



## Nutritional Composition and *In Vitro* Digestibility of Spent Mushroom Substrate Supplemented with Urea and Molasses for Ruminants

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### ABSTRACT

This study aims to evaluate the fermentation characteristics and nutritional potential of spent mushroom substrates (SMS), with or without urea and molasses supplementation, in ruminant diets. SMS derived from the cultivation of four mushroom species—*Pleurotus ostreatus* (Oyster Mushroom, OYS), *Flammulina filiformis* (Enoki mushroom, ENK), *Hypsizygus marmoreus* (Bunashimeji mushroom, SMJ), and *Lentinula edodes* (Shiitake, STK)—were analyzed for proximate composition, *in vitro* gas and methane (CH<sub>4</sub>) production, dry matter digestibility (DMD), and short-chain fatty acid (SCFA). Statistically significant differences were observed among SMS types across all incubation periods (24, 48, and 72 hours) for gas production, CH<sub>4</sub> production, DMD, and SCFA concentrations ( $p < 0.0001$ ). In addition, CH<sub>4</sub> per gram of digested dry matter (CH<sub>4</sub>/DDM) was higher in urea–molasses supplemented SMS compared to non-supplemented substrates ( $p < 0.05$ ). However, no significant differences were observed in total gas production, CH<sub>4</sub> volume, or SCFA concentrations between the two groups, suggesting that fermentation responses were influenced not only by supplementation but also by the inherent differences in substrate composition. The results indicate that different types of SMS vary significantly in their fermentation profiles and digestibility. Supplementation with urea and molasses increased CH<sub>4</sub>/DDM but did not significantly affect total gas, CH<sub>4</sub> volume, or SCFA production. These findings demonstrate the potential of SMS as a partial roughage replacement in ruminant diets, as influenced by the type of mushroom substrate, its chemical composition, and the supplementation method.

**Keywords:** *in vitro*; molasses; ruminants; spent mushroom substrate; urea

### INTRODUCTION

The increasing demand for livestock products has placed significant pressure on the availability of traditional forage resources. As global forage supplies become limited, ruminant production systems face a critical challenge in meeting animals' nutritional requirements. This scarcity of high-quality forages is particularly problematic for sustainable livestock production, as feed constitutes a major portion of production costs (Yi *et al.*, 2023).

In response to these limitations, livestock producers and researchers have explored alternative feed resources, particularly agro-industrial by-products such as crop residues, spent grain, and food processing waste (Crisostomo *et al.*, 2022; Rinne *et al.*, 2019; Shah *et al.*, 2025). These materials are abundant and cost-effective but often require processing to enhance their palatability, digestibility, and nutritional value (Andhale *et al.*, 2024; Manlapig *et al.*, 2023).

One example is spent mushroom substrate (SMS), a by-product of mushroom farming composed of

partially degraded materials like sawdust, rice straw, and wheat bran (Kalvandi *et al.*, 2018; Palangi *et al.*, 2022). In addition to supporting waste reduction, mushroom residues like SMS play an important role in improving feed availability and reducing production costs in ruminant nutrition (Antunes *et al.*, 2020; Grimm & Wösten, 2018). SMS can be used directly or ensiled as ruminant feed (Rangubhet *et al.*, 2017) and contains high levels of fiber (NDF, ADF), residual fungal biomass, protein, enzymes, and microbial communities (Leong *et al.*, 2022; Yi *et al.*, 2023). However, SMS is produced in large volumes, and its disposal—typically through landfilling or burning—poses environmental risks such as greenhouse gas emissions and land degradation (Kousar *et al.*, 2024; Royse *et al.*, 2017). The utilization of SMS contributes to agro-industrial waste reduction and aligns with circular agricultural strategies to minimize the environmental impacts of organic waste disposal (Souza *et al.*, 2024). However, untreated SMS often has low palatability and digestibility, making it less suitable for ruminant direct consumption (Rangubhet *et al.*, 2020; Yi *et al.*, 2023).

To address these challenges, several processing methods have been explored, including ensiling (Kim *et al.*, 2014), chemical treatment with molasses and urea (Zaboli *et al.*, 2023), and the addition of microbial inoculants (Kwak *et al.*, 2009), all aimed at enhancing the nutritional profile of SMS. These treatments help improve fermentability, reduce fiber content, and increase the digestibility of SMS, making it a more sustainable feed option for livestock (Yi *et al.*, 2023).

In this study, SMS derived from four commercially significant mushroom species—Oyster (*Pleurotus ostreatus*), Enoki (*Flammulina filiformis*), Bunashimeji (*Lyophyllum shimeji*), and Shiitake (*Lentinula edodes*)—was evaluated. These species were selected due to their widespread commercial cultivation in Asia, and are typically grown on different types of agricultural materials depending on the mushroom species and local farming practices (Kim *et al.*, 2014; Royse *et al.*, 2017). These compositional differences in cultivation substrates are expected to influence the nutrient profile of SMS and may subsequently affect its fermentation characteristics and CH<sub>4</sub> production when used in ruminant diets (Kalvandi *et al.*, 2018).

Thus, this study aimed to evaluate the nutritional composition and *in vitro* digestibility of SMS supplemented with urea and molasses, including its fermentation characteristics and SCFA production. The objective was to assess the feasibility of repurposing SMS from different mushroom species as a sustainable feed resource for ruminants, while contributing to the reduction of agro-industrial waste.

## MATERIALS AND METHODS

All animal experiments were approved and conducted according to the ethical guidelines of Mie University, Japan (Approval No. 2024-38).

### Sample Preparation

SMS samples were collected from the cultivation of four mushroom species. SMS derived from *Pleurotus ostreatus* (Oyster mushroom; OYS) was obtained from mushroom growers in Nueva Ecija, Philippines, while SMS from *Flammulina filiformis* (Enoki mushroom; ENK), *Hypsizygus marmoreus* (Bunashimeji; SMJ), and *Lentinula edodes* (Shiitake mushroom; STK) were sourced from commercial mushroom farms in Japan.

