RESEARCH ARTICLE Indian Journal of Animal Research, Volume 59 Issue 5: 795-803 (May 2025)

Relationship between Forage Particle Size and Rate of *in vitro* Digestion Parameters of Alfalfa-based Lamb in Complete Pelleted Diets

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10.18805/IJAR.BF-1923

ABSTRACT

Background: Intensive feeding systems for lamb production are based on high concentrations of feed, depending on the type of forage, which can cause digestive disturbances; however, forage particle length (FPL) and size (FPS) can affect the fermentation process and the productive performance of lambs. Intensive feeding systems for lamb production are based on high concentrations of feed, depending on the type of forage, which can cause digestive disturbances; however, forage particle length (FPL) and size (FPS) can affect the fermentation process and the productive performance of lambs. Understanding how the lambs meet these challenges requires a conceptual integration of forage structure, mechanical particle size of forage and microbial fermentation. This synthesis will hopefully contribute to the development of a biomimetic co-treatment process for translating the unique characteristics of digestion into an effective component of biorefineries for the industrial conversion of cellulosic in forage biomass to useful products. Therefore, this study aimed to evaluate the relationship among FPL, degradability of feed *in vitro* and digestibility of organic materials.

Methods: As a laboratory simulation of rumen functions, four alfalfa FPS lengths were studied within the components of integrated feed pellets 0.1 mm, common in the Saudi animal feed market, 5, 10 and 15 mm using the same feed composition, but with different lengths.

Result: Cumulative gas production in PSL_8 and PSL_{12} lambs was higher compared with the other two groups, indicating that lengths >15 mm and <5 mm affected gas production rates. The effect of FPL on rumen parameters had no effect on the pH of the rumen fluid. There were no significant differences in all values of the main volatile fatty acids acetate, propionate and butyrate and digestibility increased when the FPL ranged from 5 to 10 mm as in the PSL_5 and PSL_{10} groups. Hydrogen levels were significantly higher in the PSL_1 and PSL_{10} groups than in the PSL_5 and PSL_{15} groups. Based on forage size, groups PSL_5 to PSL_{10} showed the possibility of using this type of feed for growing lambs and small ruminants.

Key words: Fatty acids, Forage particle size, *In vitro* digestion, Lamb, Ruminal fermentation.

INTRODUCTION

Geopolitical changes and their impact on food supply chains in addition to global warming, high temperatures and drought (Jenkins, 1987; Izadbakhsh *et al*., 2024) may pose a threat to animal husbandry in general and growing animals in particular (Letourneau *et al*., 2024). The mechanisms of coping with heat stress are low compared to those in larger animals (Galanakis, 2023). The problem of greenhouse gas emissions is increasing, especially those produced by ruminant animals (Giamouri *et al*., 2023), which may account for up to one fifth of total production (FAO, 2022), posing many environmental and health challenges, which may be due to Overfeeding and waste (Alexander *et al*., 2017), so feeding strategies and controlling feed particle size may contribute to reducing waste and the rate of raid emission. greenhouse effect (Yan *et al*., 2024), yet both the agricultural and livestock sectors must continue to provide food to cover growing human needs (Jararweh *et al*., 2023; Fanzo and Miachon 2023); however, certain nutritional strategies and techniques can help overcome the obstacles and harmful effects of heat stress (Li *et al*., 2021). Increasing benefit levels from provided rations and reducing waste to achieve the highest return at the lowest costs (Caihong *et al*., 2023) will achieve sustainability in the production of red meat such as lamb (Zhong *et al*., 2018; Foster *et al*., 2023). Commercial livestock activities have expanded and intensive feeding has been used to produce meat from lambs such as total mixed rations (Pérez-Ruchel *et al*., 2017), which are highly concentrated to achieve nutritional goals and the highest weight gain at the lowest possible

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How to cite this article: Alharthi, A.S., Alshamiry, F.A., Alghonaim, A.A., Al-Baadani, H.H. and Alhidary, I.A. (2025). Relationship between Forage Particle Size and Rate of *in vitro* Digestion Parameters of Alfalfa-based Lamb in Complete Pelleted Diets. Indian Journal of Animal Research. 59(5): 795-803. doi: 10.18805/IJAR.BF-1923.

