

# The effect of fermented cacao pod husk using *Phanerochaete chrysosporium* on nutritive value, digestibility, fermentation parameters, and methane emissions

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**Abstract.** Pratama SM, Wajizah S, Wahyudi I, Ilham, Zulfahrizal, Munawar AA, Samadi. 2025. The effect of fermented cacao pod husk using *Phanerochaete chrysosporium* on nutritive value, digestibility, fermentation parameters, and methane emissions. *Asian J Agric* 9: 276-287. This research aimed to study the effect of fermentation using *Phanerochaete chrysosporium* with different incubation times on nutritive value, digestibility, fermentation parameters, and methane emissions of cacao pod husk. The treatments tested were: F0: without incubation; F1: 7 days of incubation time; F2: 14 days of incubation time; F3: 21 days of incubation time and F4: 28 days of incubation time. The variables observed were the quality of nutritional value, fiber fraction, in vitro rumen digestibility, gas production and methane gas concentration. The results showed that the treatment increased the crude protein value, reduced crude fiber and increased the Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) values as the incubation period increased. Treatment effectively increased digestibility with the highest obtained at F4 and the lowest at F1. The treatment also increased the total ammonia (NH<sub>3</sub>-N) and Volatile Fatty Acid (VFA) concentrations in the rumen with a pH that was still within normal levels. The total concentration of VFA or NH<sub>3</sub> in F4 was higher than in other treatments. The gas production value and methane gas concentration can be reduced by *P. chrysosporium* treatment with an incubation time of up to 28 days. It was concluded that the nutritional quality and digestibility of cocoa pod husk could be improved but were still relatively low, even though the pH, NH<sub>3</sub>-N and VFA Total values were in the normal range.

**Keywords:** Cocoa pod husk, incubation time, in vitro digestibility, methane, *Phanerochaete chrysosporium*

## INTRODUCTION

The supply of components for animal feed has become more scarce recently. This is caused, among other things, by the decreasing amount of land available for development and production of forages and local food plants such as natural field grasses and legumes as a result of habitation and land use for agriculture. In order to lessen dependence on the usage of conventional feed, it is required to explore potential new resources that might be used as alternative diets in place of all or part of the forage. One such solution is to use leftover food crops or plantation crops (Samadi et al. 2016). According to Edo et al. (2023), cocoa (*Theobroma cacao* L.) is one of the plantation commodities that produces a significant amount of biomass or by-products. After the Ivory Coast and Ghana, Indonesia is the world's third-largest cocoa producer (Witjaksono and Asmin 2016). In 2022, the total land of cocoa plantations in Indonesia was estimated to be about 1.5 million hectares with a total cocoa production of 667.300 tons (Badan Pusat Statistik 2023). Considering that the cocoa pod husk makes up roughly 75% of the total cocoa fruit (Baidoo et al. 2022), Indonesia produced over 500.475 tons of cocoa pod husk in 2022.

Cocoa pod husk, commonly discarded as agricultural waste, can be repurposed through biotechnological

processes, supporting the circular economy by reducing pollution and improving resource efficiency (Ouattara et al. 2021). Its potential use as animal feed could lower livestock production costs (Makinde et al. 2019). However, its high fiber (29.3%) and low protein (9.66%) content limits its nutritional value (Pratama et al. 2018). Additionally, it contains 12-19% lignin (DM), which is significantly higher than rice straw, making it less digestible (Laconi and Jayanegara 2015). Diets with 20-30% unprocessed cocoa pod husk reduce animal performance and digestibility in cattle, goats, and poultry (Ozung et al. 2022). Cocoa pod husk also contains theobromine (6.79 mg/100 g DM), which imparts a bitter taste, decreasing feed palatability and intake (Nguyen and Nguyen 2017; Alanon et al. 2016). High doses of theobromine may cause metabolic disturbances, such as muscle tremors, *tachycardia*, rapid breathing, and diarrhea. Therefore, to enhance its value as feed, cocoa pod husk must undergo physical, chemical, or biological treatment to improve its nutritional quality before being incorporated into animal diets (Laconi and Jayanegara 2015).