The four SMS samples were collected as representative samples from mixed cultivation batches of each mushroom species. These SMS samples were air-dried until their moisture contents were reduced to below 10%, then ground to pass through a 1 mm sieve using a Wiley mill (Sansin Industrial Co., Ltd., Yokohama, Japan) to ensure uniform particle size. For the supplemented samples, a solution containing 4% urea and 18% molasses (w/w, based on the dry matter) was prepared by dissolving the additives in 1 L of distilled water per kg of SMS. The solution was applied evenly to the substrate by spraying while continuously mixing to ensure uniform distribution following the method described by Cheva-Isarakul and

Kanjanapruthipong (1987). The supplemented SMS was subsequently air-dried at ambient temperature (25–28 °C) for 48 hours to allow the volatilization of excess ammonia from the urea. After drying, all samples were stored at –80 °C until further analysis. Each SMS type was evaluated in two forms: non-supplemented and urea–molasses supplemented.

### Chemical Analysis

The SMS used in this study were derived from the cultivation of four mushroom species with distinct substrate compositions: OYS made up of rice straw and sawdust; ENK made up of corn cob, rice bran, and wheat bran; SMJ made up of corn cob, rice bran, and wheat bran; and STK made up of sawdust and wheat bran. The exact proportions of substrate ingredients were not disclosed, as the formulations were proprietary.

All samples were dried at 60 °C for 48 hours using a forced-air oven and subsequently ground to pass through a 1 mm sieve using a Wiley mill. Triplicate samples were used for all chemical analyses.

Dry matter (DM), ash, and ether extract (EE) contents were determined according to standard procedures outlined by the Association of Official Analytical Chemists (AOAC, 2000). Nitrogen content was determined using an organic elemental analyzer (Vario EL, DKSH, Elementor Japan Co., Ltd., Yokohama, Japan), and crude protein (CP) was calculated by multiplying the nitrogen content by a factor of 6.25.

Fiber components, including neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL), were determined using the method of Van Soest *et al.* (1991). Hemicellulose was calculated as the difference between NDF and ADF, cellulose as the difference between ADF and ADL, and holocellulose as the sum of hemicellulose and cellulose (Jiang *et al.*, 2014).

### In Vitro Gas Production

The *in vitro* gas production assay was conducted following the method of Manlapig *et al.* (2024b). Three separate incubation trials were performed to evaluate the fermentation characteristics of SMS, both non-supplemented (OYS, ENK, SMJ, STK) and supplemented with urea and molasses (OYS<sup>+</sup>, ENK<sup>+</sup>, SMJ<sup>+</sup>, STK<sup>+</sup>).

Rumen fluid was obtained from three adult Corridale wethers, which were maintained on a diet consisting of 60% oat hay and a 40% concentrate mixture containing soybean meal, cracked corn, wheat bran, and alfalfa pellets in a 1:1:1:0.5 ratio. The animals had *ad libitum* access to water and mineral blocks. Ruminal contents were collected orally from each donor before the morning feeding, maintained at 39 °C, and kept under anaerobic conditions until use.

The collected rumen fluid was filtered through four layers of surgical gauze and pooled in equal volumes. The pooled fluid was mixed with pre-warmed McDougall's buffer at a 1:4 ratio (rumen fluid: buffer)

while continuously flushed with nitrogen gas to maintain anaerobic conditions.

Each substrate sample (300 mg, DM basis) was placed into 50 mL serum bottles containing 15 mL of buffered rumen fluid. Each treatment was incubated in triplicate. The bottles were sealed with rubber stoppers and aluminum caps, then incubated at 39 °C in an incubator for 24, 48, and 72 hours. To determine the actual initial dry matter (DM<sub>0</sub>), an additional set of serum bottles (0h incubation) was inoculated, immediately filtered using vacuum filtration with Whatman filter paper (Whatman plc, Maidstone, UK), and dried at 60 °C for 48 hours.

### Gas Production and Fermentation Profiles

After each incubation period (24, 48, and 72 hours), gas production was measured using a glass syringe connected to a Luer-lock three-way stopcock. Samples were immediately placed on ice to halt fermentation activity. The composition of headspace gas was analyzed using a gas chromatograph (GC-8A; Shimadzu Corporation, Kyoto, Japan) equipped with a stainless-steel column (2 m × 3 mm, Shincarbon-ST; Shinwa Chemical Industries Ltd., Kyoto, Japan) and a thermal conductivity detector (TCD), following the method of Matsui *et al.* (2013). Methane production was expressed both as volume (mL) and as a percentage of total gas produced.

For SCFA analysis, 1 mL of the fermentation fluid was transferred into 1.5 mL microcentrifuge tubes and analyzed using high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan), according to the procedure described by Manlapig *et al.* (2024a). Individual SCFAs, including acetate, propionate, and butyrate, were quantified and expressed in millimoles per liter (mmol/L).

*In vitro* dry matter digestibility (DMD) was determined by centrifuging the fermentation residue at 1,000 × g for 10 minutes at 4 °C in 50 mL centrifuge tubes. The supernatant was discarded, and the residue was collected through vacuum filtration using Whatman filter paper. The undigested residue was dried at 60 °C for 48 hours and weighed. *In vitro* DMD was calculated

as the difference between the DM<sub>0</sub> and final sample weight and expressed as a percentage. Digested dry matter (DDM, g) was calculated as the difference between the DM<sub>0</sub> and the residual dry matter after incubation. CH<sub>4</sub> per gram of DDM was calculated by dividing the total CH<sub>4</sub> production (mL) by the amount of DDM (g) for each sample.

### Statistical Analysis

Statistical analysis was performed using PROC GLM in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA, 2013). Substrate (SMS) was categorized as a fixed effect, and replications (trial run) as a random effect. CONTRAST statement was used to test the effect of urea and molasses addition on fermentation profiles. All data were presented as means ± SEM (standard error of the mean). Statistical significance was declared at p<0.05. Tukey's multiple range test was used to identify differences between treatments.

## RESULTS

### Proximate Composition and Nutrient Profiles

The nutrient compositions of unsupplemented and supplemented SMS from various mushroom species are presented in Table 1. SMS from *P. ostreatus* (OYS and OYS<sup>+</sup>) contained the highest levels of DM and ash. This substrate also exhibited the lowest NDF, ADF, ADL, cellulose, and hemicellulose. *F. velutipes* (ENK and ENK<sup>+</sup>) SMS had the highest CP, EE, and hemicellulose. In contrast, *L. edodes* (STK and STK<sup>+</sup>) showed the lowest levels of DM, CP, EE, ash, and hemicellulose, but had the highest values for NDF, ADF, and cellulose. Meanwhile, SMS from *H. marmoreus* (SMJ and SMJ<sup>+</sup>) recorded the highest ADL content.

