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Effects of the Marine Endophytic Fungus *Aspergillus terreus* on *In Vitro* Rumen Fermentation, Fiber Digestibility, and Methane Production

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Abstract

The marine endophytic fungus *Aspergillus terreus* has considerable potential to improve the utilization of lignocellulosic feed through its fibrolytic and ligninolytic activities. Therefore, the effects of *A. terreus* supplementation on rumen microbial populations, fermentation characteristics, methane production, and fiber digestibility were evaluated using an *in vitro* rumen incubation system. A completely randomized design with four treatments and five replications was used. The treatments consisted of a control diet (30:30:40 mixture of Napier grass, palm leaves, and concentrate) and the control diet supplemented with *A. terreus* at 1.5% (0.75 mL of fungal suspension at 10^6 CFU/mL^{-1}), 3.0% (1.5 mL of fungal suspension at 10^6 CFU/mL^{-1}), or 4.5% (2.25 mL of fungal suspension at 10^6 CFU/mL^{-1}). The measured variables included ruminal pH, ammonia (NH_3) concentration, volatile fatty acid (VFA) production and profiles, microbial populations, estimated methane production, dry matter digestibility (DMD), organic matter digestibility (OMD), and fiber fraction digestibility. Supplementation with *A. terreus* at 1.5%–3.0% significantly ($p < 0.05$) increased the digestibility of neutral detergent fiber, hemicellulose, cellulose, and lignin. Lignin digestibility increased from 28.22% in the control to >60% in the fungal-supplemented treatments, indicating strong ligninolytic activity. However, acid detergent fiber digestibility and microbial populations were unaffected. Most rumen fermentation parameters, including NH_3 concentration, total VFA production, VFA molar proportions, the acetate-to-propionate ratio, and estimated methane production, remained unchanged. Ruminal pH decreased slightly at the 3.0% inclusion level, whereas DMD and OMD decreased at the 4.5% supplementation level ($p < 0.05$). Overall, *A. terreus* supplementation improved structural carbohydrate degradation, particularly the digestibility of lignin, hemicellulose, and cellulose, without adversely affecting rumen fermentation. An inclusion level of 1.5%–3.0% appears to be optimal for enhancing fiber utilization under *in vitro* conditions.

Keywords:
Aspergillus terreus; marine endophytic fungus; fiber digestibility; rumen fermentation; lignin degradation

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Introduction

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Ruminant livestock, including cattle, goats, and buffalo, have a unique ability to derive energy from plant fiber through fermentation by rumen microbes. Rumen microbes produce fibrolytic enzymes, while the host animal provides an anaerobic environment for fermentation (Krause et al., 2003). Fiber is subsequently converted into volatile fatty acids (VFA), which serve as an energy source for ruminants (Gleason et al., 2022). Fiber degradation in the rumen is influenced by fiber structure, including the concentrations of pectin, hemicellulose, and cellulose; lignin content and structure; particle size; and rumen microbial populations (L. Wang et al., 2020). A key process in fiber degradation is the colonization of fiber particles by rumen microbes. Many bacterial species produce fibrolytic enzymes and are categorized as fibrolytic bacteria. In addition to bacteria, other microorganisms, including protozoa and fungi, can also degrade fiber (Gleason et al., 2022). Furthermore, altering rumen ecology can reduce ruminants from producing CH_4 (Harahap et al., 2020). Anaerobic fungi can produce enzymes that hydrolyze lignified components of plant cell walls (Callaghan et al., 2015). The anaerobic fungus *Neocallimastix* sp. YQ2 can produce ferulic acid esterase and acetyl esterase, which hydrolyze lignin–polysaccharide linkages in plant cell walls through phenolic compounds (Yue et al., 2009).

Rumen fungi can degrade plant tissues containing lignin more effectively than rumen bacteria (Akin & Borneman, 1990). Given the advantages of anaerobic fungi, their use in ruminants may increase livestock production. Marine endophytic fungi are promising fungal inoculants for improving feed fiber digestibility. Marine endophytic fungi are microscopic organisms that live within plant tissues without causing disease symptoms in their host plants, including leaves, twigs, and roots (El-Bondkly et al., 2021). In addition, marine endophytic fungi can produce potentially bioactive secondary metabolites. Our previous research showed that marine fungal extracts from mangrove leaves have antibacterial activity against *Salmonella typhimurium* (Nabila et al., 2024) and *Escherichia coli* (Kurnia et al., 2024). Marine fungal extracts also exhibit cellulolytic activity. Endophytic marine fungi can produce important enzymes, including oligosaccharide-degrading enzymes (e.g., cellulase), xylanase, mannanase, and inulinase. Marine fungi can also produce antibiotics and antimicrobial substances (Andhikawati et al., 2014). Furthermore, marine endophytic fungi exhibit high cellulolytic activity (Budiman et al., 2024).

The use of fungi as feed additives has gained substantial attention in recent years as a sustainable strategy for improving the utilization of low-quality roughage. According to a recent review by Jyothi et al. (2024), anaerobic rumen fungi represent valuable biological tools in livestock nutrition of their capacity to degrade the lignocellulosic matrix. Although conventional research has focused on native rumen fungi, such as *Neocallimastix* spp. (Agustina et al., 2024), the growing exploration of marine-derived endophytic fungi—including *A. terreus*—offers a novel perspective on biological lignin degradation. *Aspergillus terreus* facilitates biological delignification by producing oxidative extracellular enzymes, including laccase (Lac), lignin peroxidase (LiP), and manganese peroxidase (MnP). These enzymes initiate the depolymerization of complex aromatic polymers into smaller phenolic compounds, thereby increasing the accessibility of structural carbohydrates, such as cellulose and hemicellulose, to other rumen microbes. This integrated enzymatic approach aligns with recent trends in marine biotechnology that explore fungal metabolites for high-value agricultural applications (El-Bondkly et al., 2021).

