German Journal of Veterinary Research

eISSN:2703-1322

Research article

Enhanced ruminant nutrition and greenhouse gas abatement using co-ensiled corn silage, oilseeds, and spent mushroom substrate

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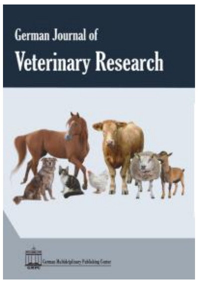

Article History:
Received: 12-Jun-2025
Accepted: 14-Aug-2025

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Abstract

This study aimed to enhance feed efficiency and mitigate greenhouse gas emissions in ruminants by co-ensiling spent mushroom substrate (SMS) and oilseeds (OS; a 1:1 ratio of canola and soybean seeds) with corn silage (CS). Treatments represented varying inclusion levels (%) of CS, SMS, and OS as follows (CS:SMS: OS): Control=100:0:0, T1=90:5:5, T2=80:10:10, T3=70:15:15, T4=60:20:20, and T5=50:25:25. Each treatment was ensiled for 0, 21, 42, or 63 days. A highly significant interaction between treatment and ensiling duration was noted for most chemical components ($p<0.001$), with crude protein, ether extract, and ash increasing, and neutral detergent fiber (NDF), acid detergent fiber (ADF), and nonfibrous carbohydrates decreasing with increasing SMS and OS levels ($p<0.001$). Acid detergent lignin (ADL), cellulose, and hemicellulose also varied significantly with treatment-time interactions ($p<0.05$). Gas production, including methane, carbon dioxide, and hydrogen sulfide (H_2S), was influenced by both treatment and ensiling duration, with an interaction effect ($p<0.05$). At 42 days, H_2S increased by 38.3% compared to the control. Methane declined linearly ($p<0.001$), and H_2S followed linear ($p=0.016$) and quadratic ($p=0.001$) trends. Total volatile fatty acids were unaffected by treatment ($p = 0.123$), but ensiling time influenced the proportions of acetate ($p=0.002$), propionate ($p=0.001$), butyrate ($p<0.001$), and valerate ($p<0.001$). At 42 days, acetate and valerate increased by 3.1% and 8.1%, respectively, while propionate and butyrate decreased by 5.5% and 4.9%, respectively, compared to the control. Nutrient degradability and microbial mass (MM) showed significant treatment and time interactions [$p=0.002$ for MM, *in vitro* apparent degradable dry matter (IVADDM), and partitioning factor (PF)], with ADL degradability increasing by 77.7% and DM degradability by 10.7%. In comparison, IVADDM and MM decreased by 6.4% and 70.6%, respectively, in T3 after 42 days. Ensiling time enhanced DM degradability ($p<0.001$), IVADDM ($p<0.001$), and MM ($p<0.001$), while *in vitro* true degradable DM and PF were not affected ($p>0.05$). The most favorable balance between digestibility, MM, and reduced emissions was achieved with treatment T3, which was ensiled for 42 days. In conclusion, co-ensiling moderate levels of SMS and OS with CS for 42 days is recommended to improve feed quality and reduce the environmental impact of ruminant production.

Keywords: Corn silage, Degradability, Ensiling duration, *In vitro* fermentation, Spent mushroom substrate, Oilseeds

Citation: Anotaenwere, C. C., Isikhuemhen, O. S., Dele, P. A., Kholif, A. E., Wuaku, M., Adelusi, O. O., Okedoyin, D. O., Alabi, J. O., Ike, K. A., Gray, D., Subedi, K., and Anele, U. Y. 2025. Enhanced ruminant nutrition and greenhouse gas abatement using co-ensiled corn silage, oilseeds, and spent mushroom. Ger. J. Vet. Res. 5 (3): 1-17. https://doi.org/10.51585/gjvr.2025.3.0144

Introduction

Ruminants are substantial contributors to anthropogenic greenhouse gas (GHG) emissions, particularly methane (CH_4) produced through enteric fermentation. Ruminants alone are responsible for approximately 16% of total global CH_4 emissions. Of the CH_4 emissions attributed to agriculture, which account for around 41% of global CH_4 emissions, livestock contribute 73%, with beef cattle responsible for 35%, dairy cattle for 30%, and small ruminants and buffaloes collectively contributing 15% (Tseten et al., 2022). This presents a major environmental challenge due to the high global warming potential of methane. Mitigating enteric CH_4 is crucial for sustainable livestock production as demand for animal products rises (Prathap et al., 2021). Dietary strategies offer a practical approach to reduce emissions while maintaining productivity (Gasparini et al., 2024; Jalal et al., 2025).

Corn silage (CS) is a staple forage in ruminant diets due to its high energy yield, typically containing 30-35% starch, which supports high feed intake. However, CS has limitations: it is relatively low in crude protein [approximately 8% of dry matter (DM)] and moderate in fiber digestibility, which can constrain animal performance unless supplemented (Scott et al., 2019). The low protein content necessitates the addition of protein sources to meet ruminant requirements. Moreover, the lignified cell walls in CS can limit rumen fiber digestion. These nutritional gaps prompt exploration of silage additives to enhance feed quality and reduce enteric CH_4 (Koakoski et al., 2024).

Spent mushroom substrate (SMS), the fibrous residue after mushroom cultivation, is a promising feed component in this context (Wuaku et al., 2025). Spent mushroom substrate is composed largely of plant cell-wall polysaccharides (cellulose, hemicellulose) with residual lignin and fungal biomass (mycelia) (Anotaenwere et al., 2024). Importantly, studies report a 60-75% reduction in lignin during mushroom growth, mediated by fungal enzymes (Martín et al., 2023; Anotaenwere et al., 2024). This biological pretreatment improves the digestibility of the remaining fiber. In addition, fungal growth enriches the substrate with protein and bioactive compounds. Ensiling SMS can further enhance its nutritional value, as fermentation breaks down fibers and increases the availability of nutrients (Wuaku et al., 2025). The inclusion of SMS in ruminant diets has been shown to improve rumen fiber degradability and feed intake, and in some cases, milk yield, without adverse effects (Kholif et al., 2014). Spent mushroom substrate also contains phenolic and antioxidant compounds from the mushroom that may modulate rumen fermentation and potentially suppress methanogenesis (Rangubhet et al., 2017).

Oilseeds (OS) such as canola (rapeseed) and soybean have gained attention as silage additives to mitigate enteric CH_4 (Hristov et al., 2022). These seeds are rich in lipids (unsaturated fatty acids) that, when released in the rumen, can reduce methanogenesis via multiple mechanisms. Unsaturated fats undergo ruminal biohydrogenation, a process that consumes hydrogen ions, which methanogens would otherwise use to produce CH_4 (Gomaa et al., 2018). Additionally, long-chain fatty acids from OS can directly inhibit methanogenic archaea and protozoa in the rumen (Sun et al., 2021). The dietary inclusion of 3–5% fat from OS has been shown to decrease enteric CH_4 emissions by approximately 10–20% *in vivo* (Beauchemin et al., 2009). Additionally, canola seed in particular has yielded persistent CH_4 reductions (~16% per unit of feed intake) without impairing fiber digestion or animal performance (Beauchemin et al., 2009). This CH_4-mitigating effect of lipids is achieved with minimal disruption to overall rumen fermentation at moderate inclusion levels (Tseten et al., 2022). However, excessive supplemental fat can suppress rumen microbial activity; thus, moderate levels are crucial for balancing fermentation and mitigating efficacy (Gomaa et al., 2018).

Combining SMS and OS is a novel approach that may yield additive or even synergistic effects. SMS offers biologically pretreated fiber, fungal protein, and potential antimethanogenic phenolics (Rangubhet et al., 2017), while OS contributes rumen-active unsaturated lipids and additional protein. Co-ensiling these components may not only improve fermentation quality but also optimize ruminal fermentation through complementary mechanisms: fiber improvement from SMS, and hydrogen suppression and methanogen inhibition from OS (Ungerfeld, 2015). While direct studies on SMS-OS interactions are scarce, ensiling them together may promote more stable preservation and enhance nutrient availability. However, potential issues such as increased risk of aerobic spoilage with high-fat inclusion, limited regional availability of SMS, and additional processing requirements (e.g., grinding or mixing), should be acknowledged as practical limitations in field applications.

