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Effects of Pelleted and Extruded Huizache–Agave Bagasse Concentrates on In Vitro Fermentation and Digestibility of Grazed Forage-Based Diets

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Simple Summary

Livestock farming is one of the most important economic activities in developing countries; however, ruminants have been linked to environmental problems, and conventional feed costs continue to rise, especially given the frequent need for supplementation. Non-conventional feed sources such as shrubs and agro-industrial residues bring the possibility of supplementing ruminant diets while reducing costs and methane emissions. This study evaluates a supplement processed by two densification methods and its inclusion at two proportions in diets for ruminants. Forages included were collected from grazing steers during two seasons of the year, and their fermentation and digestibility were assessed in vitro using ruminal inoculum from sheep. The results indicate that the two densification methods showed similar effects; moreover, the highest supplementation level increased benefits in both seasons, including improved diet digestibility; however, methane production was not reduced. In conclusion, both supplement presentations can be used to improve diet quality.

Abstract

Ruminant production in developing countries faces the challenge of reducing greenhouse gas emissions while maintaining productivity under increasingly variable climates. This study evaluated the effects of a supplement containing huizache leaves and agave bagasse, processed by pelleting or extrusion, on the in vitro fermentation of grazed forage-based diets. Forage from two seasons was mixed with the supplement at two levels (25 and 75% concentrate), resulting in eight diets. Fermentation and digestibility were assessed in a single run in triplicate using pooled ruminal inoculum from two sheep. Data were analyzed with mixed linear models. Significant interactions were found for methane percentage, ammonia nitrogen, the Gompertz A and k parameters, IVDDM and IVDOM. Increasing concentrate inclusion improved IVDDM (62.33% to 69.96%) and IVDOM (59.40% to 68.16%), total gas production (109.73 to 144.22 mL/g DM) and ammonia nitrogen (13.33 to 17.26 mg/dL), but also increased methane volume per unit of incubated DM (13.89 to 19.60 mL/g DM, +41.1%). Extrusion increased IVDDM (67.23% vs. 65.06%), IVDOM (64.70% vs. 62.59%), total gas (135.45 vs. 118.49 mL/g DM), and methane volume (17.91 vs. 15.58 mL/g DM) relative to pelleting. Because no conventional concentrate was included, these effects apply to the formulated mixtures as a whole under the tested in vitro conditions.

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1. Introduction

Global demand for meat and dairy products continues to increase, particularly in developing countries, where ruminant production contributes substantially to food security and rural livelihoods [1,2]. However, rising feed costs, seasonal fluctuations in forage availability, and environmental concerns pose significant challenges to sustainable livestock production [3–5]. These limitations are particularly relevant in northern Mexico, where irregular rainfall and prolonged droughts affect pasture productivity and nutritional quality [6–8]. Dietary supplementation can help address seasonal forage deficiencies and improve nutrient utilization in grazing cattle [9]. Previous studies have demonstrated that protein and energy supplementation can influence forage intake, digestibility, and ruminal fermentation, although selective grazing may partially compensate for seasonal variations in pasture quality [10–13]. Consequently, evaluating alternative supplementation strategies using forage actually consumed by grazing animals is essential for developing practical feeding approaches adapted to local conditions.

Martz et al. [10] suggested that cattle's selective grazing behavior may partially offset seasonal variations in pasture quality. These contrasting findings highlight the importance of evaluating supplementation strategies using forage actually consumed by grazing animals under different seasonal conditions. Despite their benefits, conventional feed supplements are often too expensive for small-scale producers because they rely heavily on energy- and protein-rich ingredients such as corn, soybean meal, and cottonseed, whose market prices continue to increase. Consequently, there is growing interest in alternative feed resources that can partially replace conventional ingredients without competing with human food production. Beyond their economic advantages, many of these resources contain secondary metabolites that could suppress ruminal methanogenesis, improving feed-use efficiency while reducing energy losses and greenhouse gas emissions [9,14]. García-Piña et al. [15] reviewed alternative feed resources for livestock production, including agricultural by-products, weeds, shrubs, forest residues, agro-industrial wastes, and aquatic macrophytes, and concluded that these resources can support more sustainable and profitable livestock production systems in developing countries such as Mexico. Among the alternative feed resources available in Mexico, *Acacia* species, commonly known as huizache, are abundant in semi-arid regions and possess several characteristics that make them attractive as ruminant feed ingredients. These species are highly adapted to drought conditions, readily colonize degraded or eroded soils, and retain foliage throughout the year. In addition, their relatively high crude protein content makes them suitable for use as alternative forage during periods of forage scarcity or as ingredients in protein supplements [16,17]. Previous studies have also shown that including huizache leaves in concentrate formulations can reduce methane production during in vitro ruminal fermentation [18].

Agave bagasse is an abundant agro-industrial by-product generated during mezcal and tequila production in Mexico. According to the Consejo Regulador del Tequila [19], approximately 2.2 million metric tons of fresh agave were processed by the tequila industry in 2023, highlighting the substantial availability of raw material from which agro-industrial residues are generated.

Because of its neutral detergent fiber content and mineral composition, agave bagasse has been proposed as an alternative feed resource for ruminants, particularly during periods of forage shortage [20]. When incorporated into concentrate formulations, it can partially replace conventional fibrous ingredients, such as wheat bran, while its naturally sweet

flavor may improve feed palatability. The physical form of a concentrate also influences its utilization by animals. Densification processes are widely used to improve feed handling, reduce ingredient sorting, enhance intake uniformity, and ensure a more consistent nutrient supply. Densified feeds also facilitate transportation, storage, and on-farm management. Pelleting and extrusion are the two most common densification methods; however, they differ substantially in their effects on feed structure. Whereas pelleting generally preserves the original physicochemical characteristics of the ingredients, extrusion subjects the feed to elevated temperature, pressure, and shear forces that can modify its structural and nutritional properties, potentially influencing nutrient availability and digestibility [21–23].

Although non-conventional feed ingredients and processing technologies have shown potential for improving ruminant nutrition, limited information is available regarding the effects of pelleting and extrusion on the in vitro fermentation characteristics of diets containing huizache leaves and agave bagasse, particularly when combined with forage consumed under different seasonal conditions. Furthermore, the effects of increasing concentrate inclusion on nutrient availability, digestibility, and methane production remain insufficiently characterized in these formulations.

We hypothesized that extrusion would improve in vitro fermentation and digestibility compared with pelleting because of the structural and physicochemical modifications induced by thermal and mechanical processing. We further hypothesized that increasing concentrate inclusion would enhance nutrient availability and digestibility, with responses varying according to seasonal forage composition.

Therefore, this study aimed to evaluate the effects of concentrate processing method (pelleting or extrusion) and inclusion level (0, 25, and 50% of dietary dry matter) on the chemical composition, in vitro gas and methane production, fermentation kinetics, ammonia nitrogen concentration, and dry matter and organic matter digestibility of diets formulated with forage collected from grazing steers during the rainy and dry seasons. All fermentation and digestibility evaluations were conducted under in vitro conditions.