The effectiveness of fungal supplementation in ruminant nutrition depends not only on the capacity of a fungus to degrade structural carbohydrates but also on how its addition influences the overall fermentation environment. Improvements in the accessibility of cellulose and hemicellulose may support the utilization of fibrous feed materials, yet changes in individual fiber fractions do not necessarily result in corresponding improvements in total dry matter or organic matter digestibility. The concentration of the fungal inoculum is therefore an important consideration when evaluating its potential as a feed additive. In addition to assessing fiber degradation, it is necessary to examine ruminal pH, ammonia concentration, volatile fatty acid production, and microbial populations to determine whether improved fiber utilization occurs without undesirable changes in fermentation. Methane production is another relevant outcome as fermentation pathways influence the distribution of metabolic hydrogen within the rumen (Bashar et al., 2024). However, estimated methane production must be interpreted cautiously when it is calculated indirectly from volatile fatty acid proportions rather than measured directly. Evaluating these parameters together provides a broader basis for determining whether a fungal isolate can improve the utilization of lignocellulosic feed under controlled in vitro conditions.

However, a notable research gap remains regarding the application of marine endophytic fungi in a complex and dynamic rumen environment. Further investigation is needed to determine the potential of marine endophytic fungi to modulate rumen fermentation, particularly the breakdown of feed fiber. Their interactions with rumen microbes and microbial enzymes suggest that they may influence feed fermentability in the rumen. However, the effects of marine endophytic fungi as potential inoculum candidates have not been widely studied. Therefore, further research is needed to assess their effects on

fermentation characteristics, rumen digestibility, and fiber degradation under *in vitro* conditions. In addition, limited information is available on whether introducing such fungi may disrupt existing microbial balances or how their ligninolytic activity translates into measurable changes in fermentation efficiency and gas production. This study provides a comprehensive evaluation of *A. terreus* as a potential rumen modifier. To our knowledge, this is the first study to simultaneously evaluate the effects of a marine endophytic fungal isolate on rumen fermentation characteristics and fiber fraction digestibility, including lignin, microbial populations, and methane production, using an *in vitro* rumen incubation system.

The evaluation of fungal-based feed additives requires consideration of how supplementation levels influence their nutritional effects. Although fungal treatment may support the breakdown of resistant plant materials, increasing the inclusion level does not necessarily lead to continuous improvements in feed utilization. Responses may depend on the characteristics of the feed substrate, interactions between fungal-derived compounds and the rumen environment, and the balance between fiber degradation and other fermentation processes. Evaluating different inclusion levels can therefore help identify treatments that support fiber utilization while maintaining overall digestibility. It is also important to distinguish improvements in individual cell-wall components from changes in the nutritional value of the feed as a whole. For marine-derived fungi, this evaluation is necessary to establish their potential usefulness as feed additives under rumen fermentation conditions.

The nutritional value of lignocellulosic feeds is influenced by the structure and composition of their plant cell walls, which can limit microbial access to structural carbohydrates. Fungal treatment may alter these materials through biological processes, including the action of extracellular enzymes, but the extent of these effects can vary with the fungal species and feed substrate. Consequently, changes in individual fiber fractions should be interpreted alongside overall digestibility and rumen fermentation characteristics. Methane-related responses also require separate consideration because changes in fiber degradation do not automatically indicate changes in methane production, as rumen microbial activity and fermentation processes are closely associated with both fiber digestion and methane emissions (Vasta et al., 2019).

The aim of this study was to evaluate the effects of marine endophytic fungal inoculation on *in vitro* rumen fermentability characteristics and estimated methane production, as well as changes in rumen microbial populations and community composition, with the goal of increasing the efficiency of rumen fermentation in livestock.

Materials and Methods

Compliance with Ethical Standards

Ethical principles for animal use and dairy fistula surgery were followed in accordance with the regulations of the Animal Ethics Committee at IPB University. The cows were housed and cared for in accordance with the guidelines of the Animal Ethics Committee at IPB University under contract no. 047/KEH/SKE/XI/2021.

Cultivation of Endophytic Marine Fungi (Tarman et al., 2011)

The marine endophytic fungus *A. terreus* isolate WB 1-2 was used throughout the study. To ensure fungal viability and consistent growth, the isolate was first cultured on Potatoes Dextrose Agar (PDA) medium at pH 7.0 and incubated at room temperature (25-27^{0}C) for 7 days. The mycelium was then transferred to 50 mL of sterile potato dextrose broth (PDB) and incubated for an additional 7 days. For mass culture, mycelial fragment (size ±1 cm) were inoculated into 500-mL Erlenmeyer flasks containing 250 mL of PDB and cultivated for 9 days. The inoculum was standardized by harvesting and homogenizing the culture broth containing fungal mycelia to obtain a uniform suspension. The concentration of the final fungal suspension was adjusted to 10^6 CFU/mL^{-1} and used as the stock suspension for all experimental treatments.

