.004$) in the O_3-processed RGP and showed quadratic effect across the pretreatment time, suggesting the best processing for 6 *versus* the unprocessed O_3 samples ($P < 0.0001$).

3.2. In vivo experiment

3.2.1. Dry matter intake, weight changes, and nutrient digestibility

No significant impact on DMI and daily weight changes by the dietary inclusion of O_3-treated RGP in newly delivered ewes was found (Table 6). However, when comparing ewes fed various quantities of O_3-processed RGP to the control, Table 7 results indicated a noteworthy increase ($P < 0.001$) in the digestibility of DM and NDF, showing linear trends ($P < 0.001$) across the O_3-pretreatment time. However, the substitution of alfalfa for RGP did not alter the digestibility of CP and EE.

3.2.2. Rumen fermentation parameter

The dietary inclusion of O_3-processed RGP at 40 % and 60 % led to a linear decrease ($P < 0.002$) in ruminal pH *versus* the control ewes (Table 8). Furthermore, the protozoa population exhibited a linear increase ($P < 0.001$) in ewes fed RGP treated with O_3 *versus* the control. Although there was no difference in the total VFA molar concentration among the different diets, the molar ratio of acetate, butyrate, and valerate showed a reduction ($P < 0.02$) with the dietary inclusion of O_3-processed RGP. The molar ratio of isovalerate and propionate increased ($P < 0.03$) at the 40 % and 60 % RGP substitution levels. The molar ratio of isovalerate and propionic acid linearly ($P < 0.01$) increased with an increasing level of RGP in the ewes' diet.

3.2.3. Milk yield and composition

The substitution of alfalfa with O_3-processed RGP led to a significant boost ($P < 0.001$) in milk production of the 40 % and 60 % RGP ewes (Table 9). Including O_3-processed RGP impacted milk components such as lactose, protein, and fat ($P < 0.05$), although it did not affect the SNF ($P = 0.79$).

3.2.4. Plasma metabolites

The dietary inclusion of O_3-processed RGP at the highest rate led to an increase ($P < 0.04$) in ewes plasma glucose and albumin levels *versus* control ewes (Table 10). However, there was a decrease ($P < 0.01$) in plasma triglyceride concentration of RGP ewes (Table 10). The dietary O_3-processed RGP did not have any effect on plasma cholesterol and NEFA (non-esterification fatty acids) concentrations, while albumin and glucose linearly increased with increasing dietary RGP levels ($P < 0.05$).

4. Discussion

4.1. In vitro and in situ experiments

The dry matter DM content of the RGP increased when exposed to Ozone. Similarly, a study by Ghorbani et al. [34], found that the addition of urea and O_3 gas during the processing of wheat straw increased its DM content. The feather meal hydrolysis using O_3 gas, caused an increase in

Table 2
Chemical composition[a] of RGP (g/100 g DM) of red grape pomace (RGP) processed with O_3 gas at different hours.

	O_3 Gas, h				SEM[b]	P-value[c]		
	Control	6	12	24		Treatment	Linear	Quadratic
DM	93.20[c]	94.75[b]	94.75[b]	96.45[a]	0.193	0.005	0.003	0.785
CP	10.14[c]	11.04[b]	11.94[a]	12.33[a]	0.071	0.008	<0.0001	0.066
Ash	4.10	4.11	4.12	4.11	0.052	0.980	0.981	0.990
EE	10.63[a]	9.84[ab]	9.26[b]	7.73[c]	0.231	0.010	0.009	0.196
NDF	44.75[a]	42.40[b]	41.85[b]	42.63[b]	0.441	0.050	0.055	0.007
ADF	28.51[a]	25.85[b]	24.73[b]	25.60[b]	0.340	0.010	0.001	0.046

[a] DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber.
[b] SEM, Standard error of the mean.
[c] In each row data with different superscripts is statistically different ($P < 0.05$).

Table 3
Phenolic compounds (g/100 g) of red grape pomace (RGP) processed with O_3 gas at different hours.

	O_3 Gas, h				SEM[a]	P-value[b]		
	Control	6	12	24		Treatment	Linear	Quadratic
Total phenolic	7.94[a]	6.53[b]	6.21[b]	5.26[b]	0.24	0.017	0.001	0.335
Total tannins	6.48[a]	4.96[b]	3.92[c]	3.83[c]	0.08	0.0007	0.002	0.007
Condensed tannins (CT)	5.33[a]	3.07[b]	2.19[c]	2.08[c]	0.02	<0.0001	<0.0001	0.0001
Protein bond CT	1.54[a]	1.09[b]	0.63[c]	0.31[d]	0.03	0.0002	0.0001	0.352
Fiber bond CT	1.47[a]	1.25[b]	0.86[c]	0.51[d]	0.03	0.0004	0.0001	0.249

[a] SEM, Standard error of the mean.
[b] In each row data with different superscripts is statistically different ($P < 0.05$).

