

Nutrient Content and *In Vitro* Digestibility of Moringa Leaves (*Moringa oleifera*) Fermented Using *Trichoderma koningiopsis*

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Abstract. *Moringa oleifera* leaves have remarkably high nutritional value, yet their use as ruminant feed remains suboptimal because high crude fiber levels limit nutrient bioavailability. This study investigated whether solid-state fermentation with a locally isolated *Trichoderma koningiopsis* AA1 (STk-AA1) strain could enhance nutrient availability and *in vitro* digestibility of moringa leaf flour. A Completely Randomized Design (CRD) was employed, with three fermentation durations (0, 4, and 8 days), each replicated three times. This experimental design yields 6 degrees of freedom, which is acceptable, although the statistical power to detect a significant difference is not particularly strong. Parameters evaluated were Dry Matter Digestibility (DMD), Organic Matter Digestibility (OMD), Gross Energy (GE), and soluble protein content. Contrary to the initial hypothesis, fermentation with STk-AA1 for 4 and 8 days significantly ($P<0.05$) reduced DMD, OMD, GE, and soluble protein compared to the unfermented control. These outcomes suggest that *T. koningiopsis* AA1 under the applied conditions does not improve, but rather diminishes, the nutritional quality of moringa leaf flour, indicating that fermentation parameters such as duration, inoculum density, and incubation conditions require further optimization before this strain can be recommended for practical feed application.

Keywords: *In vitro* digestibility, *Moringa oleifera*, Solid-state fermentation, *Trichoderma koningiopsis* AA1

Abstrak. Daun kelor (*Moringa oleifera*) dikenal memiliki kandungan nutrisi yang tinggi, namun pemanfaatannya sebagai pakan ruminansia belum optimal karena tingginya fraksi serat kasar yang membatasi ketersediaan nutrisi. Penelitian ini bertujuan mengkaji apakah fermentasi padat menggunakan isolat lokal *Trichoderma koningiopsis* AA1 (STk-AA1) dapat meningkatkan ketersediaan nutrisi dan pencernaan *in vitro* tepung daun kelor. Rancangan Acak Lengkap (RAL) diterapkan dengan tiga perlakuan lama fermentasi, yaitu 0 (kontrol), 4, dan 8 hari, masing-masing dengan tiga ulangan. Rancangan percobaan ini menghasilkan df error 6 yang masih layak meskipun statistical power untuk mendeteksi perbedaan nyata tidak terlalu kuat. Parameter yang diamati meliputi Kecernaan Bahan Kering (KcBK), Kecernaan Bahan Organik (KcBO), Gross Energy (GE), dan protein terlarut. Bertentangan dengan hipotesis awal, fermentasi dengan STk-AA1 selama 4 dan 8 hari secara signifikan ($P<0.05$) menurunkan nilai KcBK, KcBO, GE, dan protein terlarut dibandingkan dengan kontrol tanpa fermentasi. Hasil ini mengindikasikan bahwa *T. koningiopsis* AA1 dalam kondisi yang diterapkan tidak meningkatkan, melainkan menurunkan kualitas nutrisi tepung daun kelor, sehingga parameter fermentasi seperti durasi, kepadatan inokulum, dan kondisi inkubasi memerlukan optimasi lebih lanjut sebelum strain ini dapat direkomendasikan untuk aplikasi pakan secara praktis.

Kata kunci: Kecernaan *in vitro*, *Moringa oleifera*, Fermentasi padat, *Trichoderma koningiopsis* AA1

Introduction

Ruminant livestock are equipped with a multi-compartment digestive system consisting of the rumen, reticulum, omasum, and abomasum, enabling them to ferment and utilize fibrous feed materials that are unavailable to monogastric animals. The microbial ecosystem residing in the rumen facilitates the breakdown of structural carbohydrates, thereby allowing ruminants to derive energy from high-fiber substrates (Lestari et al., 2021). Despite this

capacity, when dietary fiber fractions (particularly lignin and crystalline cellulose) become excessively high, even rumen microbes encounter difficulty in degrading the feed matrix efficiently, ultimately compromising nutrient availability and animal productivity. Microbial-assisted fermentation has therefore been proposed as a cost-effective pre-treatment strategy to enhance the nutritional profile of fibrous feed materials before they are offered to livestock (Lestari et al., 2021).