Diet and Treatments

The basal diet consisted of a 40:30:30 ratio of concentrate mix, Napier grass, and palm leaves. The experiment followed a completely randomized design with four treatments and five replications: P0 (control), P1 (1.5% fungal suspension, 0.75 mL), P2 (3.0% fungal suspension, 1.5 mL^{-1}), and P3 (4.5% fungal suspension, 2.25 mL). The fungal suspension contained 1×10^6 CFU mL^{-1} of *A. terreus*. The inclusion levels of 1.5%, 3.0%, and 4.5% were selected to represent low, moderate, and high supplementation rates, respectively, to evaluate the dose-dependent response to the fungal inoculum. These inclusion rates were based on previous studies showing that fungal inoculation within this range

effectively enhances lignocellulose degradation while avoiding excessive microbial competition or adverse effects on rumen fermentation (Agustina et al., 2024; Jyothi et al., 2024). In addition, the highest inclusion level (4.5%) was included to assess whether increasing the supplementation level would result in further improvements in digestibility. The composition of the concentrate ingredients is presented in Table 1, and the nutrient composition of the concentrate mix, Napier grass, palm leaf meal, and total ration is presented in Table 2.

Table 1. Concentrate mix composition

Ingredients	Percentage (%)
Pollard	39.00
Cassava waste	36.50
Coconut cake meal	11.00
Soybean meal	7.00
$CaCO_3$	1.50
*Premix**	1.00
Molasses	4.00
Total	100.00

Note. Premix: *Rumigold Small Rum Premixture* (PT. Nutricell Pacific). $CaCO_3$ = *calcium carbonate.*

Table 2. Nutrient composition of the concentrate mix, Napier grass, palm leaf meal, and total mixed ration used in the *in vitro* study (dry matter [DM] basis)

Nutrient (%)	Concentrate mix (C)	Napier grass (NG)	Palm leaf meal (PL)	Total ration (C:NG: PL = 40:30:30)
DM	87.30	94.14	93.89	91.33
Ash	14.55	8.52	19.34	14.18
Ether extract	2.78	1.37	3.18	2.48
Crude protein	11.59	14.49	13.59	13.06
Crude fiber	24.82	30.63	18.06	24.54
Nitrogen-free extract	46.25	44.99	45.47	45.64
TDN	51.33	49.54	56.14	52.24

Note. Analysis conducted by the Biotech Center, IPB University, in 2024.

In Vitro Incubation and Quality Control

Rumen fluid was collected from a fistulated dairy cow before morning feeding to ensure high microbial activity. As a quality-control measure, the fluid was filtered through two layers of cheesecloth and transported in a preheated thermos at 39°C to the laboratory to maintain temperature stability and prevent microbial shock.

In vitro fermentation was conducted according to the method of Tilley and Terry (1963), with consideration of the methodological advantages and limitations of in vitro approaches used in ruminant nutrition (Vinyard and Faciola, 2022). Each 100-mL fermenter tube contained 0.5 g of substrate, 40 mL of McDougall's buffer, and 10 mL of filtered rumen fluid. To maintain anaerobic conditions, the tubes were flushed with CO_2 before being sealed. Incubation was conducted at a constant temperature of 39°C in a shaking water bath to simulate natural rumen motility. Samples for fermentation parameters (pH, NH_3, VFA, and microbial populations) were collected after 4 h, whereas dry matter digestibility (DMD), organic matter digestibility (OMD), and fiber fraction analyses were conducted after 48 h of incubation. Methane production was estimated from VFA molar proportions according to Moss et al. (2000), using the following equation: $CH_4 = 0.45(C_2) - 0.275(C_3) + 0.40(C_4)$. Methodological factors can influence gas and methane production measurements during in vitro rumen fermentation (Maccarana et al., 2016).

Experimental Units and Replication

The experimental design comprised four dietary treatments arranged in a completely randomized design, with five replications assigned to each treatment. The control treatment received the basal diet without fungal supplementation, whereas the other treatments received the fungal suspension at the designated inclusion levels. The experimental unit was defined according to the unit independently assigned to a

treatment during incubation. Maintaining a clear distinction between the experimental unit and repeated measurements is important for appropriate statistical analysis. The treatment comparisons were used to evaluate whether fungal supplementation was associated with differences in microbial populations, fermentation characteristics, and nutrient digestibility under the in vitro conditions of the study.

Data Analysis

The experiment was conducted using a completely randomized design with four treatments and five replications. Data were subjected to analysis of variance using SPSS version 25, and Duncan's multiple range test was used to identify specific treatment differences at a significance level of $p < 0.05$. To improve the robustness and interpretation of the findings, biological variability was reported as standard deviation accompanying the means in all results tables. In addition, effect sizes were calculated to assess the magnitude of *A. terreus* supplementation, and 95% confidence intervals were determined for key digestibility and fermentation parameters to provide more precise estimates of the observed physiological responses.

Results

Rumen Total Bacteria and Protozoa

The total bacterial and protozoal populations were not affected by *A. terreus* supplementation (Table 3).

Table 3. Effects of *A. terreus* supplementation on total rumen bacterial and protozoal populations during *in vitro* fermentation

Variables	Treatments				*p*-value
	Control (C)	C + 1.5% isolate	C + 3% isolate	C + 4.5% isolate	
Bacteria (log CFU mL^{-1})	9.87 ± 0.84	9.04 ± 1.12	8.97 ± 0.87	8.19 ± 1.07	0.10
Protozoa (log cells mL^{-1})	5.38 ± 0.09	5.40 ± 0.08	5.39 ± 0.71	5.42 ± 0.04	0.83

Effects of Aspergillus terreus Supplementation on Rumen Microbial Populations

The response of the rumen microbial populations to fungal supplementation was broadly consistent across the experimental treatments. As presented in Table 3, the total bacterial population showed a numerical decline as the inclusion level of Aspergillus terreus increased, whereas the protozoal population remained relatively similar among treatments. However, neither response reached statistical significance. These findings indicate that the supplementation treatments did not produce a statistically detectable change in the total abundance of the microbial groups measured under the experimental conditions. The results should be interpreted in relation to the scope of the measurements, because total population counts do not reveal whether the abundance or activity of particular microbial species changed during incubation.