In an effort to improve the nutritional content of cocoa pod husk, fermentation bioconversion using white rot fungi appears to be a promising strategy, especially since the small to medium scale feed industry has great potential to

adopt this technology (Syahrir et al. 2017; Yakin et al. 2020). *Phanerochaete chrysosporium*, a species of white rot fungi capable of decomposing lignin, was found to be beneficial in enhancing the nutritional content (crude fiber and crude protein) of cocoa pod husk, according to Laconi and Jayanegara (2015). According to Sun et al. (2022), *P. chrysosporium* is a microbe that can degrade lignocellulosic materials selectively. It first breaks down the lignin component before moving on to the cellulose component. The fungus employs cellulose and hemicellulose as carbon sources. This fungus can also develop at relatively low temperatures, with a temperature range of 36-40°C, making it acceptable for application in various fermentation processes that create heat (Cordero et al. 2017).

The fermentation process using *P. chrysosporium* is particularly advantageous because it targets the lignin content, which is the primary barrier to the digestibility of cocoa pod husk (Yan et al. 2022). By breaking down lignin, the fungus enhances the accessibility of cellulose and hemicellulose, which are more digestible and nutritious for livestock. This process not only improves the feed's nutritional profile but also increases its palatability, making it more acceptable to animals. Furthermore, the ability of *P. chrysosporium* to operate at relatively low temperatures makes it a cost-effective and energy-efficient solution for large-scale fermentation processes (Liu et al. 2019).

Determining the optimal fermentation period is crucial for producing effective bioprocessed products. Fermentation can reduce lignin while increasing cellulose content. The success of fermentation is measured by nutrient loss and the cellulose-to-lignin ratio. This study examines the effect of *P. chrysosporium* application timing on the nutritional value and in vitro digestibility of cocoa pod husk.

## MATERIALS AND METHODS

### Fermentation process

A total of 30 sample units of fermented cocoa pod husk ingredients with different incubation times were used in this research. The cocoa pod husk used in this research was obtained from farmers and collected from various locations in Aceh Province. Before fermentation, the cocoa pod skin is first dried in the Sun for 2 days, then ground using a cutter mill with a 1 mm sieve. All cocoa pod husk (89% dry matter) that had been ground was homogenized for fermentation. Before use, *P. chrysosporium* was first cultured by culturing on MEA (Malt Extract Agar) media, then stored in slant agar media in a test tube and stored at 4°C in the refrigerator to maintain its viability before application. The next stage was adaptation to a solid medium based on cocoa pod husk to adapt the fungus to the target substrate, which then ends with inoculation into cocoa pod husk.

For each treatment unit, 3% white rot fungus *P. chrysosporium* in liquid media with spores at  $10^6$  was added to 200 g of cocoa pod husk powder, containing 40% dry matter through the addition of water. A mixture of 3% molasses and 0.2% urea that has been dissolved is also added to the fermented cocoa pod husk, then stirred

thoroughly until homogeneous. All samples were stored at room temperature for aerobic fermentation at different times, i.e., 0, 7, 14, 21, and 28 days. Each treatment consisted of 6 repetitions. After the fermentation period, all samples were dried in an oven at 60°C for 48 h.

