



In vitro Degradability and Rumen Microbial Dynamics in Goats Supplemented with *Pleurotus* Spent Mushroom Substrate at Different Inclusion Levels

Angelica Jane M. Lluvido¹, Manuel D. Gacutan, Jr.¹, Warren D. Come¹, and Roy C. Limpangog^{1*}

¹ Faculty of Agriculture and Food Science, Visayas State University, Baybay City, Leyte, Philippines

* Correspondence: roy.limpangog@vsu.edu.ph

Citation:

Lluvido, A. J.; Gacutan, Jr., M.; Come, W.; Limpangog, R. *In vitro* degradability and rumen microbial dynamics in goats supplemented with *Pleurotus* spent mushroom substrate at different inclusion levels. *ASEAN J. Sci. Tech. Report.* **2026**, 29(6), e261393. <https://doi.org/10.55164/ajstr.v29i6.261393>

Article history:

Received: September 19, 2025

Revised: March 19, 2026

Accepted: April 11, 2026

Available online: May 10, 2026

Publisher's Note:

This article is published and distributed under the terms of the Thaksin University.

Abstract: This study evaluated the effects of *Pleurotus* spent mushroom substrate (PSMS) supplementation on *in vitro* degradability and rumen microbial dynamics in goats (*Capra hircus*). It aimed to determine the degradability of PSMS and its influence on gas production, nutrient degradability, and microbial composition. A Randomized Complete Block Design was employed with treatments: T0 – 0% PSMS (control), T1 – 3% PSMS, and T2 – 6% PSMS, all mixed with Napier grass. Parameters measured included *in vitro* gas production (IVGP), dry matter degradability (IVDMD), crude protein degradability (IVCPD), neutral detergent fiber degradability (IVNDFD), and acid detergent fiber degradability (IVADFD). Data were analyzed using Analysis of Variance (ANOVA) in SAS OnDemand, with differences determined using Tukey's Honestly Significant Difference (HSD) test at $\alpha = 0.05$. Microbial analysis showed the highest bacterial and protozoal populations in T1, whereas fungal populations were not detected in PSMS-supplemented treatments. This observation may be associated with the presence of phenolic compounds in PSMS, which originate from lignin degradation during mushroom cultivation and have been reported to exert antimicrobial or inhibitory effects on rumen microorganisms. Methodological factors, such as sampling rumen fluid and reliance on culture-based detection, may also have influenced fungal recovery. IVGP was numerically highest in T1 (52.39 cc), indicating enhanced fermentability. During the first digestion stage, the highest IVDMD occurred in T2 (54.85%), while IVCPD peaked in T1 (60.29%) but declined in T2 (37.60%), suggesting possible inhibitory effects at higher inclusion levels. IVNDFD and IVADFD were also higher in PSMS treatments, with IVADFD showing a significant linear increase ($p=0.0294$). In the second stage simulating abomasal digestion, DM degradability remained similar across treatments, while ADF degradability declined significantly in T2 ($p=0.0035$). Overall, 3% PSMS improved fermentability, digestibility, and microbial activity, indicating its potential as a sustainable ruminant feed supplement.

Keywords: *in vitro* degradability; *in vitro* gas production; *pleurotus* spent mushroom substrates; rumen microbial analysis

1. Introduction

Spent mushroom substrates (SMS) are the residual materials remaining after the harvest of edible mushroom fruiting bodies. These substrates are commonly composed of lignocellulosic agricultural by-products such as rice

straw, corn cobs, cottonseed hulls, bagasse, and other fibrous materials used as growth media during mushroom cultivation [1]. After cultivation, SMS retains appreciable amounts of nutrients, including crude protein, amino acids, minerals, partially degraded lignocellulosic components, and residual fungal biomass [1, 2]. Despite their potential value, large quantities of SMS are often incinerated or improperly disposed of, contributing to environmental problems such as pollution and eutrophication [2, 3, 4]. Therefore, the valorization of SMS as livestock feed has gained increasing attention as a sustainable strategy to recycle agricultural residues while improving feed resource availability.