Moringa oleifera, commonly known as the drumstick tree, represents one of the most nutritionally dense plants documented in tropical regions, including Indonesia, where it grows abundantly across diverse soil types without intensive cultivation requirements (Beno et al., 2022; Nisa et al., 2017). Proximate analysis of *M. oleifera* leaf meal has revealed a Crude Protein (CP) content of 26.43%, Crude Fat of 2.23%, Crude Fiber (CF) of 23.57%, ash of 6.77%, and Nitrogen-Free Extract (NFE) of 52.25% (Sumadi et al., 2017). Although these figures indicate substantial nutritional potential as a concentrate ingredient for ruminant feed formulation (Marawali et al., 2024), the CF content of 23.57% raises a critical concern: at this level, the fiber matrix may partially shield intracellular nutrients from enzymatic and microbial attack in the rumen, thereby reducing effective digestibility and overall feed value. Consequently, bio-processing techniques aimed at reducing structural fiber and improving nutrient accessibility are warranted prior to widespread incorporation of moringa leaf meal into ruminant diets (Lestari et al., 2021).

Among available bio-processing agents, fungi of the genus *Trichoderma* have attracted considerable research interest due to their prolific secretion of cellulolytic, hemicellulolytic, and proteolytic enzymes. Prior investigations employing *Trichoderma* species such as *T. reesei*, *T. viride*, and *T. harzianum* on various fibrous substrates — including corn cobs, pineapple peel, cassava pulp, and lemongrass — reported notable improvements in crude protein content and Total Digestible Nutrients (TDN) (Putri, 2020; Solehudin et al., 2024). Fermentation with *Trichoderma* has also been documented to modify the physical structure and chemical composition of feed ingredients, particularly by degrading cellulose and hemicellulose fractions (Lestari et al., 2021; Rostini et al., 2022). More specifically, *Trichoderma koningiopsis* was selected in the present study because it is a locally isolated strain (STk-AA1) that has

demonstrated cellulolytic activity under Indonesian tropical conditions (Mulyono & Husein, 2025), offering an ecologically relevant and potentially more adaptive inoculum compared to commercially available or foreign strains. However, to date, the application of *T. koningiopsis* AA1 specifically to *M. oleifera* leaf meal and its consequences for *in vitro* digestibility remain insufficiently documented (Pasue et al., 2019; Rizali et al., 2018), representing a clear research gap that merits systematic investigation.

Based on the above rationale, this study hypothesized that solid-state fermentation of *M. oleifera* leaf meal with *T. koningiopsis* AA1 for up to 8 days would significantly improve DMD, OMD, GE retention, and soluble protein content compared to unfermented controls. The primary objective was therefore to evaluate the effect of fermentation duration (0, 4, and 8 days) on the nutrient content and *in vitro* digestibility of moringa leaf flour, with the ultimate aim of determining whether this strain and protocol can be recommended as a practical feed enhancement strategy for ruminant livestock production.

Materials and Methods

Materials

The substrate utilized in this study was *Moringa oleifera* leaf flour, sourced from local smallholder farmers in Sukoharjo Regency, Central Java, Indonesia. Freshly harvested leaves were manually separated from their petioles and midribs, then sun-dried for approximately 24 hours until moisture content fell below 10%. The dried material was subsequently milled using a dry blender and sieved through a 1 mm mesh screen to obtain uniformly fine leaf flour. A 1,000 g batch of this flour was sterilized in an autoclave at 15 PSI for 15 minutes to eliminate indigenous microbial contamination prior to inoculation.

The biological agent employed was *Trichoderma koningiopsis* AA1 (STk-AA1), a local Indonesian isolate maintained at the Animal

Husbandry Study Program, Universitas Veteran Bangun Nusantara, Sukoharjo (Mulyono et al., 2021). An inoculum suspension was prepared by dissolving 20 g of STk-AA1 starter culture in a previously sterilized solution composed of 300 mL distilled water and 20 mL molasses in a 1,000 mL Erlenmeyer flask. The mixture was incubated aerobically at room temperature (approximately 28–30°C) for 24 hours to allow partial reactivation of fungal propagules. The resulting inoculum was estimated to contain approximately 1.2×10^7 colony-forming units per mL (CFU/mL) based on prior characterization of this strain (Mulyono et al., 2021).