### pH Variations during Fermentation

The pH values of cultures of unsupplemented and supplemented SMS showed significant differences (p<0.0001) across supplementation status, incubation periods, and substrate types (Figure 1). OYS and OYS<sup>+</sup>

Table 1. Proximate composition of nutrients (%) of different unsupplemented and supplemented spent mushroom substrates (SMS) with urea and molasses

Variables	Unsupplemented SMS				Supplemented SMS			
	OYS	ENK	SMJ	STK	OYS <sup>+</sup>	ENK <sup>+</sup>	SMJ <sup>+</sup>	STK <sup>+</sup>
DM	92.06±0.33	89.19±0.12	89.79±0.02	88.95±0.08	76.09±0.47	69.55±0.29	74.84±0.16	73.05±0.04
CP	8.37±0.06	14.10±0.92	11.34±0.19	5.39±0.01	13.84±0.03	16.55±0.12	16.30±0.16	11.59±0.06
EE	0.73±0.16	4.98±0.13	1.04±0.03	0.70±0.02	0.96±0.12	3.49±0.04	0.82±0.16	0.77±0.08
ASH	38.81±0.22	11.79±0.08	9.76±0.05	3.88±0.01	29.93±0.24	9.10±0.02	6.46±0.04	3.90±0.04
NDF	18.11±0.21	44.96±0.35	51.40±0.17	56.77±0.26	12.46±0.18	30.78±0.35	38.42±0.17	38.76±0.25
ADF	19.44±0.35	26.21±0.45	38.07±0.52	50.68±0.91	13.90±0.23	17.72±0.25	28.04±0.24	31.36±0.64
ADL	3.36±0.15	5.79±0.10	10.88±0.10	9.25±0.33	2.39±0.04	4.05±0.07	7.81±0.10	4.82±0.13
Hemicellulose	-	18.76±0.79	13.33±0.36	6.08±1.10	-	13.06±0.40	10.38±0.23	7.40±0.88
Cellulose	16.07±0.24	20.42±0.36	27.19±0.46	41.43±0.66	11.51±0.20	13.68±0.18	20.23±0.17	26.53±0.55
Holocellulose	16.07±0.24	39.17±0.44	40.52±0.10	47.52±0.45	11.51±0.20	26.73±0.33	30.61±0.09	33.93±0.34

Note: Unsupplemented (OYS-*Pleurotus ostreatus*, ENK-*Flammulina velutipes*, SMJ-*Hypsizygus marmoreus*, STK-*Lentinula edodes*); Supplemented with urea and molasses (OYS<sup>+</sup>-*Pleurotus ostreatus*, ENK<sup>+</sup>-*Flammulina velutipes*, SMJ<sup>+</sup>-*Hypsizygus marmoreus*, STK<sup>+</sup>-*Lentinula edodes*), DM-dry matter; CP-crude protein; EE-ether extract; NDF-neutral detergent fiber; ADF-acid detergent fiber; ADL-acid detergent lignin

exhibited significantly higher pH values than the other substrates ( $p < 0.05$ ). Among non-supplemented groups, ENK, SMJ, and STK were comparable, while within supplemented groups, ENK<sup>+</sup> and SMJ<sup>+</sup> were similar, and SMJ<sup>+</sup> was also comparable to STK<sup>+</sup>. Additionally, supplemented SMS has a significantly higher pH compared to unsupplemented SMS ( $p < 0.0001$ ). Across incubation periods, there was a clear and significant decreasing trend in pH, with the highest values observed at 0 hours, followed by 24, 48, and the lowest at 72 hours ( $p < 0.0001$ ).

### Gas Production and Dry Matter Digestibility

The *in vitro* gas production, DMD, and CH<sub>4</sub> production from unsupplemented and supplemented SMS continuously increased as the incubation period progressed (Table 2). Gas production was consistently lowest in OYS across all incubation periods, while it was highest in STK<sup>+</sup> after 24 hours and in STK after 48 and 72 hours ( $p < 0.0001$ ). After 24 hours of incubation, gas production in STK<sup>+</sup> and SMJ<sup>+</sup> was significantly higher than in OYS and OYS<sup>+</sup> ( $p < 0.0001$ ). Similarly, ENK,

Table 2. *In vitro* gas, DMD, and CH<sub>4</sub> production of unsupplemented and supplemented spent mushroom substrates (SMS) across different mushroom species