Among edible mushrooms, species of the genus *Pleurotus* are widely cultivated due to their strong lignocellulose-degrading capacity. These fungi produce extracellular enzymes such as laccases, manganese peroxidases, and cellulolytic enzymes that break down lignin, cellulose, and hemicellulose in the substrate during cultivation [5]. As a result, *Pleurotus* spent mushroom substrate (PSMS) often contains partially degraded fiber fractions that may enhance microbial accessibility and digestibility when used as ruminant feed. Previous studies have reported that SMS supplementation can influence rumen fermentation, gut microbial diversity, animal performance, and nutrient utilization [1, 3, 4]. However, SMS also contains bioactive compounds such as β -glucans, chitin, flavonoids, and phenolic compounds that may exert stimulatory or inhibitory effects on rumen microorganisms depending on their concentration [6]. These phenolic compounds originate from lignin degradation during fungal growth and may potentially influence rumen microbial populations and fermentation patterns.

Although several studies have evaluated the use of SMS in ruminant diets, limited information is available regarding the fermentability and microbial effects of *Pleurotus*-derived SMS specifically under controlled *in vitro* rumen conditions. Moreover, the optimal inclusion level of PSMS for improving rumen degradability without negatively affecting microbial activity remains unclear. Therefore, this study evaluated the effects of graded levels of PSMS supplementation (3% and 6%) on *in vitro* rumen fermentation characteristics, gas production, nutrient degradability, and microbial population dynamics in goats (*Capra hircus*). The selected inclusion levels were designed to represent low and moderate supplementation levels commonly used in exploratory feed evaluation trials. The study hypothesized that moderate inclusion of PSMS would enhance rumen fermentability and nutrient degradability by promoting partial lignocellulose degradation during mushroom cultivation.

2. Materials and Methods

2.1 Experimental Animals

The experimental animals consisted of three mature female Philippine native goats maintained at the Small Ruminant Project of Visayas State University. The animals served solely as donors of rumen fluid for the *in vitro* fermentation assays. The use of multiple donors allowed the rumen inoculum to represent biological variation among animals while maintaining consistent management conditions. All animals used in this study were handled in accordance with standard animal welfare practices and institutional guidelines for the care and use of livestock in research. The experimental procedures involved routine feeding and non-invasive rumen fluid collection, and no procedures causing pain or distress were performed.

2.2 Preparation of Feeding Materials

The basal feed, particularly Napier grass (*Pennisetum purpureum*), was collected from the Department of Animal Science pasture area at Visayas State University. Forage was collected every morning, chopped, and freshly fed to match the animal's voluntary feed intake. On the other hand, the SMS was obtained from a mushroom farm in Dagohoy, Bohol, Philippines. To improve the palatability of SMS, a considerable amount of molasses was added and thoroughly mixed into the feeding materials.

2.3 Feeding and Management of Experimental Animals

Before rumen fluid collection, the donor animals were maintained under controlled feeding conditions for a 14-day adaptation period. During this period, goats were provided Napier grass as the basal forage and small quantities of PSMS to familiarize the animals with the substrate and avoid abrupt dietary exposure. Feeding was conducted twice daily (morning and afternoon), and fresh water was provided *ad libitum*.

The adaptation period was implemented to stabilize rumen microbial activity before rumen fluid collection. However, it is acknowledged that exposure of donor animals to PSMS may influence the microbial composition of the rumen inoculum. Consequently, the results of the present study should be interpreted primarily as an evaluation of the fermentability of PSMS under controlled *in vitro* conditions rather than as a direct simulation of animals maintained on a strictly basal diet.

2.4 Collection of Rumen Fluids

Rumen fluid was collected in the morning, 1 hour before feeding, to prevent contamination with saliva. The rumen fluid collection method was conducted using an oral stomach tube [7]. The rumen fluids were carefully stored in a clean, air-tight flask. A volume of 100-150 ml of rumen fluid was collected from each of the animals. Moreover, it was filtered through a 2-layer cheesecloth, followed by the determination of rumen fluid pH using a pH meter.

2.5 Buffer Solution

The rumen fluids used in the *in vitro* degradability of the substrates were added with a buffer solution to neutralize the rumen fluid pH. A 1:2 ratio was used upon neutralizing the rumen fluids, and the buffer solution used was McDougall's buffer or phosphate-carbonate buffer.

2.6 Two-step *in vitro* Digestibility of Spent Mushroom Substrates

Two-step *in vitro* digestibility was determined using the method of Tilley and Terry with minor modifications [8]. Ground samples (3 g) of each treatment were incubated in fermentation vials containing 10 mL rumen fluid and 20 mL McDougall's buffer solution under anaerobic conditions. Carbon dioxide was flushed into each vial before sealing to maintain anaerobiosis. The vials were incubated in a shaking water bath maintained at 39–41°C for 24 h to simulate ruminal fermentation. A total of 18 incubation vials were prepared (six per treatment). After the first incubation phase, *in vitro* gas production was measured using a glass syringe. Rumen fluid samples (2 mL) were withdrawn from selected vials for microbial analysis. Following the first digestion phase, residues were recovered for the determination of ruminal degradability parameters. The remaining samples underwent the second digestion stage using acid-pepsin solution for 48 h to simulate post-ruminal digestion. The recovered residues were oven-dried at 105°C for 12 h before determination of degradability indices, including IVDMD, IVCPD, IVNDFD, and IVADFD [8, 9].