### Chemical composition and fiber fraction determination

AOAC methods refer to the analytical methods and procedures developed and validated by the Association of Official Analytical Chemists (AOAC) for the accurate and reliable analysis of various substances. Latimer (2023) employed to ascertain the nutritional composition of the samples, comprising measurements of dry matter (DM), organic matter (OM), ash, crude protein (CP), ether extract (EE), crude fiber (CF), and nitrogen-free extract (NFE). The subsequent steps outline the concise procedures for identifying each constituent in chemical analysis. The DM content was evaluated by subjecting the samples to a temperature of 105°C in an oven for 24 hours. The ash concentration was determined by incinerating samples weighing 300-500 mg in a furnace at a temperature of 500°C for duration of 3 hours. The organic matter (OM) content was determined by excluding the dry matter (DM) and ash. The CP content was determined using the standard Kjeldahl's method, which involved the operations of destruction, distillation, and titration. The CF content was determined by subjecting 0.3-0.5 g samples to boiling in 3 ml of acid and 10 mL of alkali solutions for 30 minutes each. The CF content was measured by extracting the lipids using a Soxhlet device. The NFE value was determined using the equation  $NFE = 100\% - (\text{ash content} + CP + CL + CF)$ . The determination of neutral detergent fiber (NDF) and acid detergent fiber (ADF) was conducted using the procedure described by Van Soest et al. (1991). Samples weighing 100 mg were subjected to boiling in neutral detergent and acid detergent solutions for 1 hour each, respectively. The NDF analysis in this study was conducted without the use of  $\alpha$ -amylase and sodium sulfite. The hemicellulose content was calculated by subtracting the NDF content from the ADF content. The neutral detergent insoluble CP (NDICP) and acid detergent insoluble CP (ADICP) contents were determined using the method described by Licitra et al. (1996), utilizing the remaining residue from the previous analysis of NDF and ADF. The sample analysis was performed twice to ensure accuracy and reliability.

### In vitro procedure

Theodorou et al. (1994) conducted an in vitro analysis in which cocoa pod husk treatments were subjected to McDaughall's buffer mixture and rumen fluid. The rumen fluid was collected in the morning from a cow with a rumen fistula at the Animal Science Faculty of IPB University. The cow was kept specifically for research. Before rumen fluid was collected, cows were given a controlled diet to ensure the desired rumen composition for research. The rumen fluid was strained through four layers of gauze before use. A serum tube with a volume of 125 mL was prepared for filling. It included a sample weighing 0.75 g and was supplemented with 75 mL of buffered

rumen fluid. The volumetric ratio of rumen fluid to buffer was 1:4. Afterwards, an incubation process was carried out for 48 hours at a temperature of 39°C in a water bath. Serum bottles were sealed with aluminum crimp seals and butyl rubber stoppers. The gas generation was quantified using a gas-tight syringe at certain time intervals of 2, 4, 6, 8, 12, 24, 36, and 48 hours after incubation. On each occasion when the quantity of gas generated was determined, the region was manually agitated. The quantification of methane was conducted utilizing gas chromatography (GC) Shimadzu, model GC8. The method used is in accordance with SNI 9224-1: 2023 concerning methods for measuring emissions of methane gas (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O). In order to separate the liquid portion from the solid residue, the serum bottles were subjected to centrifugation after 48 hours of incubation. The content of total volatile fatty acids (VFA) and ammonia, as well as the pH, were determined by analyzing the supernatant collected after a 48-hour incubation using the Conway microdiffusion method, as outlined in the study by Jayanegara et al. (2016). The VFA concentration of acetate, propionate, butyrate, and valerate was measured using the Gas Chromatography (GC) method, specifically the IKM 13 (GC-FID) instrument, as described by Kristensen et al. (2000). The values of in vitro dry matter digestibility (IVDMD); in vitro organic matter digestibility (IVOMD) were determined by subtracting the remaining dry matter (DM) and organic matter (OM) from the original DM and OM before the incubation process. The in vitro incubation was performed in three iterations, with two serum bottles utilized in each iteration. To implement this approach, a randomized complete block design was utilized for treating the experimental unit, with runs being treated as the blocks (replicates). For control purposes, two bottles containing buffered rumen fluid without any substrate were also incubated and regarded as blanks.

### Data analysis

The data were subjected to statistical analysis using analysis of variance (ANOVA) with the following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

The observed measure, denoted as  $Y_{ij}$ , is influenced by the general mean ( $\mu$ ), the treatment effect ( $\alpha_i$ ), and the block ( $\beta_j$ ). The study reported significant differences between treatments ( $P < 0.05$ ) and further analyzed these differences using the Duncan Multiple Range Test (DMRT).