Experimental Design

A single-factor Completely Randomized Design (CRD) was applied with fermentation duration as the sole treatment variable, comprising three levels: 0 days (unfermented control), 4 days, and 8 days. Each treatment was replicated three times, yielding nine experimental units in total. Although three replicates represent the minimum accepted threshold for one-way ANOVA, this number was adopted due to resource constraints associated with laboratory-scale fermentation. All data were analyzed using one-way Analysis of Variance (ANOVA) in SPSS version 26.0 (IBM Corp., USA). Prior to ANOVA, data normality was verified using the Shapiro–Wilk test and homogeneity of variance was confirmed by Levene's test. When treatment effects were significant ($P < 0.05$), mean separation was performed using Duncan's Multiple Range Test (DMRT).

Fermentation Procedure

One thousand grams of sterilized moringa leaf flour was combined with 300 mL of STk-AA1 inoculum suspension and 500 mL of sterile distilled water, yielding a substrate-to-moisture ratio of approximately 1:0.8 (w/v). This mixture was thoroughly blended and distributed evenly into a bamboo bioreactor measuring 13 cm in

diameter and 5 cm in depth, producing a substrate layer with a final moisture content of approximately 60–65%. The bioreactors were arranged in a stacking cabinet to allow aerobic solid-state fermentation. Incubation was maintained at an ambient temperature of 28–30°C with relative humidity of 70–80% throughout the fermentation period. Upon reaching the designated harvest time (4 or 8 days), the fermented material was oven-dried using a Memmert drying oven at 70°C for 48 hours to arrest microbial activity and stabilize the product. The dried fermented moringa leaf flour was then vacuum-sealed in polyethylene pouches and stored at room temperature until analysis.

Observed Variables

1. Dry Matter Digestibility (DMD) and Organic Matter Digestibility (OMD)

In vitro digestibility was determined following the two-stage method of Tilley and Terry (1963) as adapted by Pazla et al. (2024). Rumen fluid was obtained from a fistulated Ongole crossbred steer maintained on a basal diet of grass and concentrate at the Animal Nutrition Laboratory, Universitas Veteran Bangun Nusantara. The rumen fluid was collected before morning feeding, filtered through four layers of cheesecloth, and immediately transferred to pre-warmed thermos flasks under continuous CO₂ flushing to maintain anaerobic conditions. For each experimental unit, 10 g of the fermented sample (on a dry matter basis) was placed into a fermentation tube together with 50 mL rumen fluid and 20 mL McDougall's buffer solution (pH 6.9). Tubes were sealed with rubber stoppers fitted with one-way valves and incubated at 39°C for 48 hours in a water bath with gentle agitation. Following incubation, residues were recovered by filtration through pre-weighed sintered glass crucibles and subjected to dry matter and organic matter determination. DMD

and OMD were calculated using the following equations (Lujum et al., 2023) :

$$DMD = \frac{(DM \text{ sample}) - (DM \text{ residue} - DM \text{ blanko})}{DM \text{ sample}} \times 100\%$$

Notes:

- DM sample = Dry matter weight of sample before incubation (g)
- DM residue = Dry matter weight of sample after incubation (g)
- DM blank = Dry matter weight after incubation without treatment (g)

$$OMD = \frac{(OM \text{ sample}) - (OM \text{ residue} - OM \text{ blanko})}{OM \text{ sample}} \times 100\%$$

Notes:

- OM sample = Weight of organic matter of sample before incubation (g)
- OM residue = Weight of organic matter of sample after incubation (g)
- OM blank = Weight of organic matter after incubation without treatment (g)

Where DM sample and OM sample are the dry matter and organic matter weights before incubation, DM residue and OM residue are weights after incubation, and DM blank and OM blank are the respective weights of the blank (tubes without substrate) after incubation.