The inclusion of SMS exemplifies the circular-economy principle. SMS is often cost-free near mushroom facilities, and can lower feed cost without compromising quality (Palangi et al., 2022). On-farm production of OS for co-ensiling further integrates cropping and livestock systems, reducing reliance on external inputs. To date, most studies have evaluated SMS or OS separately, and there is a lack of research on their combined use in CS-based diets. We hypothesized that combining SMS (to improve fiber utilization) with canola or soybean seed (to supply rumen-active lipids and protein) could offer synergistic advantages for both nutrition and CH_4 mitigation. The SMS provides a pre-digested fiber component and adds protein, while OS provides a hydrogen sink and antimethanogenic fatty acids, thereby enhancing silage nutrient quality and more effectively reducing ruminal CH_4 emissions than either component used individually. Therefore, the objective of this study is to evaluate the effects of incorporating SMS with OS (canola and soybean seeds in a 1:1 ratio) into CS at varying inclusion levels (0, 10, 20, 30, 40 and 50%) and ensiled for different durations (0, 21, 42 and 63 days) on total gas and greenhouse gas production, volatile fatty acid (VFA) profiles, and nutrient degradability. By investigating this novel combination, we aim to contribute to more sustainable ruminant production systems through improved feed utilization and lower GHG emissions.

Materials and methods

Silage preparation

The experiment was carried out at the Department of Animal Sciences at North Carolina Agricultural and Technical State University. Corn was manually harvested to a stubble of 15 cm from the University farm when the kernel was at approximately one-third the milk line stage and chopped to approximately 2 cm with a forage chopper. The SMS were generated from corn stover inoculated with *Pleurotus ostreatus* grain spawn (5% wet weight) as described in Olagunju et al. (2023). Canola and soybean seeds were purchased from Nutrient Ag Solutions at Brown Summit, Greensboro, NC, USA. Corn was ensiled in plastic buckets with a 5 L capacity (cylindrical shape with 35 cm height × 16 cm diameter), and each storage time group was prepared with 4 replications. The ensiling process was completed within 6 h after harvesting. The buckets were stored in a room at an ambient temperature of 20 ± 2°C for 0, 21, 42, and 63 days. These intervals were selected to represent the early, mid, and late phases of fermentation commonly used in silage studies. The three-week intervals were chosen to capture the optimal periods of enzymatic activity and fiber degradation during silage fermentation. Spent mushroom substrate was thoroughly mixed with OS (a 1:1 ratio of canola and soybean seeds) and CS at various ratios before ensiling. Subsamples were dried at 60°C for 48 h in a forced-air oven and then milled to pass through a 1 mm sieve for analysis of their chemical composition.

Experimental design

The study was conducted using a 6 × 4 factorial arrangement, involving six inclusion levels of CS and a combined mixture of SMS, with OS (a 1:1 ratio of canola and soybean seeds) at different proportions, as well as 4 ensiling durations (0, 21, 42, and 63 days). The treatment groups included the following CS: SMS+OS ratios (%, respectively): 100:0 (Control), 90:10 (5% SMS + 5% OS, T1), 80:20 (10% SMS + 10% OS, T2), 70:30 (15% SMS + 15% OS, T3), 60:40 (20% SMS + 20% OS, T4), and 50:50 (25% SMS + 25% OS, T5). This design enabled the gradual inclusion of SMS and OS, combined from 0% to 50%, to evaluate their potential synergistic effects on reducing feed costs and improving silage quality. Each of the 24 treatments (6 inclusion levels × 4 storage durations) was replicated 4 times, resulting in a total of 96 mini bucket silos.

Sample preparation and *in vitro* batch culture

Silos were weighed after filling and at opening to determine DM recovery. After opening, the silages were mixed, and samples were collected for analysis of their chemical composition and the *in vitro* batch culture experiment. Milled silage samples (0.5 ± 0.02 g) were weighed with an analytical scale (model VWR-224AC; VWR International, Radnor, PA, USA) directly into 100 mL serum bottles (Cat. No. 223747; Wheaton Science Products, Millville, NJ, USA). Four replicates (bottles) were prepared for each treatment and incubated for 24 h. Another 4 bottles (replicates) for each treatment were prepared for the determination of nutrient degradability, where 0.5 g of the samples were weighed into Ankom bags (F57; Ankom Technology Corp, Macedon, NY, USA) and sealed using a heat impulse sealer (Model AIE-200HR, California, USA) before the bags were inserted into pre-labeled 100 mL serum bottles according to treatments.

The *in vitro* incubation procedure was based on the methods described in Anele et al. (2011) with some modifications. Artificial saliva was prepared according to McDougall's buffer recipe, containing (per L): 9.83 g $NaHCO_3$, 3.69 g Na_2HPO_4, 0.60 g KCl, 0.47 g NaCl, 0.30 g $(NH_4)_2SO_4$, 0.061 g $MgCl_2 \cdot 6H_2O$, and 0.0293 g $CaCl_2{\cdot}2H_2O$. The artificial saliva was maintained in a water bath at 39°C. Ruminal fluid was collected from two ruminally cannulated Holstein Friesian dairy cows after morning feeding. Cows were kept on pasture and supplemented with grass hay and a mineral premix. The cows were from the Dairy Research Farm of North Carolina Agricultural and Technical State University, NC. Cows were under an IACUC-approved protocol (# LA22-0019).

The artificial saliva and ruminal fluid were mixed at a 3:1 (v/v) ratio, and the pH was measured using a bench-top pH meter (model B10P, VWR International, Radnor, PA, USA). The batch culture media (60 mL) was dispensed into the 100 mL glass serum bottles. Each serum bottle received 45 mL of artificial saliva and 15 mL of rumen fluid, both of which were anaerobically flushed with carbon dioxide (CO_2) gas to maintain anaerobic conditions. The bottles were capped with a 14 mm rubber stopper and crimped with an aluminum seal cap. The bottles were incubated on an orbital shaker at 39°C and 125 rpm for 24 h. This process was repeated on a different week for a total of two independent experimental runs. At the predetermined time points, headspace gas production was measured 24 h post-incubation by inserting a 22 mm gauge needle attached to a manometer (VWR International, Radnor, PA, USA).

Estimation of greenhouse gases

Methane, ammonia (NH_3), CO_2, and hydrogen sulfide (H_2S) concentrations were estimated using a portable gas analyzer (Biogas 5000, Landtec, Dexter, MI, USA). The analyzer was calibrated according to the manufacturer's instructions. An aliquot of gas from the samples was introduced into the analyzer with a 22 G × 1.5 in (0.7 mm × 40 mm) gauge needle attached to the end of the inlet Tygon tube. The unit was purged between each sampling to eliminate any residual gas from the previous sampling.

Nutrient disappearance

After taking gas pressure and individual gas analysis readings, the Ankom bags were removed from the bottles, thoroughly rinsed under cold water until the water was clear, and dried in a 55 °C oven for 48 h. Thereafter, *in vitro* apparent degradable DM (IVADDM) and *in vitro* true degradable DM (IVTDDM) were estimated as described by Anele et al. (2011). The degradation of DM, neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) was calculated by subtracting the dried residue weight from the initial weight of dried substrate after each analysis, as follows: Nutrient degradation = [(Initial nutrient weight − Final nutrient weight) / Initial nutrient weight] × 100, and expressed as *d*DM, *d*NDF, *d*ADF, and *d*ADL, respectively.

Microbial mass estimation

Microbial mass determination was based on Blümmel and Lebzien (2001), with slight modifications. Feed substrates were weighed directly into the serum bottles, and fermentation was terminated after 24 h. The contents of the bottles were poured into pre-weighed centrifuge tubes (Thermo Fisher Scientific, Rochester, NY, USA). The samples were centrifuged at 20,000 × g at 4°C for 15 min. Blanks were centrifuged as a correction factor for the buffered ruminal inoculum residues. After removing the tubes from the centrifuge, the supernatant was poured off, and the pellets were placed in a freezer for 24 h. The frozen pellets were placed in a freeze-dryer (L-200, Buchi Lyovapor, New Castle, DE, USA) for approximately 72 h. After freeze-drying, the lyophilized samples were weighed to determine the weight of the pellets, and MM was calculated as described by Blümmel and Lebzien (2001). The partitioning factor (PF) at 24 h of incubation (mg *d*DM/mL gas) was calculated (Blümmel et al., 1997).

Volatile fatty acid analysis

Volatile fatty acid concentration was measured based on the protocol of Ruiz-Moreno et al. (2015). The samples were analyzed using a gas chromatography (Agilent 7890B GC system/5977B GC-MSD/7693 autosampler, Agilent Technologies, Santa Clara, CA, USA) with a capillary column (Zebron ZB-FFP, Phenomenex Inc., Torrance, CA, USA). An internal standard mixture of metaphosphoric acid and crotonic acid (trans-2-butenoic acid) was employed, while acetate (C_2), propionate (C_3), butyrate (C_4), isobutyrate (iso-C_4), valerate (C_5), and isovalerate (iso-C_5) served as quantitative external standards (Ruiz-Moreno et al., 2015).