2. Materials and Methods

2.1. Study Area

The study was conducted at several locations in Durango, Mexico, including the Postgraduate Laboratory of the Faculty of Veterinary Medicine and Animal Science, Universidad Juárez del Estado de Durango (FMVZ-UJED); the Microbial Biotechnology Laboratory of the Graduate, Research, and Technological Development Unit, Instituto Tecnológico de Durango (UPIDET-TecNM/ITD); the Interdisciplinary Research Center for Regional Integral Development, Instituto Politécnico Nacional, Durango Unit (CIDIIR-IPN); the Valle del Guadiana Experimental Field of the North Central Regional Research Center, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (CIRNOC-INIFAP); and an open grassland located in the Malpaís de la Breña region.

All surgical procedures and animal management practices (steers and sheep) were approved by the Committee for the Foment and Animal Protection of Durango State in accordance with the Official Mexican Standard NOM-062-ZOO-1999 [24].

2.2. Collection of Forage Intake by Season

Two rumen-fistulated Criollo steers, weighing approximately 680 kg each, were used in this study.

Samples of the diet consumed by cattle were obtained in an open grassland located in Malpaís de la Breña, Durango (at 24°19′22.20″ N, 104°45′57″ W). An adaptation phase of 14 days was implemented prior to collection of rumen contents; on day 15, rumens of animals were manually evacuated through the rumen fistula; with the rumen empty, they

were allowed to graze freely for 60 min [6]. The forage consumed during this period was collected in plastic bags and immediately transported to the laboratory to be rinsed with distilled water and frozen (−20 °C) until processing. Then, it was dried in a forced-air oven at 55 °C for 72 h and ground using a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA; Bear Cat #1A-S, Westerns Land and Roller Co., Hastings, NE, USA) equipped with a 1 mm screen. Sampling was performed at the beginning and end of the rainy season (August and October 2023) and the dry season (April and June 2023); these samples were pooled by season and designated as Season 1 and Season 2 intake, respectively.

2.3. Formulation of the Concentrate

The concentrate used as a dietary supplement was formulated to contain 21% crude protein (CP). It consisted of leaves from two huizache species, *Acacia farnesiana* (15%) and *Acacia schaffneri* (5%), collected from suburban areas of Durango with abundant huizache vegetation; *Agave duranguensis* bagasse (6%), obtained from a mezcal distillery in Nombre de Dios, Durango; and commercial feed ingredients commonly used in ruminant nutrition, including cottonseed meal (15%), soybean meal (18%), dried distillers grains (15%), ground corn (9%), and wheat bran (17%), all purchased from local suppliers. All ingredient proportions are expressed on an as-fed basis.

Leaves of *Acacia farnesiana* and *Acacia schaffneri* were obtained from two suburban areas of the city of Durango, Dgo. (Mexico), with abundant huizache vegetation. Once identified by species-specific morphological characteristics, branches of approximately 1 m were cut from randomly selected shrubs. The branches were left to dry for 5 days, after which the leaves were manually separated and pooled by species. *Agave duranguensis* bagasse was donated by a mezcal plant located in Nombre de Dios, Dgo. (Mexico). Samples of huizache and bagasse were dried in a forced-air oven at 55 °C (Riossa mod. HS, series HSMN, 127 V, 50/60 cicles, D.G.E.-S.I.C 9672, Rios Rocha S.A., Mexico City, Mexico) until constant weight, processed in a mill (Arthur H. Thomas Wiley, Philadelphia, PA, USA), and sieved through a 1 mm mesh.

A single concentrate mixture was prepared and subsequently divided into two portions, each of which was processed using a different densification method.

2.3.1. Pelletized Concentrate

The ingredients were thoroughly mixed and ground using a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA; Bear Cat #1A-S, Westerns Land and Roller Co., Hastings, NE, USA) equipped with a 1 mm screen. Moisture content was adjusted to 13% by adding distilled water 48 h before processing.

Pelleting was performed using a pilot-scale pellet Mill (Model ZSLP-R300, ZhengZhou ZhongDeBao Industrial Co., Ltd., Zhengzhou, China) fitted with a 6 mm die and operated at an inlet temperature of 50–55 °C. After processing, the pellets were allowed to cool and stored in hermetic plastic bottles protected from direct light and away from heat sources at room temperature (18 °C min.–28 °C max.) until further analysis within the following four weeks.

2.3.2. Extruded Concentrate

The ingredients were mixed, ground using an OEM industrial high speed grinder, (Keiter IC60B com., 32,000 rpm, 110 V, Model 2000A, China) and passed through a 0.5 mm sieve. Moisture content was adjusted to 14% by adding distilled water 48 h before extrusion.

Extrusion was carried out using a Brabender single-screw extruder (Duisburg, Germany) with a 1:1 compression ratio, a screw speed of 150 rpm, a feed rate of 45 rpm, a 6 mm die, and four heating zones set at 90, 100, 110, and 150 °C, respectively, following the procedure described previously [25]. The extruded concentrate was allowed to cool and stored in hermetic plastic

bottles protected from direct light and away from heat sources at room temperature (18 °C min.–28 °C max.) until further analysis within the following four weeks.

Table 1 presents the chemical composition of pelleted and extruded concentrates and forages from seasons 1 and 2.

Table 1. Chemical composition of the concentrates and forages.

%	Pelleted Concentrate	Extruded Concentrate	Season 1 Forage	Season 2 Forage
DM	94.41 ± 0.058	93.96 ± 0.031	93.55 ± 0.029	94.01 ± 0.116
OM	88.14 ± 0.100	88.55 ± 0.006	83.27 ± 0.003	80.98 ± 0.080
CP	21.29 ± 0.463	20.2 ± 0.178	10.40 ± 0.088	10.45 ± 0.121
NDF	41.12 ± 0.160	51.69 ± 0.518	64.61 ± 0.459	62.85 ± 0.123
ADF	18.24 ± 0.018	18.95 ± 0.029	42.07 ± 0.423	40.93 ± 0.031
HEM	22.88 ± 0.178	32.75 ± 0.548	22.54 ± 0.036	21.92 ± 0.092
LIG	3.09 ± 0.053	3.92 ± 0.052	2.54 ± 0.049	2.67 ± 0.392
CEL	14.17 ± 0.056	14.42 ± 0.049	34.94 ± 0.329	34.31 ± 0.075
EE	3.85 ± 0.141	3.28 ± 0.006	2.09 ± 0.009	1.25 ± 0.062
NFC	27.47 ± 0.381	19.39 ± 0.0350	12.61 ± 0.378	12.42 ± 0.101
TC	68.59 ± 0.442	71.08 ± 0.187	77.22 ± 0.084	75.27 ± 0.023
TP	0.62 ± 0.009	0.45 ± 0.004	ND	ND
CT	0.27 ± 0.015	0.23 ± 0.001	ND	ND

Data expressed as Mean ± Standard Error. Sample size, n = 3 (technical replicates per sample). DM, dry matter (g/100 g fresh matter); OM, organic matter (g/100 g dry matter); CP, crude protein (g/100 g dry matter); EE, ether extract (g/100 g dry matter); NDF, neutral detergent fiber (g/100 g dry matter); ADF, acid detergent fiber (g/100 g dry matter); HEM, hemicellulose (g/100 g dry matter); CEL, cellulose (g/100 g dry matter); LIG, lignin (g/100 g dry matter); NFCs, non-fiber carbohydrates (g/100 g dry matter); TCs, total carbohydrates (g/100 g dry matter); TPs, total phenols (gallic acid equivalents, g/100 g dry matter); CTs, condensed tannins (catechin equivalents, g/100 g dry matter). ND: non-detectable values, where concentrations were below the detection limit of the analytical method used.