Submitted: 10-11-2024 **Accepted:** 16-12-2024 **Online:** 22-01-2025

cost (Mahgoub *et al*., 2000). In addition, the use of concentrated pellets instead of whole grains may improve the productive characteristics of growing lambs, such as growth rate and carcass quality (Alshamiry *et al*., 2023).

Previous studies on the effect of forage particle size (FPS) considered one of the more modern methods confirm that it may affect the activity of the digestive system and microbial flora in the rumen (Ichihara and Fukubayashi, 2010; Qin *et al*., 2023) and consequently absorption (Cui *et al*., 2019; Foster *et al*., 2023). Any change in FPS may lead to digestive disturbances and fermentation in the rumen. Alterations in FPS may result in digestive disturbances and fermentation inside the rumen (Chen *et al*., 2021), the activity of the microbial flora in the rumen correlates with the surface area and the rate of forage material decomposition (Zebeli *et al*., 2007; Weimer and Hall, 2020). Consequently, affecting the fattening rate and general performance of lambs (Francisco *et al*., 2020). A comparison of ration substrates on rumen microbial fermentation does not depend solely on fiber content (Mirheidari *et al*., 2020), although it is necessary to maintain the health, dynamism and activity of the rumen and to maintain the internal environment for the functioning of different types of bacterial communities (Letourneau *et al*., 2024).

Understanding the biomass accumulation in ruminants is critical for improved earnings and long-term livestock production (Ekeocha *et al*., 2023). Similarly, the protein and fat content of the rations, FPS and forage particle length (FPL) and the rate of cutting and grinding, are important factors (Li *et al*., 2021).

According to Aïssa *et al*. (2021), the maturation stage of forage material can impact digestibility and gas generation by influencing the percentage of dry matter and nutritional content, including protein and fiber.

The nutritional value of animal forage depends on its chemical composition, digestibility coefficient, fiber content and particle size (López *et al*., 2005). To achieve this, there are many ways to estimate nutritional value but *in vitro* methods may be the best (Foster *et al*., 2023) and have been used to estimate nutrient concentrations, digestibility (Gomide *et al*., 2023) and the extent of particle size effects. Furthermore, the ratio of concentrate to roughage may influence fermentation efficiency, gas production and microbial protein synthesis (Zhang *et al*., 2022). To predict digestion efficiency and make assumptions about the feed used, nutritional value and quality should be compared with *in vivo* experiments (Foster *et al*., 2023).

In vitro techniques are also considered simple and relatively inexpensive compared to other forage evaluation methods (Vinyard and Faciola, 2022). Subsequently, there is a need to know more about the effects of the FPS and FPL on the intake, growth rate and refusal and wasted forage. Therefore, this study aimed to evaluate the relationship among FPL, degradability of feed *in vitro* and digestibility of organic materials.

MATERIALS AND METHODS

Animal welfare and ethics clearance

This study was undertaken at King Saud University (KSU), Riyadh, Saudi Arabia and the animals and procedures adopted in this study were in accordance with the Animal Welfare Act of Practice for the Care and Use of Animals for Scientific Purposes and approved by the Research Ethics Committee, KSU (Ethics Reference No: KSU-SE-22-26).

Animals and experimental design

A total of 40 Naimi male lambs, with an average body weight of 23±2 kg and ~3 months old, were used in the 84-day trial. Initially, there was a 14-day adaptation period. The lambs were weighed, ear-tagged, vaccinated against clostridial diseases and treated for internal and external parasites as well as other vaccines against the most common infectious diseases in Saudi Arabia. Thereafter, the animals were randomly divided into four groups of 24 lambs each and housed in shaded pens, provided with a feed trough and a 10-L plastic water bucket. All lambs were offered the same complete pelleted diet (the basal diet) mostly corn and alfalfa hay containing 2.7 Mcal of MEm and 13.5% CP/kg. Dry matter (DM) content at maintenance level (2.5% of the initial body weight) was measured twice daily at 08:00 and 15:00. On day 1 of the experimental period, lambs were randomly assigned to one of four dietary treatments (four groups in each treatment): complete pelleted diet contained chopped alfalfa hay with lengths either 1 mm (the basal diet; PSL_1; control), 5 mm ((PSL_5), 10 mm (PSL_{10}) or 15 mm (PSL_{15}), Forage particle size is controlled industrially by controlling the distance between the drills or knives by the Mesh size and it is difficult to achieve completely equal sizes, but it represents 70% of the target according to the Pennsylvania scale.

