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Improvement of the nutritional value of *Lupinus mutabilis* sweet foliage meal by solid-state fermentation with *Aspergillus niger* J1 and *Trichoderma viride* M5-2 strains

Mejora del valor nutritivo de harina de follaje de *Lupinus mutabilis* Sweet mediante fermentación en estado sólido con las cepas *Aspergillus niger* J1 y *Trichoderma viride* M5-2

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Abstract

To increase the nutritive value of *Lupinus mutabilis* Sweet (chocho) foliage meal by solid-state fermentation with *Aspergillus niger* J1 and *Trichoderma viride* M5-2 strains, two laboratory experiments were carried out. A completely randomized design with 2×8 factorial arrangement and three replicates was used. The two strains of lignocellulolytic fungi and the fermentation times (0, 24, 48, 72, 96, 120, 144 and 168 h) were selected as factors. Samples were taken every 24 h for enzymatic analyses (exo β1-4 glucanase) and chemical composition (neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose and lignin). Substrate pH and moisture, as well as phenolic and flavonoid content composition were measured. Variations in the physicochemical properties of the flour studied were observed, with decreases in NDF, flavonoids and phenolic content by both strains, reaching a maximum of 12, 75 and 84% respectively in a maximum time of 168 hours in the fermentation with *A. niger* J1 ($P<0.01$). In enzyme kinetics, interaction was observed in all factors ($P<0.01$). High values of exo β 1-4 glucanase enzymes were recorded in *L. mutabilis* Sweet with strain *T. viride* M5-2 at 96 h and sustained this activity over time for *A. niger* J1 with 0.189 UPF/mL. *T. viride* M5-2 and *A. niger* J1 strains improve the nutritive value of legume meal.

Keywords: Legume, Solid Fermentation, antinutrients, monogastrics.

Resumen

Para incrementar el valor nutritivo de la harina de follaje de *Lupinus mutabilis* Sweet (chocho) por medio de una fermentación en estado sólido con las cepas *Aspergillus niger* J1 y *Trichoderma viride* M5-2 se efectuaron 2 experimentos a nivel de laboratorio. Se utilizó un diseño completamente aleatorizado, con arreglo factorial 2×8 y tres repeticiones. Como factores se seleccionaron las dos cepas de hongos lignocelulolíticos y los tiempos de fermentación (0, 24, 48, 72, 96, 120, 144 y 168 h). Se tomaron muestras cada 24 h para los análisis enzimáticos (exo β1-4 glucanasa) y composición química (fibra neutro detergente (FND), fibra ácido detergente (FAD), celulosa y lignina). Se midió el pH y la humedad en el sustrato, así como la composición de contenido fenólico y flavonoides. Se observaron variaciones en las propiedades físico-químicas de la harina estudiada, con disminución de la FND, flavonoides y el contenido fenólico por ambas cepas, alcanzando un máximo de 12, 75 y 84% respectivamente en un tiempo máximo de 168 horas en la fermentación con *A. niger* J1 ($P<0,01$). En la cinética enzimática se observó interacción en todos los factores ($P<0,01$). Se registraron valores altos de enzimas exo β 1-4 glucanasa en *L. mutabilis* Sweet con la cepa *T. viride* M5-2 a las 96 h y sostenida esta actividad en el tiempo para *A. niger* J1 con 0,189 UPF/mL. Las cepas *T. viride* M5-2 y *A. niger* J1 mejoran el valor nutritivo de la harina de leguminosa.

Palabras clave: Leguminosa, Fermentación sólida, antinutrientes, monogástricos.

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1 Introduction

Lupinus mutabilis Sweet (chocho) is a crop with low nutritional requirements that grows in marginal soils, and its grains have high nutritional value, providing valuable proteins in the human diet. This legume also preserves soil fertility through nitrogen fixation, it is rich in calcium and proteins, and its cultivation has spread throughout Ecuador, becoming a key component in development projects in indigenous regions of the country (Martínez Flores et al., 2016).