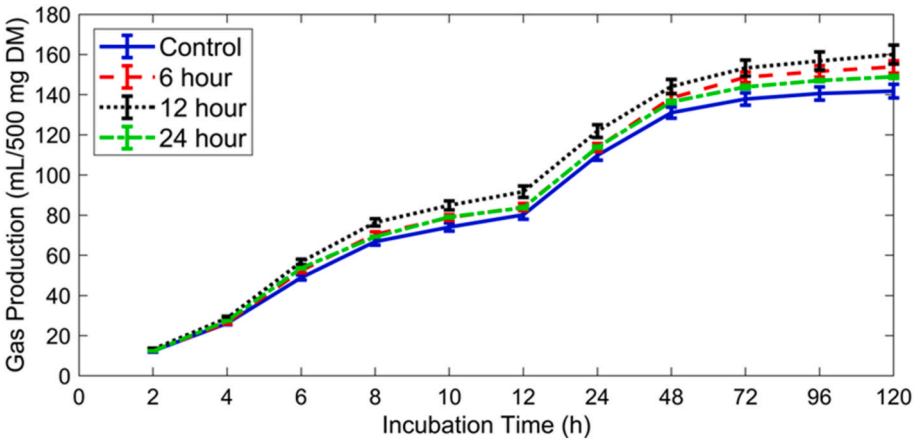

Fig. 2. The effect of red grape pomace (RGP) processed with O_3 gas at different times on the kinetics of *in vitro* gas production. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Ruminal fermentation and gas production *in vitro* of red grape pomace (RGP) processed with O_3 gas at different hours.

Parameter[a]	O_3 Gas, h				SEM[b]	P-value[c]		
	Control	6	12	24		Treatment	Linear	Quadratic
B, ml/500 mg DM	139.49[b]	150.13[a]	154.92[a]	145.61[ab]	3.28	0.46	<0.0001	<0.0001
C, ml/h	0.078[b]	0.073[c]	0.081[a]	0.079[b]	0.008	0.0001	<0.0001	<0.0001
Lag time, h	0.59[b]	0.48[c]	0.68[a]	0.58[b]	0.042	0.004	<0.0001	<0.0001
CH_4, ml/500 mg DM	58.49	63.13	63.02	59.90	0.772	0.054	0.774	0.006
pH_{24}	6.74[a]	6.66[ab]	6.62[b]	6.73[a]	0.011	0.034	0.536	0.001
Protozoa population$_{24}$, $\times 10^5$/mL	17.25[b]	18.20[a]	19.10[a]	18.15[ab]	0.010	0.015	0.070	0.042
ME_{24}, MJ/kg DM	11.52	11.61	12.07	11.74	0.124	0.151	0.091	0.132
DOM_{24}, %	52.07[b]	54.06[ab]	57.54[a]	54.59[ab]	0.637	0.033	0.012	0.012
$SCFA_{24}$, mmol/200 mg DM	0.98[b]	1.02[ab]	1.08[a]	1.03[ab]	0.014	0.047	0.035	0.014

[a] A, potential cumulative GP (measured in mL); C, the rate at which gas is being produced (expressed as h^{-1}); T, the duration of fermentation (measured in hours); and L, time delay before GP begins (Lag phase; measured in hours); DM, dry matter, ME, metabolizable energy; DOM, *in vitro* degradability organic matter; SCFA, short chain fatty acids.
[b] SEM, Standard error of the mean.
[c] In each row data with different superscripts is statistically different ($P < 0.05$).

the CP content, mainly as a result of the oxidative properties of the O_3 Asadnezhad et al. [15] and Ghorbani et al. [34] reported higher CP content in O3-processed wheat straw.

Furthermore, in a study by Ben Salem et al. [10], processing *Acacia cyanophylla Lindl* (shrub foliage) with oxidizing substances increased CP content. Ghorbani et al. [34] mentioned that the O_3 processing of wheat straw caused a decrease in NDF and ADF. Additionally, Barros et al. [35] reported that O_3 breaks down lignin and cross-links between lignin and carbohydrates in cell walls. This decomposition of lignin and chemical bonds contributes to a significant decrease in NDF and ADF contents, as well as a lower rumen fluid pH, indicating extensive hydrolysis of cell wall components and increased production of VFA in O_3-processed RGP.