2.4. Diet Formulation

After processing, both presentations of the concentrate were dried at 55 °C until constant weight and ground to a final particle size of 1 mm. The concentrates were then mixed with forages to elaborate the diets, considering dry matter weights. Diets were stored in hermetic plastic bottles at room temperature (18 °C min.–28 °C max.), protected from light and away from heat sources. Proximal analysis and fermentation assays were performed within the following four weeks.

Forage collected during two seasons (rainy and dry), two concentrate presentations (pelleted and extruded), and two forage-to-concentrate ratios (75:25, and 50:50) were combined to formulate eight experimental diets.

2.5. Chemical Analysis and Secondary Metabolite Determinations

All experimental diets were analyzed for dry matter (DM; AOAC Method 930.15), organic matter and ash (OM, ASH; AOAC Method 942.05), ether extract (EE; AOAC Method 920.39), and crude protein (CP; AOAC Method 990.03) according to AOAC procedures [26]. Neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (LIG), hemicellulose (HEM), and cellulose (CEL) were determined following the method of Van Soest et al. [27] using an ANKOM Fiber Analyzer 200 (ANKOM Technology, Macedon, NY, USA) according to the manufacturer's instructions.

Non-fiber carbohydrates (NFCs) and total carbohydrates (TCs) were calculated using Formulas (1) and (2) respectively:

$$NFC = [100 - (CP + EE + ASH + NDF)] \quad (1)$$

$$TC = (NFC + NDF) \quad (2)$$

Condensed tannins (CTs) were extracted and estimated according to Heimler et al. [28] using a UV-VIS spectrophotometer (Spectronic Instruments, Genesys 10S, Mod. 336003,

Waltham, MA, EUA); CTs were expressed in Catechin equivalents. However, no previous studies were found for forages, nor the formulated diets, thereby these values were omitted in subsequent tables.

All determinations were performed in triplicate on each of the three sections obtained after grinding each sample.

2.6. In Vitro Fermentations

In vitro fermentations were performed using ruminal fluid collected from rumen-fistulated sheep. The donor animals were fed alfalfa hay and a commercial concentrate in a 75/25 ratio. This diet was permanent and was kept during the experimental period; as a consequence, no adaptation period was required.

The in vitro fermentation assay was conducted in one independent run by triplicate. On each run, ruminal fluid was collected from the two sheep, pooled, and used to inoculate three fermentation vessels per treatment. Three more flasks were prepared identically to perform the gas production assays.

Approximately equal volumes of ruminal contents were collected from each sheep at 7:00 a.m. and immediately filtered through four layers of cheesecloth to obtain the ruminal fluid. The filtered ruminal fluid was transferred to a pre-warmed insulated container and transported immediately to the laboratory, where it was mixed with two volumes of buffer solution containing Na_2HPO_4 (1.4 g/L), KH_2PO_4 (1.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.14 g/L), $CaCl_2 \cdot 2H_2O$ (1.58 mg/L), $MnCl_2 \cdot 4H_2O$ (1.2 mg/L), $CoCl_2 \cdot 6H_2O$ (0.12 mg/L), $FeCl_2 \cdot 6H_2O$ (0.1 mg/L), $NaHCO_3$ (8.4 g/L), NH_4HCO_3 (0.1 g/L), resazurin (1.22 mg/L), NaOH (0.08 g/L), and $Na_2S \cdot 7H_2O$ (0.336 g/L). The buffered inoculum was continuously flushed with CO_2 to ensure anaerobic conditions during preparation and transfer to the incubation flasks for all in vitro assays [29].

Next, 0.5 g of substrate and 40 mL of buffered inoculum were placed in a 120 mL anaerobic flask, flushed with CO_2, and sealed for incubation in a water bath at 39 °C for 24 h, following the procedure described by Theodorou et al. [29]. For the determination of gas production parameters, incubation of the flasks was extended to 96 h.

To ensure a reproducible in vitro fermentation performance, two flasks with alfalfa hay were included in each assay. In addition, two blanks without substrate were used to correct the obtained values for gas and ammonia nitrogen.

2.6.1. In Vitro Gas Production Patterns

After incubation, total gas was collected with a 150 mL syringe. Methane production was measured according to Fievez et al. [30]. The gas was transferred through a two-way valve into a second syringe containing 4 mL of 40% (w/v) NaOH. After gentle mixing, the remaining gas volume was recorded and considered as methane. Carbon dioxide production was calculated by subtracting the methane volume from total gas production. It is important to note that methane value includes minor gases such as H_2, O_2, and N_2, which account for less than 1% of total gas; nevertheless, this estimate is considered adequate for comparing methanogenic potential [30].

2.6.2. Ammonia Nitrogen

Ammonia nitrogen (NH_3-N) concentration in filtered fermentation fluid after 24 h of incubation was measured using the method of Galyean [31].

Ten-milliliter aliquots were acidified with 0.3 mL of 50% sulfuric acid and frozen until analysis. For measurement, 0.5 mL of each diluted sample (1/3 dilution in distilled water) was incubated at 95 °C for 5 min with 2.5 mL of phenol reagent (0.15 g of sodium nitroferricyanide and 33 mL of phenol 90% w/v in a 3 L solution with distilled water) and 2 mL of hypochlorite reagent (15 g of sodium hydroxide, 113.6 g of disodium phosphate,

and 150 mL of 5.25% sodium hypochlorite in 3 L solution with distilled water). Absorbance was read at 630 nm using a spectrophotometer. NH_3-N concentration was calculated from a standard curve (R = 0.998) prepared with ammonium solutions of known concentration and the working standards were as follows: 1, 2, 4, 6, and 8 mM made by diluting aliquots of a stock solution 100 mM (0.6607 g of ammonium sulfate in 100 mL with HCl 0.1 N) [31].

Ammonia nitrogen concentration values were corrected by subtracting the concentration in the fermentation fluid used in each run.

2.6.3. Gas Production Parameters

Cumulative gas production was recorded at 0, 3, 6, 9, 12, 24, 48, 72, and 96 h of incubation using a pressure transducer (Delta OHM, HGD 2124.2). Gas production kinetics were fitted to the Gompertz model proposed by Lavrenčič et al. [32] using Formula (3).

$$GP(t) = G_{max}[-A(-kt)] \quad (3)$$

where GP(t) is the cumulative gas production at incubation time t (h), Gmax is the asymptotic gas production, A is a dimensionless shape parameter, and k is the rate constant (h^{-1}). The parameter A was not interpreted as lag time.

