

Effects of Multi-Strain Microbial Inoculants on Fermentation Quality and *In Vitro* Digestibility of Rice Straw Silage

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ABSTRACT

Rice straw is one of the most widely available agricultural by-products in Thailand. However, its direct use as ruminant feed is constrained by low crude protein concentration, high structural fiber content, and the presence of a lignocellulosic matrix that limits microbial degradation in the rumen. This study evaluated the effects of selected microbial inoculant combinations on fermentation quality, chemical composition, fiber fractions, and *in vitro* gas production of rice straw silage. Chopped rice straw was assigned to five microbial treatments in a completely randomized design with three replications: T1, *Trichoderma reesei*; T2, *Saccharomyces cerevisiae*; T3, *T. reesei* + *S. cerevisiae*; T4, *T. reesei* + *S. cerevisiae* + *Bacillus licheniformis*; and T5, *T. reesei* + *S. cerevisiae* + *Lactobacillus plantarum*. The treated materials were ensiled under anaerobic conditions and evaluated after 7, 14, 21, and 28 days of fermentation. Microbial inoculation significantly influenced fiber degradation, particularly neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) ($P < 0.001$). Among the treatments, T5 showed the most favorable response after 28 days of fermentation, with the lowest NDF (69.47%), ADF (47.66%), and ADL (19.02%) values. The lowest pH value was also observed in T5 at 21 days (4.65), suggesting improved acidification during ensiling. *In vitro* gas production differed significantly among treatments at 28 days ($P < 0.01$), with T5 producing the highest gas volume (74.5 mL), indicating greater ruminal fermentability. These results suggest that the combined application of cellulolytic fungi, yeast, and lactic acid bacteria can enhance lignocellulose degradation and improve the feeding value of rice straw silage. Therefore, the inoculant combination of *T. reesei* + *S. cerevisiae* + *L. plantarum* may be considered a promising biological strategy for improving the utilization of rice straw in ruminant production systems.

Keywords: Rice straw silage; Multi-strain Inoculants; Lignocellulose degradation; *In vitro* gas production; Rumen fermentation

INTRODUCTION

Rice straw is a major crop residue generated from rice production in Thailand and other rice-producing regions. Owing to the large annual volume of rice cultivation, rice straw represents an important potential roughage resource for ruminant production. Nevertheless, its practical use as feed remains limited because untreated rice straw is characterized by low nutritive value, poor palatability, and low digestibility. In many production areas, rice straw is left unused or burned in the field, which contributes to air pollution and the loss of potentially valuable biomass. Improving the feeding value of rice straw is therefore relevant not only for reducing feed costs but also for promoting more efficient resource use and sustainable livestock production.

The main nutritional limitation of rice straw is its high concentration of lignocellulosic fiber and low crude protein content. The plant cell wall of rice straw consists primarily of cellulose, hemicellulose,

and lignin. Among these components, lignin is particularly resistant to microbial degradation and forms complex physical and chemical associations with cellulose and hemicellulose. These associations restrict the access of rumen microorganisms and fibrolytic enzymes to fermentable carbohydrates, resulting in reduced voluntary intake and poor digestibility. For this reason, several physical, chemical, and biological treatments have been investigated to improve the feeding value of low-quality roughages. Among these approaches, biological treatment using microorganisms has received increasing attention because it is relatively safe, environmentally friendly, and potentially suitable for farm-level application.

Microbial inoculants may improve silage quality and fiber utilization through different mechanisms. *Trichoderma reesei* is a cellulolytic fungus capable of producing cellulase and hemicellulase enzymes, which can hydrolyze

structural carbohydrates in plant cell walls. *Saccharomyces cerevisiae* may contribute to the fermentation process by consuming residual oxygen, supporting the establishment of anaerobic conditions, and stimulating beneficial microbial activity. *Bacillus licheniformis* is known to produce extracellular enzymes, including protease and xylanase, which may assist in protein and hemicellulose degradation. In contrast, *Lactobacillus plantarum* is a homofermentative lactic acid bacterium that rapidly produces lactic acid, reduces pH, and inhibits undesirable microorganisms during ensiling. These distinct functional characteristics indicate that combinations of microorganisms with complementary activities may be more effective than single-strain inoculation.