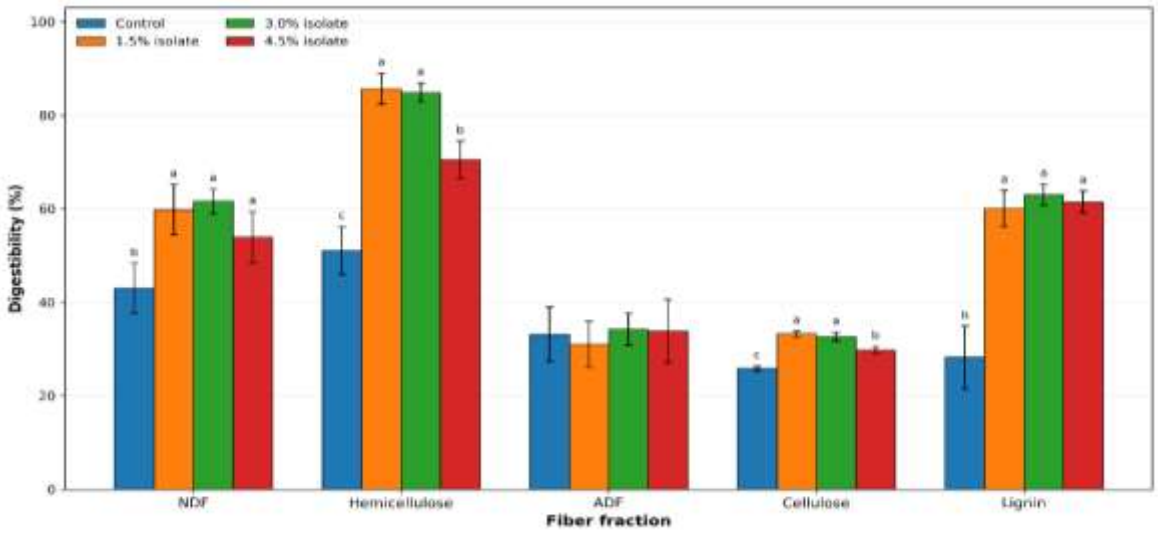
Fiber and Nutrient Digestibility

The inclusion of marine fungi (up to 4.5%) did not affect crude fiber digestibility. The addition of the marine endophytic fungal isolate increased the digestibility of structural carbohydrates, particularly at the 1.5% and 3.0% inclusion levels. Neutral detergent fiber (NDF) digestibility increased significantly ($p < 0.01$) in the 1.5% and 3.0% fungal isolate treatments. Similarly, hemicellulose digestibility increased, and cellulose digestibility also increased significantly in the 1.5% and 3.0% fungal isolate treatments ($p < 0.01$). Lignin digestibility was significantly higher in all fungal isolate treatments than in the control ($p < 0.01$). In contrast, acid detergent fiber (ADF) digestibility was not significantly affected by fungal isolate supplementation ($p = 0.12$) (Table 4).

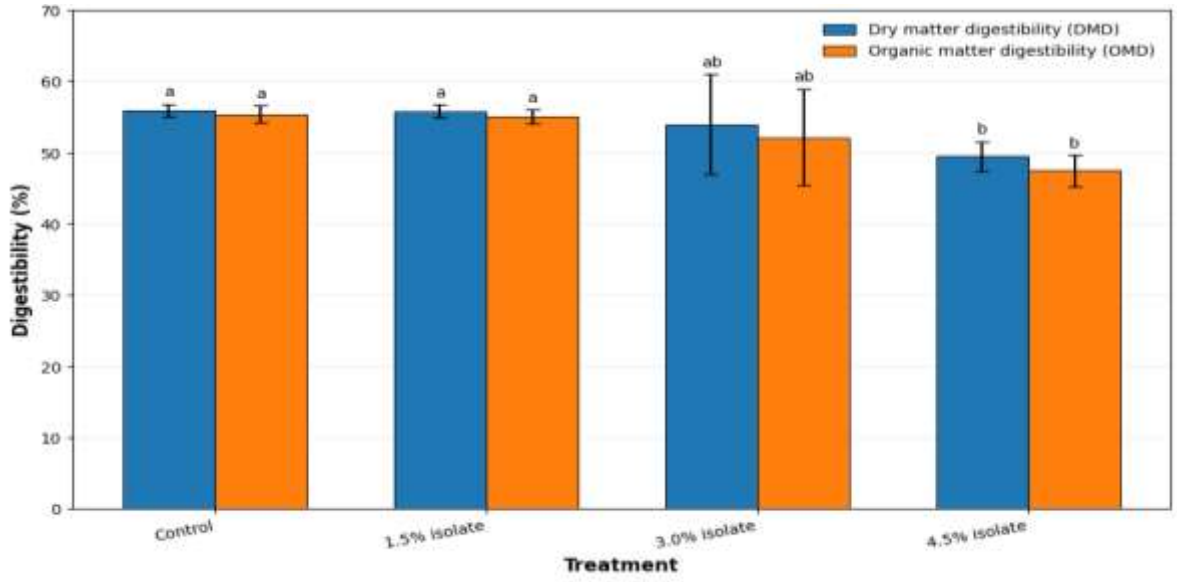
Table 4. Effects of *A. terreus* supplementation on the digestibility of fiber fractions and nutrients following 48-h *in vitro* incubation