Chemical analysis

The dietary samples were analyzed for DM (#930.15), N (#954.01), and ether extract (EE; #920.39) according to AOAC (2019). Nitrogen was determined using an organic elemental analyzer (2400 CHNS, PerkinElmer, Waltham, MA, USA). Ether extract was determined using the Ankom XT15 extractor (Ankom, Macedon, NY, USA). Neutral detergent fiber and ADF were analyzed using an Ankom 200 Fiber Analyzer (Ankom, Macedon, NY, USA) according to AOAC (method 973.18) (AOAC, 2019). The NDF content was determined as described by Van Soest et al. (1991) using heat-stable α-amylase with sodium sulfite. Acid detergent lignin was determined by soaking in concentrated sulfuric acid (72%) using the analytical methods of ANKOM Technologies. The concentrations of nonfibrous carbohydrates (NFC=1000−NDF−CP−EE−ash), cellulose (ADF-ADL), hemicellulose (NDF-ADF), and organic matter (OM=1000-ash) in treatments were calculated.

Statistical analysis

The data generated were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC, USA) in a 6 × 4 factorial arrangement. The model used was: $Y_{ijk} = \mu + P_i + S_j + (PS)_{ij} + e_{ijk}$, where Yijk is each observation for a given variable, μ is the overall mean, P_i is the proportion effect, S_j is the effect of ensiling duration, $(PS)_{ij}$ is the interaction between proportion and ensiling duration, and e_{ijk} is the residual error.

Results

Chemical composition

The interaction between diet and ensiling duration was significant ($p<0.001$) for OM and ash contents (Table 1). No significant interactions were observed for the other parameters. Organic matter had the highest values in the control group, particularly at 42 days of ensiling, while the lowest OM values were associated with higher SMS and oilseed inclusion (T5), regardless of duration. Ash content exhibited a consistent increase with greater inclusion levels of SMS and oilseeds, especially in T5, across all ensiling periods.

Regarding dietary effects, increasing the proportion of SMS and OS had a profound and mostly linear effect on several chemical constituents. Significant linear responses were observed ($p<0.001$) for OM, ash, CP, EE, NFC, NDF, ADF, ADL, hemicellulose, and cellulose. As SMS and OS increased from the control to T5, the contents of ash, CP, EE, ADF, ADL, and cellulose also increased, whereas the contents of OM, NFC, NDF, and hemicellulose tended to decrease. Similarly, EE was significantly higher in T5 (maximum ~11.88%) compared to the control (minimum ~7.85%). Notably, ADF and ADL also increased with the inclusion of SMS.

Ensiling duration had a significant effect on nearly all parameters except NFC and ADL. The linear p-values for DM, OM, ash, CP, EE, NDF, ADF, hemicellulose, and cellulose were all significant ($p<0.001$). Additionally, quadratic effects were significant for several variables, including OM, ash, CP, NFC, and NDF. The CP content increased progressively with ensiling time, peaking around day 42 in all diets, with a slight decline or stabilization by day 63. Ash content followed a similar quadratic pattern, with early decreases followed by later increases. Fiber components (ADF and cellulose) decreased significantly during the ensiling process (linear, $p<0.001$), reflecting the potential degradation of structural carbohydrates. Meanwhile, DM content did not follow a strictly linear increase across treatments or durations; instead, slight variations were observed depending on diet and fermentation time.

Gas and greenhouse gas production

The interaction between treatment and ensiling duration significantly influenced GP ($p=0.006$), CH_4 ($p=0.014$), CO_2 ($p=0.006$), and H_2S ($p=0.028$) (Table 2). The control treatment had a consistent increase in GP and CH_4 over time, whereas treatments like T5 demonstrated a less pronounced or more variable response, particularly in CH_4 and H_2S. Treatment composition had a significant impact on all fermentation parameters assessed. GP, CH_4, and CO_2 responded linearly and significantly ($p<0.001$) to the increasing replacement of CS with SMS and OS. Quadratic trends were also evident for GP ($p=0.026$), NH_3-N ($p=0.014$), and H_2S ($p=0.001$).

Table 1: Analyzed chemical composition (%, DM basis, unless otherwise stated) of experimental treatments composed of varying proportions of corn silage (CS), spent mushroom substrate (SMS) and oilseeds (OS; canola and soybean seeds in a 1:1 ratio) ensiled for different durations (0, 21, 42, or 63 days).

Treatment[1]	Ensiling duration (day)	DM, fresh weight	OM	Ash	CP	EE	NFC	NDF	ADF	ADL	Hemicellulose[2]	Cellulose[2]
Control	0	94.3^{j}	95.7^{fg}	4.31^{kl}	6.5^{g}	7.85^{n}	36.5^{a-c}	44.9^{a}	26.0^{e-h}	2.77^{ij}	18.9^{a}	23.2^{a}
	21	94.5^{f-j}	96.6^{a}	3.39^{q}	12.7^{a-d}	8.19^{mn}	34.7^{b-d}	41.1^{hi}	24.0^{mn}	2.59^{ij}	17.1^{e-i}	21.4^{g-j}
	42	94.8^{a-f}	96.7^{a}	3.30^{q}	16.8^{a}	7.96^{n}	29.2^{f-i}	42.7^{d-f}	25.0^{i-l}	2.38^{j}	17.8^{c-e}	22.6^{a-d}
	63	95.0^{a}	96.4^{b}	3.58^{p}	9.6^{d-g}	8.28^{l-n}	39.9^{a}	38.6^{l}	22.8^{o}	2.61^{ij}	15.8^{j}	20.2^{l}
T1	0	94.3^{ij}	95.4^{hi}	4.62^{ij}	7.5^{fg}	8.62^{k-m}	34.4^{b-e}	44.9^{a}	26.3^{c-f}	3.41^{g-i}	18.6^{ab}	22.9^{ab}
	21	94.5^{d-j}	96.2^{c}	3.79^{o}	13.1^{a-d}	8.91^{jk}	32.8^{c-f}	41.5^{g-i}	24.5^{k-m}	3.26^{g-i}	17.0^{f-i}	21.3^{h-j}
	42	94.8^{a-e}	96.3^{bc}	3.71^{op}	16.8^{a}	8.70^{kl}	27.9^{g-k}	43.0^{c-e}	25.4^{g-k}	3.06^{h-j}	17.6^{c-f}	22.3^{b-e}
	63	95.0^{a}	96.0^{d}	3.96^{n}	10.2^{c-g}	8.99^{k-j}	37.6^{ab}	39.3^{kl}	23.5^{no}	3.27^{g-i}	15.8^{j}	20.2^{l}
T2	0	94.4^{h-j}	95.1^{j}	4.93^{h}	8.4^{e-g}	9.38^{h-j}	32.3^{c-g}	44.9^{a}	26.7^{b-e}	4.05^{e-g}	18.2^{a-c}	22.6^{a-c}
	21	94.6^{c-i}	95.8^{ef}	4.19^{lm}	13.4^{a-d}	9.65^{gh}	30.9^{d-h}	41.9^{f-h}	26.7^{b-e}	3.92^{e-g}	16.8^{g-i}	21.2^{ij}
	42	94.8^{a-e}	95.9^{e}	4.12^{m}	16.7^{a}	9.46^{hi}	26.5^{h-l}	43.2^{b-d}	25.9^{e-i}	3.74^{f-h}	17.4^{d-g}	22.1^{c-f}
	63	95.0^{ab}	95.7^{fg}	4.34^{k}	10.9^{b-f}	9.72^{f-h}	35.1^{b-d}	39.9^{jk}	24.1^{l-n}	3.92^{e-g}	15.8^{j}	20.2^{l}
T3	0	94.5^{g-j}	94.8^{lm}	5.24^{ef}	9.4^{d-g}	10.14^{e-g}	30.3^{e-i}	45.0^{a}	27.0^{a-d}	4.69^{c-e}	17.9^{b-d}	22.3^{b-e}
	21	94.6^{c-i}	95.4^{hi}	4.59^{ji}	13.8^{a-c}	10.38^{e}	28.9^{f-j}	42.3^{e-g}	25.6^{f-j}	4.58^{c-e}	16.8^{g-i}	21.0^{ij}
	42	94.9^{a-e}	95.5^{h}	4.53^{j}	16.7^{a}	10.21^{ef}	25.2^{j-l}	43.5^{b-d}	26.3^{c-g}	4.42^{d-f}	17.2^{e-h}	21.9^{d-h}
	63	95.0^{ab}	95.3^{i}	4.73^{i}	11.6^{b-f}	10.43^{de}	32.7^{d-f}	40.6^{ij}	24.8^{j-m}	4.59^{c-e}	15.8^{j}	20.2^{kl}
T4	0	94.5^{e-j}	94.5^{p}	5.55^{b}	10.4^{b-g}	10.90^{cd}	28.2^{f-k}	45.0^{a}	27.4^{ab}	5.34^{a-c}	17.6^{c-f}	22.0^{c-g}
	21	94.7^{b-h}	95.0^{jk}	4.99^{gh}	14.1^{a-c}	11.10^{c}	27.1^{h-l}	42.7^{d-f}	26.2^{d-g}	5.24^{a-d}	16.5^{hi}	20.9^{kj}
	42	94.9^{a-d}	95.1^{j}	4.94^{h}	16.6^{a}	10.96^{c}	23.8^{kl}	43.7^{bc}	26.8^{b-e}	5.11^{b-d}	17.0^{f-i}	21.6^{e-i}
	63	94.9^{ab}	94.9^{kl}	5.11^{fg}	12.2^{b-e}	11.16^{bc}	30.3^{e-i}	41.2^{hi}	25.5^{f-j}	5.24^{a-c}	15.8^{j}	20.2^{kl}
T5	0	94.6^{c-i}	94.1^{q}	5.86^{a}	11.4^{b-f}	11.67^{ab}	26.1^{i-l}	45.0^{a}	27.7^{a}	5.98^{a}	17.3^{d-g}	21.7^{e-i}
	21	94.7^{a-g}	94.6^{no}	5.40^{cd}	14.5^{ab}	11.83^{a}	25.2^{j-l}	43.1^{b-e}	26.7^{b-e}	5.90^{ab}	16.4^{ij}	20.8^{j-l}
	42	94.9^{abc}	94.7^{mn}	5.35^{de}	16.5^{a}	11.71^{a}	22.4^{l}	43.9^{b}	27.2^{a-c}	5.79^{ab}	16.7^{g-i}	21.4^{f-j}
	63	94.9^{ab}	94.5^{op}	5.49^{bc}	12.9^{a-d}	11.88^{a}	27.8^{g-k}	41.9^{f-h}	26.1^{d-g}	5.90^{ab}	15.8^{j}	20.2^{kl}
SEM		0.12	0.05	0.048	1.49	0.183	1.65	0.32	0.33	0.291	0.25	0.25
p value												
Diet												
Linear		0.027	<0.001	<0.001	0.008	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Quadratic		0.999	0.999	0.999	1.000	0.999	1.000	1.000	0.999	0.999	0.999	0.999
Ensiling duration												
Linear		<0.001	<0.001	<0.001	0.0004	0.023	0.218	<0.001	<0.001	0.326	<0.001	<0.001
Quadratic		0.791	<0.001	<0.001	<0.001	0.939	<0.001	0.354	0.747	0.209	0.447	0.061
Diet × Ensiling duration		0.994	0.001	0.001	0.993	1.000	0.997	0.008	0.871	1.000	0.552	0.466