2.7 Proximate Analysis of SMS

Proximate analysis was conducted to determine the nutritional value of PSMS. Analyses on dry matter, crude protein, crude fiber, ash, and crude fat were conducted at the Visayas State University- Department of Animal Science - Animal Nutrition Laboratory.

2.8 Rumen Microbial Analysis

The withdrawn rumen fluids from each vial after the first digestion phase were stored in sterile, sealed test tubes. Analysis of the rumen microbial population resulting from incubation with varying treatments was conducted through a series of laboratory processes. Hence, in the analysis of microbial population dynamics, a total of 9 rumen fluid samples from the three treatments, with three replicates each, underwent gross microscopy to determine and differentiate microbial shapes. The identified rumen bacteria were subjected to Gram staining to facilitate thorough isolation from the diverse consortium. Furthermore, the bacterial species were counted as colony-forming units (CFU/ml), while protozoal and fungal species were counted as cells/ml.

2.9 Experimental Design and Treatment

The study used a Randomized Complete Block Design (RCBD), in which rumen fluid from each donor goat served as a biological block to account for variation among rumen inoculum sources. Three dietary treatments were evaluated:

T0: 0% PSMS (control; Napier grass only)

T1: 3% PSMS supplementation

T2: 6% PSMS supplementation

Each treatment was incubated in six fermentation vials, yielding 18 incubation units in total. The selected inclusion levels (3% and 6%) were chosen to represent low and moderate supplementation levels commonly used in exploratory feed evaluation studies to assess the potential effects of novel feed resources without drastically altering substrate composition.

2.10 Data Analysis

Data were analyzed using Analysis of Variance (ANOVA) in SAS OnDemand, with differences determined by Tukey's Honestly Significant Difference Test (HSD) at $\alpha=0.05$. The present study utilized rumen fluid obtained from three donor goats, which represents a relatively small number of biological sources. While this approach is commonly used in preliminary *in vitro* fermentation studies, the limited number of donors may limit statistical power and the generalizability of the results. Furthermore, donor animals were exposed to PSMS during the adaptation period, which may have influenced the rumen inoculum's microbial composition. Future studies should consider using a larger number of donor animals maintained on a uniform basal diet to minimize potential confounding effects and improve the robustness of the findings.

3. Results and Discussion

3.1 Chemical Composition of PSMS

Displayed in Table 1 is the chemical composition of the *Pleurotus* spent mushroom substrate (PSMS). The material exhibited a relatively high dry matter content (80.38%), while crude protein (1.79%) and ether extract (4.74%) were comparatively low. Notably, the ash content reached 61.59%, which is substantially higher than values commonly reported for most forages and agricultural by-products. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were 19.77% and 18.49%, respectively.

Table 1. Chemical composition of PSMS

Material	Composition (%)					
	DM	EE	CP	Ash	NDF	ADF
PSMS	80.38	4.74	1.79	61.59	19.77	18.49

The crude protein level observed in the present study was lower than values reported in other SMS studies [10]. This difference may be attributed to the composition of the substrate materials used in the mushroom production process. In the present study, the PSMS was primarily composed of rice straw (approximately 75%), supplemented with sawdust, rice bran, molasses, and agricultural lime. These materials are inherently low in nitrogen, and the available nitrogen may have been further utilized by the growing fungal mycelium during mushroom cultivation. Previous studies have reported that nitrogen present in mushroom substrates can be incorporated into fungal biomass during fruiting body formation, thereby reducing the residual crude protein content of the spent substrate [11]. The ash content recorded in this study (61.59%) was considerably higher than values typically reported for lignocellulosic feed materials. This unusually high ash fraction may partly reflect the accumulation of inorganic residues in the substrate during mushroom cultivation. The addition of agricultural lime during substrate preparation likely increased the substrate's mineral content. At the same time, the dominance of rice straw as a substrate component may also have contributed significant silica content, which is known to occur at relatively high levels in rice straw [12]. Additionally, progressive degradation and fungal metabolism of organic matter may concentrate the remaining inorganic components in the spent substrate. However, it should be noted that the present study did not include a detailed mineral fraction analysis (e.g., silica or individual mineral determination), and therefore, the precise contributors to the elevated ash fraction remain uncertain.