Variables	Unsupplemented SMS				Supplemented SMS				SEM	p-value
	OYS	ENK	SMJ	STK	OYS <sup>+</sup>	ENK <sup>+</sup>	SMJ <sup>+</sup>	STK <sup>+</sup>		
<b>24h</b>										
Gas, mL	13.33±1.33 <sup>c</sup>	21.11±1.11 <sup>ab</sup>	21.78±0.59 <sup>ab</sup>	21.78±1.18 <sup>ab</sup>	16.67±1.15 <sup>bc</sup>	21.56±0.97 <sup>ab</sup>	23.33±1.33 <sup>a</sup>	24.67±1.33 <sup>a</sup>	0.80	***
CH <sub>4</sub> , %	3.73±0.53 <sup>b</sup>	9.28±0.59 <sup>a</sup>	9.41±0.77 <sup>a</sup>	9.24±0.74 <sup>a</sup>	8.12±0.82 <sup>a</sup>	10.33±0.96 <sup>a</sup>	11.33±0.54 <sup>a</sup>	10.41±0.70 <sup>a</sup>	0.50	***
CH <sub>4</sub> , mL	0.48±0.04 <sup>c</sup>	1.95±0.08 <sup>ab</sup>	2.05±0.16 <sup>ab</sup>	2.00±0.13 <sup>ab</sup>	1.35±0.13 <sup>b</sup>	2.22±0.19 <sup>a</sup>	2.65±0.22 <sup>a</sup>	2.56±0.13 <sup>a</sup>	0.14	***
DMD, %	4.13±0.85 <sup>b</sup>	5.62±0.54 <sup>ab</sup>	5.51±1.30 <sup>ab</sup>	7.26±0.58 <sup>ab</sup>	6.58±1.24 <sup>ab</sup>	6.82±0.35 <sup>ab</sup>	7.05±1.44 <sup>ab</sup>	10.52±1.87 <sup>a</sup>	0.49	*
CH <sub>4</sub> /DDM, mL/g	54.45±17.21 <sup>b</sup>	142.13±7.59 <sup>ab</sup>	161.08±23.73 <sup>ab</sup>	110.38±14.10 <sup>ab</sup>	120.94±43.97 <sup>ab</sup>	169.18±8.99 <sup>ab</sup>	235.39±50.37 <sup>a</sup>	142.13±23.86 <sup>ab</sup>	13.06	*
<b>48h</b>										
Gas, mL	15.11±0.80 <sup>d</sup>	33.11±0.44 <sup>b</sup>	34.67±1.39 <sup>b</sup>	40.22±0.97 <sup>a</sup>	17.78±1.18 <sup>d</sup>	25.11±1.11 <sup>c</sup>	31.56±1.24 <sup>b</sup>	36.67±1.39 <sup>ab</sup>	1.80	***
CH <sub>4</sub> , %	5.62±0.68 <sup>c</sup>	13.89±0.74 <sup>a</sup>	13.98±0.53 <sup>a</sup>	13.38±0.72 <sup>a</sup>	9.38±0.51 <sup>b</sup>	12.97±0.55 <sup>a</sup>	14.30±0.45 <sup>a</sup>	13.60±0.50 <sup>a</sup>	0.62	***
CH <sub>4</sub> , mL	0.85±0.09 <sup>c</sup>	4.60±0.20 <sup>ab</sup>	4.86±0.36 <sup>a</sup>	5.40±0.42 <sup>a</sup>	1.66±0.08 <sup>c</sup>	3.27±0.28 <sup>b</sup>	4.53±0.32 <sup>ab</sup>	5.00±0.37 <sup>a</sup>	0.34	***
DMD, %	7.39±1.93 <sup>d</sup>	19.82±1.72 <sup>b</sup>	21.21±1.00 <sup>b</sup>	30.55±1.30 <sup>a</sup>	8.95±2.34 <sup>d</sup>	11.65±1.77 <sup>cd</sup>	19.20±1.10 <sup>bc</sup>	30.40±0.69 <sup>a</sup>	1.80	***
CH <sub>4</sub> /DDM, mL/g	57.84±24.59 <sup>b</sup>	94.63±10.92 <sup>ab</sup>	92.49±7.16 <sup>ab</sup>	68.20±3.46 <sup>ab</sup>	108.11±36.38 <sup>ab</sup>	153.53±13.43 <sup>a</sup>	131.49±11.38 <sup>ab</sup>	90.52±5.76 <sup>ab</sup>	8.01	*
<b>72h</b>										
Gas, mL	16.67±0.67 <sup>e</sup>	43.11±1.60 <sup>bc</sup>	42.89±0.59 <sup>bc</sup>	48.44±0.44 <sup>a</sup>	20.00±1.02 <sup>e</sup>	30.67±0.77 <sup>d</sup>	38.89±1.24 <sup>c</sup>	43.78±0.59 <sup>b</sup>	2.32	***
CH <sub>4</sub> , %	6.61±0.49 <sup>c</sup>	16.56±0.91 <sup>a</sup>	16.41±0.45 <sup>a</sup>	15.38±0.67 <sup>a</sup>	10.01±0.62 <sup>b</sup>	14.72±0.01 <sup>a</sup>	15.95±0.47 <sup>a</sup>	15.08±0.51 <sup>a</sup>	0.72	***
CH <sub>4</sub> , mL	1.10±0.05 <sup>c</sup>	7.17±0.65 <sup>a</sup>	7.04±0.27 <sup>a</sup>	7.46±0.40 <sup>a</sup>	2.00±0.16 <sup>c</sup>	4.52±0.11 <sup>b</sup>	6.22±0.39 <sup>a</sup>	6.61±0.31 <sup>a</sup>	0.49	***
DMD, %	8.24±2.19 <sup>c</sup>	30.79±2.83 <sup>a</sup>	29.97±2.04 <sup>ab</sup>	37.32±0.27 <sup>a</sup>	10.64±2.31 <sup>c</sup>	21.33±1.93 <sup>b</sup>	30.18±0.63 <sup>ab</sup>	36.86±1.28 <sup>a</sup>	2.25	***
CH <sub>4</sub> /DDM, mL/g	64.77±24.34	93.31±2.90	94.30±3.88	77.35±5.06	109.20±39.42	111.13±4.63	117.02±11.58	99.62±8.31	6.18	NS

Note: <sup>a,b,c,d,e</sup>Different superscript letters within the column indicate a significant difference. \*Significant at  $p < 0.05$ . \*\*\*Significant at  $p < 0.0001$ . NS-not significant. Unsupplemented (OYS-*Pleurotus ostreatus*, ENK-*Flammulina velutipes*, SMJ-*Hypsizygyus marmoreus*, STK-*Lentinula edodes*); Supplemented with urea and molasses (OYS<sup>+</sup>-*Pleurotus ostreatus*, ENK<sup>+</sup>-*Flammulina velutipes*, SMJ<sup>+</sup>-*Hypsizygyus marmoreus*, STK<sup>+</sup>-*Lentinula edodes*); CH<sub>4</sub>-methane; DMD-dry matter digestibility; CH<sub>4</sub>/DDM-methane per digested dry matter.