The grain of *L. mutabilis* Sweet is considered highly nutritious, with proteins and oils comprising more than half of its weight. Based on bromatological analyses, it contains an average of 35.5% protein, 16.9% oil, 7.65% crude fiber, 4.15% ash, and 35.77% carbohydrates (Carvajal-Larenas, 2019). Additionally, it contains certain proteins that can help reduce blood glucose levels (Vargas-Guerrero et al., 2014; Gulisano et al., 2019).

Being a short-cycle crop, after harvesting, *L. mutabilis* Sweet leaves behind residues consisting of foliage and pods, which can represent up to 75% of the plant's weight. These residues are often reincorporated into the soil, where natural degradation returns nutrients to the soil. Alternatively, this waste can be used as animal feed. This residue typically contains a high fiber content, consisting of crude fiber (CF), neutral detergent fiber (NDF), and acid detergent fiber (ADF).

Fiber can be defined as the plant components that have low digestibility and promote ruminal balance. Because of this, its use has mainly been directed toward ruminants, although it can be included in the diets of monogastric animals (Savón et al., 2005). However, its use requires an appropriate transformation strategy to achieve socially desirable and economically viable production systems (Savón et al., 2005; Trujillo and Escobar, 2012; Rodríguez García, 2017) due to the presence of antinutritional compounds (fiber, phenolic content, flavonoids, tannins) in such feedstuffs (Molina-Poveda et al., 2013; Díaz Sánchez et al., 2017; Martínez-Pérez et al., 2018). It is recommended that NDF content does not exceed 65% and that ADF does not exceed 45% (Linn and Martin, 1991). While polyphenols are known to have beneficial effects on animal health, a high content of these compounds can negatively affect the nutritional value of the substrate (Siddhuraju et al., 2000; Bessada et al., 2019).

Solid-state fermentation (SSF) is a transformation method involving the growth of microorganisms on a substrate under certain moisture, pH, and temperature conditions, using the substrate as a nutrient source to facilitate growth, development, and reproduction (Pandey, 2003). In this study, the microorganisms utilized the antinutritional compounds (ADF, NDF, polyphenols) as a food source for their growth, thereby reducing their levels. The use of SSF has demonstrated an increase in the nutritional value of fermented substrates and their safety for animal feed.

Valiño et al. (2015) increased the nutritional value of flour from four legumes using solid fermentation with *T. viride* M5-2 for use in monogastric species. Varadyova et al. (2018) published a review showing that substrates enriched through solid fermentation can increase the concentration of polyunsaturated fatty acids in the rumen. In 2019, Sugiharto and Ranjitkar demonstrated that fermentation, in addition to being an economical means of enhancing the nutritional value of ingredients used in broiler chicken feed, also has a beneficial influence on gut morphology, immune function, and bird growth performance (Valiño et al., 2015; Varadyova et al., 2018; Sugiharto and Ranjitkar, 2019).

The objective of this research is to improve the nutritional value of *L. mutabilis* Sweet foliage meal through solid fermentation with the strains *Aspergillus niger* J1 and *Trichoderma viride* M5-2.

2 Materials and Methods

2.1 Experimental Design and Statistical Analysis

A completely randomized design was used, with three replications and two inoculants (*A. niger* J1 and *T. viride* M5-2), with 8 sampling times (0, 24, 48, 72, 96, 120, 144, and 168 hours). Each biodigester was considered an experimental unit. Statistical analyses were performed using STATGRAPHICS XV CENTURION software.

2.2 Microorganisms

Two strains of lignocellulolytic fungi were used: *Aspergillus niger*, isolated from sugarcane bagasse at the Faculty of Chemical Engineering at the Technological University of Havana "José Antonio Echeverría" (CUJAE), and *Trichoderma viride* M5-2, from the strain bank of the Animal Science Institute (Mayabeque, Cuba). These strains are known for their hydrolytic activity and secretion of cellulase enzymes in substrates with high fiber content, and both strains were evaluated through solid-state fermentation (Valiño et al., 2004a).

2.3 Fermentation Substrate

The plant substrate consisted of post-harvest *L. mutabilis* Sweet plants (leaves and stems) from the "Lupita" farm in the Riobamba canton, Chimborazo province. The material was dried at the Biotechnology Research Center of Ecuador (CIBE) in a HAFO SERIES 1 600 oven at 65 °C for 48 hours and then ground using a manual CORONA mill.