All experimental diets were formulated in the facility at the Experimental Station of Animal Production Department, College of Food and Agriculture Sciences, King Saud University, to meet or exceed the nutritional requirements (National Research Council, 2007) of growing lambs and then provided as complete pelleted diets (Table 1).

Measurements and sample collection

Feeding analysis

Feed from each treatment was sampled before the study and monthly during the study and samples were frozen at -4°C for analysis. At the end of the study, feed samples were pooled (5%) and analyzed for nutrient composition at KSU laboratories. DM and ash content were determined by drying samples in an oven at 100°C for 24 h and by incinerating samples at 550°C for 3 h in a muffle furnace, respectively. The crude protein content of each sample was measured using an elemental analyzer. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to methods described by (Van Soest *et al*., 1991) and AOAC method no. 973.18 C (2000), respectively.

Rumen fermentation profile

Rumen fluid samples (~50 mL) from six lambs/treatment were collected using an oral stomach tube before the morning feeding (0 h) and 3 h after feeding on day 45. The samples were analyzed immediately for pH using a microprocessor pH-meter (Model pH 211, Hanna® Instruments, Woonsocket, RI, USA) and then strained through four layers of cheesecloth. They were then transferred into plastic tubes and acidified with 2 mL of concentrated sulfuric acid (H_2SO_4) and frozen at -20°C for further analysis. The samples were then thawed and analyzed for ammonia (NH_3-N) using a TECO® Diagnostics kit and total proportion volatile fatty acids (VFAs) as described by (Jenkins, 1987), using 2-ethylbutyric acid (EBA) as an internal standard and gas chromatography mass spectrometry.

***In vitro* batch culture fermentation measurements**

Feed samples were collected, dried to process DM and stored in sterile bags at 4°C until apparent digestibility could be estimated using the method of (Prachansuwan *et al*., 2019) In four ANKOM gas jars per treatment as replicates (20 gas jars/treatment), 70 mL of strained rumen fluid was combined with 130 mL of pre-warmed buffer medium as batch rumen cultures. Finely powdered feed (200 g; based on DM) was weighed in a Dacron bag (Ankom Inc., Fairport, NY, USA), added to each ANKOM jar and incubated for 24 h to determine the apparent digestibility of DM, OM, ADF and NDF. The samples and feed treatments were analyzed for DM, ash analysis for calculating OM (100_Ash) and NDF according to the methods of AOAC, 2000. All dietary treatment jars, which were previously sealed, were filled with CO_2 and then connected to a Tedlar gas collection bag (Santa®, CA, USA.). The jars were placed in a water bath (Thermo® Fisher Scientific, Model 2873.0, USA) at 39°C for 24 h until the release process of the gases in the collection bag was completed. The jars were shaken every 2 h for 30 s. All CO_2 GP values are expressed as milliliters per time (2 h) and total GP (24 h) (Johnson *et al*., 2009).

VFAs and Ammonia-N (NH3-N) in the culture jars were determined at the end of each experiment. Samples (5 mL) from each jar were taken and placed directly placed in an ice bath and then stored at -20°C until analysis. The samples were mixed with 1 mL of 25% metaphosphoric acid and centrifuged at 20,000×g for 10 min at 4°C to produce a clear supernatant. The supernatant (1 mL) was filtered with a PTFE syringe filter (0.2 µm) and transferred to a 1.5 mL glass chromatography vial (Agilent). Acetic acid (C2), propionic acid (C3), butyric acid (C4), isobutyric acid (iso-C4), valeric acid (Val) and isovaleric acid (iso-Val) were analyzed by gas chromatography (Shimadzu Scientific Instruments Inc., Columbia, MD, USA), using EBA as an internal standard (Ichihara and Fukubayashi, 2010). VFA separation was performed as described previously (Cui *et al*., 2019). VFAs are expressed as mM/1.0 ml sample. A sample was collected from each of the jars and centrifuged at 12.000×g for 15 min at 4°C to measure the ammonia N concentration (NH_3-N) using a spectrophotometer (Perkin® Elmer, Waltham, USA.). The pH values of the culture jar sample were determined directly during sampling using a digital pH meter (Hanna® Instruments pH 211; Woonsocket, RI, USA-2014).