The fiber fractions of PSMS were relatively low compared with those typically reported for raw lignocellulosic materials. The NDF and ADF values were also very close to each other, suggesting a minimal hemicellulose fraction (approximately 1.3%). Such a narrow difference between NDF and ADF may indicate extensive hemicellulose degradation during mushroom cultivation. *Pleurotus* species are known to secrete lignocellulolytic enzymes such as laccases, lignin peroxidases, manganese peroxidases, and cellulases, which

collectively degrade lignin and associated polysaccharides in lignocellulosic substrates [13]. Through this enzymatic process, hemicellulose and other easily degradable structural carbohydrates may be partially hydrolyzed during fungal colonization, potentially explaining the reduced fiber fractions observed in the present study. Nevertheless, the possibility of analytical variation in fiber determination cannot be completely excluded. It should also be noted that lignin concentration was not determined in the present study because acid detergent lignin (ADL) analysis was not included in the proximate evaluation. Since lignin plays a critical role in determining the structural characteristics and degradability of lignocellulosic substrates, future studies should include detailed fiber fractionation (NDF, ADF, and ADL) to characterize better the structural carbohydrate composition of PSMS and its implications for rumen fermentation.

3.2 Rumen Microbial Analysis

The data presented in Table 2 show the observed dynamics in rumen microbial populations following incubation with different levels of PSMS. Three major microbial groups typically present in the rumen, which include bacteria, protozoa, and fungi, were evaluated. Bacterial populations were quantified as colony-forming units (CFU/mL), whereas protozoal and fungal populations were expressed as cells/mL. Morphological observations were conducted using microscopy and Gram staining to characterize general microbial features. As shown in Table 2, bacterial populations were numerically higher than protozoal and fungal populations across treatments, consistent with the general microbial hierarchy in the rumen ecosystem, in which bacteria are the dominant microbial group. Microscopic observations indicated that most bacterial isolates were Gram-negative and predominantly rod-shaped or coccoid forms. Similar observations have been reported in rumen microbial ecology, where Gram-negative anaerobic bacteria dominate the microbial community and degrade structural and non-structural carbohydrates [14].

Table 2. Dynamics in rumen microbial population

Treatment	Rumen Microorganism			Description
	Bacteria (cfu/ml)	Protozoa (cells/ml)	Fungi (cells/ml)	
0% PSMS	6267.00	4000.00	3000.00	Bacteria: Gram-negative (long rod-shaped, short rod-shaped, coccus-shaped) Protozoa: Not indicated Fungi: Bi-flagellate fungi zoospores
3% PSMS	21866.67	5750.00	0.00	Bacteria: Gram-negative (long rod-shaped) Protozoa: Not indicated Fungi: Not detected
6% PSMS	2233.33	3000.00	0.00	Bacteria: Gram-negative (Streptobacillus) and Gram-positive Protozoa: Not indicated Fungi: Not detected

Numerically, the treatment receiving 3% PSMS (T1) showed the highest bacterial and protozoal counts compared with the control and the 6% supplementation level. In contrast, microbial counts declined in T2, suggesting that higher PSMS inclusion may not favor microbial proliferation under the present incubation conditions. However, it should be noted that no statistical comparisons were performed for microbial population counts; therefore, the observed differences should be interpreted only as descriptive trends rather than statistically validated treatment effects. The absence of detectable fungal cells in T1 and T2 may be associated with several factors. One possible explanation is the presence of phenolic compounds in PSMS. Phenolic metabolites can be generated during lignin degradation by ligninolytic enzymes such as laccases and peroxidases produced by *Pleurotus* species during mushroom cultivation [15, 16]. Phenolic compounds have been reported to exert antimicrobial effects and may influence rumen microbial populations under certain conditions [17, 18]. Nevertheless, this explanation remains speculative, as phenolic concentrations were not quantified in the present

study. Alternative explanations must also be considered. Rumen anaerobic fungi are known to strongly attach to feed particles during colonization and fiber degradation [19, 20]. Since rumen fluid samples were collected from the liquid phase of the incubation vials while avoiding feed residues, fungal structures attached to the substrate particles may not have been adequately captured during sampling. Consequently, the apparent absence of fungi in PSMS treatments may reflect sampling limitations rather than the complete absence of fungal populations.