2.4 Fermentation process

For this experiment, 42 glass jars of 250 ml were used as biodigesters (21 for fermentation with*A. niger* J1 and 21 for fermentation with *T. viride* M5-2). Each was filled with 10 g of the dried substrate and then moistened with distilled water to achieve an initial moisture content of 70 % (25 ml). The mixture was enriched with 2.5 % urea (0.25 g), 5 % potassium phosphate (KH_2PO_4, 0.5 g), and 10 % ammonium sulfate ($(NH_4)_2SO_4$, 1 g) (Roussos et al., 1991), and the pH of the substrate was adjusted to 6. The wet substrate was sterilized with saturated steam in an autoclave for 20 minutes at 121 °C. Two spore suspensions were prepared for substrate inoculation: *A. niger* J1 was inoculated into the biodigesters containing the substrate at a concentration of 10^6 spores/g of dry substrate (Villena and Gutiérrez-Correa, 2003), and *T. viride* M5-2 was inoculated into the remaining biodigesters at a concentration of 10^7 spores/g of dry substrate (Valiño et al., 2004b).

The inoculated and homogenized jars were incubated at 30 °C for 168 hours. Samples were taken every 24 hours for the corresponding analyses, and fungal growth and substrate colonization were visually observed.

2.5 Chemical and Enzymatic Analyses

Three grams of fermented material were weighed at 0, 24, 48, 72, 96, 120, 144, and 168 hours, and 30 ml of distilled water was added. The sample was placed on a mechanical shaker at 140 rpm for 20 minutes, centrifuged at 5,000 rpm for 10 minutes, and filtered through Whatman No. 40 filter paper to obtain the enzymatic extract. The pH was measured, and the enzymatic analysis of exo 1,4 β-D glucanase activity (filter paper activity) was performed using the technique from the National Renewable Energy Laboratory NREL/TP-510-42628, January 2008 (Adney and Baker, 2008). This enzymatic activity was expressed in filter paper units per milliliter (FPU/ml).

The bromatological indicators studied included dry matter conversion (DM), calculated by gravimetry as the difference between the initial and final dry mass (Oliva et al., 2018), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (LIG) using the ANSI/ASTM D1106-56 method, and cellulose (CEL) using the ANSI/ASTM D1103-60 method (1977). The content of phenolic compounds (mg of gallic acid/100 g of dry substrate) was analyzed according to the Folin-Ciocalteu method (Vera et al., 2022), and the flavonoid content was determined by the spectrophotometric method (mg of catechins/100 g of dry substrate) (Xu et al., 2017).

3 Results and discussion

The bromatological composition and granulometry of the substrate are detailed in Tables 1 and 2, respectively. Fiber, particularly that derived from forages, constitutes the main component of ruminant diets, although its use in monogastrics is limited due to the morphology of their digestive systems. Despite this, several studies have demonstrated the feasibility of using this type of substrate in their diets (Savón et al., 2005; Jha et al., 2019).

Phenolic compounds, particularly tannins, can precipitate certain proteins, thus reducing their digestibility and limiting the availability of amino acids, compromising the nutritional value of the substrate (Bessada et al., 2019).

Table 1. Composition of *L. mutabilis* Sweet foliage meal.

Component	%	mg/100 gr of DM*
Moisture	3.12	
Neutral Detergent Fiber (NDF)	71.05	
Acid Detergent Fiber (ADF)	53.84	
Lignin	13.28	
Cellulose	40.56	
Hemicellulose	17.21	
Flavonoids		55.53
Phenolic Content		9.78

* Dry Matter

Table 2. Granulometric Characterization of *L. mutabilis* Sweet Foliage Meal.