Means in the same column with different superscripts differ, p<0.05. P-value is the observed significance level of the F-test for treatment; SEM = standard error of the mean. Abbreviations: ADF, acid detergent fiber; ADL, acid detergent lignin; crude protein; DM, dry matter; EE, ether extract; NDF, neutral detergent fiber; NFC, nonfibrous carbohydrates; OM, organic matter.[1]Treatments: Control = 100:0:0, T1 = 90:5:5, T2 = 80:10:10, T3 = 70:15:15, T4 = 60:20:20, and T5 = 50:25:25 (CS:SMS:OS). [2]Calculated values: Hemicellulose = NDF – ADF; Cellulose = ADF – ADL.

Table 2: Productions of gas (mL/g DM), CH_4 (mg/g DM), CO_2 (mg/g DM), NH_3 (mmol/g DM), and H_2S (mmol/g DM) from treatments containing varying proportions of corn silage (CS), spent mushroom substrate (SMS) and oilseeds (OS; canola and soybean seeds in a 1:1 ratio) ensiled for different durations (0, 21, 42, or 63 days) and 24 h of incubation.

Treatment[1]	Duration (day)	GP	CH_4	CO_2	NH_3	H_2S
Control	0	147.9^{efg}	5.62^{f-j}	29.1^{ij}	79.4^{cdef}	364.0^{e-h}
	21	159.2^{cd}	7.52^{bcd}	40.9^{c-g}	94.5^{bcd}	382.5^{d-g}
	42	175.5^{b}	8.08^{abc}	44.8^{bc}	78.7^{c-f}	401.2^{b-g}
	63	186.5^{a}	9.24^{a}	54.2^{a}	121.6^{ab}	529.2^{abc}
T1	0	133.9^{hij}	6.30^{d-i}	34.4^{ghi}	108.1^{a-d}	480.6^{b-e}
	21	153.9^{de}	7.35^{bcd}	41.2^{c-f}	82.1^{c-f}	369.8^{e-h}
	42	169.9^{b}	8.75^{ab}	51.0^{ab}	133.0^{a}	678.0^{a}
	63	166.8^{bc}	6.34^{d-i}	37.4^{d-h}	89.5^{b-e}	383.5^{c-g}
T2	0	132.1^{ij}	5.89^{e-j}	35.2^{e-i}	99.5^{bcd}	424.2^{b-f}
	21	145.3^{fg}	6.82^{c-f}	38.9^{c-h}	108.3^{a-d}	538.1^{ab}
	42	159.1^{cd}	7.48^{bcd}	43.6^{cd}	110.7^{abc}	526.0^{bcd}
	63	159.4^{cd}	7.18^{cde}	44.0^{bcd}	82.5^{c-f}	412.5^{b-g}
T3	0	126.7^{jk}	5.97^{e-j}	34.6^{f-i}	88.4^{c-f}	405.7^{b-g}
	21	123.3^{kl}	5.68^{f-j}	32.7^{hij}	82.8^{cdef}	422.0^{b-f}
	42	149.5^{ef}	7.44^{bcd}	44.8^{bc}	112.5^{abc}	554.8^{ab}
	63	150.6^{def}	7.11^{cde}	43.1^{cd}	89.0^{b-f}	444.8^{b-f}
T4	0	112.0^{lm}	5.20^{ij}	29.9^{ij}	94.5^{bcd}	342.5^{fgh}
	21	120.3^{kl}	5.24^{hij}	30.3^{ij}	79.3^{c-f}	359.5^{e-h}
	42	149.9^{ef}	7.05^{cde}	43.5^{cd}	107.7^{a-d}	508.9^{bcd}
	63	147.9^{efg}	6.64^{c-g}	41.7^{cde}	86.0^{c-f}	447.4^{b-f}
T5	0	113.0^{lm}	5.41^{g-j}	29.0^{ij}	61.0^{ef}	232.0^{h}
	21	114.6^{lm}	4.56j	26.8^{j}	55.7^{f}	265.5^{gh}
	42	136.0^{hi}	5.34^{g-j}	33.7^{hij}	94.5^{bcd}	453.9^{b-f}
	63	140.6^{gh}	6.56^{d-h}	41.1^{c-g}	79.3^{c-f}	435.8^{b-f}
SEM		2.94	0.486	2.43	10.97	49.24
p value						
Treatment						
Linear		<0.001	<0.001	<0.001	0.001	0.016
Quadratic		0.026	0.576	0.095	0.014	0.001
Ensiling duration						
Linear		<0.001	<0.001	<0.001	0.058	0.001
Quadratic		0.011	0.131	0.142	0.159	0.043
Treatment × Duration		0.006	0.014	0.006	0.091	0.028

Means in the same column with different superscripts differ, P<0.05. P-value is the observed significance level of the F-test for treatment; SEM = standard error of the mean. Abbreviations: CH_4, methane; CO_2, carbon dioxide; NH_3, ammonia; H_2S, hydrogen sulfide. [1]Treatments: Control = 100:0:0, T1 = 90:5:5, T2 = 80:10:10, T3 = 70:15:15, T4 = 60:20:20, and T5 = 50:25:25 (CS:SMS:OS).