Model parameters were estimated by nonlinear least-square regression using the NLIN procedure of SAS (SAS Institute Inc., Cary, NC, USA) with the Gauss–Newton algorithm. Initial parameter estimates were Gmax = 18, A = 0.5, and k = 0.5. Each regression was performed using nine observations. Convergence was assessed using the SAS estimation output. Model performance was evaluated using residual sums of squares and visual comparisons between observed and predicted cumulative gas production.

2.7. In Vitro True Digestibility

In vitro true digestibility of dry matter (IVDDM) was determined by measuring the weight reduction after 48 h of fermentation at 39 °C using ruminal fluid and buffer solution in a 1:4 ratio. This assay was performed in triplicate using a Daisy II incubator following the manufacturer's instructions (ANKOM Technology, Macedon, NY, USA). For this, 0.5 g of each sample was placed in a filter bag (ANKOM F57; 25 µm porosity; ANKOM Technology) previously dried to constant weight, and the bag was placed into a Daisy digestion jar containing 1600 mL of buffer consisting of solution A (KH_2PO_4, 10 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; NaCl, 0.5 g/L; $CaCl_2 \cdot 2H_2O$, 0.1 g/L; urea 0.5 g/L) adjusted to pH 6.8 with solution B (Na_2CO_3, 15.0 g/L; $Na_2S \cdot 9H_2O$), and 400 mL of rumen inoculum obtained as previously described for in vitro fermentations (Section 2.6). The container was purged with CO_2 to ensure anaerobic conditions, and digestion took place under agitation and at a constant temperature for 48 h. Afterwards, filter bags were rinsed with cold tap water, and the NDF procedure was followed to remove microbial debris and soluble remaining fractions. The filter bags were dried at 55 °C for 24 h and weighed to determine IVDDM according to Equation (4). Blank corrections were applied using a bag without samples processed along with the rest of the bags. The run was performed in parallel with the in vitro fermentation assay.

$$\text{IVDDM} = 100 - \frac{(\text{W3} - (\text{W1} \times \text{C1}))}{\text{W2} \times \text{DM}} \times 100 \quad (4)$$

where C1 is blank bag correction (final weight of processed bag without sample/original blank bag weight), W1 is bag tare weight, W2 is sample weight, W3 is the final bag weight after incubation and sequential NDF treatment, and DM is the dry matter content of each sample.

In vitro digestibility of organic matter (IVDOM) was determined using the weight of ash after incineration, as it is considered inorganic matter according to Formula (5).

$$\text{IVDOM} = 100 - \frac{\text{W5}}{\text{W2} \times \text{DM} \times \text{OM}} \times 100 \quad (5)$$

where W5 is the ash-free residue weight calculated according to Formula (6).

$$W5 = (W3 - W1 \times C1) - W4 \quad (6)$$

where W4 is the weight of ash after incineration minus the weight of the incinerated bag without sample.

2.8. Statistical Analysis

The study followed a completely randomized 2 × 2 × 2 factorial design with three replicates.

For dietary chemical composition, because the samples analyzed were technical replicates obtained from a single formulated mixture per treatment, data are reported descriptively as means ± standard deviation (n = 3 technical replicates). No inferential statistical analysis was performed for these variables.

Data for biological parameters were analyzed using a linear mixed model (MIXED procedure, SAS version 9.0, SAS Institute Inc., Cary, NC, USA). The experimental unit was defined as the individual fermentation flask or ANKOM F57 bag, and each treatment was performed in triplicate (n = 3, total N = 24). The model included season, concentrate presentation, forage-to-concentrate ratio, and their interactions as fixed effects. To account for the shared experimental run structure, the replicate was included as a random effect. The mixed model used is shown in Formula (7):

$$Yijkl = \mu + \alpha i + \beta j + \gamma k + (\alpha\beta)ij + (\alpha\gamma)ik + (\beta\gamma)jk + (\alpha\beta\gamma)ijk + Rl + \varepsilon ijkl \quad (7)$$

where Yijkl is the dependent variable, μ is the overall mean, αi , βj , and γk are the fixed effects of the main factors, (αβ)ij , (αγ)ik , (βγ)jk, and (αβγ)ijk are the fixed effects of the two-way and three-way interactions between the main factors, Rl ~ N(0, σ^2r) is the random effect of the l-th replicate, and εijkl ~ N(0, σ^2e) is the residual error.

Variance components were estimated using Restricted Maximum Likelihood (REML) with a Variance Components covariance structure. Denominator degrees of freedom for the Type 3 tests of fixed effects were calculated using the Kenward–Roger adjustment (Den DF = 14). The level of significance was set at ($p < 0.05$).

Due to the distinct nature of the evaluated parameters, different modeling strategies were applied. For dietary chemical composition, data were fitted using a main effect-only (additive) model, as interaction terms among ingredient mixing factors lack biological relevance when characterizing the baseline nutrient profile of formulated diets. Conversely, for the rest of the evaluated parameters, a full factorial model including all main effects and their interactions was utilized to account for the dynamic and associative biological responses to the diets.

When significant differences were detected ($p < 0.05$), means were compared using the Tukey–Kramer adjustment for multiple comparisons.

3. Results

3.1. Chemical Analysis

Table 2 summarizes the chemical composition of the formulated diets.

Percentages of DM and OM were also close among samples. Higher values of OM were obtained during Season 1, which was also the case for the extruded concentrate presentation and the 50/50 forage-to-concentrate ratio, indicating that these levels of each evaluated factor provided more organic matter to the formulated diets.

Table 2. Main effects of intake season, concentrate presentation and forage-to-concentrate ratio on the chemical composition of formulated diets.