Mesh No. (U.S. STD. Sieve)	%
< 5	0.28
5 - 16	22.67
16 - 25	37.39
25 - 60	33.71
> 60	

In Table 3, it can be observed that both strains exhibited growth on the selected substrate, with differences in sporulation. *A. niger* J1 showed mycelial growth at 24 hours, began sporulation at 48 hours, and fully colonized the surface of the substrate by 72 hours. In contrast, *T. viride* M5-2 began sporulation at 72 hours and did not fully cover the substrate with spores throughout the experiment. An important factor influencing the observed mycelial growth could be particle size, as noted by Haldar and Purkait (2020) and Gao et al. (2020). Substrate colonization and sporulation are enhanced by the surface area available for contact, with larger surface areas favoring better colonization.

The pH variation during the 168-hour fermentation process is shown in Figure 1. For both strains, pH values between 6 and 8 were observed, corresponding to the optimal range for cellulolytic enzyme activity in fibrous substrates. It is known that the optimal initial pH values for the hydrolytic action of cellulase enzymes are between 5 and 6, although these may vary depending on the substrate being fermented, the incubation temperature, and even the strain from which the enzyme originates (Kaschuk et al., 2020).

In this study, the *T. viride* M5-2 strain showed low cellulolytic activity between pH 6 and 8 during the fermentation of *L. mutabilis* Sweet at 30°C, differing from results obtained in other studies with legumes (*Vigna unguiculata*), where the pH remained between 5 and 7 (Valiño et al., 2004b) (Figure 2). It was also observed that during fermentation with the *T. viride* M5-2 strain, the pH rose to levels above 8 after the third day of fermentation. This could be related to the α-amino groups present in the substrate's proteins, which ionize when dissolved in water, releasing a proton from the functional group that could raise the pH. Additionally, the substrate is rich in nitrogenous compounds that could also contribute to the pH increase (Villacrés et al., 2020).

Table 4 shows the fermentation process of *L. mutabilis* Sweet in relation to dry matter (DM) conversion, where an interaction between the studied factors is observed. The *A. niger* J1 strain converts up to 7.68 percentage points during the 168 hours of fermentation. On the other hand, the *T. viride* M5-2 strain converts only 1.3 percentage points in the same period. These values indicate the fermentation activity, with substrate conversion observed by the fungi. The difference in conversion between the strains may be attributed to the metabolism of the fungi (Lameiras et al., 2018), as well as the composition of soluble carbohydrates and, consequently, the functional food properties of legumes (Dustet and Izquierdo, 2004).

The determinations of exo β-1,4-glucanase activity (Figure 2) showed that the fungi produce cellulase enzymes, with most enzyme activity occurring during the initial hours of fermentation and remaining active throughout the 168-hour fermentation period. This hydrolytic activity presents a marked difference between strains. The *A. niger* J1 strain exhibited greater cellulolytic activity, increasing from 0.064 FPU/mL to 0.189 FPU/mL by the end of fermentation of *L. mutabilis*, which corresponds to the fungal colonization of the substrate and its sporulation. However, for the *T. viride* M5-2 strain, there was no significant increase in enzymatic activity, reaching a maximum of 0.106 FPU/mL on the second day of fermentation, followed by a decline to as low as 0.074 FPU/mL. These enzyme activity values were very low. This behavior could

be related to the type of substrate, which may not have favored hydrolytic action (Malgas et al., 2017), and may also be influenced by the elevated pH, which can affect enzymatic activity (Villacrés et al., 2020).

Table 3. Growth of *A. niger* J1 and *T. viride* M5-2 during the fermentation dynamics of *L. mutabilis* Sweet at Temperature = 30°C, pH = 6, Humidity = 70%.

Strain	Growth (Hours)						
	24	48	72	96	120	144	168
A. niger J1	X	XX	XXX	XXX	XXX	XXX	XXX
T. viride M5-2		X	XX	XX	XX	XX	XX