Methane declined linearly with dietary changes ($p<0.001$), while NH_3-N followed a quadratic pattern, initially rising before decreasing ($p=0.014$). H_2S exhibited both linear ($p=0.016$) and quadratic ($p=0.001$) responses. Ensiling duration had a significant effect on all fermentation parameters, especially GP and gas profiles. GP, CH_4, and CO_2 increased linearly with longer ensiling times ($p<0.001$), with GP ($p=0.011$) and H_2S ($p=0.043$) also showing significant quadratic effects. H_2S levels increased over time, following both linear ($p=0.001$) and quadratic ($p=0.043$) trends.

Volatile fatty acids

No significant interactions were detected between treatment and ensiling duration for total or individual VFA ($p>0.05$ for all; Table 3). Dietary treatments did not significantly affect total VFA concentrations in either the linear ($p=0.123$) or quadratic form ($p=0.325$). Similarly, the proportions of C_2, C_3, the C_2:C_3 ratio, C_4, and iso-C_5 were not influenced by treatment in either trend ($p>0.05$). However, treatment exerted a significant linear effect on C_5 ($p=0.008$) and iso-C_5 ($p=0.005$), with both decreasing as the levels of SMS and OS inclusion increased.

Ensiling duration significantly impacted several fermentation parameters. Total VFA concentrations declined linearly over time ($p=0.017$), though the quadratic effect was not significant ($p=0.663$). Concentrations of C_2 and C_3 increased linearly with longer ensiling times ($p=0.002$ and 0.001, respectively). At the same time, C_4 decreased markedly in a linear fashion ($p<0.001$) and exhibited a quadratic pattern as well ($p=0.012$), suggesting a peak at intermediate durations. No significant linear or quadratic effects were found for iso-C_4 ($p>0.05$). Valerate declined linearly with extended ensiling ($p<0.001$), and iso-C_5 exhibited a marginal quadratic response ($p=0.030$). The C_2:C_3 ratio remained unaffected by ensiling duration.

Table 3. Total (mmol/g DM) and individual volatile fatty acid proportions (%) produced from treatments containing varying proportions of corn silage (CS), spent mushroom substrate (SMS) and oilseeds (OS; canola and soybean seeds in a 1:1 ratio) ensiled for different durations (0, 21, 42, or 63 days) and 24 h of incubation.

Treatment[1]	**Duration (day)**	**Total**	**C_2**	**C_3**	**C_2:C_3**	**C_4**	**Iso-C_4**	**C_5**	**Iso-C_5**
Control	0	73.0^{bcd}	59.8^{cde}	20.5^{cde}	3.06^{ab}	17.7^{ab}	0.37^{a-d}	1.49^{abc}	0.20^{a-f}
	21	94.6^{a}	62.5^{a-d}	21.3^{b-e}	3.55^{a}	14.6^{ef}	0.29^{e}	1.17^{c-g}	0.15^{g}
	42	67.8^{bcd}	61.2^{a-e}	25.3^{a}	2.42^{b}	12.2^{gh}	0.36^{a-e}	0.74^{fg}	0.20^{a-g}
	63	64.5^{bcd}	61.6^{a-d}	25.2^{a}	2.44^{b}	11.9^{gh}	0.34^{a-e}	0.73^{g}	0.19^{c-g}
T1	0	67.0^{bcd}	62.1^{a-d}	20.2^{de}	3.18^{ab}	15.9^{b-e}	0.32^{cde}	1.31^{a-f}	0.17^{efg}
	21	67.0^{bcd}	60.6^{b-e}	22.2^{a-d}	2.76^{ab}	15.6^{cde}	0.29^{e}	1.23^{b-g}	0.15^{g}
	42	61.5^{bcd}	62.3^{a-d}	24.1^{abc}	2.59^{ab}	12.2^{gh}	0.37^{a-d}	0.79^{fg}	0.20^{a-g}
	63	69.3^{bcd}	65.6^{ab}	22.1^{a-e}	3.24	11.1^{gh}	0.31^{cde}	0.70^{g}	0.18^{c-g}
T2	0	77.2^{ab}	56.0^{e}	23.8^{abc}	2.45^{b}	17.9^{a}	0.38^{a-d}	1.71^{ab}	0.21^{a-e}
	21	72.9^{bcd}	61.0^{b-e}	22.1^{a-d}	2.78^{ab}	15.1^{de}	0.31^{de}	1.32^{a-e}	0.16^{fg}
	42	57.1^{bcd}	62.5^{a-d}	23.9^{abc}	2.62^{ab}	12.1^{gh}	0.40^{ab}	0.86^{efg}	0.22^{a-d}
	63	57.1^{bcd}	61.8^{a-d}	24.1^{abc}	2.58^{ab}	12.5^{gh}	0.40^{abc}	0.92^{d-g}	0.24^{a}
T3	0	69.3^{bcd}	57.7^{de}	21.7^{a-e}	2.81^{ab}	18.1^{a}	0.40^{ab}	1.77^{a}	0.24^{ab}
	21	66.6^{bcd}	59.3^{cde}	22.3^{a-d}	2.69^{ab}	16.4^{a-e}	0.34^{b-e}	1.46^{abc}	0.18^{d-g}
	42	61.0^{bcd}	63.1^{abc}	23.9^{abc}	2.64^{ab}	11.6^{gh}	0.38^{a-d}	0.80^{fg}	0.21^{a-e}
	63	55.4^{bcd}	61.3^{a-e}	24.5^{ab}	2.51^{ab}	12.7^{fg}	0.39^{a-d}	0.91^{efg}	0.23^{a-d}
T4	0	71.4^{bcd}	59.2^{cde}	21.2^{b-e}	2.88^{ab}	17.3^{abc}	0.39^{a-d}	1.67^{abc}	0.22^{a-e}
	21	76.7^{abc}	60.6^{b-e}	21.0^{b-e}	2.94^{ab}	16.4^{a-e}	0.36^{a-e}	1.45^{a-d}	0.20^{a-f}
	42	58.3^{bcd}	63.8^{abc}	23.6^{a-d}	2.72^{ab}	11.2^{gh}	0.37^{a-d}	0.78^{fg}	0.21^{a-f}
	63	76.2^{abc}	66.9^{a}	21.5^{a-e}	3.52^{ab}	10.3^{h}	0.37^{a-d}	0.77^{fg}	0.20^{a-g}
T5	0	59.9^{bcd}	63.0^{abc}	18.7^{e}	3.45^{ab}	16.4^{a-e}	0.35^{a-e}	1.40^{a-e}	0.19^{b-g}
	21	78.2^{ab}	59.3^{cde}	22.2^{a-d}	2.72^{ab}	16.2^{a-e}	0.40^{ab}	1.64^{abc}	0.21^{a-e}
	42	53.9^{d}	64.4^{abc}	22.8^{a-d}	2.83^{ab}	11.3^{gh}	0.40^{ab}	0.85^{efg}	0.23^{abc}
	63	59.1^{bcd}	63.3^{abc}	23.5^{a-d}	2.70^{ab}	11.6^{gh}	0.42^{a}	0.91^{efg}	0.24^{ab}
SEM		7.35	1.88	1.25	0.356	0.66	0.028	0.183	0.017
p value Treatment									
Linear		0.123	0.468	0.171	0.741	0.873	0.008	0.101	0.005
Quadratic		0.325	0.243	0.341	0.273	0.209	0.881	0.388	0.417
Ensiling duration									
Linear		0.017	0.002	0.001	0.318	<0.001	0.280	<0.001	0.104
Quadratic		0.663	0.810	0.220	0.400	0.012	0.251	0.195	0.030
Treatment × Duration		0.668	0.599	0.751	0.687	0.206	0.690	0.987	0.433

Means in the same column with different superscripts differ, P<0.05. P-value is the observed significance level of the F-test for treatment; SEM = standard error of the mean. Abbreviations: C_2, acetate; C_3, propionate; C_2:C_3, acetate-to-propionate ratio; C_4, butyrate; Iso-C_4, isobutyrate; C_5, valerate; Iso-C_5, isovalerate. [1]Treatments: Control = 100:0:0, T1 = 90:5:5, T2 = 80:10:10, T3 = 70:15:15, T4 = 60:20:20, and T5 = 50:25:25 (CS:SMS:OS).

Nutrient degradability and fermentation parameters

Significant interactions between treatment and ensiling duration were observed for MM ($p=0.002$), undegraded materials ($p=0.013$), IVADDM ($p=0.002$), IVTDDM ($p=0.010$), and PF ($p=0.002$) (Table 4). The increase in MM over time was more notable in treatments containing intermediate levels of SMS and OS. Similarly, enhancements in IVADDM and IVTDDM with prolonged ensiling were more evident in treatments with moderate SMS inclusion, such as T2 and T3.