Digestibility variables	Treatments				
	Control (C)	C + 1.5% isolate	C + 3% isolate	C + 4.5% isolate	*p-Value*
Crude fiber (%)	60.81 ± 0.76^{a}	60.58 ± 0.62^{a}	58.88 ± 5.84^{ab}	54.80 ± 1.94^{b}	0.03
NDF (%)	42.97 ± 5.36^{b}	59.79 ± 5.37^{a}	61.57 ± 2.63^{a}	53.91 ± 5.43^{a}	<0.01
Hemicellulose (%)	51.03 ± 5.09^{c}	85.62 ± 3.32^{a}	84.84 ± 1.97^{a}	70.47 ± 3.97^{b}	<0.01
ADF (%)	33.17 ± 5.76	31.08 ± 4.87	34.24 ± 3.51	33.83 ± 6.73	0.12
Cellulose (%)	25.8 ± 0.50^{c}	33.20 ± 0.60^{a}	32.60 ± 0.90^{a}	29.80 ± 0.70^{b}	<0.01
Lignin (%)	28.22 ± 6.70^{b}	60.05 ± 3.86^{a}	62.96 ± 2.30^{a}	61.46 ± 2.32^{a}	<0.01
DMD (%)	55.96 ± 0.85^{a}	55.86 ± 0.92^{a}	54.01 ± 7.03^{ab}	49.49 ± 2.09^{b}	0.04
OMD (%)	55.42 ± 1.22^{a}	55.08 ± 0.94^{a}	52.17 ± 6.78^{ab}	47.50 ± 2.17^{b}	<0.01

Note: *Different superscripts within the same row indicate significant differences ($p \leq 0.05$)*



Note: *Bars show mean ± SD. Different letters within a fiber fraction indicate significant differences ($p \leq 0.05$); ADF treatment effect was not significant ($p = 0.12$).*



Note. *Bars represent means ± standard deviations (SD). Different letters within each measure indicate significant differences among treatments ($p \leq 0.05$). DMD, dry matter digestibility; OMD, organic matter digestibility.*

The digestibility results showed different responses among the measured fiber and nutrient fractions. Neutral detergent fiber, hemicellulose, and cellulose digestibility responded more favorably to the lower fungal inclusion levels than to the highest inclusion level. Lignin digestibility was higher in all supplemented treatments than in the control, whereas acid detergent fiber digestibility did not differ significantly among treatments. In contrast, dry matter and organic matter digestibility were lower at the highest supplementation level. This pattern demonstrates that the response of individual structural components was not identical to the response of the complete feed substrate. The results therefore support evaluating fiber fraction digestibility together with broader measures of nutrient utilization when assessing fungal supplementation.

Rumen Fermentation Characteristics

Supplementation with the marine fungal isolate had limited effects on overall rumen fermentation characteristics. Among the measured variables, only ruminal pH was significantly affected by the treatments ($p = 0.04$), whereas NH_3 concentration, total VFA production, VFA molar proportions, the acetate-to-propionate ratio (C2:C3), and methane production remained statistically unchanged ($p > 0.05$).

The pH values ranged from 6.96 to 7.03, remaining within the optimal range for rumen microbial activity and fiber degradation. The 3.0% fungal isolate treatment resulted in a slightly lower pH (6.96) than the control and other treatments (Table 5).

Table 5. Rumen fermentation characteristics and estimated methane production at varying levels of *A. terreus* isolate supplementation

Variables	Treatments				
	Control (C)	C + 1.5% isolate	C + 3% isolate	C + 4.5% isolate	*p-value*
pH	7.03 ± 0.01[b]	7.03 ± 0.04[b]	6.96 ± 0.34[a]	7.01 ± 0.05[b]	0.04
NH_3 (mM)	12.35 ± 1.96	9.46 ± 2.50	9.39 ± 2.89	8.62 ± 1.39	0.08
Total VFA (mM)					
4 h incubation	85.54 ± 24.71	77.85 ± 16.13	85.79 ± 19.32	91.93 ± 14.22	0.77
8 h incubation	107.80 ± 9.75	101.79 ± 9.20	101.29 ± 14.78	112.74 ± 25.36	0.64
Molar proportion of VFA (% mM)					
Acetate/C2 (%)					
4 h incubation	63.81 ± 10.47	57.98 ± 6.85	51.92 ± 3.45	62.68 ± 11.62	0.25
8 h incubation	67.08 ± 5.80	66.56 ± 8.30	67.06 ± 4.25	67.31 ± 7.26	0.99
Propionate/C3 (%)					
4 h incubation	27.72 ± 10.80	32.43 ± 4.43	37.90 ± 4.49	29.81 ± 10.43	0.36
8 h incubation	26.19 ± 5.07	25.00 ± 7.76	24.57 ± 4.71	25.81 ± 7.07	0.97
Butyrate/C4 (%)					
4 h incubation	6.65 ± 0.77	7.98 ± 2.06	8.90 ± 1.75	6.57 ± 1.14	0.14
8 h incubation	5.77 ± 0.73	7.38 ± 1.70	7.34 ± 0.52	6.04 ± 1.25	0.07
Valerate/C5 (%)					
4 h incubation	1.81 ± 0.76	1.59 ± 0.87	1.28 ± 0.33	0.92 ± 0.10	0.23
8 h incubation	0.95 ± 0.09	1.04 ± 0.19	1.00 ± 0.09	0.83 ± 0.15	0.13
C2:C3 ratio					
4 h incubation	2.70 ± 1.44	1.83 ± 0.46	1.39 ± 0.25	2.62 ± 1.99	0.42
8 h incubation	2.69 ± 0.88	3.01 ± 1.52	2.84 ± 0.74	2.85 ± 1.16	0.97
CH_4 (mM)					
4 h incubation	19.15 ± 4.65	16.11 ± 5.27	14.19 ± 3.89	22.63 ± 7.66	0.20
8 h incubation	27.48 ± 6.18	26.26 ± 4.65	26.36 ± 2.42	28.69 ± 7.57	0.88

Note. Different superscripts within the same row indicate significant differences ($p \leq 0.05$). Methane production was estimated stoichiometrically from VFA molar proportions.