X: onset of mycelial growth, XX: sporulation, XXX: complete sporulation

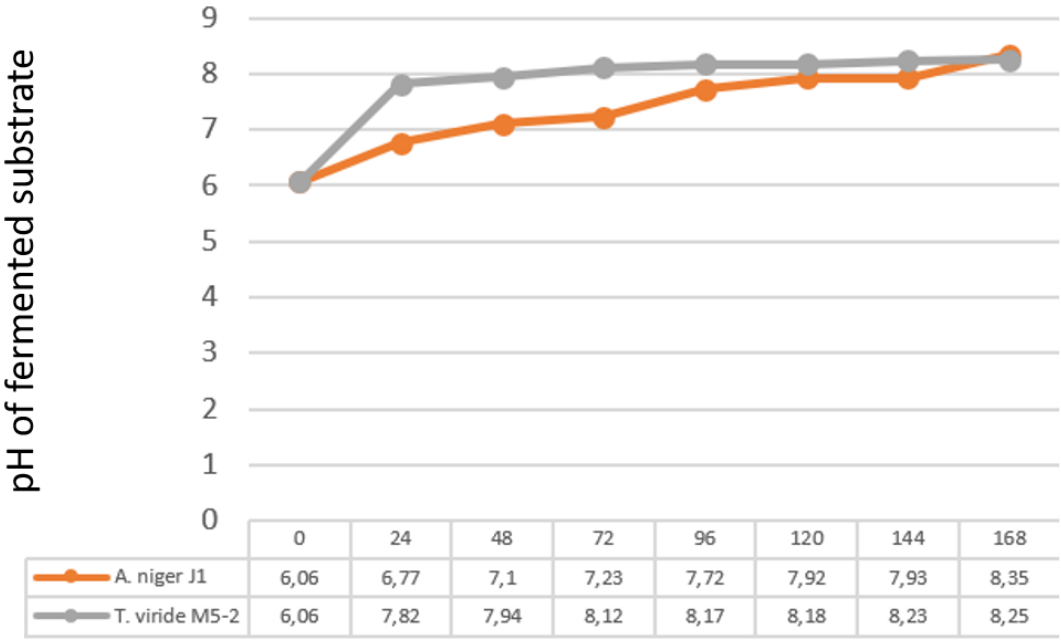

Figure 1. pH variation during the fermentation dynamics of *L. mutabilis* Sweet by *A. niger* J1 and *T. viride* M5-2. SE (±) 0.168, P< 0.001.

Table 5 shows the effect of the fermentation process on the fibrous fraction of the substrate. A notable reduction in NDF values by both strains and in ADF values by *A. niger* J1 was observed. This reduction could be related to the high nitrogen content associated with this indicator (Villacrés et al., 2020), as the studied strains, in addition to using the components of the cell wall, may have metabolized part of the nitrogen associated with fiber as a nutrient before beginning to degrade lignin (Valiño et al., 2015).

NDF, also known as insoluble fiber, is a good predictor of the substrate's energy value. Very low levels slow down intestinal transit, reduce productive yields, and increase the risk of digestive pathologies (Jiménez-Moreno et al., 2019). Conversely, excessive levels are related to poor digestibility, particularly in monogastric species whose digestive systems are not adapted to high-fiber feeds. Valiño et al. (2015) demonstrated that the *T. viride* M5-2 strain has valuable potential for the biotrans-

formation of fibrous substrates that could later be included in animal feed.