It is also important to acknowledge that microbial characterization in this study relied exclusively on culture-dependent techniques, including colony counting, Gram staining, and microscopic observation. While these methods provide useful preliminary information on culturable microbial populations, they capture only a small fraction of the total rumen microbiota, as a large proportion of rumen microorganisms are not readily culturable under standard laboratory conditions. Therefore, the microbial results presented here should be interpreted with caution, and future studies employing culture-independent molecular techniques (e.g., 16S rRNA sequencing or metagenomic analysis) would provide a more comprehensive characterization of microbial community dynamics associated with PSMS supplementation. Overall, the observed microbial patterns suggest that moderate PSMS inclusion (3%) may support microbial proliferation under *in vitro* fermentation conditions, possibly due to the availability of partially degraded lignocellulosic components. However, given the methodological limitations described above, these findings should be regarded as preliminary observations requiring confirmation through more comprehensive microbial analyses.

3.3 *In vitro* Gas Production (IVGP)

Presented in Table 3 are the *in vitro* gas production (IVGP) values obtained from the different treatments after the first digestion phase. The control treatment (T0) produced a mean gas volume of 46.90 cc, while treatments supplemented with PSMS produced slightly higher gas volumes of 52.39 cc (T1) and 51.54 cc (T2). Statistical analysis indicated no significant differences among treatments ($p=0.4542$). Linear and quadratic contrasts were also not significant.

Table 3. *In vitro* gas production

Parameter	Treatment			SEM	p-value	p-value	
	0% PSMS	3% PSMS	6% PSMS			Linear	Quadratic
IVGP (cc)	46.90	52.39	51.54	3.6421	0.4542	0.3359	0.4377

In vitro gas production is commonly used as an indirect indicator of rumen substrate fermentability and microbial activity, as gas is produced during microbial degradation of carbohydrates and other fermentable feed components [21]. Although the treatments supplemented with PSMS showed numerically higher gas production compared to the control, these differences were not statistically significant, indicating that the inclusion of PSMS at 3% and 6% did not significantly alter fermentation activity under the conditions of this experiment. Previous studies have reported an increase in *in vitro* gas production from lignocellulosic substrates treated with *Pleurotus* species, attributed to fungal degradation of structural carbohydrates, such as cellulose, hemicellulose, and lignin, during solid-state fermentation [21, 22]. Such degradation can increase microbial accessibility to fermentable substrates. However, in the present study, the lack of significant treatment effects suggests that PSMS supplementation at the evaluated inclusion levels did not substantially modify the fermentability of the basal diet. Gas produced during *in vitro* fermentation generally consists primarily of carbon dioxide (CO₂) and methane (CH₄), which are generated during microbial fermentation processes in the rumen. While higher gas production may reflect increased fermentative activity, *in vitro* gas volume alone does not distinguish between CO₂ and methane production and therefore cannot be directly interpreted as an indicator of enteric methane emissions without additional measurements. Consequently, the environmental implications of the observed gas production in the present study remain uncertain and would require further investigation using methods specifically designed to quantify methane production.

3.4 *In vitro* Dry Matter Degradability (IVDMD)

Table 4 presents the IVDMD of the treatments at the 24-hour *in vitro* 1st-stage nutrient digestibility. The results show no significant differences among the treatments ($p=0.6988$), with IVDMD ranging from 51%

to 54%. These results suggest that treatments have a similar degree of DM degradability and are sufficiently degradable by rumen microbes. Although statistically the same, T2 showed a slightly higher IVDMD (54.58%), followed by T1 (51.59%) and T0 (51.45%). The increase in IVDMD with PSMS inclusion may be attributed to its pre-digested nature, as indicated by its NDF and ADF values, which have been degraded by fungal biomass. Residual ligninolytic enzymes present in PSMS [23] may also have contributed to increased substrate accessibility for microbial degradation. Overall, the results suggest that PSMS supplementation did not significantly affect DM degradability of the basal feed. The second-stage IVDMD, simulating abomasal digestion (Table 5), also showed no significant differences ($p=0.8061$). In this stage, T0 had the highest IVDMD (65.30%), followed by T1 (62.54%) and T2 (59.26%), indicating that overall DM degradability remains comparable across treatments after the acid-pepsin phase.