The fermentation measurements indicated that the effects of fungal supplementation were limited for most of the variables examined. The treatment-related difference in ruminal pH was statistically significant, while ammonia concentration, total volatile fatty acid production, individual volatile fatty

acid proportions, the acetate-to-propionate ratio, and estimated methane production did not differ significantly among treatments. Although some numerical variation occurred between treatments and incubation times, these differences should not be interpreted as confirmed treatment effects in the absence of statistical significance. In addition, because methane production was estimated from volatile fatty acid proportions, the findings describe predicted methane responses rather than direct measurements of methane gas. These distinctions are important when interpreting the fermentation results and considering the potential application of the fungal isolate.

Discussion

The addition of the marine fungal isolate did not affect total bacterial or protozoal populations. This finding indicates that *A. terreus* supplementation did not alter the total populations of these microbial groups. The absence of an effect on total bacterial and protozoal populations indicates that the marine fungal isolate does not substantially alter the overall abundance of these microbial groups in the rumen. Although endophytic marine fungal extracts have exhibited antibacterial activity against *Salmonella typhimurium* (Nabila et al., 2024) and *Escherichia coli* (Kurnia et al., 2024), *A. terreus* supplementation did not affect total rumen bacterial or protozoal populations. As rumen bacteria are necessary for feed digestion, the stability of their total population suggests that fungal supplementation did not disrupt the overall microbial balance or bacterial fermentative activity. Previous studies have shown that fungal supplementation can have varied effects on rumen microbial populations. Due to their high hydrogen production, anaerobic fungi and rumen methanogenic archaea can form relatively stable cocultures (Dehority & Tirabasso, 2000). We acknowledge that the lack of molecular analyses, such as 16S rRNA sequencing or qPCR, limits our ability to identify specific changes in the abundance of key fibrolytic microorganisms. Such high-resolution techniques could provide deeper mechanistic insights into whether the observed improvements are attributable solely to fungal enzymatic activity or involve synergistic stimulation of specific bacterial groups. Therefore, future research incorporating molecular profiling is warranted to further characterize the interaction between *A. terreus* and the complex rumen microbial community.

The significant improvements in fiber digestibility observed in this study, particularly at the 1.5% and 3.0% supplementation levels, are likely driven by the enzymatic profile of the marine endophytic fungus *A. terreus*. NDF digestibility increased significantly from 42.97% in the control treatment to 59.79% and 61.57% in the 1.5% and 3.0% fungal isolate treatments, respectively. Hemicellulose digestibility also improved markedly, reaching >84% in both treatments compared with 51.03% in the control. Cellulose digestibility also increased markedly from 25.8% in the control to approximately 33% in the 1.5% and 3.0% fungal isolate treatments. These results suggest that the fungal isolate may produce fibrolytic enzymes that enhance the degradation of plant cell wall components during ruminal fermentation (Khan et al., 2025). Previous research has demonstrated that *Aspergillus terreus* can produce cellulolytic enzymes, including endoglucanase and β-glucosidase, which contribute to cellulose degradation (Kumar and Parikh, 2015; Sohail et al., 2016).

Although direct enzyme assays were not conducted in this trial, the marked increase in lignin digestibility from 28.22% in the control to >60% in the fungal-supplemented treatments may indicate ligninolytic activity. Similarly, previous research has shown that white-rot fungal treatment can improve lignin degradation and the in vitro ruminal degradability of lignocellulosic feedstocks (Nayan et al., 2019). The ability to partially degrade lignin may have facilitated microbial access to cellulose and hemicellulose fractions, thereby improving overall fiber utilization. Lignin degradation by fungi is associated with the production of oxidative extracellular enzymes, including Lac, LiP, and MnP (Imran, 2016). Genomic analysis of *Aspergillus terreus* has also identified genes associated with lignocellulolytic activity, including β-glucosidases involved in cellulose degradation (Dadheech et al., 2019).These enzymes initiate lignin depolymerization through redox reactions, converting complex aromatic polymers into smaller phenolic compounds. Lignin degradation is an oxidative process driven by radical-mediated reactions. Research has shown that fungi can efficiently degrade lignin using their mycelia to penetrate plant cell walls. The mycelia secrete enzymes that catalyze the one-electron oxidation of lignin units, generating various radical intermediates that ultimately break down into quinones, aromatic aldehydes, alcohols, and ring-opened products (Gao et al., 2025).

In contrast, ADF digestibility was not markedly affected by fungal isolate supplementation, indicating that the fungal treatment had a greater effect on hemicellulose and cellulose degradation than on the more recalcitrant ADF fraction.

Although fiber digestibility generally improved at the lower supplementation levels, supplementation at 4.5% was associated with lower overall feed digestibility. Crude fiber digestibility decreased compared

with that in the control treatment, suggesting that higher inclusion levels of the fungal isolate may have altered fermentation dynamics or exerted inhibitory effects on rumen microbial activity, resulting in reduced nutrient utilization. Such alterations may be associated with the enzymatic activity of the fungus, particularly its ability to produce ligninolytic and hemicellulolytic enzymes that can disrupt the lignocellulosic matrix (Durães Sette & Costa Bonugli Santos, 2013; Mäkelä et al., 2016).