Although previous studies have reported beneficial effects of individual microbial inoculants on silage fermentation, information on the combined use of cellulolytic fungi, yeast, *Bacillus* spp., and lactic acid bacteria for improving rice straw silage remains limited. In particular, few studies have examined how different microbial combinations influence fiber fractions and *in vitro* ruminal fermentability across different fermentation periods. Therefore, further evaluation is needed to clarify whether multi-strain inoculation can improve lignocellulose degradation and enhance the digestibility of rice straw silage.

This study was based on the hypothesis that multi-strain microbial inoculation would improve fermentation quality, reduce fiber fractions, and increase *in vitro* gas production of rice straw silage compared with single-strain inoculation. The objective of this study was to evaluate the effects of different microbial inoculant combinations on chemical composition, pH, NDF, ADF, ADL, and *in vitro* gas production of rice straw silage after 7, 14, 21, and 28 days of fermentation.

MATERIALS AND METHODS

Experimental design and treatments

The experiment was arranged in a completely randomized design (CRD) with five microbial treatments and three replications per treatment. Rice straw was chopped into approximately 3–5 cm lengths to improve mixing uniformity and increase the contact surface between the substrate and microbial inoculants. The chopped rice straw was thoroughly mixed and randomly allocated to the following treatment groups.

T1: *Trichoderma reesei*

T2: *Saccharomyces cerevisiae*

T3: *Trichoderma reesei* + *Saccharomyces cerevisiae*

T4: *Trichoderma reesei* + *Saccharomyces cerevisiae* + *Bacillus licheniformis*

T5: *Trichoderma reesei* + *Saccharomyces cerevisiae* + *Lactobacillus plantarum*

Preparation and ensiling of rice straw

Each microbial inoculant was prepared as a suspension and applied uniformly to the rice straw at approximately 1×10^6 CFU/g fresh matter. After inoculation, the treated rice straw was mixed thoroughly to ensure an even distribution of microorganisms throughout the material. The samples were then packed tightly into airtight plastic containers or fermentation bags to minimize oxygen exposure and promote anaerobic conditions. All silage samples were stored at room temperature and opened for analysis after 7, 14, 21, and 28 days of fermentation.

Chemical composition and fiber analysis

At each fermentation period, representative silage samples were collected from each replicate. The samples were analyzed for dry matter, ash, crude protein, and ether extract according to standard AOAC procedures. Fiber fractions, including neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL), were determined using the detergent fiber method. Silage pH was measured by mixing a representative sample with distilled water, filtering the extract, and measuring the filtrate using a calibrated pH meter.

In vitro gas production

In vitro gas production was used as an indicator of ruminal fermentability and potential digestibility. The procedure was conducted according to the principle of the *in vitro* gas production technique. Rumen fluid was collected before morning feeding and maintained under anaerobic conditions. The rumen fluid was filtered through warm cheesecloth and mixed with buffer solution at a ratio of 1:2 under continuous CO₂ flushing. Approximately 200 mg of dried and ground silage sample was incubated with buffered rumen fluid in calibrated incubation bottles or syringes at 39 °C. Gas production was recorded after incubation using

a gas-measuring device or pressure transducer. Gas volume was expressed as milliliters per sample and used to compare the fermentability of rice straw silage among treatments.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) according to a completely randomized design. When significant treatment effects were detected, treatment means were compared using an appropriate multiple comparison test. Statistical significance was declared at $P < 0.05$, while highly significant differences were declared at $P < 0.01$ or $P < 0.001$. Results are presented as mean \pm standard deviation.