Season/Presentation	Forage-to-Concentrate Ratio	Component (%)										
		DM	OM	CP	NDF	ADF	HEM	LIG	CEL	EE	NFC	TC
Season 1/pellet	75/25	93.84 ± 0.15	84.57 ± 0.16	12.16 ± 0.0.1	55.98 ± 0.45	34.48 ± 0.48	21.50 ± 0.03	3.51 ± 0.07	29.08 ± 0.30	2.30 ± 0.31	20.30 ± 0.29	76.28 ± 0.15
	50/50	93.65 ± 0.05	85.68 ± 0.08	15.01 ± 0.64	51.90 ± 0.741	29.64 ± 0.21	22.27 ± 0.50	2.91 ± 0.15	24.61 ± 0.17	3.97 ± 0.14	21.14 ± 0.19	73.04 ± 0.58
Season 1/extruded	75/25	93.55 ± 0.25	84.73 ± 0.11	11.75 ± 0.07	61.03 ± 0.17	36.70 ± 0.41	24.33 ± 0.24	3.21 ± 0.28	30.12 ± 0.36	2.35 ± 0.19	16.04 ± 0.31	77.07 ± 0.15
	50/50	93.46 ± 0.35	86.07 ± 0.24	14.67 ± 0.67	55.09 ± 1.22	30.39 ± 2.05	24.70 ± 3.28	3.81 ± 0.71	24.57 ± 1.39	2.66 ± 0.05	20.18 ± 0.85	75.27 ± 0.51
Season 2/pellet	75/25	93.96 ± 0.15	82.92 ± 0.01	13.69 ± 0.86	56.09 ± 0.43	35.03 ± 0.38	26.06 ± 0.81	2.41 ± 0.75	29.40 ± 0.25	2.87 ± 0.13	16.27 ± 1.16	72.36 ± 0.73
	50/50	93.69 ± 0.01	84.55 ± 0.09	15.35 ± 1.26	52.37 ± 0.23	30.35 ± 0.59	22.01 ± 0.36	3.01 ± 0.41	24.62 ± 0.41	2.04 ± 0.09	21.11 ± 1.66	73.48 ± 1.44
Season 2/extruded	75/25	94.10 ± 0.001	83.06 ± 0.20	12.15 ± 0.46	62.41 ± 0.10	36.96 ± 0.60	25.45 ± 0.70	3.11 ± 0.09	30.28 ± 0.72	1.92 ± 0.21	12.47 ± 0.97	74.88 ± 0.87
	50/50	94.40 ± 0.20	85.04 ± 0.05	14.01 ± 0.51	58.84 ± 0.63	32.36 ± 1.67	26.48 ± 1.04	4.36 ± 0.30	26.06 ± 1.33	2.37 ± 0.39	15.43 ± 0.69	74.27 ± 0.08

Data expressed as Mean ± Standard Error. Sample size, n = 3 (technical replicates per diet). DM, dry matter (g/100 g fresh matter); OM, organic matter (g/100 g dry matter); CP, crude protein (g/100 g dry matter); EE, ether extract (g/100 g dry matter); NDF, neutral detergent fiber (g/100 g dry matter); ADF, acid detergent fiber (g/100 g dry matter); HEM, hemicellulose (g/100 g dry matter); CEL, cellulose (g/100 g dry matter); LIG, lignin (g/100 g dry matter); NFCs, non-fiber carbohydrates (g/100 g dry matter); TCs, total carbohydrates (g/100 g dry matter).

As expected, considering the protein content of the supplement, CP content was higher for the forage-to-concentrate ratio 75/25 and the proportion of concentrate increased CP due to a higher protein percentage in the supplement. CP content was also higher for diets including the pelleted concentrate mainly due to the higher value of crude protein in this presentation, as observed in Table 1.

Different values in NDF, ADF, HEM, CEL and LIG for different concentrate presentations in the same season and ratio indicate that different concentrate processing and conditioning methods cause variation in the proportions of fiber components and overall carbohydrate distribution.

In general, NDF, ADF, CEL and TC decreased as concentrate inclusion increased; conversely, LIG and NFC increased.

Given that NDF and ADF represent the major structural fractions that fall within the broader category of total carbohydrates, TCs exhibited a pattern that closely mirrored the behavior previously observed for both variables, which adds coherence to the dataset.

In Season 1, NDF and TC contents were bigger than in Season 2 (2%), but NDF was smaller. This indicates that the conditions present at that stage, such as higher levels of sunlight and water availability, lead to an increase in the total carbohydrate content of the forage consumed by animals, especially non-fibrous carbohydrates.

EE increased in Season 1, which was expected because grasses develop better nutritional content under favorable environmental conditions. The forage-to-concentrate ratio 25/75 improved EE due to supplement fat content (as observed in Table 1); a similar explanation applies to the higher percentage in the pellet concentrate as this presentation has a slightly higher value of EE than the extruded.

3.2. In Vitro Fermentation Variables

Ammonia nitrogen concentration and in vitro gas production data are presented in Table 3. Significant interactions of evaluated factors were detected for NH_3-N ($p < 0.0001$). The highest NH_3-N values were obtained in both seasons, with the pellet concentrate at a 50/50 forage-to-concentrate; this can be associated with higher CP contents in these diets (Table 2). However, data may also be related to differences in protein availability, especially considering that extruded concentrate in the same forage-to-concentrate ratios led to about 4 mg/dL NH_3-N lower concentrations (19.93 vs. 15.77 in Season 1, and 18.63 vs. 14.57 in Season 2).

Table 3. Interactions and main effects of intake season, concentrate presentation and forage-to-concentrate ratio on in vitro total gas, methane and carbon dioxide production, and ammonia nitrogen concentration.

	Season/Concentrate Presentation	Forage-to-Concentrate Ratio	Variable				
			NH_3-N (mg/dL)	Total Gas (mL/g DM)	CH_4 (mL/g DM)	CO_2 (mL/g DM)	CH_4 (%)
Interaction Effects	Season 1/pellet	75/25	8.27 ^{d}	110.12	11.94	98.18	10.95 ^{b}
		50/50	19.93 ^{a}	137.45	19.36	118.09	14.04 ^{ab}
	Season 1/extruded	75/25	15.03 ^{b}	128.13	16.15	111.98	12.71 ^{b}
		50/50	15.77 ^{b}	154.30	21.11	133.19	13.62 ^{ab}
	Season 2/pellet	75/25	17.70 ^{a}	85.34	14.07	71.28	16.59 ^{a}
		50/50	18.63 ^{a}	141.06	16.93	124.13	12.01 ^{b}
	Season 2/extruded	75/25	12.33 ^{c}	115.31	13.41	101.90	11.56 ^{b}
		50/50	14.57 ^{b}	144.08	20.99	123.09	14.72 ^{ab}
	Season × Presentation × Ratio ($p < 0.05$)		**<0.0001**	0.2904	0.1135	0.1474	**0.0082**
	Season × Presentation ($p < 0.05$)		**<0.0001**	0.9383	0.5585	0.9747	0.2738
	Season × Ratio ($p < 0.05$)		**<0.0001**	0.2073	0.6560	0.1479	0.1145
	Presentation × Ratio ($p < 0.05$)		**<0.0001**	0.2506	0.6042	0.1798	0.1066
	SEM1		**0.5550**	8.3351	1.6185	7.6552	1.2195
Main effects	Season	Season 1	14.75	132.20	17.14	115.36	12.83
		Season 2	15.81	121.45	16.35	105.10	13.72
		($p < 0.05$)	0.0020	0.0792	0.4695	0.0762	0.2865
	Concentrate presentation	Pellet	16.13	118.49 ^{b}	15.58 ^{b}	102.92 ^{b}	12.24
		Extruded	14.43	135.45 ^{a}	17.91 ^{a}	117.54 ^{a}	12.07
		($p < 0.05$)	<0.0001	**0.0109**	**0.0451**	**0.0157**	0.7647
	Forage-to-concentrate ratio	75/25	13.33	109.73 ^{b}	13.89 ^{b}	95.83 ^{b}	12.95
		50/50	17.26	144.22 ^{a}	19.60 ^{a}	124.62 ^{a}	13.60
		($p < 0.05$)	<0.0001	**<0.0001**	**<0.0001**	**<0.0001**	0.4384
	SEM2		0.4365	4.1675	0.9604	3.8276	0.7170

Values are Least Squares Means of the mixed model. SEM1 = Standard error of the mean for interaction effects (two-way and three-way interactions). SEM2 = Standard error of the mean for main effects. abcd: Superscript letters indicate significant differences (Tukey–Kramer, $p < 0.05$) according to the highest-order significant interaction for each variable. Significant *p*-values are shown in bold. For variables with significant interaction effects, letters are restricted strictly to the interaction rows. For variables without significant interactions letters are restricted strictly to the main effect subgroups. The model included the fixed effects of season, presentation, ratio, and their interactions, with replicate as a random effect (n = 3 per treatment combination; total N = 24). NH_3-N = ammonia nitrogen, CH_4 = methane and CO_2 = carbon dioxide.