2. Gross Energy (GE)

The GE content of each fermented sample was quantified by complete combustion in an adiabatic bomb calorimeter (Mbali et al., 2024). Samples were oven-dried to near-zero moisture content and then precisely weighed prior to loading into the combustion vessel. High-pressure oxygen was introduced into the sealed

bomb, and ignition was initiated by electrical discharge. The resulting temperature rise in the surrounding water jacket was recorded and converted to energy units (kcal/kg DM) based on the calorimeter's heat capacity constant.

3. Soluble Protein

Soluble protein concentration was quantified using the Lowry colorimetric method (Muyassaroh et al., 2020). Briefly, 5 g of each fermented sample was homogenized in 100 mL distilled water, and the homogenate was centrifuged to yield a clear supernatant. One milliliter of the supernatant was combined with 1 mL Lowry D reagent and 3 mL Lowry E reagent, vortexed to achieve homogeneity, and the absorbance was read at 590 nm using a UV-Vis spectrophotometer. Protein concentration was calculated against a standard curve prepared with Bovine Serum Albumin (BSA) as the reference protein. Reagent compositions were as follows: Lowry D = Lowry A : Lowry B : Lowry C (10 : 0.5 : 0.5); Lowry A = 10% Na₂CO₃ in 0.5 N NaOH; Lowry B = 1% CuSO₄; Lowry C = 2% Potassium Sodium Tartrate; Lowry E = 2 N Folin–Ciocalteu reagent.

Results and Discussion

Four key nutritional indicators were assessed to evaluate the outcomes of *M. oleifera* leaf flour fermentation with STk-AA1: DMD, OMD, GE, and soluble protein. As presented in Table 1, all parameters declined progressively with increasing fermentation duration, and the differences were statistically significant (P<0.05) for all variables.

Table 1. Nutrient content and *in vitro* digestibility of fermented moringa leaves using STk-AA1

Parameters	0 Days	4 Days	8 Days
Dry Matter Digestibility (%)	81.17 ± 0.42 ^b	77.61 ± 0.38 ^a	77.49 ± 0.35 ^a
Organic Matter Digestibility (%)	78.99 ± 0.51 ^b	74.45 ± 0.44 ^a	74.53 ± 0.47 ^a
Gross Energy (kcal/kg DM)	4189.67 ± 12.34 ^a	4050.67 ± 10.87 ^b	4040.00 ± 11.22 ^b
Soluble Protein (%)	6.20 ± 0.18 ^c	5.23 ± 0.15 ^b	4.06 ± 0.12 ^a

^{abc} = on the same line shows a significant difference (P<0.05). This experimental design yields 6 degrees of freedom, which is acceptable, although the statistical power to detect a significant difference is not particularly strong.

Effect of STk-AA1 Fermentation on Dry Matter Digestibility (DMD)

DMD declined from 81.17% in the unfermented control to 77.61% after 4 days of fermentation and further to 77.49% following 8 days of fermentation. Although the absolute reduction between day 4 and day 8 was marginal (~0.12 percentage points), both fermented treatments differed significantly from the control ($P < 0.05$), while no significant difference was detected between the two fermented groups. This outcome is consistent with the finding of Mulyono et al. (2021), who documented that solid-state fermentation of rice straw with *Trichoderma* AA1 tended to reduce rather than improve *in vitro* DMD values.

From a mechanistic perspective, the observed decline in DMD can be attributed to at least two interconnected phenomena associated with fungal growth on the substrate. First, *Trichoderma* preferentially catabolizes readily fermentable carbohydrate fractions — including simple sugars and soluble polysaccharides — to fuel its own biomass production, thereby reducing the pool of easily digestible substrates available to rumen microorganisms during *in vitro* incubation (Utama, 2021). Second, and critically, the accumulation of *Trichoderma* biomass per se introduces chitin, the primary structural polysaccharide of fungal cell walls, into the substrate matrix. Chitin consists of linearly linked N-acetylglucosamine (GlcNAc) units joined through $\beta(1,4)$ glycosidic bonds — a configuration comparable to cellulose but biochemically more resistant due to the presence of acetamide groups (Corneliyawati et al., 2018; Kappel et al., 2024; Putra et al., 2014). Because rumen microbiota and mammalian digestive enzymes possess negligible chitinolytic activity, the accumulation of chitin-rich fungal biomass translates directly into an increase in the indigestible fiber fraction, reducing DMD (Dalle et al., 2022; Mbali et al., 2024). The absence of post-fermentation proximate