The findings of the study indicate that the effects of *Aspergillus terreus* supplementation differed according to the digestibility parameter evaluated. Improvements in several fiber fractions did not correspond to a consistent improvement in overall feed digestibility, highlighting the importance of considering individual fiber components alongside broader measures of nutrient utilization. The lower dry matter and organic matter digestibility observed at the highest supplementation level further emphasizes that improved degradation of selected structural components does not necessarily translate into greater utilization of the complete feed substrate. However, the experiment does not establish the mechanism responsible for this response. Further investigation of fungal enzyme activity and feed composition would help clarify the relationship between fungal supplementation, structural carbohydrate degradation, and overall digestibility. These findings support a careful evaluation of the fungal inclusion level and indicate that its nutritional effects should be assessed using multiple digestibility measures rather than a single parameter. Previous research has also examined fungal-treated lignocellulosic biomass as a potential feed ingredient for ruminants, highlighting the importance of evaluating both the benefits of biological treatment and its effects on feed quality (Van Kuijk et al., 2015).

Marine-derived fungi are potent producers of extracellular enzymes, such as cellulases, xylanases, and Lac, which can degrade complex polysaccharides and phenolic polymers (Bonugli Santos et al., 2015). The increased digestibility of lignin and hemicellulose suggests partial modification of the lignocellulosic matrix, which could enhance the accessibility of cellulose to microbial degradation in the rumen. The increase in cellulose digestibility in the treated samples may reflect a relative concentration effect resulting from the breakdown of hemicellulose and lignin fractions rather than an absolute increase in cellulose concentration. Related research has demonstrated that β-mannanase produced by Aspergillus terreus can hydrolyze pretreated lignocellulosic biomass, supporting its potential role in carbohydrate breakdown (Soni et al., 2016). Similarly, xylanase production by *Aspergillus terreus* has been investigated for the saccharification of rice straw, supporting the potential of this species in lignocellulosic biomass conversion (Sharma and Bajaj, 2018).

By breaking down plant cell walls with their rhizoids, anaerobic fungi facilitate access to these walls for other microorganisms. Within the rumen microbial community, they also possess strong enzymatic activity for cell wall degradation (Agustina et al., 2024). The marine endophytic fungus *A. terreu*s exhibits ligninolytic activity, which may enhance the nutritional value of lignocellulosic feed materials. This fungus produces oxidative extracellular enzymes, such as Lac, LiP, and MnP, which initiate lignin depolymerization through redox reactions, converting complex aromatic polymers into smaller phenolic compounds (Durães Sette & Costa Bonugli Santos, 2013). Through partial delignification, *A. terreus* increases the accessibility of structural carbohydrates, such as cellulose and hemicellulose, to rumen microbes, thereby enhancing fiber degradability and nutrient utilization. Marine isolates of *A. terreus* also exhibit halotolerance and broad enzymatic stability across a range of temperatures and pH values, making them potentially suitable for the biological pretreatment of agricultural byproducts, such as palm leaves, used in ruminant diets. Hence, lignin modification by *A. terreus* offers a sustainable strategy to improve the digestibility of low-quality roughages and reduce feed costs in ruminant production systems. Although statistically significant, the decrease in pH was small and remained within the normal physiological range for rumen fermentation. A lower pH typically indicates increased feed degradation and microbial fermentation, leading to the production of acidic metabolites such as VFAs. The fungi could promote the growth or activity of acid-producing microbes or contribute to the breakdown of fiber or other substrates (Jyothi et al., 2024), generating acids that reduce pH. The decrease in rumen pH may have contributed to the numerical reduction in ruminal ammonia concentration. Ammonia (NH_3) in the rumen is primarily produced through microbial degradation of dietary protein and deamination of amino acids, processes that are optimal at a neutral to slightly alkaline pH. When rumen pH declines, typically of rapid fermentation of readily fermentable carbohydrates and subsequent accumulation of VFAs, the activity of proteolytic and deaminating microorganisms is inhibited (Russell & Hino, 1985).

Ammonia (NH_3) concentrations tended to decrease as fungal isolate supplementation increased. Although this decrease was not statistically significant, the trend may indicate improved nitrogen utilization for microbial protein synthesis, thereby reducing ammonia accumulation in the fermentation

medium. Total VFA production at both 4 and 8 h of incubation was not substantially influenced by fungal isolate supplementation. However, the highest total VFA concentration at both incubation times was observed in the 4.5% fungal isolate treatment at 8 h. These results indicate that the fungal isolate did not impair fermentative activity and may have supported fermentation intensity at higher inclusion levels, although the differences were not statistically significant. The molar proportions of individual VFAs were also unaffected by treatment. Nevertheless, numerical differences were observed after 4 h of incubation. The 3.0% fungal isolate treatment had the lowest acetate proportion and the highest propionate proportion, resulting in the lowest C2:C3 ratio (1.39). Such a shift toward propionate production may be considered favorable propionate serves as a glucogenic precursor, and its formation can be associated with reduced hydrogen availability for methanogenesis.

In this study, methane (CH_4) production was estimated from the stoichiometric proportions of VFAs, a method that reflects the balance between hydrogen donors and sinks during fermentation Meta-analysis and machine-learning approaches have also been applied to predict methane production from in-vitro rumen fermentation data, providing an alternative approach for evaluating methane outcomes (Ellis et al., 2020). Although no statistically significant differences were observed among treatments, the 3.0% *A. terreus* treatment exhibited a favorable numerical trend, with the lowest estimated methane production during the early (4 h) incubation stage. This numerical trend coincided with the lowest acetate-to-propionate (C2:C3) ratio (1.39) observed in the same treatment. Biologically, increased propionate production provides a competing pathway for hydrogen, thereby reducing the hydrogen available to methanogenic archaea for CH_4 production (Janssen, 2010; K. Wang et al., 2023; Beauchemin et al., 2020). Although these stoichiometric estimates provide a useful screening approach for evaluating the antimethanogenic potential of *A. terreus*, indirect calculations may not capture the full complexity of gas dynamics, including direct inhibition of methanogens or changes in total gas volume (Martins et al., 2024). Therefore, while the current findings suggest that *A. terreus* supplementation did not substantially alter total rumen bacterial or protozoal populations, future validation through direct methane quantification, such as gas chromatography, is required to confirm the effects of this marine isolate on greenhouse gas emissions and its potential role as a rumen modifier (O'Donnell et al., 2024).