Total CH_4 production was strongly affected by the forage-to-concentrate ratio, with the highest values observed for the 50/50 ratio (17.91 mL/g DM vs. 15.58 mL/g DM; $p < 0.0001$), and by concentrate presentation ($p = 0.0451$), with approximately 2.33 mL/g DM less produced

for the pellet. However, there were significant interactions among the evaluated factors for methane percentage values ($p = 0.0082$), indicating a combined effect; the highest percentages were statistically equivalent in most diets with a 50/50 forage-to-concentrate ratio.

Total gas production was affected by concentrate presentation ($p = 0.0109$). The extruded concentrate led to greater gas production than the pellet (135.45 vs. 118.49 mL/g DM). The forage-to-concentrate ratio significantly influenced all gas production variables ($p < 0.0001$); total gas, carbon dioxide and methane production increased when concentrate inclusion was the highest.

The gas production kinetics and the in vitro digestibility parameters are shown in Table 4.

Table 4. In vitro gas production parameters, digestibility of dry matter (IVDDM) and in vitro digestibility of organic matter (IVDOM).

	Season/Concentrate Presentation	Forage-to-Concentrate Ratio	Variable				
			Gmax	A	k	IVDDM (%)	IVDOM (%)
Interaction Effects	Season 1/pellet	75/25	150.20	2.44 ^{b}	0.051 ^{b}	61.46 ^{e}	58.29 ^{d}
		50/50	177.47	2.26 ^{c}	0.086 ^{a}	68.14 ^{c}	66.21 ^{b}
	Season 1/extruded	75/25	139.77	2.28 ^{bc}	0.047 ^{b}	61.13 ^{e}	58.24 ^{d}
		50/50	166.17	2.21 ^{c}	0.089 ^{a}	70.80 ^{b}	69.02 ^{b}
	Season 2/pellet	75/25	157.90	3.42 ^{a}	0.073 ^{a}	62.00 ^{e}	59.23 ^{d}
		50/50	161.90	2.37 ^{bc}	0.070 ^{a}	68.63 ^{c}	66.63 ^{b}
	Season 2/extruded	75/25	136.07	2.58 ^{b}	0.045 ^{b}	64.73 ^{d}	61.84 ^{c}
		50/50	166.27	2.22 ^{c}	0.069 ^{a}	72.27 ^{a}	70.77 ^{a}
	Season × Presentation × Ratio ($p < 0.05$)		0.3270	0.1664	0.2593	0.1594	0.3227
	Season × Presentation ($p < 0.05$)		0.8754	0.709	0.2593	**0.0125**	**0.0087**
	Season × Ratio ($p < 0.05$)		0.4776	**0.0121**	0.1460	0.1440	0.0889
	Presentation × Ratio ($p < 0.05$)		0.3581	0.0685	**0.0072**	**0.0151**	**0.0046**
	SEM1		0.3270	0.1447	0.0066	0.5964	0.6194
Main effects	Season	Season 1	158.40	2.298	0.0684	65.38	62.59
		Season 2	155.53	2.650	0.0637	66.91	64.92
		($p < 0.05$)	0.6741	0.0034	0.3166	0.0007	<0.0001
	Concentrate presentation	Pellet	161.87	2.624	0.0696	65.06	62.59
		Extruded	152.07	2.324	0.06244	67.23	64.70
		($p < 0.05$)	0.1625	0.0098	0.1322	<0.0001	0.0001
	Forage-to-concentrate ratio	75/25	145.9 ^{b}	2.680	0.0540	62.33	59.40
		50/50	167.95 ^{a}	2.268	0.0781	69.96	68.16
		($p < 0.05$)	**0.0047**	0.0010	<0.0001	<0.0001	<0.0001
	SEM2		4.7326	0.0724	0.00357	0.4126	0.4736

Values are Least Squares Means of the mixed model. SEM1 = Standard error of the mean for interaction effects (two-way and three-way interactions). SEM2 = Standard error of the mean for main effects. abcde: Superscript letters indicate significant differences (Tukey–Kramer, $p < 0.05$) according to the highest-order significant interaction for each variable. Significant *p*-values are shown in bold. For variables with significant interaction effects, letters are restricted strictly to the interaction rows. For variables without significant interactions letters are restricted strictly to the main effect subgroups. The model included the fixed effects of season, presentation, ratio, and their interactions, with replicate as a random effect (n = 3 per treatment combination; total N = 24). Significant *p*-values are shown in bold. Gmax = Asymptotic gas production (mL/0.5 g DM), k = Rate constant (h^{-1}), A = Dimensionless shape parameter, IVDDM = in vitro digestibility of dry matter and IVDOM = in vitro digestibility of organic matter.

There was a Season × Ratio interaction for A, Station × Presentation interactions for IVDDM, and IVDOM, and Presentation × Ratio interactions for k, IVDDM, and IVDOM.

Due to these interactions, the effects of processing and inclusion level must be interpreted conditionally; no triple interactions were detected for these variables. Season and concentrate presentation significantly affected the dimensionless Gompertz shape parameter (A), in vitro dry matter digestibility (IVDDM), and in vitro organic matter digestibility (IVDOM). In contrast, forage-to-concentrate ratio strongly affected all evaluated variables, with digestibility improving as concentrate inclusion increased (62.33% vs. 69.96% IVDDM, and 59.40% vs. 68.16% IVDOM).

The dimensionless Gompertz shape parameter (A) was lower for diets containing extruded concentrate (2.32) than for those containing pelleted concentrate (2.624; $p = 0.0098$). However, this parameter does not directly represent fermentation lag time. The Season × Ratio interaction was significant ($p = 0.121$). When evaluating the individual treatment combinations, the pellet concentrate at a 75/25 ratio during Season 2 exhibited the highest A value (3.42). In Season 1, the 25/75 inclusion level resulted in significantly higher values; in Season 2, this effect was improved by the pelleted presentation (3.4234 vs. 2.5776).