Increasing the levels of SMS and OS in the treatment significantly influenced the degradability of *d*NDF ($p<0.001$), *d*ADF ($p=0.002$), and *d*ADL ($p<0.001$), all of which showed clear linear trends. Treatment also had a significant linear effect on undegraded materials ($p=0.038$), IVADDM ($p=0.002$), and IVTDDM ($p=0.001$), with values increasing as SMS and OS inclusion increased. Additionally, IVTDDM displayed a significant quadratic effect ($p=0.038$). In contrast, PF and MM were not significantly affected by treatment alone ($p>0.05$).

With respect to ensiling duration, *d*DM increased linearly with time ($p<0.001$), as did *d*ADF ($p=0.005$) and *d*ADL ($p = 0.004$), indicating improved fiber degradability with longer storage periods. *d*NDF followed a significant quadratic pattern ($p=0.003$). The microbial mass also rose linearly over time ($p<0.001$). The IVADDM increased significantly with ensiling duration ($p<0.001$), while IVTDDM and PF remained unaffected ($p>0.05$).

Discussion

Chemical composition

The present study evaluated the chemical composition of silages produced with varying proportions of CS, SMS, and OS over different ensiling durations. The findings revealed that both diet formulation and ensiling period significantly influenced most of the chemical constituents of the treatments, while interactions between diet and ensiling duration were generally limited. Among all measured variables, only OM and ash exhibited significant interactions between diet and ensiling duration. This suggests that, for most parameters, compositional changes were primarily additive and predictable based on diet or time alone. However, OM and ash responded more dynamically to the combined effects of diet and storage time, likely due to the mineral-rich nature of SMS and the complex microbial and chemical transformations of these components during fermentation (Okoye et al., 2023).

Dry matter content increased linearly with the inclusion of SMS and OS, reflecting the inherently lower moisture content of these ingredients compared to fresh CS. A minor but consistent increase in DM over the ensiling period likely reflects the concentration effect caused by microbial fermentation and associated moisture losses (Anotaenwere et al., 2024). Organic matter content, on the other hand, decreased with increasing SMS inclusion, which can be attributed to the high ash (i.e., inorganic) content of SMS (Wuaku et al., 2025). This trend was particularly evident in treatments with greater SMS inclusion, consistent with reports highlighting elevated mineral content (e.g., calcium, phosphorus, potassium) in fungal residues (Anotaenwere et al., 2024). The decline in OM during ensiling further supports the notion that microbial catabolism of organic substrates, coupled with mineral solubilization and redistribution, leads to a relative increase in ash proportion over time (Borreani et al., 2018a). The significant interaction observed between diet and ensiling duration for both OM and ash confirms that mineral-related transformations during fermentation are sensitive to both substrate composition and fermentation time, especially in high-SMS treatments (Wuaku et al., 2025).

Crude protein content increased linearly with the inclusion of SMS and OS, due to their relatively higher protein concentrations. Temporal changes in CP followed a consistent pattern across treatments, increasing during early ensiling phases, likely due to carbohydrate degradation and relative concentration of remaining nutrients. However, CP plateaued or declined slightly by day 63, potentially reflecting proteolysis or conversion of true protein into non-protein nitrogen fractions (Aloba et al., 2022). Despite these dynamics, the absence of interaction effects indicates that the CP response to ensiling was independent of dietary treatment.

Table 4: Nutrient degradability (%), undegraded residual (g), dry matter disappearance, and microbial mass (g) of treatments with varying proportions of corn silage (CS), spent mushroom substrate (SMS) and oilseeds (OS; canola and soybean seeds in a 1:1 ratio) ensiled for different durations (0, 21, 42, or 63 days) and 24 h of incubation.

Treatment[1]	Duration (day)	*d*DM	*d*NDF	*d*ADF	*d*ADL	Microbial mass (%)	Undegraded materials (g)	IVADDM[2]	IVTDDM[2]	PF[2]
Control	0	42.0^{a-e}	77.2^{ab}	59.3^{e-g}	18.9^{fg}	0.05^{gh}	0.22^{c}	45.3^{b-f}	55.2^{ab}	1.87^{ef}
	21	44.0^{a-d}	73.2^{c-g}	61.3^{b-f}	19.4^{e-g}	0.14^{b-e}	0.20^{c}	29.7^{g-i}	59.0^{ab}	1.83^{f}
	42	41.2^{c-e}	77.0^{a-c}	58.9^{e-g}	14.8^{g}	0.17^{abc}	0.50^{a}	54.5^{a-c}	15.8^{c}	3.42^{a}
	63	44.5^{abc}	73.6^{b-f}	58.5^{fg}	19.3^{fg}	0.06^{f-h}	0.27^{bc}	57.0^{a}	48.7^{ab}	2.12^{c-f}
T1	0	41.8^{b-e}	77.6^{a}	59.1^{e-g}	23.1^{c-g}	0.11^{c-h}	0.23^{c}	33.2^{gh}	54.8^{b}	2.05^{c-f}
	21	41.8^{b-e}	68.0^{ij}	61.3^{b-f}	24.8^{b-f}	0.12^{c-g}	0.20^{c}	36.9^{f-h}	60.5^{ab}	1.95^{ef}
	42	46.8^{ab}	74.8^{a-e}	59.8^{c-g}	18.6^{fg}	0.06^{f-h}	0.27^{bc}	56.1^{a}	49.1^{b}	2.26^{b-f}
	63	46.2^{ab}	73.5^{b-g}	58.8^{fg}	18.4^{fg}	0.07^{e-h}	0.26^{bc}	55.3^{ab}	49.9^{b}	2.22^{b-f}
T2	0	42.3^{a-e}	71.6^{d-i}	58.2^{g}	26.9^{a-f}	0.13^{b-f}	0.21^{c}	32.4^{gh}	58.6^{ab}	2.24^{b-f}
	21	42.9^{a-e}	69.8^{f-j}	61.4^{b-f}	28.7^{a-e}	0.16^{b-d}	0.20^{c}	28.2^{hi}	60.1^{ab}	2.06^{c-f}
	42	45.1^{abc}	74.6^{a-e}	61.3^{b-f}	21.5^{d-g}	0.04^{h}	0.21^{c}	51.9^{abc}	58.9^{ab}	1.85^{ef}
	63	45.0^{abc}	72.3^{d-g}	62.0^{a-e}	20.0^{e-g}	0.08^{d-g}	0.27^{bc}	52.2^{a-c}	49.5^{ab}	2.30^{b-f}
T3	0	41.8^{b-e}	75.1^{a-d}	60.7^{b-g}	26.3^{a-f}	0.12^{c-g}	0.22^{c}	32.4^{gh}	56.2^{ab}	2.23^{b-f}
	21	42.8^{a-e}	66.6^{jk}	62.0^{a-e}	29.8^{a-d}	0.11^{c-g}	0.19^{c}	40.0^{e-g}	62.0^{ab}	2.60^{bc}
	42	45.6^{ab}	73.5^{b-f}	61.0^{b-g}	26.3^{a-f}	0.05^{gh}	0.20^{c}	51.0^{a-d}	59.9^{ab}	2.03^{d-f}
	63	44.7^{abc}	70.6^{e-i}	63.3^{ab}	24.1^{b-g}	0.08^{d-h}	0.27^{bc}	53.0^{a-c}	49.9^{ab}	2.37^{b-f}
T4	0	38.8^{e}	70.9^{f-i}	59.6^{c-g}	34.4^{a}	0.21^{a}	0.22^{c}	16.1^{j}	56.7^{ab}	2.70^{b-d}
	21	39.0^{de}	68.7^{h-j}	62.3^{a-d}	32.7^{ab}	0.07^{e-h}	0.21^{c}	43.5^{c-f}	58.1^{ab}	2.42^{b-e}
	42	44.5^{abc}	69.5^{g-j}	62.5^{a-d}	27.2^{a-f}	0.05^{gh}	0.20^{c}	50.7^{a-d}	61.3^{ab}	2.06^{c-f}
	63	47.0^{a}	68.2^{ij}	62.7^{a-c}	25.5^{a-f}	0.09^{d-h}	0.16^{c}	50.5^{a-d}	68.2^{a}	2.29^{b-f}
T5	0	38.8^{e}	69.0^{h-j}	58.9^{d-g}	33.0^{ab}	0.17^{ab}	0.21^{c}	19.7^{ij}	57.5^{ab}	2.57^{b-d}
	21	40.0^{c-e}	62.8^{k}	61.1^{b-g}	28.5^{a-e}	0.15^{b-e}	0.36^{b}	40.4^{d-g}	50.6^{b}	2.81^{ab}
	42	42.3^{a-e}	68.6^{h-j}	64.4^{a}	31.0^{a-c}	0.07^{e-h}	0.20^{c}	46.5^{a-f}	60.2^{ab}	2.23^{b-f}
	63	45.4^{ab}	68.7^{h-j}	62.3^{a-d}	26.7^{a-f}	0.06^{f-h}	0.19^{c}	49.6^{a-e}	62.3^{ab}	2.22^{b-f}
SEM		1.82	1.38	1.04	3.22	0.026	0.045	3.66	5.77	0.203
p value										
Treatment										
Linear		0.140	<0.001	0.002	<0.001	0.549	0.038	0.002	0.001	0.064
Quadratic		0.119	0.522	0.277	0.328	0.305	0.025	0.923	0.038	0.098
Ensiling duration										
Linear		<0.001	0.360	0.005	0.004	<0.001	0.305	<0.001	0.243	0.932
Quadratic		0.799	0.003	0.010	0.681	0.718	0.363	0.082	0.682	0.722
Treatment × Duration		0.782	0.153	0.200	0.972	0.002	0.013	0.002	0.010	0.002