RESULTS AND DISCUSSION

Chemical composition and pH

The effects of microbial inoculation on chemical composition and pH of rice straw silage are presented in Table 1. Dry matter content ranged from approximately 94.35% to 96.92% across treatments and fermentation periods. Significant differences among treatments were observed at all sampling times ($P < 0.01$ or $P < 0.001$). Ash content also differed significantly among treatments throughout fermentation ($P < 0.001$). Crude protein content showed a significant treatment effect only at 14 days

($P < 0.01$), whereas no significant differences were detected at 7, 21, or 28 days. Ether extract differed significantly at 7, 14, and 28 days ($P \leq 0.01$), but not at 21 days. The pH values varied among treatments, with significant differences observed at 14 days ($P < 0.01$). Although treatment differences were not significant at 21 days, the lowest pH value was recorded in T5 (4.65), indicating a stronger acidification response in this treatment

Table 1. Effects of multi-strain microbial inoculation on chemical composition and pH of rice straw silage during different fermentation periods

Item	Time	T1	T2	T3	T4	T5	P-value
Dry matter	7 d	96.92 \pm 0.21 ^a	95.72 \pm 0.70 ^b	96.20 \pm 0.03 ^c	95.24 \pm 0.09 ^d	95.11 \pm 0.20 ^d	<0.001
	14 d	95.07 \pm 0.27 ^a	95.67 \pm 0.02 ^b	95.45 \pm 0.17 ^{ab}	96.44 \pm 0.21 ^c	95.51 \pm 0.23 ^{ab}	<0.001
	21 d	96.24 \pm 0.31 ^a	94.35 \pm 0.92 ^b	95.41 \pm 0.22 ^{ab}	96.15 \pm 0.02 ^a	96.14 \pm 0.01 ^a	<0.01
	28 d	95.66 \pm 0.54 ^a	95.31 \pm 0.48 ^{ab}	95.27 \pm 0.08 ^{ab}	94.41 \pm 0.33 ^b	95.73 \pm 0.24 ^a	<0.01
Ash	7 d	3.38 \pm 0.56 ^a	4.28 \pm 0.07 ^{bc}	3.80 \pm 0.03 ^{ab}	4.76 \pm 0.09 ^{cd}	5.02 \pm 0.95 ^d	<0.001
	14 d	4.93 \pm 0.27 ^a	4.33 \pm 0.02 ^b	4.56 \pm 0.18 ^{ab}	3.56 \pm 0.21 ^c	4.49 \pm 0.23 ^{ab}	<0.001
	21 d	3.76 \pm 0.31 ^a	5.99 \pm 0.71 ^b	4.59 \pm 0.22 ^a	3.85 \pm 0.02 ^a	3.86 \pm 0.01 ^a	<0.001
	28 d	3.56 \pm 0.25 ^a	4.69 \pm 0.48 ^b	4.59 \pm 0.26 ^b	5.59 \pm 0.33 ^c	4.27 \pm 0.24 ^{ab}	<0.001
Crude protein	7 d	2.87 \pm 0.00	2.58 \pm 0.34	2.75 \pm 0.33	3.16 \pm 0.42	2.63 \pm 0.39	0.28
	14 d	3.10 \pm 0.10 ^a	2.31 \pm 0.13 ^b	2.98 \pm 0.41 ^{ab}	2.36 \pm 0.06 ^b	2.38 \pm 0.34 ^b	<0.01
	21 d	2.67 \pm 0.14	2.43 \pm 0.24	2.52 \pm 0.22	2.45 \pm 0.41	2.15 \pm 0.16	0.22
	28 d	2.54 \pm 0.44	2.63 \pm 0.29	2.33 \pm 0.30	2.69 \pm 0.03	2.49 \pm 0.19	0.61
Ether extract	7 d	0.76 \pm 0.00 ^a	0.78 \pm 0.09 ^a	0.82 \pm 0.07 ^a	0.77 \pm 0.15 ^a	0.53 \pm 0.03 ^b	0.01
	14 d	1.89 \pm 0.44 ^a	1.68 \pm 0.24 ^{ab}	0.83 \pm 0.20 ^c	1.81 \pm 0.23 ^{ac}	1.03 \pm 0.08 ^{bc}	<0.001
	21 d	0.88 \pm 0.08	0.79 \pm 0.21	0.98 \pm 0.17	1.19 \pm 0.39	1.37 \pm 0.50	0.21
	28 d	0.83 \pm 0.09 ^a	0.67 \pm 0.14 ^{ab}	0.48 \pm 0.01 ^b	0.77 \pm 0.01 ^a	0.81 \pm 0.01 ^a	<0.001
pH	7 d	6.90 \pm 0.17	5.42 \pm 1.44	6.35 \pm 0.80	6.74 \pm 0.11	6.65 \pm 0.83	0.27
	14 d	6.04 \pm 0.61 ^{ab}	5.82 \pm 0.77 ^{ab}	5.59 \pm 0.70 ^a	7.05 \pm 0.11 ^b	7.11 \pm 0.10 ^b	<0.01
	21 d	4.97 \pm 0.15	5.15 \pm 0.60	5.46 \pm 1.23	5.03 \pm 0.89	4.65 \pm 0.08	0.75
	28 d	5.80 \pm 0.18	5.12 \pm 0.31	5.75 \pm 1.61	5.53 \pm 1.51	5.51 \pm 0.66	0.93