The rate constant (k) was influenced by the forage-to-concentrate ratio, with the highest value observed at the 50:50 ratio (0.078 h^{-1}; $p < 0.0001$), and by the Presentation × Ratio interaction ($p = 0.0072$). In Season 1, the higher inclusion level (50/50) resulted in significantly higher k values compared to 75/25 in both presentations. However, in Season 2, this pattern was modified: while the level of 50/50 remained significantly higher than 75/25 in the extruded diets, no significant differences were detected between levels in the pelleted diets.

The IVDDM and IVDOM were significantly affected by season ($p = 0.0007$, $p < 0.0001$), concentrate presentation ($p < 0.0001$, $p = 0.0001$), and forage-to-concentrate ratio ($p < 0.0001$, $p < 0.0001$). However, significant Season × Presentation ($p = 0.0125$, $p = 0.0087$) and Presentation × Level ($p = 0.0151$, $p = 0.0046$) interactions were detected. Overall, extruded diets resulted in higher digestibility than pelleted diets, but this difference was more pronounced in Season 2. Specifically, the extruded diet at 50/50 ratio in Season 2 achieved the highest digestibility (72.27% IVDDM and 70.77% IVDOM), which was significantly greater than all other treatments. The lowest digestibilities were observed at a 75/25 ratio. The Presentation × Ratio interaction indicates that the increase in digestibility from a 75/25 to 50/50 ratio was steeper in extruded diets compared to pelleted diets.

4. Discussion

Among the chemical components presented in Table 2, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and non-fiber carbohydrates (NFCs) are particularly relevant because of their crucial roles in ruminal fermentation and animal performance.

As expected, crude protein concentration increased with increasing concentrate inclusion because the formulated supplement contained substantially more protein than the grazed forage. Concentration presentations also affected CP content, with slightly lower values observed in the extruded diets.

This difference is unlikely to be a direct consequence of the extrusion process itself and more likely reflects the sieving step performed before extrusion, during which larger particles from protein-rich ingredients, such as cottonseed meal, may have been partially removed.

The decrease in NDF with increasing concentrate inclusion primarily reflects the chemical composition of the formulated supplement, which contained a greater proportion of readily fermentable ingredients than the grazed forage. Consequently, replacing forage with concentrate reduced the diet's overall proportion of structural carbohydrates.

Diets containing the extruded concentrate consistently exhibited higher NDF values than their pelleted counterparts, and a similar pattern was observed for other cell wall components, including ADF, cellulose, and total carbohydrates.

Several mechanisms explain these differences; during extrusion, thermal and mechanical processing could modify structures such as carbohydrates and proteins, increasing the measured NDF fraction [32]. In addition, the intense shear forces generated during extrusion could disrupt the plant cell wall, increase hemicellulose extractability, and modify its physicochemical properties [33,34]. These structural changes could influence the concentrate's nutritional characteristics and, consequently, its fermentative behavior.

The increase in NFC with increasing concentrate inclusion was expected because the supplement contained a greater proportion of readily fermentable carbohydrates than the grazed forage. Higher NFC values were observed during Season 1, reflecting the greater availability of digestible forage associated with higher rainfall during that period [35]. Diets containing the pelleted concentrate also exhibited higher NFC values, suggesting that part of the soluble carbohydrate fraction may have been lost during extrusion, as previously reported [36]. Furthermore, increasing concentrate inclusion increased dietary starch by incorporating ingredients such as ground corn.

A similar pattern was observed for ether extract, reflecting both the superior nutritional quality of the forage collected during the rainy season [13] and potential lipid changes during extrusion due to exposure to elevated temperatures [37,38]. Collectively, these results indicate that concentrating processing influenced the availability of fermentable nutrients, in addition to the observed fermentation responses.

Across treatments, the distribution between structural and non-fibrous carbohydrate fractions varied considerably, reflecting changes in diet composition associated with concentrate inclusion level and processing methods. These differences may influence ruminal fermentation efficiency by altering the availability of fermentable substrates. Overall, the results indicate that both concentrate inclusion level and physical presentation interact with seasonal forage conditions to shape the nutritional characteristics of the experimental diets.

The significant interaction among season, concentrate presentation, and inclusion level indicates that NH_3-N concentration was influenced by the combined effects of these factors. Under the pelleted presentation, NH_3-N concentrations were higher during Season 2, whereas no seasonal differences were observed for the extruded concentrate. The highest values were obtained for diets with Season 2 forage and the pellet presentation. NH_3-N is the final product of protein and non-protein nitrogen degradation. Previous studies have established a relationship between ruminal ammonia concentration and dietary crude protein availability; this may be observed in this study, as higher ammonia nitrogen values were obtained for diets with higher CP contents in most cases [39]. However, an increase in NH_3-N does not always indicate an improvement in fermentative performance, and other variables such as gas production and digestibility must be studied in further analyses, especially considering that this pattern was not observed for all diets. Although most diets with higher CP content resulted in higher NH_3-N concentrations, the Season 2/pellet diet at the 75/25 ratio resulted in one of the highest values for this variable. In this regard, NH_3-N may be analyzed by considering total gas production and digestibility: the Season 2/pellet diet at the 75/25 ratio led to the lowest gas production (85 mL/g DM) and lower digestibilities (62.00% IVDDM and 59.23% IVDOM) than the 50/50 diets, whereas the Season 2/pellet diet at the 50/50 ratio led to 141.06 mL/g DM and better digestibility percentages (68.63% IVDDM and 66.63% IVDOM). This may indicate that the high NH_3-N values for this diet are caused by an undesirable accumulation of this compound due to inefficient nitrogen use by rumen microorganisms and an unbalanced fermentation, in contrast with the 50/50 diet results, which indicate improved degradation. Interestingly, the increase was more pronounced in the pelleted diets, suggesting differences in nitrogen availability or ruminal degradability between the two processing methods. Because NH_3-N serves as the primary nitrogen source for microbial protein synthesis, these differences may have nutritional implications. However, because in vitro systems do not

account for nitrogen absorption and recycling within the rumen, additional in vivo studies are needed to confirm these responses under practical feeding conditions.

Total gas production increased as concentrate inclusion increased, reflecting the greater availability of readily fermentable nutrients, particularly NFC and crude protein, in the supplemented diets [40]. This is consistent with Jalal et al. [41], who reported that substrates with higher NFC content produced greater gas volumes. Gas production (mL/g DM) was also higher for the extruded presentation; in this case, the effects of extrusion processing conditions on nutrients such as starch may explain these values, as they increase accessibility and fermentability by rumen microorganisms [33,34].

The increase in methane volume (mL/g DM) for the 50/50 ratio may be associated with the higher availability of fermentable carbohydrates in the concentrate, which can enhance hydrogen production, thereby promoting methanogenesis [42]. However, higher methane percentages are indicative of a greater proportion of fermentative energy diverted toward the production of methane as a waste gas, and in consequence, lower energy efficiency in the fermentation process [43]. This may be supported by the present study considering that the highest methane percentage was recorded in Season 2/pellet at the 75/25 forage-to-concentrate ratio—the diet previously indicated as the least efficient considering gas production, ammonia nitrogen and digestibility values. It is important to mention that the significant triple interaction ($p = 0.0082$) observed for methane percentage indicates that this variable was influenced by the combined effects of season, concentrate presentation, and supplementation level. In addition, further in vivo approaches are required to determine whether these responses translate into improvements in animal performance and methane efficiency under practical production conditions.