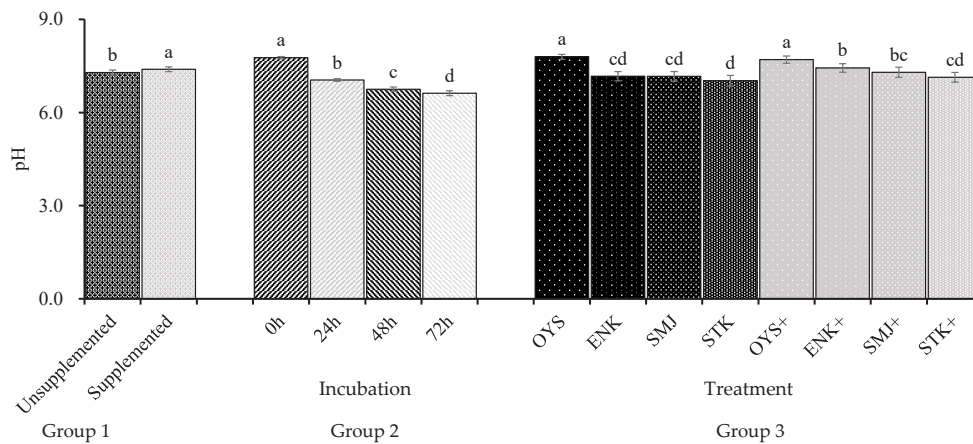


Figure 1. pH level in unsupplemented and supplemented spent mushroom substrates (SMS) from the cultivation of different mushroom species across incubation periods. <sup>a,b,c,d</sup> Different superscript letters within the same group indicate a significant difference ( $p < 0.0001$ ). Group 1 – Comparison of pH between unsupplemented and supplemented SMS; Group 2 – Overall SMS pH after 0, 24, 48, and 72 hours of incubation; Group 3 – Comparison of overall SMS pH among different mushroom types: OYS, ENK, SMJ, STK, OYS<sup>+</sup>, ENK<sup>+</sup>, SMJ<sup>+</sup>, and STK<sup>+</sup>. Unsupplemented (OYS-*Pleurotus ostreatus*, ENK-*Flammulina velutipes*, SMJ-*Hypsizygyus marmoreus*, STK-*Lentinula edodes*); Supplemented with urea and molasses (OYS<sup>+</sup>-*Pleurotus ostreatus*, ENK<sup>+</sup>-*Flammulina velutipes*, SMJ<sup>+</sup>-*Hypsizygyus marmoreus*, STK<sup>+</sup>-*Lentinula edodes*).

ENK<sup>+</sup>, and SMJ had significantly higher gas production and concentration than OYS (p<0.0001). After 48 and 72 hours, gas production and concentration in ENK, ENK<sup>+</sup>, SMJ, and SMJ<sup>+</sup> were significantly higher compared to OYS and OYS<sup>+</sup> at both incubation periods (p<0.0001).

After 24 hours, CH<sub>4</sub> production (both % and mL) was significantly lower in OYS compared to ENK, ENK<sup>+</sup>, SMJ, SMJ<sup>+</sup>, STK, and STK<sup>+</sup>, with SMJ<sup>+</sup> producing the highest CH<sub>4</sub> values (p<0.0001). After 48 and 72 hours, CH<sub>4</sub> production and concentration were significantly higher in ENK, SMJ, STK, and their supplemented forms (ENK<sup>+</sup>, SMJ<sup>+</sup>, STK<sup>+</sup>) compared to OYS and OYS<sup>+</sup> (p<0.0001).

DMD increased over time, with STK<sup>+</sup> highest after 24 hours (p<0.05). After 48 and 72 hours, DMD were significantly higher in ENK, SMJ, SMJ<sup>+</sup>, STK, and STK<sup>+</sup> compared to OYS and OYS<sup>+</sup> (p<0.0001). CH<sub>4</sub>/DDM were significantly higher in SMJ<sup>+</sup> than OYS after 24 hours and in ENK<sup>+</sup> than OYS after 48 hours (p<0.05), while no significant differences were observed after 72 hours.

**SCFA Production and Fermentation**

The production of SCFA from unsupplemented and supplemented SMS during *in vitro* fermentation progressively increased with the incubation period (Table 3). After 24 hours, total SCFA concentrations were significantly higher in ENK, SMJ, STK, and their supplemented forms (ENK<sup>+</sup>, SMJ<sup>+</sup>, STK<sup>+</sup>) compared to OYS (p<0.001), with ENK<sup>+</sup> recording the highest value. After 48 hours, STK and STK<sup>+</sup> exhibited the highest SCFA production, while OYS had the lowest (p<0.0001). After 72 hours, STK again produced the highest total SCFA production, whereas both OYS and OYS<sup>+</sup> had the lowest values (p<0.0001).

Acetate production followed a similar trend. After 24 hours, acetate levels in ENK, ENK<sup>+</sup>, SMJ, and SMJ<sup>+</sup> were significantly higher than in OYS (p<0.001). After 48 and 72 hours, STK recorded the highest acetate

concentrations (p<0.0001), with ENK, ENK<sup>+</sup>, and SMJ remaining significantly higher than OYS and OYS<sup>+</sup>.

Propionate production was significantly higher in ENK, SMJ, STK, and their supplemented forms (ENK<sup>+</sup>, SMJ<sup>+</sup>, STK<sup>+</sup>) compared to OYS after 24 hours (p<0.001). After 48 and 72 hours, STK and STK<sup>+</sup> produced the highest propionate concentrations, while ENK, ENK<sup>+</sup>, SMJ, and SMJ<sup>+</sup> remained significantly higher than OYS and OYS<sup>+</sup> (p<0.001).

Butyrate production after 24 and 48 hours followed a consistent trend, ENK<sup>+</sup>, SMJ, SMJ<sup>+</sup>, STK, and STK<sup>+</sup> produced significantly higher butyrate than OYS and OYS<sup>+</sup> (p<0.001), with ENK<sup>+</sup> showing the highest concentrations during both incubation periods. After 72 hours, butyrate concentrations remained significantly higher in ENK, SMJ, STK, and their supplemented forms (ENK<sup>+</sup>, SMJ<sup>+</sup>, STK<sup>+</sup>) compared to OYS and OYS<sup>+</sup> (p<0.0001).

***In Vitro* Fermentation of Supplemented SMS after 72 Hours**

The *in vitro* gas and CH<sub>4</sub> production, DMD, and SCFA profiles between unsupplemented and supplemented SMS after 72 hours of incubation are presented in Table 4. Gas production, CH<sub>4</sub> percentage and volume, DMD, total SCFA, as well as the concentrations of acetate, propionate, and butyrate, were comparable between unsupplemented and supplemented substrates, with no significant differences observed. However, CH<sub>4</sub>/DDM was significantly higher in supplemented SMS than in unsupplemented SMS (p<0.05).