Means in the same column with different superscripts differ, P<0.05. P-value is the observed significance level of the F-test for treatment; SEM = standard error of the mean. Abbreviations: *d*DM, degraded dry matter; *d*NDF, degraded neutral detergent fiber; *d*ADF, degraded acid detergent fiber; *d*ADL, degraded acid detergent lignin; IVADDM, in vitro apparent degradable dry matter; IVTDDM, in vitro true degradable dry matter; PF, partitioning factor (mg degraded DM per mL gas). [1]Treatments: Control = 100:0:0, T1 = 90:5:5, T2 = 80:10:10, T3 = 70:15:15, T4 = 60:20:20, and T5 = 50:25:25 (CS:SMS:OS). [2]Calculated values.

Similarly, EE content increased linearly with the dietary inclusion of OS, particularly canola and soybean seeds, both of which are rich in lipids. A modest increase in EE during the ensiling period may result from the disruption of cellular membranes and the liberation of bound lipids. The lack of a significant interaction between diet and storage time suggests that fermentation-related lipid changes were consistent across all diets. Additionally, microbial lipolysis under anaerobic conditions is generally limited, especially in oilseed-rich diets, which may help preserve lipid fractions during storage (Llamas et al., 2025).

In contrast, NFC content decreased with increasing SMS and OS levels, indicating a dilution effect due to the replacement of the highly fermentable, carbohydrate-rich CS with lower NFC components. Ensiling duration did not significantly affect NFC, implying that the majority of soluble carbohydrate fermentation occurred early and reached a stable level thereafter (Du et al., 2020). The absence of an interaction effect further reinforces the idea that carbohydrate fermentation followed a similar pattern regardless of dietary formulation. The dietary inclusion of SMS also led to a consistent increase in structural carbohydrate fractions, including NDF, ADF, hemicellulose, and cellulose, due to the fibrous nature of the fungal residues. However, all these fiber components decreased over the ensiling period, reflecting progressive microbial degradation and acid hydrolysis (Wróbel et al., 2023). The decline was most pronounced in hemicellulose, which exhibited a quadratic response characterized by rapid degradation during the initial ensiling period, followed by stabilization. This is likely due to the more accessible, amorphous structure of hemicellulose compared to cellulose. In contrast, cellulose declined more gradually, likely due to its crystalline structure and lower susceptibility to microbial attack (Zhao et al., 2025). Notably, while the dietary effect on fiber fractions was significant, no interaction with ensiling duration was observed, suggesting that fiber degradation followed a uniform trajectory across treatments regardless of initial content The reduction in NDF and ADF over time aligns with previous findings on the fermentation dynamics of high-fiber substrates (Aloba et al., 2022), indicating an improvement in the digestibility of the silage over time. Interestingly, despite its fiber-like nature, ADL exhibited different behavior. Lignin content increased significantly with dietary SMS level, as fungal biomass contains heavily lignified cell walls. However, ADL was not significantly affected by ensiling duration or its interaction with diet. This aligns with the known recalcitrance of lignin to both microbial and chemical degradation under anaerobic conditions. Although some reports have suggested a relative increase in lignin concentration over time due to the degradation of more digestible components (Anotaenwere et al., 2024), such a pattern was not observed in this study, possibly due to a combination of a stable lignin structure and limited microbial colonization in these treatments. Still, treatments with higher SMS (Long et al., 2024) and OS levels (Oldick and Firkins, 2000) may be prone to reduced digestibility over time due to the retention of lignin-bound fiber fractions, as microbial access to structural polysaccharides can be physically and chemically impeded.

The general trends observed in this study are consistent with those reported in similar work. For example, Anotaenwere et al. (2024) reported that increasing SMS inclusion elevated fiber fractions and reduced cellulose and CP concentrations. Their work also reported significant interactions between diet and storage time, particularly in higher inclusion levels of SMS. These observations, along with our findings, underscore that while ensiling enhances certain aspects of feed quality (e.g., reducing fiber content), challenges related to lignin accumulation and protein stability persist, particularly in diets containing high levels of fibrous or oilseed-based ingredients.

Gas production and greenhouse-gas emissions

The significant interaction between treatment and ensiling duration on GP and associated fermentation gases (CH_4, CO_2, and H_2S) underscores the complex biochemical interplay between substrate composition and fermentation kinetics during the in vitro fermentation (Morsy et al., 2022; Olagunju et al., 2024). These interactions suggest that the metabolic activity of the ensiled biomass is highly dependent on both the initial formulation and the biochemical transformations that occur during fermentation. control treatment (100% CS) exhibited a consistent increase in GP and CH_4 over the ensiling period, reflecting the typical fermentation dynamics of CS, which is rich in fermentable carbohydrates (Giagnoni et al., 2024). With an increasing ensiling period, microbial fermentation is likely to have intensified, leading to enhanced GP (Anotaenwere et al., 2024).

In contrast, treatments with higher inclusion levels of SMS and OS, such as T5, showed a more attenuated or inconsistent response, particularly in CH_4 and H_2S production. This variability may be linked to the presence of bioactive compounds, higher fat and fiber fractions, or antimicrobial metabolites in SMS and OS, which could inhibit methanogenesis or alter microbial community dynamics during storage (Gomaa et al., 2018; Wuaku et al., 2025).

The observed linear decline in CH_4 with increasing levels of SMS and OS substitution suggests that replacing CS with these unconventional feedstuffs can suppress methanogenic activity. This aligns with previous findings indicating that SMS contains phenolic compounds and other secondary metabolites capable of modulating rumen microbial populations, including methanogens (Rangubhet et al., 2017). Moreover, OS often contributes high lipid content, which has been reported to reduce CH_4 emissions through inhibition of protozoa and associated archaea (Wang et al., 2017). Therefore, the reduction in CH_4 is likely a synergistic outcome of substrate limitation, microbial inhibition, and altered fermentation pathways.

Gas production and CO_2 output also increased significantly with the inclusion of SMS and OS, albeit with a linear trend, indicating enhanced microbial fermentation activity and carbohydrate degradation (Anotaenwere et al., 2024). This suggests that, despite reduced CH_4 output, total fermentation activity may remain robust or even enhanced, possibly shifting toward more C_3-dominated fermentation pathways, which are less associated with hydrogen accumulation and thus CH_4 synthesis (Beauchemin et al., 2020).

H_2S production was another critical parameter affected by both treatment and storage time. Its production during the in vitro fermentation was increased over prolonged ensiling, particularly in SMS- and OS-based treatments, suggesting the presence of sulfur-rich compounds or enhanced microbial sulfur metabolism. H_2S is often associated with the degradation of sulfur-containing amino acids and can be influenced by the balance between protein degradation and microbial utilization of sulfur (Areniello et al., 2024). The quadratic response of H_2S with dietary treatment suggests that moderate inclusion of SMS and OS may initially stimulate sulfur-reducing activity, while excessive inclusion might suppress it due to microbial inhibition or sulfur limitation. The role of ensiling duration alone was also significant, with linear increases in GP, CH_4, and CO_2 suggesting that prolonged fermentation enhances overall microbial activity and substrate breakdown. The quadratic response in GP and H_2S may point to optimal fermentation efficiency at intermediate time points, followed by stabilization or decline as substrates become depleted or inhibitory metabolites accumulate. The consistent rise in H_2S over time further supports the hypothesis that prolonged storage may lead to increased proteolysis or microbial turnover (Aloba et al., 2022), resulting in the release of sulfurous compounds into the fermentation matrix. Anotaenwere et al. (2024) stated that treatments with higher levels of SMS resulted in reduced GP, CH_4, and CO_2 productions. In contrast, extending the ensiling period increased CH_4 and CO_2 production.