Tannins from different sources have been proposed to affect methane production and their effects are highly dependent on concentration and dietary source [44]. In the present study, however, tannin concentrations in the experimental diets were below the detection limit of the analytical method used. Therefore, although tannins may have contributed to the fermentation responses observed in other studies, they cannot be considered a sufficient factor to mitigate methane production under the conditions evaluated here. This is consistent with the fact that the diets with the highest methane output (50/50 ratio) were also those with the greatest concentrate inclusion, indicating that the increase in methane was driven primarily by the greater availability of fermentable carbohydrates rather than by any tannin-mediated effect.

Although this hypothesis requires direct confirmation, the lower NH_3-N concentrations observed in the extruded diets may be consistent with a reduction in ruminal protein degradability. However, alternative explanations must be considered, including reduced protein intake and increased microbial ammonia uptake previously proposed [41]. Furthermore, similar effects have been reported following thermal–mechanical processing, where protein denaturation and the formation of protein–carbohydrate complexes reduce ruminal degradation [45]. Increasing the supply of bypass protein is generally considered beneficial because it enhances the flow of absorbable amino acids to the small intestine, thereby improving protein utilization and helping meet the nutritional requirements of high-producing animals [46]. Nevertheless, because the present study did not directly measure bypass protein, it is not possible to distinguish whether the reduced NH_3-N reflects increased bypass protein or a reduction in total nitrogen availability. Direct measurements of protein degradability and subsequent intestinal availability are required to confirm an increase in bypass protein, since reduced protein degradation cannot automatically be interpreted as improved protein utilization, as previously described [47].

No significant main effect of concentrate presentation on the rate constant k indicates that neither pelleting nor extrusion substantially altered the intrinsic speed of microbial

fermentation of the diets. However, the significant Season × Ratio interaction suggests that the rate at which the substrate was fermented depended on the proportion of concentrate, but this response was modulated by the season. The higher values observed at a 50/50 ratio indicate a faster fermentation rate, which is typically associated with a higher proportion of readily fermentable carbohydrates in the concentrate [40]. According to the results, the fermentation rate was primarily driven by the concentrate inclusion level and the seasonal forage characteristics, rather than by the processing of the concentrate.

The increase in dimensionless parameter A in the Gompertz model observed at the lowest inclusion level during Season 2 suggests a synergistic effect between the concentrate and the seasonal characteristics of forage. According to the results, at the lowest inclusion level during Season 2, the concentrate combined with forage decreased the rapid availability of fermentable carbohydrates; in contrast, Season 1 resulted in more stable A values across inclusion levels. The presence of higher proportions of structural carbohydrates at Season 2 diets with the 75/25 ratio reduces the feed quality and may explain higher A values, since this parameter is associated with the colonization time of microorganisms to substrates that increases when there is the need for fibrinolytic enzyme secretion before fermentation accelerates [32].

The highest in vitro digestibility values (IVDDM, IVDOM, Table 4) were obtained with diets containing 50% extruded concentrate; this response is consistent with the greater accessibility of substrates to be degraded due to the effects of extrusion on nutrients [33,34]; differences between processing methods indicated a less pronounced improvement for the pellet presentation. The highest digestibility value was obtained in the Season 2/extruded diet at a 50/50 ratio (72.27% IVDDM and 70.77% IVDOM), while the higher improvements were obtained for the Season 1/extruded diets (15.8% increase in IVDDM and 18.5% increase in IVDOM). The significant Season × Presentation interaction indicates that the magnitude of this processing effect was modulated by the season, possibly due to differences in the basal forage quality between seasons. Similarly, the significant Presentation × Level interaction suggests that the benefit of extrusion was more evident at the higher concentrate inclusion level (50/50). At the lower inclusion level (75/25), the high proportion of forage might have masked the positive effects of extrusion on the concentrate fraction, resulting in the lack of significant differences among these treatments.

These in vitro results suggest that diets with forage from Season 1 responded more favorably to concentrate supplementation than diets with forage collected during Season 2. This observation is consistent with in vivo studies showing that supplementation generally elicits greater responses when the basal forage is of higher nutritional quality [48]. Nevertheless, the marked improvements observed in Season 2 also demonstrate that concentrate supplementation can substantially enhance the digestibility of lower-quality forage during periods of reduced pasture availability.

Limitations

A limitation of this study is that forage samples were pooled by season and animal; consequently, independent biological replicates of the forage were not available. Thus, the results reflect the pooled diet of each season rather than individual animal variability; this must be considered when extrapolating the results to other conditions. Nevertheless, the use of diet samples consumed by grazing animals at two seasons strengthens the practical relevance of the findings.

In addition, fermentations were performed in a single run with technical replicates (three bottles per treatment), rather than independent runs on separate days. Thus, the design does not allow independent replication of run-to-run or donor-to-donor variation, and inferences are restricted to the specific conditions of this single fermentation run; although alfalfa controls were included, future studies should include independent runs

to confirm the robustness of these findings. Another constraint of the present study that must be acknowledged is the use of sheep as rumen fluid donors. Although evidence indicates that sheep can serve as ruminal inoculum donors for evaluation of cattle diets [49], differences in fermentation kinetics have been documented [50]. Therefore, the results of the present study should be extrapolated to cattle with caution.

Finally, as the design did not include a conventional concentrate as a comparator, these effects are attributed to the formulated diets as a whole and are not attributed to the alternative ingredients per se.

5. Conclusions

The non-conventional concentrate improved in vitro fermentation and digestibility of grazed forage-based diets under the tested conditions. Diets containing a higher concentrate inclusion showed greater in vitro digestibility. Increasing concentrate inclusion consistently enhanced nutrient availability, gas production, and digestibility, whereas extrusion generally produced greater improvements in digestibility and total gas production than pelleting. Nevertheless, concentrate increase was accompanied by a higher methane volume per unit of incubated DM, which represents a trade-off that warrants consideration. Because no conventional concentrate was included as a comparator, these effects are attributed to the formulated mixtures as a whole. These laboratory findings provide a basis for future studies to evaluate animal performance, feeding costs, environmental sustainability, and methane emission intensity under practical production conditions.

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Abbreviations

The following abbreviations are used in this manuscript:

DM	Dry matter
OM	Organic matter
CP	Crude protein
NDF	Neutral detergent fiber
ADF	Acid detergent fiber
HEM	Hemicellulose
CEL	Cellulose
LIG	Lignin
NFCs	Non-fiber carbohydrates
TCs	Total carbohydrates
EE	Ether extract
NH_3-N	Ammonia nitrogen
CH_4	Methane
CO_2	Carbon dioxide
Gmax	Asymptotic gas production (mL/0.5g DM)
k	Rate constant (h^{-1})
A	Dimensionless shape parameter
IVDDM	In vitro digestibility of dry matter
IVDOM	In vitro digestibility of organic matter

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