3.5 *In vitro* Crude Protein Degradability (IVCPD)

The IVCPD of the first-stage *in vitro* digestion (Table 4) was highest in T1 (60.29%), followed by T0 (40.07%) and T2 (37.60%). The lower IVCPD in T2 than in T1 may be associated with a higher inclusion rate of PSMS. Lignocellulosic residues such as spent mushroom substrate may contain bioactive compounds, including phenolic constituents derived from plant materials or fungal metabolism, which have been reported to interact with digestive enzymes and potentially influence protein degradability. However, the phenolic content of the PSMS used in this study was not determined; therefore, this explanation should be considered speculative and requires further investigation. After the second-stage *in vitro* digestion (Table 5), IVCPD values were relatively stable across treatments (~53–55%), reflecting the effect of pepsin and HCl on enzymatic digestion at the abomasal phase. The observed pattern suggests that crude protein degradability in the substrates is relatively robust to PSMS inclusion, even though structural fiber fractions may vary.

3.6 *In vitro* Neutral Detergent Fiber Degradability (IVNDFD)

The IVNDFD (Tables 4 and 5) was slightly lower in the first stage (rumen simulation) than in the second stage. No significant differences were observed (1st stage $p=0.7795$; 2nd stage $p=0.6530$). Although statistically the same, T1 showed higher IVNDFD in both stages (30.78% in stage 1 and 38.69% in stage 2) than T0 (21.05% and 26.52%) and T2 (22.30% and 22.26%). Higher degradability during the first stage can be attributed to active rumen microbial fermentation before the addition of HCl and pepsin. The inclusion of PSMS, with its partially degraded fiber components, likely increased the accessibility of cellulose and hemicellulose to microbial breakdown, enhancing NDF degradability. In the second stage, the addition of acid-pepsin may have contributed to slightly lower fiber degradability due to partial degradation of microbial cells.

3.7 *In vitro* Acid Detergent Fiber Degradability (IVADFD)

First-stage IVADFD (Table 4) showed a non-significant trend ($p=0.0525$) but a significant linear increase with PSMS inclusion: T0 = 45.46%, T1 = 46.34%, T2 = 54.84%. This may reflect the effect of residual ligninolytic enzymes in PSMS, which degrade lignified fiber fractions, thereby increasing ADF digestibility during ruminal simulation [22]. In contrast, second-stage IVADFD (Table 5) showed a significant quadratic effect ($p=0.0035$), with T0 = 61.33%, T1 = 60.33%, and T2 = 43.33%. The decline in IVADFD at the highest PSMS inclusion may be related to bioactive compounds present in PSMS that interact with fiber-degrading enzymes under acidic conditions. However, the exact mechanism remains hypothetical due to the absence of measured phenolic content. Differences in the simulated digestive environments may explain the contrasting patterns across stages. The first stage represents ruminal fermentation under near-neutral pH (~6.8), where microbial cellulolytic activity predominates, and PSMS inclusion may improve fiber accessibility. The second stage simulates abomasal digestion under acidic conditions (pH 2–4), where enzyme activity and interactions with potential bioactive compounds may differ, leading to decreased ADF degradation at higher PSMS inclusion levels. Finally, the reduction in second-stage IVADFD at T2 may contribute to the slight numerical decline in second-stage IVDMD, since the ADF fraction represents the more resistant structural components of plant cell walls. In contrast, IVCPD remained relatively stable across treatments, indicating that the protein fraction was less affected by changes in structural fiber degradation.

Table 4. 1st stage *in vitro* nutrient degradability of substrates

Parameter (%)	Treatment			SEM	p-value	p-value	
	0% PSMS	3% PSMS	6% PSMS			Linear	Quadratic
DM	51.45	51.59	54.85	3.0677	0.6988	0.4778	0.6987
CP	40.07	60.29	37.60	0.2845	<0.0001	0.0004	<0.0001
NDF	21.05	30.75	22.30	10.4559	0.7795	0.9350	0.5096
ADF	45.46	46.34	54.84	2.0750	0.0525	0.0294	0.1946

Table 5. 2nd stage *in vitro* nutrient degradability of substrates

Parameter (%)	Treatment			SEM	p-value	p-value	
	0% PSMS	3% PSMS	6% PSMS			Linear	Quadratic
DM	65.30	62.54	59.26	6.9175	0.8061	0.5373	0.9747
CP	55.48	52.95	55.38	6.9142	0.9584	0.9918	0.7842
NDF	26.52	38.69	22.65	12.1485	0.6530	0.8325	0.3967
ADF	61.33	60.33	43.33	18.7098	0.0035	0.0021	0.0221