Statistical analysis

Statistical analyses were performed using SAS software v. 9.4 (SAS Institute Inc., Cary, NY, USA-2022). The gas production dataset was scaled down to a 1-h resolution and nonlinear regression analysis was performed using the SAS MIXED procedure. Significant differences in estimated parameters of gas production kinetics among the substrates or treatments were assessed using likelihood ratio 95 % confidence intervals. The variables were compared among the four forage particle sizes using the Kruskal-Wallis test with the NPAR1_WAY procedure in the gas component dataset. $P<0.05$ was considered statistically significant and Duncan's multiple range test was used to analyze these statistical differences.

RESULTS AND DISSCUSION

***In vitro* digestion and fermentation**

The estimated nutrient digestibility, pH, ruminal gas production and fermentation for dietary treatments with

Table 1: Ingredients and chemical composition of the basal diet[1].

Item, unit	Contain
Ingredients, % of dietary DM	
Corn	34.89
Alfalfa hay	23.29
Soybean meal	9.13
Wheat bran	17.34
Wheat straw	6.76
Limestone	2.67
Molasses	2.12
Dicalcium phosphate	1.62
Salt	1.12
Acid buffer	0.91
Vitamins and minerals premix[2]	0.15
Nutrient composition, DM basis	
Dry Matter, (%)	90.34
Ash, %	8.17
Crude protein, %	13.93
Ether extract, %	1.54
Neutral detergent fiber, %	30.21
Acid detergent fiber, %	14.11
Metabolizable energy, Mcal/kg	2.79

[1]Alfalfa hay was chopped to either 1, 5, 10 or 15-mm lengths and incorporated into the complete pelleted diets.

[2]Contained per kg, 10000 IU vitamin A, 1000 IU, vitamin D, 20 IU, vitamin E, 300 mg Mg, 24 mg Cu, 0.6 mg Co, 1.2 mg I, 60 mg Mn, 0.3 mg Se, 60 mg Zn.

different lengths of alfalfa hay during the *in vitro* digestion process are presented in Table 2 and Fig 1. The apparent OM digestibility was affected ($P<0.05$) by dietary treatment. The mixture of loose alfalfa hay with concentrate pellets (PSL_{15}) resulted in a decrease in apparent digestibility for OM of 70.84% ($P<0.01$) compared to those of other experimental diets. The total gas production over the 24 h incubation period and ruminal fermentation (VFAs or Ammonia-N) did not differ ($P<0.05$) between treatments and they were similar across treatments, averaging 201.25 mL, 33.39 mM and 8.22 mM, respectively. The ruminal pH value was greater ($P<0.01$) in PSL_{10} and PSL_{15} than in other treatments.

The addition of forage at lengths of 5-10 mm to the complete pelleted diet increased ($P<0.05$) Iso-valerate and valerate molar percentages, respectively, during the *in vitro* digestion compared to the PSL_{10} diet. However, the PSL_1 and PSL_{15} diets had intermediate iso-valerate and valerate molar percentages (Table 2).

Fiber digestibility

The mean ruminal fluid pH and fermentation profiles of growing lambs fed pelleted diets containing different staple lengths of alfalfa hay are shown in Table 3. The pH values of ruminal fluid, total VFA concentrations and proportions of the majority of individual VFAs in the ruminal fluid were similar across dietary treatments and did not differ ($P>0.05$) between treatments. Feeding in the group control diet (PSL_1) resulted in an increase ($P>0.05$) in butyric acid molar percentage of VFAs (9.17%) compared with other groups. In terms of polynomial contrast effects, there was a quadratic response ($P>0.05$) found in propionic and acetic acid molar percentages, whereas a linear response was observed ($P>0.05$) in acetate to propionate ratio as alfalfa hay length increased (Table 3 and Fig 1).