The addition of the marine fungal isolate maintained normal rumen fermentation conditions within the normal range, with no adverse effects on pH, VFA production, or nitrogen metabolism Other in-vitro studies have also evaluated feed supplementation for its effects on rumen fermentation, nutrient degradability, microbial populations, and methane mitigation (Muslykhah et al., 2024). Although most fermentation parameters were not significantly altered, the 3.0% fungal isolate treatment showed favorable numerical trends, including lower pH, a higher propionate proportion, a lower C2:C3 ratio, and lower estimated methane production during the early fermentation stage. Although 1.5% and 3.0% *A. terreus* supplementation levels appeared more favorable, the 4.5% level significantly reduced overall feed utilization, with DMD and OMD decreasing to 49.49% and 47.50%, respectively. This threshold effect may be attributed to two factors: first, extensive lignin degradation (>60%) accumulates phenolic monomers that inhibit sensitive cellulolytic bacteria; second, antimicrobial secondary metabolites produced by *A. terreus* could potentially alter microbial metabolic activity without substantially affecting total bacterial counts. In addition, the numerically higher total VFA concentration at 8 h in the 4.5% treatment may indicate greater fermentation activity at this time point, although the difference was not statistically significant. Thus, precise dosing of *A. terreus* is essential to reduce disruption of the rumen ecosystem (Fhonna et al., 2026).

The findings also highlight the importance of distinguishing changes in structural carbohydrate degradation from changes in overall fermentation efficiency.

The findings of this study should be interpreted in light of several methodological limitations. First, the experiment was conducted under *in vitro* conditions, which cannot fully reproduce the complex physiological processes occurring in live ruminants. Factors such as voluntary feed intake, digesta passage rate, nutrient absorption, and animal metabolism were not evaluated. Therefore, the improvements observed in selected fiber fractions cannot yet be assumed to produce equivalent benefits in animal performance or total-tract digestibility.

Second, methane production was estimated from volatile fatty acid proportions rather than measured directly. Although this approach provides an indication of potential changes in fermentation pathways, it does not establish the actual quantity of methane released. Future experiments should include direct gas measurements to determine whether the numerical differences observed among treatments represent meaningful changes in methane production.

Third, the study did not directly measure the extracellular enzyme activities of *Aspergillus terreus*. Consequently, the observed changes in lignin, hemicellulose, and cellulose digestibility cannot be

attributed conclusively to specific fungal enzymes. Further work should evaluate relevant fibrolytic and ligninolytic enzyme activities and examine changes in feed composition to clarify the mechanisms underlying the digestibility responses.
Finally, total bacterial and protozoal counts do not provide detailed information about changes in individual microbial groups or their functions. Future research incorporating molecular microbial profiling could help characterize the interactions between the fungal isolate and the rumen microbial community. Studies involving additional inoculum levels and subsequent *in vivo* validation would also help establish the reproducibility, safety, and practical relevance of the observed responses. These investigations are necessary before recommending a specific supplementation level for routine livestock feeding.

Fungal supplementation may improve the accessibility of selected fiber components without producing a consistent improvement in the utilization of the complete feed substrate. This response may depend on the balance between beneficial enzymatic activity and other changes in the fermentation environment. Consequently, the evaluation of fungal feed additives should include multiple indicators of nutrient utilization rather than relying exclusively on fiber digestibility. The present findings also emphasize the need for caution when interpreting methane estimates derived from volatile fatty acid profiles. Such estimates can indicate possible changes in fermentation pathways, but they cannot independently establish the extent of methane mitigation. Future studies should also consider the methodological limitations of in vitro gas-production and rumen-digestibility assessments when interpreting experimental findings and comparing results across studies (Chávez-Solis et al., 2026). This integrated approach would help determine whether the benefits observed under in vitro conditions can be translated into practical improvements in ruminant nutrition.
In conclusion, *A. terreus* supplementation at 1.5%–3.0% improved the digestibility of fiber fraction, including lignin, hemicellulose, and cellulose, without adversely affecting overall rumen fermentation, showing strong potential for improving the utilization of low-quality roughages. However, these findings were obtained under strictly *in vitro* conditions, they do not account for *in vivo* factors such as feed passage rate, voluntary intake, or metabolic responses. Consequently, *in vivo* trials are necessary to determine the optimal inclusion level and evaluate its effects on animal performance, total-tract nutrient utilization, and direct methane emissions.

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Author Contributions
Sri Suharti and Kustiariyah Tarman conceived and designed the study. Sri Suharti, Sheren Nabila, and Ade Dimas Kurnia conducted the experiments, collected the data, performed the statistical analyses, and prepared the initial draft of the manuscript. Sri Suharti, Ali Bain, Rahman, and Komang G. Wiryawan supervised the research, contributed to data interpretation, and critically revised the manuscript. All authors reviewed, edited, and approved the final version of the manuscript.

Data Availability Statement
All data generated or analyzed during this study are included in this published article.

Conflict of Interest
The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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