analysis (particularly crude fiber, NDF, and ADF) in the present study represents a limitation, as direct fiber data would have substantiated this proposed mechanism more rigorously. These findings carry a practical implication: fermentation of moringa leaf flour with STk-AA1 under the current protocol does not offer a digestibility advantage, and its unrestricted application in ruminant feed formulations should be approached cautiously. Optimization of inoculum concentration, fermentation substrate moisture, and incubation duration is recommended before any scale-up.

Effect of STk-AA1 Fermentation on Organic Matter Digestibility (OMD)

OMD declined from 78.99% in the control to 74.45% at day 4, with a negligible recovery to 74.53% at day 8. The marginal upswing between day 4 and day 8 was not statistically significant, suggesting that the fermentation-induced changes in organic matter composition reached a plateau by day 4. The reduction in OMD is mechanistically linked to alterations in the chemical architecture of the substrate imposed by fungal metabolic activity (Zahera et al., 2022). Specifically, (Kabeakan et al., 2020) demonstrated that microbial fermentation of forage-based substrates can intensify lignification of residual cell wall material, as enzymatic breakdown of amorphous cellulose and hemicellulose exposes and concentrates the more recalcitrant lignin network. Lignin and its condensation products are notoriously refractory to both enzymatic and rumen microbial degradation, thereby reducing the proportion of organic matter that is accessible for digestion (Kabeakan et al., 2020).

The divergence of the present results from those of Muhammad et al. (2020) and Muawahid (2021), who reported OMD improvements following *Trichoderma*-based fermentation of moringa and rice straw, respectively, may be attributed to differences in strain identity, inoculum preparation, substrate composition,

and fermentation duration. In the present study, the relatively short incubation periods (4 and 8 days) may have been insufficient for STk-AA1 to achieve meaningful lignocellulosic degradation, yet long enough to permit substantial depletion of soluble organic fractions and accumulation of indigestible fungal biomass. Mulyono et al. (2021) similarly reported that their *Trichoderma* AA1 strain reduced OMD values during rice straw fermentation, attributing this to the limited efficacy of *Trichoderma* cellulases against the silica–lignin complex present in the substrate cell wall. From the standpoint of ruminant nutrition, a reduction in OMD from nearly 79% to approximately 74.5% represents a non-trivial decline in the energy-yielding potential of the feed, since organic matter digestibility is directly correlated with metabolizable energy supply to the animal. This underlines the need for a refined fermentation strategy that can preserve or enhance organic matter digestibility while simultaneously reducing anti-nutritional fiber fractions.

Effect of STk-AA1 Fermentation on Gross Energy (GE)

GE declined from 4,189.67 kcal/kg DM in the unfermented control to 4,050.67 kcal/kg DM at day 4 and 4,040.00 kcal/kg DM at day 8. The significant reduction ($P<0.05$) observed at both fermentation time points relative to the control, without a statistically significant difference between the two fermented groups, mirrors the trend observed for DMD and OMD. The primary driver of GE reduction during aerobic solid-state fermentation is the respiratory catabolism of carbohydrate substrates by *Trichoderma*, which releases chemical energy stored in organic bonds as heat and CO₂ rather than conserving it in fermentation end-products available to the host animal (Hasanah & Haryuni, 2024; Lekı et al., 2024). Ibarruri et al. (2021) reported an analogous mechanism during fermentation of green fodder, wherein the conversion of carbohydrate fractions to gaseous metabolites

by fermentative microorganisms progressively diminished total combustible energy.