In vitro fermentation may play an important role in the study of feed efficiency and highlight the differences between feed types that affect the quantity and quality of feed available to ruminants (Bueno *et al*., 2020; Vastolo *et al*., 2022). Estimates and measurements related to the kinetics of nutrient decomposition in the rumen of ruminants are important indicators through which nutrients are classified as well as their carbohydrate and protein contents. This may then aid our understanding of digestion rates (Basto-Silva *et al*., 2022; Paixão *et al*., 2022). Compatibility between the decomposition of carbohydrates and nitrogen in the rumen increases the level of microbial protein synthesis and reduces energy loss during fermentation in the rumen. Accordingly, through laboratory digestion, they may be able to predict animal performance based on the nutritional composition of the feed (Santos *et al*., 2016; Wei *et al*., 2021).

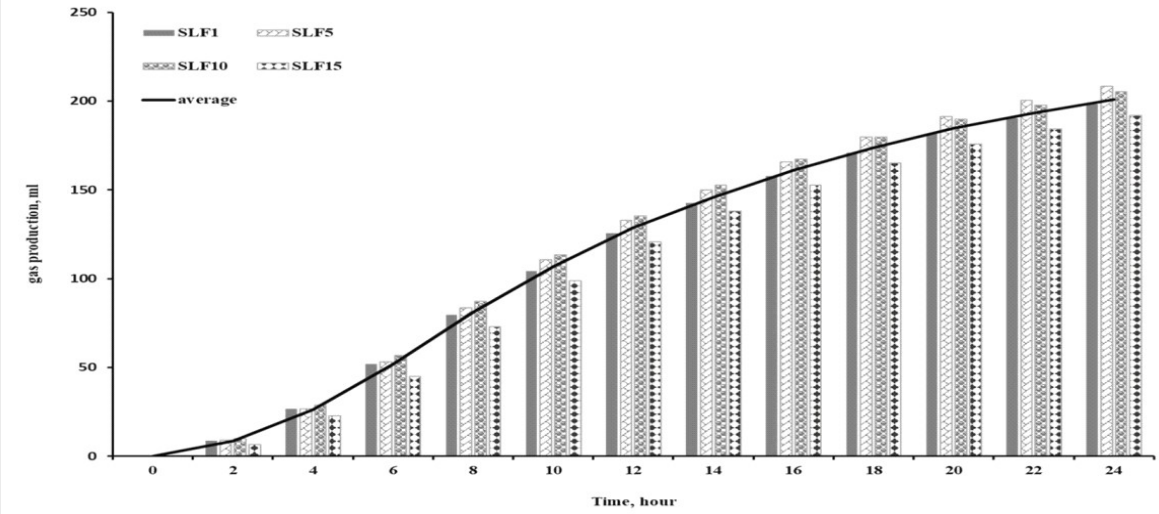
Alfalfa feed is one of the most common types of feed used when manufacturing pelleted feed because of its high protein content (Al-Saiady *et al*., 2010; Blume *et al*., 2021; Srisaikham and Rupitak, 2021) and palatability in animals (Vahdani *et al*., 2014). To determine the nutritional value of alfalfa with the rest of the feeds included in the formulation

Table 2: Nutrient digestibility and fermentation characteristics produced from the *in vitro* incubation of dietary treatments with rumen fluid of growing lambs fed pelleted diets containing different staple lengths of alfalfa hay.