The cell wall becomes more fragile and easier to digest through oxidative processing methods, leading to an increase in GP [34]. Lignin and phenolic substances can be oxidized by oxidative agents [36], resulting in the breaking of chemical bonds between these substances and cell wall carbohydrates and proteins. This breakdown makes them available for fermentation. It has been reported that condensed tannins negatively affect cellulolytic bacteria in the rumen [37].

The processing of RGP with O_3 reduced the phenolic substances and made the cell wall fragile, therefore the cumulative GP and GP rate were increased. However, it should be noted that even with longer processing times of 24 h, the cumulative GP did not increase significantly, and there was no improvement in fermentation parameters despite the reduction of phenolic compounds concentration. The nonlinear impact of ozonation on the digestibility and fermentability of the RGP can be explained by the formation of various cyclic compounds and carboxylic acids, which display antimicrobial properties, due to the reaction between

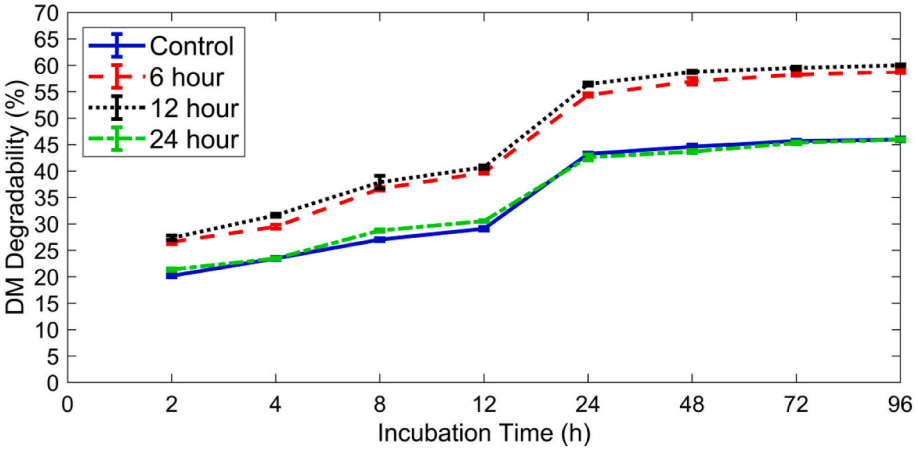

Fig. 3. The effect of red grape pomace (RGP) processed with O_3 gas at different times on the kinetics of in situ dry matter (DM) degradability. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

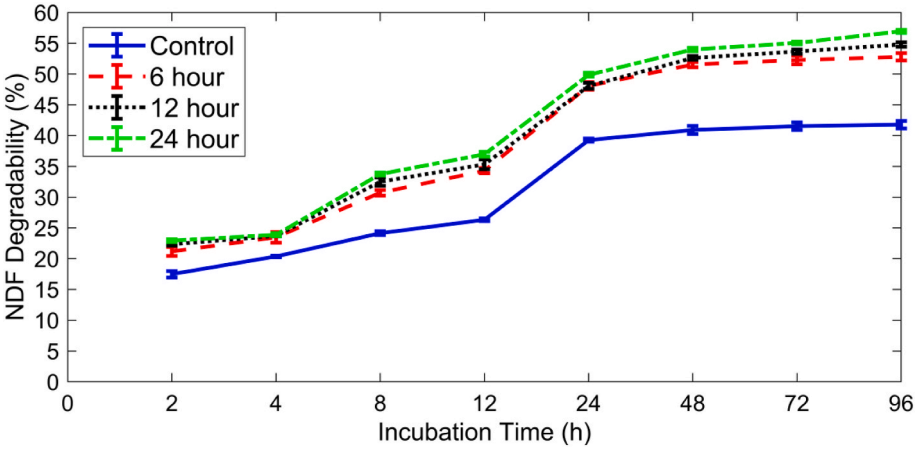

Fig. 4. The effect of red grape pomace (RGP) processed with O_3 gas at different times on the kinetics of in situ neutral detergent fiber (NDF) degradability. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Degradability of DM and NDF parameters (g/100g DM) of red grape pomace (RGP) processed with O_3 gas at different hours.