Values are presented as mean \pm standard deviation. Different superscript letters within the same row indicate significant differences among treatments ($P < 0.05$). T1 = *Trichoderma reesei*; T2 = *Saccharomyces cerevisiae*; T3 = *T. reesei* + *S. cerevisiae*; T4 = *T. reesei* + *S. cerevisiae* + *Bacillus licheniformis*; T5 = *T. reesei* + *S. cerevisiae* + *Lactobacillus plantarum*.

Fiber fractions

The effects of microbial inoculation on NDF, ADF, and ADL are shown in Table 2. Significant differences among treatments were observed for all fiber fractions at all fermentation periods (P < 0.05 to P < 0.001). At 28 days, T5 had the lowest NDF value (69.47%), followed by T4

(72.82%) and T2 (72.93%). For ADF, T5 and T4 had lower values than T1 and T2 at 28 days. A particularly clear reduction was observed in ADL, where T5 showed the lowest value at 28 days (19.02%). These results indicate that the T5 combination was the most effective treatment for reducing structural fiber components in rice straw silage.

Table 2. Effects of multi-strain microbial inoculation on fiber fractions of rice straw silage during different fermentation periods.

Item	Time	T1	T2	T3	T4	T5	P-value
NDF	7 d	77.68±0.04 ^a	75.11±0.04 ^b	76.18±0.04 ^c	76.82±0.04 ^d	76.81±0.04 ^d	<0.001
	14 d	75.01±0.04 ^a	75.71±0.04 ^b	75.68±0.04 ^b	75.28±0.04 ^c	78.34±0.04 ^c	<0.001
	21 d	77.11±0.04 ^a	73.92±0.04 ^b	72.38±0.04 ^c	72.65±0.04 ^d	70.14±0.05 ^e	<0.001
	28 d	75.73±0.04 ^a	72.93±0.04 ^b	74.12±0.04 ^c	72.82±0.04 ^b	69.47±0.04 ^d	<0.001
ADF	7 d	53.64±0.04 ^a	52.31±0.04 ^{bc}	51.48±0.36 ^b	49.36±0.04 ^d	52.68±0.31 ^c	0.016
	14 d	52.18±0.04 ^a	52.18±0.06 ^{ab}	50.66±0.04 ^c	51.07±0.04 ^d	51.92±0.04 ^b	<0.001
	21 d	51.67±0.14 ^a	50.67±0.00 ^b	49.18±0.06 ^c	45.01±0.01 ^d	44.06±0.03 ^e	<0.001
	28 d	50.81±0.24 ^a	50.19±0.16 ^a	48.68±0.40 ^b	47.69±0.41 ^b	47.66±0.13 ^b	<0.001
ADL	7 d	33.54±0.01 ^a	38.27±0.30 ^b	34.52±0.52 ^a	35.93±0.15 ^c	30.28±0.34 ^d	<0.001
	14 d	26.27±0.02 ^a	28.12±0.50 ^b	27.54±0.10 ^b	26.52±0.35 ^{ac}	23.44±0.24 ^d	<0.001
	21 d	25.23±0.54 ^a	21.68±0.03 ^b	21.32±0.11 ^b	24.51±0.14 ^a	21.18±0.00 ^b	<0.001
	28 d	22.18±0.36 ^a	20.17±0.07 ^{bc}	20.41±0.24 ^b	22.55±0.44 ^a	19.02±0.34 ^c	<0.001