4. Conclusions

The study indicates that supplementation of PSMS at moderate levels (3% of the diet) does not significantly alter overall ruminal fermentation, *in vitro* gas production, or nutrient degradability in goats. While some numerical trends, such as slightly higher bacterial and protozoal counts and marginal increases in IVGP and degradability parameters at 3% PSMS, were observed, these differences were not statistically significant. Therefore, claims of enhanced fermentation or nutrient utilization based solely on numerical trends should be interpreted cautiously. Notably, the second-stage IVADFD showed a significant decrease at the highest PSMS inclusion (6%), suggesting that higher levels of PSMS may reduce the degradability of structural fiber components, potentially due to bioactive compounds such as phenolics. This is consistent with the observed absence of rumen fungi at 6% PSMS, indicating that excessive inclusion may adversely affect specific microbial groups involved in fiber degradation. Based on these findings, PSMS supplementation up to 3% of the diet appears safe and unlikely to affect rumen microbial populations or nutrient degradability negatively, whereas higher levels may inhibit fiber-degrading microbes and enzymatic activity. The mineral-rich composition and partially pre-digested nature of PSMS make it a potentially valuable feed ingredient, but care should be taken to avoid exceeding the suggested inclusion rate. Overall, PSMS can be considered a supplemental feed resource for small ruminants at moderate inclusion levels. Its effects on rumen fermentation, microbial dynamics, and fiber degradability warrant further investigation, particularly with direct measurements of phenolic compounds and their interaction with rumen microbial enzymes.

Author Contributions: Conceptualization, A.J.L, R.L., and M.G.; methodology, A.J.L, R.L. and M.G.; validation, A.J.L, R.L. and M.G.; formal analysis, A.J.L. and R.L.; investigation, A.J.L. and R.L.; resources, A.J.L; data curation, A.J.L and R.L.; writing—original draft preparation, A.J.L.; writing—review and editing, A.J.L., R.L., M.G., and W.C.; visualization, A.J.L., and R.L.; supervision, A.J.L., and R.L.; project administration, A.J.L. and R.L.; funding acquisition, A.J.L.. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

- [1] Zhang, Y.; Dou, M.; Chen, X.; Huang, X.; Huang, Q.; Huang, X. Effects of Fermented Mushroom Residue Feed on Growth Performance and Rumen Microorganisms of Goats. **2021**. <https://doi.org/10.21203/rs.3.rs-139648/v1>