Parameter[a]	O_3 Gas, h				SEM[c]	P-value[d]		
	Control	6	12	24		Treatment	Linear	Quadratic
A_{DM}	13.08	13.96	18.70	13.94	1.583	0.147	0.267	0.074
A_{NDF}	5.59	8.81	9.75	9.74	1.770	0.071	0.304	0.588
B_{DM}	32.84^{bc}	44.26^{a}	40.74^{ab}	31.40^{c}	1.652	0.004	0.257	<0.0001
B_{NDF}	35.60	43.13	43.84	45.75	2.371	0.085	0.021	0.284
C_{DM}	0.075	0.092	0.082	0.080	0.005	0.285	0.845	0.097
C_{NDF}	0.079	0.089	0.083	0.085	0.011	0.864	0.444	0.657
$ED_{DM} = 0.05$[b]	32.86^{c}	42.69^{b}	44.03^{a}	33.32^{c}	0.011	<0.0001	0.002	<0.0001
$ED_{NDF} = 0.05$[b]	29.29^{b}	36.45^{a}	36.66^{a}	38.19^{a}	0.640	0.0001	<0.0001	0.022
$ED_{DM} = 0.08$[b]	29.05^{c}	37.70^{b}	39.36^{a}	29.71^{c}	0.176	<0.0001	0.007	<0.0001
$ED_{NDF} = 0.08$[b]	25.34^{b}	31.60^{a}	31.72^{a}	33.08^{a}	0.470	0.0001	<0.0001	0.0006

[a] A, Soluble fraction; B, potentially degradable fraction; C, degradation rate constant, ED, effective degradability.
[b] Rumen outflow rate.
[c] SEM, Standard error of the mean.
[d] In each row data with different superscripts is statistically different ($P < 0.05$).

excess ozone and oxidized substances, as well as the degradation products of lignin [13].

According to a study Travaini et al. [13], the degradation of sugar during O_3 processing results in the production of varying quantities of oxalic, formic, acetic, and levulinic acids. These acids can hinder the degradability process. The limited effect of O_3 processing on overall GP during early incubation could be attributed to the fact that raw grain products contain a significant amount of easily digestible carbohydrates [2], which can undergo fermentation even without being processed. This aligns with the findings of Getachew et al. [37], who observed that oxidizing chemicals increase the fermentability of plants containing tannins. Results regarding the effects of O_3 on CT concentration were

Table 6
Performance of ewes fed with rations containing different levels of O_3-12 h-processed red grape pomace (RGP).

Variable	Ozone-12-h treated RGP, %[a]				SEM[c]	P-value[d]			
	0	20	40	60		Treatment	Treatment x time	Linear	Quadratic
Weight Changes, kg/Week	−1.12	−1.04	−0.99	−0.95	0.041	0.07	0.08	0.638	0.944
DMI, kg/d[b]	2.38	2.37	2.38	2.39	0.022	0.92	0.68	0.961	0.957

[a] Dietary level of the ozone-12-h treated red grape pomace (RGP) replaced by alfalfa hay at 0, 20, 40, and 60 % of RGP in the total mixed ration.
[b] DMI, Dry matter intake.
[c] SEM, Standard error of the mean.
[d] In each row data with different superscripts is statistically different ($P < 0.05$).

Table 7
Apparent digestibility (g/kg) of ewes fed with rations containing different levels of O_3-12 h-processed red grape pomace (RGP).

Variable[b]	Ozone-12-h treated RGP, %[a]				SEM[c]	P-value[d]		
	0	20	40	60		Treatment	Linear	Quadratic
DM	796.1[c]	817.5[b]	830.1[a]	833.9[a]	5.05	<0.0001	<0.0001	0.0004
CP	707.4	711.9	714.2	716.4	2.11	0.06	0.416	0.772
OM	795.1	797.3	796.4	794.2	6.12	0.34	0.672	0.254
EE	641.7	641.8	639.2	640.2	4.09	0.33	0.471	0.838
NDF	648.4[d]	657.8[c]	671.3[b]	688.9[a]	1.08	<0.0001	<0.0001	0.016

[a] Dietary level of the ozone-12-h treated red grape pomace (RGP) replaced by alfalfa hay at 0, 20, 40, and 60 % of RGP in the total mixed ration.
[b] DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; OM, organic matter.
[c] SEM, Standard error of the mean.
[d] In each row data with different superscripts is statistically different ($P < 0.05$).

Table 8
Rumen metabolite of ewes fed with rations containing different levels of O_3-12 h-processed red grape pomace (RGP).