Values are presented as mean ± standard deviation. Different superscript letters within the same row indicate significant differences among treatments (P < 0.05). T1 = *Trichoderma reesei*; T2 = *Saccharomyces cerevisiae*; T3 = *T. reesei* + *S. cerevisiae*; T4 = *T. reesei* + *S. cerevisiae* + *Bacillus licheniformis*; T5 = *T. reesei* + *S. cerevisiae* + *Lactobacillus plantarum*.

In vitro gas production

The effects of microbial inoculation on in vitro gas production are presented in Table 3. No significant differences among treatments were observed at 7, 14, or 21 days of fermentation (P > 0.05). However, treatment effects became significant

at 28 days (P < 0.01). At this time, T5 produced the highest gas volume (74.5 mL), whereas T2 and T3 produced comparatively lower values. These results suggest that the influence of microbial inoculation on ruminal fermentability became more evident after a longer fermentation period.

Table 3. Effects of multi-strain microbial inoculation on in vitro gas production of rice straw silage during different fermentation periods

Item	Time	T1	T2	T3	T4	T5	P-value
Gas production	7 d	71.0±1.41	70.5±0.71	70.5±0.71	70.0±0.00	71.0±0.00	0.69
	14 d	74.5±2.12	74.0±2.83	77.5±0.71	74.0±2.83	72.0±0.00	0.25
	21 d	73.0±1.41	73.0±4.24	75.0±1.41	72.0±0.00	71.0±1.41	0.52
	28 d	71.0±1.41 ^{ab}	70.0±0.00 ^b	69.0±1.41 ^b	72.0±0.00 ^{ab}	74.5±0.71 ^a	<0.01

Values are presented as mean ± standard deviation. Different superscript letters within the same row indicate significant differences among treatments (P < 0.05). T1 = *Trichoderma reesei*; T2 = *Saccharomyces cerevisiae*; T3 = *T. reesei* + *S. cerevisiae*; T4 = *T. reesei* + *S. cerevisiae* + *Bacillus licheniformis*; T5 = *T. reesei* + *S. cerevisiae* + *Lactobacillus plantarum*.

Effects of microbial inoculation on fermentation quality and chemical composition

The results of the present study indicate that microbial inoculation influenced several chemical characteristics of rice straw silage. Dry matter content remained within a relatively narrow range during fermentation, suggesting that the ensiling process did not result in excessive material loss. This finding is important because substantial dry matter loss can reduce the amount of recoverable feed and may reflect undesirable fermentation. The significant differences in ash content among treatments may be associated with proportional changes in organic matter utilization during fermentation rather than direct changes in mineral concentration. Therefore, ash responses should be interpreted cautiously as part of the overall compositional changes occurring during ensiling.

Crude protein content differed significantly only at 14 days. The higher crude protein value observed in some treatments during this period may be related to microbial biomass accumulation in the early stage of fermentation. However, the subsequent decline or stabilization of crude protein content may be explained by microbial protein turnover, proteolysis, and the use of soluble nitrogen by fermentative microorganisms. The crude protein response was therefore less consistent than the fiber response, indicating that the primary contribution of microbial inoculation in this study was associated more with structural fiber modification than with protein enrichment.

Silage pH is an important indicator of fermentation quality because rapid acidification helps suppress undesirable microorganisms and preserve nutrients. The lowest pH value observed in T5 at 21 days suggests that the inclusion of *Lactobacillus plantarum* promoted lactic acid fermentation. Lactic acid bacteria convert available water-soluble carbohydrates into lactic acid, resulting in pH reduction and improved fermentation stability. Although rice straw contains relatively low levels of readily fermentable carbohydrates, the combined activity of *T. reesei* and *S. cerevisiae* may have increased substrate availability and created a more favorable environment for lactic acid production in T5.