**DISCUSSION**

**Proximate Composition and Nutrient Profile of SMS**

SMS is increasingly being explored as a sustainable alternative feed ingredient for ruminants, considering global feed shortage and the need to utilize agro-

Table 3. SCFA profiles of unsupplemented and supplemented spent mushroom substrates (SMS) from different mushroom species during *in vitro* fermentation

Variables	Unsupplemented SMS				Supplemented SMS				SEM	p-value
	OYS	ENK	SMJ	STK	OYS <sup>+</sup>	ENK <sup>+</sup>	SMJ <sup>+</sup>	STK <sup>+</sup>		
<b>24h</b>										
Total SCFA, mmol/L	17.48±0.84 <sup>c</sup>	34.60±2.34 <sup>ab</sup>	35.37±2.68 <sup>ab</sup>	30.10±1.55 <sup>b</sup>	27.77±1.98 <sup>bc</sup>	40.82±2.86 <sup>a</sup>	36.67±2.82 <sup>ab</sup>	30.10±1.55 <sup>b</sup>	1.53	***
Acetate, mmol/L	12.98±0.76 <sup>b</sup>	22.96±1.73 <sup>a</sup>	23.08±2.50 <sup>a</sup>	20.64±1.74 <sup>ab</sup>	19.23±1.81 <sup>ab</sup>	26.41±2.52 <sup>a</sup>	23.29±2.43 <sup>a</sup>	20.64±1.74 <sup>ab</sup>	1.28	**
Propionate, mmol/L	2.69±0.06 <sup>c</sup>	7.95±0.55 <sup>a</sup>	8.87±0.20 <sup>a</sup>	6.07±0.20 <sup>b</sup>	5.60±0.11 <sup>b</sup>	9.95±0.47 <sup>a</sup>	9.14±0.37 <sup>a</sup>	6.07±0.20 <sup>b</sup>	0.55	***
Butyrate, mmol/L	1.81±0.03 <sup>d</sup>	3.07±0.07 <sup>bc</sup>	3.41±0.03 <sup>b</sup>	3.39±0.01 <sup>b</sup>	2.94±0.07 <sup>c</sup>	4.46±0.13 <sup>a</sup>	4.24±0.02 <sup>a</sup>	3.39±0.01 <sup>b</sup>	0.21	***
<b>48h</b>										
Total SCFA, mmol/L	20.22±1.36 <sup>d</sup>	57.54±2.50 <sup>ab</sup>	58.19±4.24 <sup>ab</sup>	66.44±3.76 <sup>a</sup>	30.58±1.38 <sup>cd</sup>	59.61±3.66 <sup>ab</sup>	45.29±2.99 <sup>bc</sup>	66.44±3.76 <sup>a</sup>	3.46	***
Acetate, mmol/L	14.80±1.11 <sup>c</sup>	38.44±2.42 <sup>a</sup>	38.67±2.92 <sup>a</sup>	40.73±3.11 <sup>a</sup>	21.12±1.41 <sup>bc</sup>	38.27±2.97 <sup>a</sup>	29.03±2.50 <sup>ab</sup>	40.73±3.11 <sup>a</sup>	2.44	***
Propionate, mmol/L	3.32±0.15 <sup>e</sup>	14.46±0.06 <sup>b</sup>	14.84±1.08 <sup>b</sup>	20.39±0.51 <sup>a</sup>	6.40±0.05 <sup>d</sup>	15.63±0.77 <sup>b</sup>	11.37±0.53 <sup>c</sup>	20.39±0.51 <sup>a</sup>	1.30	***
Butyrate, mmol/L	2.10±0.10 <sup>e</sup>	4.64±0.14 <sup>c</sup>	4.68±0.24 <sup>bc</sup>	5.32±0.14 <sup>ab</sup>	3.06±0.09 <sup>d</sup>	5.71±0.08 <sup>a</sup>	4.89±0.04 <sup>bc</sup>	5.32±0.14 <sup>ab</sup>	0.30	***
<b>72h</b>										
Total SCFA, mmol/L	21.76±1.06 <sup>e</sup>	76.02±2.41 <sup>ab</sup>	76.37±1.86 <sup>ab</sup>	83.74±0.80 <sup>a</sup>	31.44±2.11 <sup>d</sup>	69.02±2.24 <sup>b</sup>	53.23±0.46 <sup>c</sup>	73.16±2.48 <sup>b</sup>	4.49	***
Acetate, mmol/L	16.01±0.88 <sup>c</sup>	51.13±2.74 <sup>a</sup>	51.28±1.27 <sup>a</sup>	51.63±1.64 <sup>a</sup>	21.90±1.80 <sup>c</sup>	45.08±2.33 <sup>a</sup>	34.94±0.62 <sup>b</sup>	44.50±2.55 <sup>a</sup>	3.16	***
Propionate, mmol/L	3.55±0.10 <sup>f</sup>	18.91±0.48 <sup>c</sup>	18.95±0.40 <sup>c</sup>	25.26±0.80 <sup>a</sup>	6.57±0.18 <sup>e</sup>	17.65±0.06 <sup>c</sup>	12.93±0.01 <sup>d</sup>	22.09±0.19 <sup>b</sup>	1.58	***
Butyrate, mmol/L	2.21±0.08 <sup>d</sup>	5.99±0.14 <sup>bc</sup>	6.14±0.19 <sup>abc</sup>	6.85±0.04 <sup>a</sup>	2.98±0.13 <sup>d</sup>	6.29±0.16 <sup>ab</sup>	5.36±0.16 <sup>c</sup>	6.57±0.26 <sup>ab</sup>	0.40	***

Note: <sup>a,b,c,d,e,f</sup>Different superscript letters within the column indicate a significant difference. \*\*Significant at p<0.01. \*\*\*Significant at p<0.0001. Unsupplemented (OYS-*Pleurotus ostreatus*, ENK-*Flammulina velutipes*, SMJ-*Hypsizygyus marmoreus*, STK-*Lentinula edodes*); Supplemented with urea and molasses (OYS<sup>+</sup>-*Pleurotus ostreatus*, ENK<sup>+</sup>-*Flammulina velutipes*, SMJ<sup>+</sup>-*Hypsizygyus marmoreus*, STK<sup>+</sup>-*Lentinula edodes*). SCFA-short chain fatty acid.