	Ozone-12-h treated RGP, %[a]				SEM[c]	P-value[d]		
Parameter	0	20	40	60		Treatment	Linear	Quadratic
pH	6.59[a]	6.57[ab]	6.52[bc]	6.50[c]	0.02	0.004	0.002	0.847
Protozoa population, $\times 10^5$/mL	19.10[c]	21.70[b]	24.20[a]	24.50[a]	0.20	<0.0001	<0.0001	0.001
Valeric acid, mol/100mol	2.92[a]	2.51[b]	2.49[b]	2.36[b]	0.05	0.017	0.073	0.416
Iso valeric acid, mol/100mol	3.61[b]	4.23[ab]	4.41[a]	4.50[a]	0.10	0.025	0.005	0.081
Butyric acid, mol/100mol	2.80[a]	2.52[b]	2.57[b]	2.33[c]	0.02	0.003	0.010	0.801
Propionic acid, mol/100mol	33.80[c]	36.11[bc]	36.07[ab]	39.98[a]	0.37	0.005	0.0009	0.712
Acetic acid, mol/100mol	56.84[a]	54.60[b]	52.43[c]	50.81[c]	0.29	0.002	<0.0001	0.335
Total VFA, mol/100mol[b]	112.24	113.18	113.14	113.59	1.15	0.863	0.612	0.887

[a] Dietary level of the ozone-12-h treated red grape pomace (RGP) replaced by alfalfa hay at 0, 20, 40, and 60 % of RGP in the total mixed ration.
[b] VFA = Volatile fatty acids.
[c] SEM, Standard error of the mean.
[d] In each row data with different superscripts is statistically different ($P < 0.05$).

Table 9
Milk yield (kg/d) and composition of ewes fed with rations containing different levels of O_3-12 h-processed red grape pomace (RGP).

	Ozone-12-h treated RGP, %[a]				SEM[c]	P-value[d]			
	0	20	40	60		Treatment	Treatment x time	Linear	Quadratic
Milk yield	1.02[b]	1.01[b]	1.08[a]	1.11[a]	0.021	<0.0001	0.34	0.551	0.879
Milk composition, %									
Fat	4.42[b]	3.73[a]	3.78[a]	3.58[a]	0.182	<0.001	–	0.131	0.143
Protein	3.75[b]	3.03[a]	2.96[a]	3.10[a]	0.234	0.05	–	0.065	0.204
Lactose	4.71[b]	4.14[a]	4.98[c]	5.17[c]	0.141	<0.001	–	0.218	0.071
SNF[b]	9.53	9.17	9.02	9.15	0.370	0.79	–	0.530	0.586

[a] Dietary level of the ozone-12-h treated red grape pomace (RGP) replaced by alfalfa hay at 0, 20, 40, and 60 % of RGP in the total mixed ration.
[b] SNF = Total Solid non fat.
[c] SEM, Standard error of the mean.
[d] In each row data with different superscripts is statistically different ($P < 0.05$).

consistent with expectations as GP increased. However, combining O_3 and urea for wheat straw processing led to reduced GP from potentially fermentable matter but resulted in a higher GP rate [34]. Processing with O_3 has resulted in an enhanced fermentability and fermentation rate, consequently increasing the estimated concentration of $SCFA_{24}$ and DOM_{24} 37]. Thus, it is anticipated that the 12-h treatment will provide a greater energy supply for the rumen bacteria.

The application of O_3 gas in the pretreatment of sugarcane bagasse and straw for enzymatic saccharification led to the decomposition of lignin, breaking the links between lignin and cell wall carbohydrates [35]. This process weakens the chemical bonds between cellulose micro-fibers, resulting in the release of cellulose. Additionally, it

Table 10
Blood metabolites of ewes fed with rations containing different levels of O_3-12 h-processed red grape pomace (RGP).

Variable	Ozone-12-h treated RGP, %[a]				SEM[c]	P-value[d]		
	0	20	40	60		Treatment	Linear	Quadratic
Glucose, mmol/L	4.26[b]	4.28[ab]	4.32[ab]	4.36[a]	0.011	0.04	0.048	0.762
Albumin, g/L	1.76[b]	1.78[ab]	1.79[ab]	1.80[a]	0.060	0.04	0.008	0.320
Triglyceride, g/L	3.38[a]	3.35[b]	3.30[c]	3.28[c]	0.043	0.01	0.024	0.501
Cholesterol, g/L	1.85	1.84	1.83	1.83	0.014	0.89	0.579	0.856
Total protein, g/L	38.4[b]	38.5[b]	40.2[ab]	41.5[a]	0.412	0.06	0.08	0.243
NEFA, mmol/L[b]	0.33	0.30	0.29	0.30	0.061	0.59	0.350	0.512