Superior fiber degradation in T5

The most notable finding of this study was the greater reduction of NDF, ADF, and ADL in T5, particularly at 21 and 28 days of fermentation. This response can be explained by the complementary activities of *T. reesei*, *S. cerevisiae*, and *L. plantarum*.

T. reesei is known for its ability to produce cellulolytic and hemicellulolytic enzymes, which can break down cellulose and hemicellulose in plant cell walls. Partial degradation of the fiber matrix may increase the availability of fermentable substrates during ensiling and improve the accessibility of rumen microorganisms to structural carbohydrates.

S. cerevisiae may further support the fermentation process by consuming residual oxygen and helping establish anaerobic conditions. Oxygen removal during the early stage of ensiling is essential because prolonged aerobic conditions can delay lactic acid fermentation and promote the growth of undesirable microorganisms. In addition, yeast metabolites such as vitamins, peptides, and growth factors may stimulate beneficial microbial populations. Thus, the inclusion of *S. cerevisiae* may have contributed to a more favorable microbial environment for both enzymatic fiber modification and acid fermentation.

The addition of *L. plantarum* in T5 likely provided a further advantage over treatments that did not include lactic acid bacteria. *L. plantarum* is a strong lactic acid producer and can rapidly reduce pH, stabilize silage fermentation, and limit nutrient losses. A lower pH may also help preserve the products of fungal and yeast activity during fermentation. Therefore, the superior performance of T5 was likely the result of a synergistic interaction among the three microorganisms: *T. reesei* promoted fiber hydrolysis, *S. cerevisiae* improved the anaerobic environment and supported microbial activity, and *L. plantarum* stabilized fermentation through lactic acid production.

The reduction in ADL is particularly meaningful because lignin is a major barrier to ruminal degradation of rice straw. Lower ADL values suggest modification of the lignified structure, which may allow rumen microorganisms greater access to cellulose and hemicellulose. This interpretation is consistent with the observation that T5 had both the lowest ADL value and the highest *in vitro* gas production at 28 days.

Relationship between fiber fractions and *in vitro* gas production

In vitro gas production reflects the fermentation of soluble and structural carbohydrates by rumen microorganisms. The absence of significant differences at 7, 14, and 21 days suggests that the early fermentation period was not sufficient to create clear differences in ruminal fermentability among treatments. However, after 28 days, T5 produced a significantly higher gas volume than several other

treatments. This result indicates that a longer fermentation period allowed the multi-strain inoculant to modify the fiber structure more effectively, thereby improving the fermentability of rice straw silage.

The relationship between reduced fiber fractions and increased gas production supports the interpretation that microbial pretreatment improved substrate availability. As NDF, ADF, and ADL decreased, rumen microorganisms likely gained greater access to fermentable carbohydrates. Therefore, the higher gas production observed in T5 at 28 days is consistent with its lower NDF, ADF, and ADL values. These findings also support the usefulness of *in vitro* gas production as a practical indicator of improved digestibility in biologically treated rice straw.

Optimal fermentation period

The results suggest that a fermentation period of 21–28 days is appropriate for rice straw treated with multi-strain microbial inoculants. At 21 days, pH reduction was most evident in T5, indicating active lactic acid fermentation. At 28 days, fiber degradation and *in vitro* gas production were most improved. Therefore, fermentation for at least 21 days may be required to achieve effective acidification, whereas 28 days appears more suitable when the primary objective is to maximize fiber modification and ruminal fermentability.

Conclusions

Multi-strain microbial inoculation improved fermentation characteristics, fiber degradation, and *in vitro* fermentability of rice straw silage. Among the tested treatments, T5 (*T. reesei*, *S. cerevisiae*, and *L. plantarum*) showed the most favorable response, as indicated by the lowest NDF, ADF, and ADL values and the highest *in vitro* gas production at 28 days of fermentation. The superior performance of T5 was likely associated with synergistic interactions among cellulolytic fungi, yeast, and lactic acid bacteria. These findings suggest that multi-strain microbial inoculation is a promising biological approach for improving the nutritive value of rice straw and increasing its potential use as ruminant feed. For practical application, fermentation for 21–28 days is recommended, with 28 days providing the clearest improvement in fiber degradation and ruminal fermentability.

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