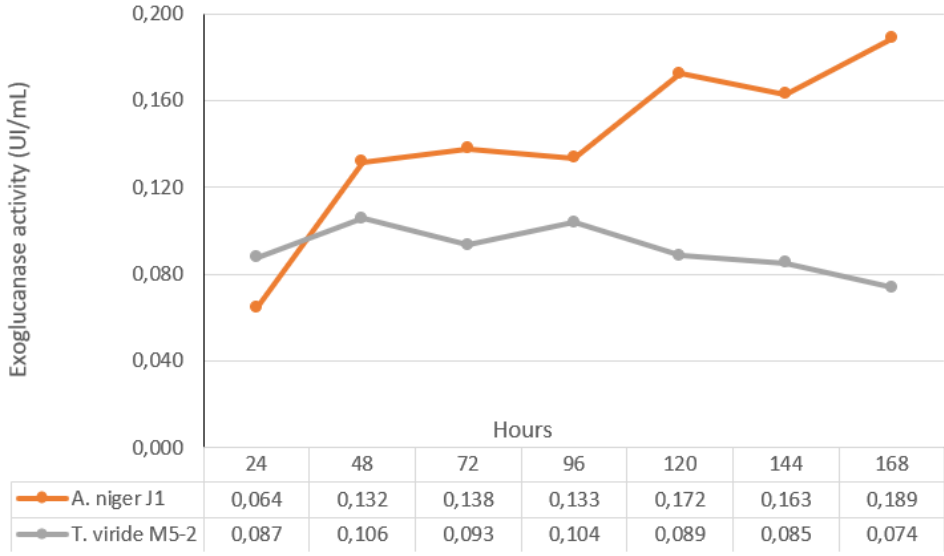

Figure 2. Exo β-1,4 glucanase enzymatic activity of *A. niger* J1 and *T. viride* M5-2 during the solid-state fermentation dynamics of *L. mutabilis* Sweet. SE (±) 0.038, P<0.001.

Table 4. Dry matter conversion during the solid fermentation process of *L. mutabilis* Sweet with *A. niger* J1 and *T. viride* M5-2. Initial conditions: Temperature = 30 °C, pH = 6, Humidity = 70%.

Indicator	Strain	Fermentation Time (hours)								SE (±)
		0	24	48	72	96	120	144	168	Sign.
Dry Matter	*A. niger* J1	0^{a}	0.17^{a}	0.34^{a}	3.02^{c}	4.46^{d}	5.71^{e}	7.48^{f}	7.68^{f}	0.679
Conversion (%)	*T. viride* M5-2	0^{a}	0.9^{b}	0.93^{b}	0.94^{b}	0.95^{b}	1.16^{b}	1.23^{b}	1.3^{b}	P<0.001

^{a,b,c,d,e,f} Different letters indicate significant differences at P<<0.05, according to Duncan.

Both strains demonstrated the ability to degrade the fibrous fraction of the substrate. *A. niger* J1 reduced NDF and ADF by 12 percentage points, while *T. viride* M5-2 reduced NDF by 12 percentage points but only reduced ADF by 6 percentage points. The cellulolytic activity was greater in *A. niger*, which degraded cellulose by 11 percentage points, while *T. viride* M5-2 only degraded 4 percentage points.

The use of *L. mutabilis* Sweet as a substrate for solid fermentations has been scarcely reported in the literature. Other varieties of *Lupinus* have been used in fermentations with bacteria to improve their nutritional values (Starkute et al., 2016; Bartkiene et al., 2018).

Table 6 shows the effect of fermentation on secondary metabolites present and associated with the fibrous fraction of the analyzed substrate. From the analysis of phenolic content and its transformation, it was observed that the *A. niger* J1 strain reduced the phenolic content from 9.78 mg to 1.83 mg per 100 grams of dry substrate, with the greatest reduction occurring during the first three days. This reduction is consistent with the findings of Molina et al. (1990), who reported up to a 75% reduction in polyphenol content using a different strain of *A. niger*.

On the other hand, *T. viride* M5-2 only reduced the phenolic content from 9.78 mg to 4.49 mg throughout the process. The *A. niger* J1 strain reduced

flavonoids by 53% compared to the unfermented substrate, while the *T. viride* M5-2 strain reduced them by 22% during the first four days of fermentation. However, after the fifth day, the concentration of flavonoids likely increased due to the reduction in dry matter during fermentation.

It was observed, unlike other lignocellulosic substrates, that no pretreatment was required for the fermentation of Lupinus, as previously noted in laboratory experiments with other legumes (Pérez et al., 2016), which is not the case when sugarcane bagasse is used (De la Cruz et al., 2016).

Phytochemical studies on phenolic compounds conducted by Scull et al. (2015) on forage from other legumes with various fungal strains yielded similar results. However, the enzymatic potential of the T. viride M5-2 strain had a greater impact on the transformation of this seasonal legume, with a reduction of 32% in polyphenols, 18% in flavonoids, and 3% in fiber, without the addition of other mineral sources.

Table 5. Effect of the fermentation process on the fibrous fraction of the substrate with both strains. Temperature = 30°C, pH = 6, Humidity = 70%.