- [2] Martín, C.; Zervakis, G. I.; Xiong, S.; Koutrotsios, G.; Strætkevørn, K. O. Spent Substrate from Mushroom Cultivation: Exploitation Potential toward Various Applications and Value-Added Products. *Bioengineered* **2023**, *14*, 2252138. <https://doi.org/10.1080/21655979.2023.2252138>
- [3] Huang, X.; Zhou, L.; You, X.; Han, H.; Chen, X.; Huang, X. Production Performance and Rumen Bacterial Community Structure of Hu Sheep Fed Fermented Spent Mushroom Substrate from *Pleurotus eryngii*. *Sci. Rep.* **2023**, *13*, 8696. <https://doi.org/10.1038/s41598-023-35828-8>
- [4] Lu, M. L.; Yuan, G. H.; Li, C. C.; Hu, L. H.; Feng, X. W.; Jiang, H.; Liu, L. L.; Rehemujiang, H.; Xu, G. S. Effects of Spent Substrate of Oyster Mushroom (*Pleurotus ostreatus*) on Feed Utilization and Liver Serum Indices of Hu Sheep. *Animals* **2024**, *14*, 3416. <https://doi.org/10.3390/ani14233416>
- [5] van Kuijk, S. J.; Sonnenberg, A. S.; Baars, J. J.; Hendriks, W. H.; Cone, J. W. Fungal Treatment of Lignocellulosic Biomass: Importance of Fungal Species, Colonization, and Time. *Anim. Feed Sci. Technol.* **2015**, *209*, 40–50. <https://doi.org/10.1016/j.anifeedsci.2015.07.026>
- [6] Törös, G.; El-Ramady, H.; Prokisch, J.; Velasco, F.; Llanaj, X.; Nguyen, D. H.; Peles, F. Modulation of the Gut Microbiota with Prebiotics and Antimicrobial Agents from *Pleurotus ostreatus*. *Foods* **2023**, *12*, 2010. <https://doi.org/10.3390/foods12102010>
- [7] Muizelaar, W.; Bani, P.; Kuhla, B.; Larsen, M.; Tapio, I.; Yáñez-Ruiz, D.; van Gastelen, S. Rumen Fluid Sampling via Oral Stomach Tubing Method. **2020**. <https://doi.org/10.5680/MCPB008>
- [8] Tilley, J. M. A.; Terry, D. R. A Two-Stage Technique for the *in Vitro* Digestion of Forage Crops. *Grass Forage Sci.* **1963**, *18*, 104–111. <https://doi.org/10.1111/j.1365-2494.1963.tb00335.x>
- [9] Zewdie, A. K. The Different Methods of Measuring Feed Digestibility: A Review. *EC Nutr.* **2019**, *14*, 68–74.
- [10] Silva, S. O.; Da Costa, S. M. G.; Clemente, E. Chemical composition of *Pleurotus pulmonarius* (Fr.) Quél., substrates and residue after cultivation. *Brazilian Archives of Biology and Technology* **2002**, *45*(4), 531–535. <https://doi.org/10.1590/s1516-89132002000600018>
- [11] McGinnis, M. R.; Tyring, S. K. Introduction to Mycology. In *Medical Microbiology*, 4th ed.; **1996**.
- [12] Khoshnood Motlagh, E.; Asasian-Kolur, N.; Sharifian, S. A Comparative Study on Rice Husk and Rice Straw. *Biomass Convers. Biorefin.* **2022**, *12*, 5729–5738. <https://doi.org/10.1007/s13399-020-01145-7>
- [13] Adebayo, E. A.; Martinez-Carrera, D. Oyster Mushrooms (*Pleurotus*) and Lignocellulosic Biomass Utilization. *Afr. J. Biotechnol.* **2015**, *14*, 52–67. <https://doi.org/10.5897/AJB2014.14249>
- [14] Nagaraja, T. G. Microbiology of the Rumen. In *Rumenology*; Springer: Cham, **2016**; pp 39–61. https://doi.org/10.1007/978-3-319-30533-2_2
- [15] Kumla, J.; Suwannarach, N.; Sujarit, K.; Penkhrue, W.; Kakumyan, P.; Jatuwong, K.; Vadthanarat, S.; Lumyong, S. Cultivation of Mushrooms and Enzyme Production. *Molecules* **2020**, *25*, 2811. <https://doi.org/10.3390/molecules25122811>
- [16] Klausen, S. J.; Falck-Ytter, A. B.; Strætkevørn, K. O.; Martin, C. Valorization of Spent Mushroom Substrate. *Molecules* **2023**, *28*, 5140. <https://doi.org/10.3390/molecules28135140>
- [17] de Paula, E. M.; Samensari, R. B.; Machado, E.; Pereira, L. M.; Maia, F. J.; Yoshimura, E. H.; Franzolin, R.; Faciola, A. P.; Zeoula, L. M. Effects of Phenolic Compounds on Ruminal Protozoa. *Livest. Sci.* **2016**, *185*, 136–141. <https://doi.org/10.1016/j.livsci.2016.01.021>
- [18] Baptista, F.; Campos, J.; Costa-Silva, V.; Pinto, A. R.; Saavedra, M. J.; Ferreira, L. M.; Rodrigues, M.; Barros, A. N. Nutraceutical Potential of *Lentinula edodes* Spent Substrate. *J. Fungi* **2023**, *9*, 1200. <https://doi.org/10.3390/jof9121200>
- [19] Bauchop, T. Rumen Anaerobic Fungi of Cattle and Sheep. *Appl. Environ. Microbiol.* **1979**, *38*, 148–158. <https://doi.org/10.1128/aem.38.1.148-158.1979>
- [20] Perez, H. G.; Stevenson, C. K.; Lourenco, J. M.; Callaway, T. R. Understanding Rumen Microbiology. *Encyclopedia* **2024**, *4*, 148–157. <https://doi.org/10.3390/encyclopedia4010013>
- [21] Akinfemi, A.; Ogunwole, O. A. Chemical Composition and Digestibility of Rice Straw Treated with *Pleurotus*. *Slovak J. Anim. Sci.* **2012**, *45*, 14–20.

-
- [22] Sufyan, A.; Ahmad, N.; Shahzad, F.; Embaby, M. G.; AbuGhazaleh, A.; Khan, N. A. Improving Nutritional Value of Crop Residues Using *Pleurotus*. *J. Sci. Food Agric.* **2002**, *102*, 2445–2453. <https://doi.org/10.1002/jsfa.11584>
- [23] Kirk, T. K.; Farrell, R. L. Enzymatic “Combustion”: Microbial Degradation of Lignin. *Annu. Rev. Microbiol.* **1987**, *41*, 465–505. <https://doi.org/10.1146/annurev.mi.41.100187.002341>