An additional contributing mechanism involves the enzymatic production of β -(1,3)-glucanase by *Trichoderma*, which, combined with the deposition of chitin-rich fungal cell wall material in the substrate, cumulatively elevates the structural carbohydrate fraction (Corneliyawati et al., 2018; Kappel et al., 2024; Putra et al., 2014). Since structural carbohydrates such as chitin and crystalline cellulose are resistant to combustion at the same energy density as readily fermentable substrates, their relative enrichment in the fermented matrix contributes to an apparent reduction in GE per unit of dry matter. Budiyanto et al. (2021) This corroborates this pathway, reporting that tofu dregs fermented with *Trichoderma viridae* showed increasing proportions of lignin and crystalline cellulose alongside a reduction in energy density. The progressive GE decline observed across fermentation durations has direct implications for ration balancing: feed formulations that include fermented moringa leaf flour processed under the present protocol would need to account for a reduced energy contribution per kilogram of inclusion.

Effect of STk-AA1 Fermentation on Soluble Protein Content

Soluble protein exhibited the most pronounced and consistent decline across all three fermentation time points, decreasing from 6.20% in the control to 5.23% at day 4 and 4.06% at day 8. All three means were statistically distinct from one another ($P<0.05$), indicating that protein degradation continued progressively throughout the 8-day incubation. This trajectory implicates the ongoing secretion and activity of extracellular proteolytic enzymes by STk-AA1 as the principal mechanism of protein catabolism. *Trichoderma* is well documented as a producer of aspartate and serine proteases that cleave substrate proteins

into peptides and free amino acids (Revania et al., 2023). These liberated nitrogen-containing compounds serve as the primary nitrogen source sustaining fungal growth, biomass expansion, and sporulation during solid-state fermentation (Amara & El-Baky, 2023). Consequently, the net soluble protein content of the substrate declines as fungal assimilation outpaces any potential protein biosynthesis within the fermented matrix.

Furthermore, fermentation-associated biochemical processes including deamination of amino acids and volatilization of nitrogenous compounds (e.g., ammonia) contribute additional pathways of protein loss that are independent of fungal anabolism (Adawyah et al., 2021; Leki et al., 2024; Raharjo et al., 2019). Rahman et al. (2018) also observed that fermentative conditions tend to accelerate the breakdown of structural proteins, reducing the fraction present in soluble, readily assimilable form. The reduction in soluble protein is particularly concerning from a nutritional standpoint because soluble protein fractions are among the most rapidly degraded in the rumen and contribute disproportionately to microbial protein synthesis and overall nitrogen utilization efficiency in ruminants. A 34.5% decline in soluble protein over 8 days of fermentation (from 6.20% to 4.06%) therefore represents a meaningful reduction in the feed's capacity to support rumen microbial activity, which may secondarily impair fiber digestion and volatile fatty acid production. Future studies should evaluate whether co-inoculation with nitrogen-fixing bacteria or supplementation of the fermentation substrate with inorganic nitrogen could offset the nitrogen losses incurred during STk-AA1-driven solid-state fermentation.

Conclusions

Solid-state fermentation of *Moringa oleifera* leaf flour with *Trichoderma koningiopsis* AA1 (STk-AA1) for 4 and 8 days did not yield the anticipated nutritional improvements; instead, it

resulted in a consistent and significant deterioration of all measured parameters — DMD, OMD, GE, and soluble protein — compared to the unfermented control. These findings indicate a fundamental limitation of the current fermentation protocol and suggest that the accumulation of chitin-rich fungal biomass, combined with the respiratory consumption of fermentable carbohydrates and proteolytic nitrogen assimilation by STk-AA1, collectively undermines rather than enhances the nutritional quality of moringa leaf flour. From a practical ruminant nutrition standpoint, moringa leaf flour fermented under the present conditions is not recommended as a superior feed ingredient in its current form, as its incorporation into ruminant rations at significant inclusion levels could reduce overall diet digestibility and energy density. Future research should explore alternative fermentation approaches, such as shorter incubation windows, reduced inoculum loading, combined microbial consortia including cellulolytic bacteria, or pre-treatment of the substrate to disrupt the lignin barrier, to achieve genuine improvements in fiber degradation and nutrient bioavailability before recommending *T. koningiopsis* AA1 as a viable fermentation agent for moringa-based ruminant feed.

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