[a] Dietary level of the ozone-12-h treated red grape pomace (RGP) replaced by alfalfa hay at 0, 20, 40, and 60 % of RGP in the total mixed ration.
[b] NEFA, non-esterification fatty acids.
[c] SEM, Standard error of the mean.
[d] In each row data with different superscripts is statistically different ($P < 0.05$).

enhances the accessibility of cellulose and hemicellulose to cellulolytic enzymes. Tannins present in animal feed decrease the degradability of DM and NDF. However, the use of O_3 gas resulted in an increased ruminal degradability of nutrients, indicating the deactivation of phenolic compounds and greater hydrolysis of cell wall carbohydrates in the samples treated with O_3 gas for 6 and 12 h. It is important to note that extending the processing time from 12 to 24 h decreased the degradability of DM and NDF. This decline can be attributed to the formation of inhibitory carboxylic compounds [13,38]. When wheat straw is processed with oxidizing agents like ammonia, the NDF content is reduced, and its degradability is increased [39]. Combining the processing of rice straw with O_3 and liquid ammonia enhances its degradability and promotes biogas production [36].

4.2. In vivo experiment

The DMI did not show a significant difference, but in the study of Ghaffari et al. [40] replacing 30 % of alfalfa hay with pistachio by-products in the diet of growing lambs resulted in a decrease in feed intake. Generally, it is believed that when the tannin concentration in the diet exceeds 50 g/kg, it leads to a reduction in animal feed intake [41]. Tannins have adverse effects on ruminants' feed intake by reducing palatability, slowing down rumen turnover rates, and decreasing digestion rate [9].

Deactivating tannins increases the effective rumen degradability of NDF, which subsequently accelerates the passage rate of rumen contents to the intestine and promotes higher feed intake. In the present study, incorporating increasing levels of RGP instead of alfalfa hay did not negatively impact feed intake due to the efficient deactivation of CT, which enhanced the palatability of RGP [42]. Limited research has been conducted on the impact of O_3 gas-treated agricultural waste on the blood parameters of animals. Rezaeenia [43] reported that including 15 % ensiled pistachio by-products in the diet of early lactation dairy cows had no impact on serum glucose and blood urea nitrogen. Similarly, Gholizadeh et al. [44] noted that including 10 % pistachio by-products in the diet of dairy cows did not affect glucose and blood urea nitrogen levels.

Some researchers have indicated that diets containing high levels of non-degradable protein sources in the rumen can lead to increased blood glucose concentration due to enhanced passage of protein, glycogenic amino acids, and gluconeogenesis [45]. In the current study, the elevated blood glucose levels in the O_3-pretreated RGP diets compared to the control group suggest improved digestibility and nutrient absorption of RGP as a result of the treatment. Total protein, an important blood parameter, is directly associated with feed protein degradability and microbial protein production in the rumen [46].

Incorporating 15 % grape pomace into the diet of dairy cows did not cause significant changes in milk fat and protein levels, which are key factors in dairy products [4]. However, it was effective in boosting lactose content, which is a disaccharide produced by mammary gland cells and is known for its ability to bind calcium, thus enhancing its absorption.

5. Conclusions

Based on these results, the degradability and nutritional content of RGP can be enhanced by treating it with O_3 gas. The most significant improvement was observed when the RGP was processed with O_3 gas for 12 h, making this method highly recommended for RGP processing. The *in vivo* phase of the experiment revealed that using O_3 gas-processed RGP in ewes' diets does not negatively affect their performance and it can be recommended to use ozone-processed RGP as a substitute for alfalfa in ewes' diets.

Disclaimer/publisher's note

The views expressed in this article are those of the authors.

Ethics approval

The Animal Care and Use Committee of Urmia University (IACUC Protocol #IR2018011) approved all animal procedures in advance (Iranian Council of Animal Care, 1995).

CRediT authorship contribution statement

B.A.: Writing – original draft; Investigation; **R.P.:** Writing – original draft; Investigation; **Y.A.:** Design of experiments, Literature survey; **H.K. B.:** Validation, Literature survey; **H.A.:** Supervision; **D.K.T.N., P.E.H. R., V.V.O:** Validation, Editing – review and editing; **M.L. and A.Z.M. Salem:** Writing – review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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