Volatile fatty acid profiles

The absence of significant interactions between treatment and ensiling duration on total and individual VFA concentrations indicates that the effects of these two factors operated independently in modulating fermentation end-products. This finding suggests that neither the substitution of CS with SMS and OS, nor the progressive stages of ensiling, synergistically or antagonistically altered the overall VFA profile. Instead, each factor exerted its influence in isolation.

Dietary treatments had limited influence on VFA concentrations overall, with no significant changes observed in total VFAs, major fermentation acids (C_2, C_3, and C_4), or the C_2:C_3 ratio. These results suggest that replacing CS with SMS and OS did not substantially alter the fermentability of the treatments in terms of VFA output. However, two branched-chain fatty acids, iso-C_4 and iso-C_5, did respond significantly to dietary changes, both decreasing linearly with increasing levels of SMS and OS. Since isoacids are typically derived from microbial deamination of leucine, isoleucine, and valine (Firkins et al., 2024), a treatment lower in degradable protein or rich in fibrous and less fermentable protein sources (as SMS often is) may result in reduced isoacid production. However, Anotaenwere et al. (2024) reported that increasing the proportion of SMS in the treatment at the expense of CS led to higher isoacid production. Thus, the observed decline in C_5 and iso-C_5 points to a shift in microbial deamination pathways and amino acid catabolism (Gao and Geng, 2022), accompanied by an increase in SMS and OS inclusion.

In contrast, ensiling duration had a more pronounced effect on several fermentation characteristics. Total VFA concentrations declined linearly over time, likely reflecting a gradual depletion of readily fermentable carbohydrates as ensiling progressed (Anotaenwere et al., 2024). This trend is consistent with typical silage fermentation dynamics, where an initial burst of microbial activity consumes simple sugars, producing acids that subsequently lower the pH and suppress further fermentation (Bernardes et al., 2018). Interestingly, while total VFAs declined, concentrations of C_2 and C_3 increased linearly with longer storage durations (Gordo et al., 2023). This paradox may be explained by a shift in fermentation pathways from rapid lactic acid production to slower secondary fermentations that favor C_2 and C_3 formation as hemicellulose and other complex carbohydrates become available through prolonged microbial activity (Owens and Basalan, 2016).

Butyrate displayed a contrasting pattern, with concentrations decreasing linearly and following a quadratic trend. The decline in C_4 over time may indicate a reduction in clostridial activity, which typically dominates early in silage fermentation and is associated with poor silage quality (Borreani et al., 2018b). The quadratic pattern suggests that C_4 might have peaked at an intermediate storage time before declining, possibly as lactic acid bacteria outcompeted clostridia under increasingly anaerobic and acidic conditions (Okoye et al., 2023).

Notably, the C_2:C_3 ratio remained stable throughout the ensiling period, suggesting that the balance between C_2- and C_3-producing microbial populations was not significantly disturbed over time. This stability could reflect a steady state of carbohydrate fermentation pathways or a consistent microbial community structure despite changes in substrate availability. Anotaenwere et al. (2024) reported that total VFA and C_4 concentrations peaked in the treatment with 50% SMS inclusion, and that extending the ensiling period resulted in increased proportions of C_2 and C_3.

Nutrient disappearance and fermentation

The interactive effects of treatment composition and ensiling duration significantly influenced key parameters related to ruminal digestibility and fermentation efficiency. Notably, significant interactions were found for MM, undegraded materials, IVADDM, IVTDDM, and PF. These interactions highlight the intricate interplay between dietary composition, particularly the inclusion of SMS and OS, and the biochemical transformations that occur during the ensiling process.

The increase in MM over time was most pronounced in treatments with intermediate SMS and OS inclusion levels (e.g., T2 and T3). This suggests that moderate supplementation optimally supported microbial proliferation during fermentation and in vitro digestion, likely due to a more balanced availability of fermentable substrates and nutrients (Belanche et al., 2012). In contrast, higher inclusion levels (as in T5) may have introduced more indigestible components such as lignin from the SMS or fats phenolic compounds from the OS, which could inhibit microbial activity (Sun et al., 2021). Similarly, the enhancements in IVADDM and IVTDDM with extended ensiling were particularly notable in T2 and T3, reinforcing the idea that moderate substitution of CS with SMS and OS can improve feed digestibility when coupled with sufficient ensiling time.

Dietary effects alone revealed a clear linear increase in the degradability of fiber fractions (*d*NDF, *d*ADF, and *d*ADL) with increasing inclusion of SMS and OS. These trends likely reflect the partial breakdown of cell wall components in SMS during the fermentation (Anotaenwere et al., 2024), as well as the nutritional richness of OS, which can enhance microbial fermentation (Kholif et al., 2018). Additionally, undegraded material decreased significantly with higher SMS and OS levels. At the same time, both IVADDM and IVTDDM improved, indicating that these alternative feed components contributed to better nutrient availability and digestion potential (Wuaku et al., 2025). Interestingly, IVTDDM also exhibited a significant quadratic response, suggesting that beyond a certain threshold of SMS and OS inclusion, digestibility gains may plateau or even decline, likely due to increased lignification or reduced availability of fermentable carbohydrates. Anotaenwere et al. (2024) stated that treatments with higher levels of SMS resulted in enhanced *d*DM, *d*NDF, *d*ADF, and *d*ADL. In contrast, extending the ensiling period increased *d*DM, and decreased *d*NDF and *d*ADF.

On the other hand, PF and MM were not significantly influenced by treatment alone. The lack of a dietary effect on PF suggests that GP per unit of IVTDDM remained relatively stable across treatments (Blümmel et al., 1997), pointing to a consistent fermentation efficiency regardless of the CS replacement level. Similarly, the static MM response across treatments implies that microbial biomass yield was more influenced by fermentation time and interactive effects rather than feed composition alone (Wuaku et al., 2025).

Ensiling duration played a crucial role in modifying digestibility traits. All of *d*DM, *d*ADF, and *d*ADL all increased linearly with time, indicating progressive improvements in fiber accessibility and breakdown with prolonged storage (Cueva et al., 2023). This enhancement may result from enzymatic and microbial activities during ensiling that soften the plant cell wall matrix, improving the exposure of cellulose and hemicellulose to ruminal microbes (Weimer, 2022). The *d*NDF followed a quadratic pattern, possibly reflecting an initial increase followed by stabilization, as readily degradable fiber fractions are exhausted and more recalcitrant components dominate the substrate pool (Hoffman et al., 2001).

Microbial mass also increased linearly over ensiling time, suggesting that the ensiling process progressively created a more favorable environment for microbial colonization and growth during in vitro incubation (Aloba et al., 2022). The enhancement in IVADDM with time supports this view, as improved fermentation substrates likely contributed to more effective digestion. However, IVTDDM and PF were unaffected by storage duration, indicating that while apparent digestibility increased, the proportion of gas produced relative to digested substrate and the absolute digestible matter yield remained stable. This suggests a potential ceiling effect, where additional ensiling time does not translate into proportionally higher fermentability or efficiency gains.

Conclusions

The present study demonstrated that co-ensiling SMS and OS (canola and soybean) with CS significantly affected the chemical composition, fermentation profile, nutrient degradability, and greenhouse gas emissions of the silage. Both the treatment composition and ensiling duration played key roles, with several notable interactions observed. Among the tested treatments, treatment T3, which comprised moderate levels of SMS and OS, enhanced nutrient degradability (IVADDM, IVTDDM), resulted in greater microbial mass, and led to lower methane and hydrogen sulfide emissions compared with the other treatments. These benefits were maximized at 42 days of ensiling, a duration that provided a balance between improved fermentation and fiber breakdown while minimizing undesirable gas emissions and nutrient losses. Although higher SMS and OS levels (e.g., T5) improved CP and EE contents, they were associated with increased ash and less favorable gas and fermentation profiles. Ensiling for shorter periods (0–21 days) was inadequate for achieving stable fermentation or optimal feed value. In conclusion, co-ensiling SMS and OS with CS at moderate inclusion levels (T3) and for 42 days offers a practical and sustainable approach to enhancing ruminant feed efficiency and reducing greenhouse gas emissions.

Article Information

Funding. This research was funded by USDA National Institute of Food and Agriculture, Evans-Allen Project # NC.X 350-5-22-120-1.

Conflict of interest. The author declared no conflict of interest.

Authors contribution. All authors contributed equally to the work.

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