Indicators (%)	Strain	Fermentation Time (hours)								SE (±)
		0	24	48	72	96	120	144	168	Signific.
NDF	*A. niger* J1	71.05^{b}	61.26^{a}	59.25^{a}	61.60^{a}	60.73^{a}	60.55^{a}	57.62^{a}	59.37^{a}	0.773
	T. viride M5-2	71.05^{b}	61.67^{a}	59.87^{a}	61.26^{a}	60.14^{a}	60.70^{a}	60.64^{a}	58.97^{a}	P<0.001
ADF	*A. niger* J1	53.84^{d}	50.84^{d}	47.29^{abcd}	47.94^{abcd}	48.43^{abcd}	42.75^{ab}	43.04^{abc}	41.77^{a}	0.827
	T. viride M5-2	53.84^{d}	49.34^{bcd}	49.08^{abcd}	50.23^{cd}	48.60^{abcd}	49.19^{abcd}	47.87^{abcd}	47.89^{abcd}	P<0.001
Lignin	*A. niger* J1	13.28^{c}	10.80^{abc}	11.36^{abc}	12.62^{bc}	13.75^{c}	12.15^{abc}	13.01^{c}	12.01^{abc}	0.320
	T. viride M5-2	13.28^{c}	9.25^{a}	9.83^{ab}	11.16^{abc}	10.92^{abc}	11.62^{abc}	11.06^{abc}	11.06^{abc}	P<0.001
Cellulose	*A. niger* J1	40.56^{c}	40.04^{c}	35.93^{abc}	35.32^{abc}	34.68^{abc}	30.60^{ab}	30.03^{a}	29.76^{a}	0.939
	T. viride M5-2	40.56^{c}	40.09^{c}	39.25^{c}	39.07^{c}	37.68^{c}	37.57^{c}	36.81^{bc}	36.83^{bc}	P<0.001

^{a,b,c,d} Different letters indicate significant differences at P<0.05, according to Duncan.

Table 6. Effect of fermentation on flavonoids and polyphenols.

Indicator (mg / 100 g DM)	Strain	Fermentation time (hours)								SE (±)
		0	24	48	72	96	120	144	168	Signific.
Flavonoids	*A. niger* J1	55.53^{de}	46.82^{bc}	30.25^{a}	27.57^{a}	30.62^{a}	27.05^{a}	26.53^{a}	26.01^{a}	3.890
	T. viride M5-2	55.53^{de}	53.53^{de}	50.31^{cd}	44.57^{b}	43.28^{b}	56.35^{e}	64.23^{f}	72.17^{g}	P<0.001
Phenolic content	*A. niger* J1	9.78^{h}	5.35^{d}	1.96^{ab}	1.83^{a}	2.61^{ab}	2.16^{ab}	2.52^{b}	2.52^{b}	0.730
	T. viride M5-2	9.78^{h}	8.74^{g}	8.60^{g}	8.45^{g}	6.90^{f}	6.10^{e}	5.02^{d}	4.49^{c}	P<0.001

^{a,b,c,d,e,f,g,h} Different letters indicate significant differences at P<0.05, according to Duncan.

4 Conclusions

The results demonstrate that using solid-state fermentation can reduce antinutritional factors in *L. mutabilis* Sweet foliage meal to acceptable levels, thereby increasing its potential nutritional value. In this study, there was a significant reduction in NDF, ADF, flavonoids, and phenolic content during fermentation, improving its nutritional quality and enhancing its potential for use in monogastric species, while facilitating further processing. It is important to optimize the process for potential scaling.

The *T. viride* M5-2 and *A. niger* J1 strains enabled the development of a biologically feasible fermentation process for the foliage meal under study, improving its nutritional value, with the best results obtained using the *A. niger* J1 strain. It is recommended to optimize the process for scaling.

Authors' contribution

D.J.C.M.: Conceptualization, Formal analysis, Data processing, Funding acquisition, Investigation,

Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft. J.C.D.M: Conceptualization, Project administration, Supervision, Writing –review & editing. E.V.C.: Conceptualization, Project administration, Supervision, Writing –review & editing.

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