Table 4. Comparison of fermentation characteristics between unsupplemented and supplemented spent mushroom substrates (SMS) after 72-hour incubation

Variables	Unsupplemented SMS	Supplemented SMS	SEM
Gas, mL	37.78±3.76	33.33±2.75	2.32
CH <sub>4</sub> , %	13.74±1.28	13.94±0.73	0.72
CH <sub>4</sub> , mL	5.69±0.82	4.84±0.56	0.49
DMD, %	26.58±3.42	24.75±3.05	2.25
CH <sub>4</sub> /DDM, mL/g	82.43±6.54 <sup>b</sup>	109.24±9.19 <sup>a</sup>	6.18
Total SCFA, mmol/L	64.47±7.53	56.71±5.01	2.77
Acetate, mmol/L	42.51±4.68	36.60±2.96	1.47
Propionate, mmol/L	16.67±2.42	14.81±1.74	0.34
Butyrate, mmol/L	5.30±0.55	5.30±0.43	4.49

Note: <sup>a,b</sup>Different superscript letters within the column indicate a significant difference ( $p < 0.05$ ). Supplemented with urea and molasses; CH<sub>4</sub>-methane; DMD-dry matter digestibility; CH<sub>4</sub>/DDM-methane per digested dry matter; SCFA-short chain fatty acid.

industrial by-products more efficiently. Like other crop residues and agro-industrial by-products, it is typically high in NDF and ADF, contributing to its low digestibility (Kwak *et al.*, 2009; Rangubhet *et al.*, 2020). Reducing fiber content is essential to enhance nutrient availability and digestibility, both critical for improving ruminant productivity. Supplementation with urea has been shown to facilitate the breakdown of fiber fractions in fibrous residues, while molasses is commonly added to enhance palatability and increase the water-soluble carbohydrate content of the feed (Akinfemi *et al.*, 2018; Zaboli *et al.*, 2023).

In the present study, NDF, ADF, and ADL were reduced, while CP substantially increased following urea and molasses supplementation. The reduction in fiber fractions may be attributed to the solubilization of hemicellulose, which also decreased post-treatment. This observation is consistent with Sundstol and Coxworth (1984), who reported decreased NDF content in urea-treated straw due to hemicellulose solubilization. Chen *et al.* (2025) likewise observed that alkali-treated *Pleurotus eryngii* SMS supplemented with condensed molasses solubles improved fiber digestibility, increased CP levels, and enhanced microbial protein synthesis *in vitro*. Ramirez *et al.* (2007) also reported reductions in NDF content in urea-treated corn residues and grass hays. In general, doses of 4% to 6% urea in DM are effective in reducing NDF in rice straw, wheat straw, and corn residues (Akinfemi *et al.*, 2020; Sallam *et al.*, 2024). The increase in CP observed in this study aligns with results from Ramirez *et al.* (2007), who reported similar improvements in hay and corn residues supplemented with 4% urea. Comparable results were also observed by Akinfemi *et al.* (2018) and Aruwayo (2018), highlighting the efficacy of adding urea in enhancing CP content in corn cobs and rice straw, thereby improving their potential as ruminant feed resources.

The variation in nutrient composition among SMS types can be attributed to differences in their initial substrate composition. ENK, SMJ, and STK all contained

wheat bran; however, the exact proportion included in each formulation was not specified. Wheat bran is known for its relatively high CP and fermentable carbohydrate content, which likely contributed to the elevated CP levels and enhanced fermentability observed in these SMS types (Getachew *et al.*, 2002; Wang *et al.*, 2022). In contrast, OYS, composed primarily of rice straw and sawdust, exhibited higher levels of structural fiber and lower nutrient density due to the high lignin and silica content typical of such lignocellulosic materials (Ma *et al.*, 2020). These compositional differences emphasize the influence of initial substrate composition on the nutritional profile and fermentability of SMS, even prior to urea-molasses supplementation.

The observed reduction in DM and EE in the supplemented SMS may be attributed to a dilution effect from the addition of urea and molasses. Goyal *et al.* (2023) similarly reported reduced EE concentrations in rice straw treated with these additives. Molasses, being primarily composed of sugars and water, has a low EE content and thus reduces the overall nutrient density of the substrate (Mordenti *et al.*, 2021). The decline in DM content is also attributable to the high moisture content of molasses (approximately 25%) (Mordenti *et al.*, 2021). This pattern is consistent with findings by Zaboli *et al.* (2023), who observed reductions in DM when molasses was added to spent mushroom compost.

#### pH Variations during Fermentation

Ruminal pH plays a critical role in determining nutrient availability and microbial fermentation efficiency (Faniyi *et al.*, 2019). In this study, pH varied significantly across substrates, supplementation, and incubation periods, reflecting fermentation dynamics. Supplemented substrates consistently exhibited higher pH values, likely due to the buffering effect of urea, as also reported in an *in vitro* fermentation trial by Shen *et al.* (2023). Wahyono *et al.* (2022) and Zheng *et al.* (2024) similarly reported that urea, due to its alkaline nature, increased rumen pH and improved fermentation efficiency in fattening sheep. The highest pH level observed after 0 hours of incubation can be attributed to the neutral conditions at the start of the fermentation process, where minimal metabolic activity has occurred. As fermentation progresses, pH gradually decreases after 24, 48, and 72 hours due to an active fermentation phase. Similar studies have shown that extended incubation leads to significant pH declines due to ongoing substrate metabolism (Zheng *et al.*, 2024). This decline corresponds with increasing SCFA production, a by-product of microbial fermentation, which contributes to the acidification of the medium. Among the substrates, OYS maintained the highest pH, indicating a slower acidification process compared to the others. This may be attributed to its base composition of rice straw and sawdust, which are high in lignocellulosic fiber and low in readily fermentable carbohydrates. These components are less accessible to microbial degradation, resulting in slower fermentation and reduced SCFA production. In contrast, substrates such

as ENK, SMJ, and STK, which contain more fermentable materials like corn cob, rice bran, and wheat bran, supported more rapid microbial fermentation and acid accumulation. These differences align with previous findings that the compositional difference and fiber structure of agro-industrial residues significantly influence fermentation dynamics and acidification rates (Kwak *et al.*, 2009; Ridla *et al.*, 2025).