Variables, unit[2]	Dietary treatments[1]				SEM	*P* value
	SLF_1	SLF_5	SLF_{10}	SLF_{15}		
DM digestibility, %	78.73	81.84	79.64	77.12	1.37	0.20
OM digestibility, %	77.13[a]	80.45[a]	78.51[a]	70.84[b]	1.50	0.01
NDF digestibility, %	57.31	55.52	58.14	55.49	2.83	0.88
Ruminal pH	6.50[b]	6.51[b]	6.58[a]	6.61[a]	0.04	0.01
Total gas production, mL	199	209	205	192	5.41	0.22
CO_2, ml	155	165	160	150	4.23	0.16
CH_4, ml	40.44	41.40	41.95	39.58	1.10	0.48
H_2, ml	2.92[a]	1.99[b]	2.97[a]	2.10[b]	0.07	0.01
Total VFA, mM	33.55	37.29	32.07	32.79	2.21	0.40
Acetate	15.83	17.47	15.31	15.33	0.91	0.35
Propionate	8.91	9.87	8.50	8.90	0.71	0.59
Butyrate	4.72	5.33	4.41	4.39	0.02	0.29
Iso-Butyrate	0.42	0.47	0.39	0.43	0.36	0.29
Iso-Valerate	0.81[ab]	0.93[a]	0.74[b]	0.88[ab]	0.04	0.02
Valerate	0.64[ab]	0.76[a]	0.60[b]	0.71[ab]	0.04	0.04
Acetate: Propionate ratio	1.78	1.77	1.80	1.72	0.05	0.81
Ammonia-N, mM	7.30	7.89	8.15	9.50	0.55	0.11

[a,b] Within a row, means without a common superscript differ ($P<0.05$).[1]SLF_1 = A complete pelleted diet contained 1 mm length of alfalfa hay (Control); SLF_5 = A complete pelleted diet contained 5 mm length of alfalfa hay; SLF_{10} = A complete pelleted diet contained 10 mm length of alfalfa hay; and SLF_{15} = A complete pelleted diet contained 15 mm length of alfalfa hay. VFA = Volatile fatty acid.

when their lengths and sizes differ and to determine the amount of gas resulting from fermentation that may lead to fixed indicators and standards for evaluating the quality of manufactured feeds, this study showed that modification of FPL and FPS without changing the chemical composition may affect cumulative gas production (Zebeli *et al*., 2008). The obtained results showed that the values of cumulative gas production in PSL_5 and PSL_{10} lambs were higher compared with the other two groups, indicating that lengths greater than 5 and less than 15 mm affected the rate of gas production, which is consistent with the findings of Li *et al*. (2018). There is a relationship between the FPS and the time spent in the rumen; the larger the FPS, the more it delays in the digestive system and the smaller the FPS, the faster it exits and in both cases it affects the efficiency of digestion, fermentation and decomposition of the feed, so logically it seems that the average FPS combines between them in achieving the appropriate time while increasing the rate of efficiency of forage utilization (Beauchemin *et al*., 2003; Jiang *et al*., 2019; Chelotti *et al*., 2024). Rapid fermentation may increase gas build-up but simultaneously reduce nitrogen supply, which can negatively affect microbial activity (Magalhães *et al*., 2019). FPL effects on rumen parameters had no effect on the pH of rumen fluid at different time points, which is consistent with the results reported by (Costa *et al*., 2017) and (Santos-



The y-axis represents the amount of cumulative gas production (mL/g DM). [1]SLF_1 = A complete pelleted diet contained 1 mm length of alfalfa hay (Control); SLF_5 = A complete pelleted diet contained 5 mm length of alfalfa hay; SLF_{10} = A complete pelleted diet contained 10 mm length of alfalfa hay; and SLF_{15} = A complete pelleted diet contained 15 mm length of alfalfa hay.

Fig 1: Cumulative CO_2 gas production during the *in vitro* digestion for FPS from 2 to 24 hours of incubation (the x-axis).

Table 3: Effects of feeding different staple lengths of alfalfa hay on pH values of rumen fluid and ruminal fermentation characteristics of growing lambs fed pelleted diets.