### Gas Production and Dry Matter Digestibility

The fermentation profile in the rumen is a key indicator of nutrient availability, as it directly influences total gas and CH<sub>4</sub> production, as well as digestibility (Etelea *et al.*, 2018). The *in vitro* gas production technique remains a widely applied and cost-effective approach for assessing feed fermentability and digestibility, particularly in the evaluation of agro-industrial by-products and crop residues (Bashar *et al.*, 2024; Parchami *et al.*, 2024).

In this study, gas production was consistently lowest in OYS and OYS<sup>+</sup>, likely due to the high proportion of rice straw and sawdust in their composition. These materials are known for their high lignin, ash, and silica content, which reduces fermentation activity by rumen microorganisms and subsequently lowers gas production (Ma *et al.*, 2020; Oladosu *et al.*, 2016). In contrast, STK and STK<sup>+</sup> exhibited the highest gas production, possibly due to the inclusion of wheat bran, a highly fermentable substrate that enhances microbial activity and gas production (Bashar *et al.*, 2024). Although ENK, SMJ, and their supplemented forms also contained wheat bran, differences in fermentation response may be attributed to the initial substrate composition. The combination of sawdust and wheat bran in STK may have provided a more favorable fermentation environment than the corn cob-based substrates in ENK and SMJ.

CH<sub>4</sub> production, both in volume and percentage, followed a similar trend. OYS and OYS<sup>+</sup> showed the lowest CH<sub>4</sub> values across incubation periods, likely due to their poor fermentability and higher ash content, which limits microbial activity. Substrates with higher gas production, such as STK, ENK, SMJ, and their supplemented forms, also yielded higher CH<sub>4</sub> production, consistent with the positive correlation between fermentable carbohydrate content and CH<sub>4</sub> production (Kim *et al.*, 2013; Li *et al.*, 2021). This may be attributed to the higher concentrations of OM and fermentable carbohydrates in these SMS types (Anotaenwere *et al.*, 2025).

DMD was lowest in OYS and OYS<sup>+</sup>, reflecting their high levels of indigestible fiber and ash, which reduce microbial access to nutrients (Aquino *et al.*, 2020; Mandey *et al.*, 2017). In contrast, STK, ENK, SMJ, and their supplemented forms exhibited higher DMD, likely due to the inclusion of wheat bran, which facilitates fiber degradation and improves nutrient availability (Akinfemi *et al.*, 2018; Kim *et al.*, 2013). These digestibility trends were consistent with gas production patterns, as gas volume is often positively correlated with digestibility *in vitro* (Widodo *et al.*, 2023).

CH<sub>4</sub>/DDM is a useful indicator of methane yield relative to nutrient utilization. In this study, OYS and OYS<sup>+</sup> showed the lowest CH<sub>4</sub>/DDM values, reflecting their lower fermentability and digestibility, while SMJ and SMJ<sup>+</sup> exhibited the highest values. This pattern supports the interpretation that higher digestibility can be associated with increased CH<sub>4</sub> output—a known trade-off in ruminant nutrition (Løvendahl *et al.*, 2018).

After 72 hours of incubation, CH<sub>4</sub> production and DMD were generally similar between supplemented and non-supplemented groups; however, CH<sub>4</sub>/DDM tended to be higher in supplemented SMS. This may reflect enhanced fiber degradation and microbial activity due to urea–molasses treatment, although differences in base substrate composition likely also contributed to variation. These results are consistent with previous studies showing that improved digestibility from nitrogen and energy supplementation can elevate CH<sub>4</sub> yield per unit of a digested substrate (Akinfemi *et al.*, 2020; Zaboli *et al.*, 2023).

### SCFA Production and Fermentation

SCFAs are crucial energy sources in ruminants, serving as the primary end-products of microbial fermentation in the rumen and directly influencing animal productivity (Amirault *et al.*, 2024). Produced through carbohydrate fermentation, SCFAs—particularly acetate, propionate, and butyrate—account for about 70% of the metabolic energy required by ruminants (Reddy & Hyder, 2023). In this study, SMS containing wheat bran supported higher SCFA production, likely due to the availability of fermentable carbohydrates that stimulate microbial activity. The increased SCFA production observed in wheat bran-containing SMS was consistent across individual SCFA components, including acetate, propionate, and butyrate. This observation aligns with the findings of Wang *et al.* (2022), who reported that the inclusion of wheat bran enhances rumen microbial growth and increases total SCFA concentration.

Conversely, OYS and OYS<sup>+</sup>, composed of rice straw and sawdust, produced the lowest levels of both total SCFA and individual SCFA components (acetate, propionate, and butyrate). This is likely due to its high ash content, which restricts microbial degradation and thus limits fermentation efficiency (Aquino *et al.*, 2020; Ma *et al.*, 2020). Studies indicate that substrates with high ash content, such as rice straw, tend to yield lower SCFAs due to reduced microbial accessibility and slower fermentation rates (Oladosu *et al.*, 2016). The SCFA values observed in this study align with the trend in gas volume, reflecting a close association between SCFA and gas production during fermentation, as previously noted by Akinfemi *et al.* (2020) and Getachew *et al.* (2002). Gas production results from the fermentation of carbohydrates into SCFAs, including acetate, propionate, and butyrate, making it a reliable indicator of substrate fermentation and an estimate of potential digestibility in the rumen (Getachew *et al.*, 2002; Muqier *et al.*, 2023).

In this study, the addition of urea and molasses did not significantly influence SCFA production. This

contrasts with previous findings by Akinfemi *et al.* (2020) on rice straw, Akinfemi *et al.* (2018) on corn cob, and Zaboli *et al.* (2023) on spent mushroom compost, where SCFA concentrations increased following urea-molasses supplementation. This difference may be attributed to variations in substrate composition and microbial fermentation dynamics across studies.

## CONCLUSION

Supplementation with urea and molasses increased crude protein content and reduced fiber fractions; however, effects on digestibility and fermentation characteristics, including SCFA production, varied among substrates. These variations were primarily attributed to differences in the initial substrate composition of the SMS. The findings demonstrate the potential of SMS as a nutritionally viable feed resource for ruminants and highlight its relevance in sustainable livestock production. Moreover, the utilization of SMS contributes to agro-industrial waste reduction and aligns with circular agricultural strategies aimed at minimizing the environmental impacts of organic waste disposal.

## CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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