Measurement, unit[2]	Dietary treatments[1]				SEM	*P* value
	SLF_1	SLF_5	SLF_{10}	SLF_{15}		
Ruminal pH						
Before feeding, 0 h	6.19	6.17	6.29	6.69	0.17	0.14
After feeding, 3 h	5.99	5.95	5.93	5.90	0.09	0.91
VFA composition, %						
Total VFA, mM	32.86	34.87	31.03	33.14	1.43	0.14
Lactic acid	42.01	43.42	43.31	44.49	0.98	0.34
Formic acid	1.57	1.65	1.62	1.76	0.17	0.07
Acetic acid	28.40	29.99	28.50	27.41	1.09	0.23
Propionic acid	18.84	17.60	18.63	19.08	0.87	0.18
Butyric acid	9.17[a]	7.34[b]	7.94[b]	7.26[b]	1.21	0.05
Acetate: Propionate ratio	1.51[b]	1.70[a]	1.53[b]	1.44[b]	0.12	0.02

[a,b]Within a row, means without a common superscript differ ($P<0.05$). [1]SLF_1 = A complete pelleted diet contained 1 mm length of alfalfa hay (Control); SLF_5 = A complete pelleted diet contained 5 mm length of alfalfa hay; SLF_{10} = A complete pelleted diet contained 10 mm length of alfalfa hay and SLF_{15} = A complete pelleted diet contained 15 mm length of alfalfa hay.. VFA = Volatile fatty acid.

Silva *et al*., 2019). There were no significant differences in the main VFAs acetate, propionate and butyrate nor in gas productivity. OM digestibility was significantly higher in the PSL_1, PSL_5 and PSL_{10} groups than in PSL_{15} group. This may be due to the liberation of microbial flora activity as the length of the forage and the percentage of fibers in the ration affect the digestibility of OM, by shifting the flow of the hydrogenation pathways towards VFAs, which results in the liberation of part of the laboratory according to the reported (Wang *et al*., 2018). As shown by (Shen *et al*., 2017), digestibility increased when the length of the forage particles ranged from 8 to 12 mm, as in the PSL_8 and PSL_{12} groups, this result is consistent with what the researchers reported (Vidya *et al*., 2024). The hydrogen levels were significantly higher in the PSL_1 and PSL_{10} groups than in the PSL_5 and PSL_{15} groups. The higher hydrogen values may be due to the liberation of H_2 as the particle length and the percentage of fibers in the ratio affected the production of methane by shifting the H_2 flow towards propionate, resulting in the liberation of part of the hydrogen in the laboratory (Wang *et al*., 2018; Bhatt *et al*., 2023). Ramirez *et al*. (2016) stated that dietary modifications may modify rumen kinetics, which may affect the synthesis and deposition of fats in ruminants that feed on particles of different forage lengths. Efforts to control forage particle size, when formulating animal diets, may help reduce methane production, as well as improve animal productivity, by reducing the amount of waste, residue and feces and succeed in mitigating animal gas emissions (Lakhani *et al*., 2023).

CONCLUSION

Based on feed size, the values of PSL_5 to PSL_{10} showed that these feed types could be used for feeding growing lambs and small ruminants, which can be explained by the amount of time required for the microbial flora, its ability to deteriorate and decompose the feed and by increasing the surface area. However, further studies are necessary to determine the particle size and length of forage before the manufacture of complete pelleted feed in growing animal diets, the study furthermore recommends conducting it on *in vivo* animals and other breeds of sheep and goats. Further experimental studies are required, to investigate the effects of various particle sizes of different forage on different production parameters in ruminants.

ACKNOWLEDGEMENT

The authors extend their appreciation to the National Plan for Science, Technology and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia (award number 2-17-04-001-0038).

Author contributions

Faisal A. Alshamiry, Hani H. Al-Baadani and Ahmed A. Alghonaim: Methodology, formal analysis and writing original draft. Abdulrahman S. Alharthi, Faisal A. Alshamiry and conceptualization, methodology and data curation; Ibrahim A. Alhidary: Investigation, review and editing and project administration. All the authors have read and agreed to the published version of the manuscript.

Funding

The authors extend their appreciation to the National Plan for Science, Technology and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia (award number 2-17-04-001-0038).

Institutional Review Board Statement

The study was conducted at King Saud University, Riyadh, Saudi Arabia and the use of animals and procedures adopted here were in accordance with the Animal Welfare Act of Practice for the Care and Use of Animals for Scientific Purposes and approved by the Research Ethics Committee of King Saud University (Ethics Reference No: KSU-SE-22-26).

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Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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