## RESULTS AND DISCUSSION

### Chemical composition

Table 1 presents the nutritional quality of cocoa pod husk that was fermented using *P. chrysosporium* for varying lengths of time. Statistical study revealed that the treatment of *P. chrysosporium* had a highly significant impact on the crude protein content of cocoa pod husk ( $P < 0.01$ ). The F4 treatment, with a fermentation duration of 28 days, had the highest crude protein concentration, specifically measuring 13.85% (Table 1). The crude protein content showed a linear increase, with a slight decrease observed in the F2 treatment. Syahrir et al. (2017) found that the crude protein content of cocoa pod husk could be increased through fermentation treatment employing a combination of *P. chrysosporium* and *Pleurotus ostreatus* at a ratio of 8 and 15 g/kg, respectively. According to a study conducted by Laconi and Jayanegara (2015), the inclusion of *P. chrysosporium* at a concentration of 3% can enhance the crude protein content of cocoa pod husk. The rise in crude protein content during solid-state fermentation is a result of microorganisms being cultivated in lignocellulosic material. This cultivation allows yeast to utilize carbohydrates from starch, which are broken down into glucose during the degradation process. The microorganisms use the glucose to synthesize mycelium biomass (Terefe et al. 2021).

According to Kumla et al. (2020) there is a direct relationship between the amount of mycelium and the protein content. Increasing the number of mycelia leads to an increase in protein content. Among the cultivated strains, *P. chrysosporium* exhibited the highest mycelial biomass concentration in submerged fermentation (*H. annosum*, *P. chrysosporium*, *P. ostreatus*, *T. versicolor*, and *L. lepideus*) (1.03 g 100 mL<sup>-1</sup>) after 14 days (Irbe et al. 2023). The decline in crude protein seen over the 14-day incubation period is attributed to protein degradation occurring during the fermentation process. Proteolytic bacteria were found to be active and responsible for breaking down the protein present in the feed. Figaj et al. (2019) suggested that certain proteins in the substrate undergo degradation, or proteolysis, by both plant and microbial protease enzymes, resulting in the formation of non-protein nitrogen (NPN) molecules, such as amino acids and ammonia. Supplementing the fermentation media with nitrogen can enhance the growth of mycelium in *P. chrysosporium*.

**Table 1.** Nutritive value of cocoa pod husks fermented by using *Phanerochaete chrysosporium* at different incubation times

Nutritive values	F0	F1	F2	F3	F4	P-value	SEM
DM (%)	88.33±0.07 <sup>c</sup>	88.91±0.09 <sup>b</sup>	85.96±0.10 <sup>d</sup>	89.68±0.38 <sup>a</sup>	90.21±0.11 <sup>d</sup>	<0.01	0.29
OM (%)	89.47±0.06 <sup>a</sup>	88.16±0.08 <sup>b</sup>	87.29±0.10 <sup>c</sup>	85.83±0.10 <sup>d</sup>	85.01±0.09 <sup>e</sup>	<0.01	0.30
CP (% DM)	10.16±0.04 <sup>e</sup>	13.44±0.14 <sup>d</sup>	11.14±0.18 <sup>c</sup>	12.98±0.20 <sup>b</sup>	13.85±0.08 <sup>a</sup>	<0.01	0.27
CF (% DM)	30.09±0.51 <sup>a</sup>	34.44±0.91 <sup>a</sup>	32.96±1.27 <sup>a</sup>	30.06±0.42 <sup>b</sup>	30.55±0.70 <sup>b</sup>	<0.01	0.47
EE (% DM)	1.25±0.05 <sup>b</sup>	1.25±0.03 <sup>b</sup>	1.01±0.04 <sup>c</sup>	1.74±0.08 <sup>a</sup>	1.13±0.10 <sup>bc</sup>	<0.01	0.05
Ash (% DM)	9.31±0.05 <sup>e</sup>	10.53±0.07 <sup>d</sup>	10.92±0.08 <sup>c</sup>	12.71±0.10 <sup>b</sup>	13.53±0.07 <sup>a</sup>	<0.01	0.28
NFE (% DM)	49.19±0.51 <sup>a</sup>	42.88±0.79 <sup>a</sup>	41.4±1.37 <sup>b</sup>	42.55±0.53 <sup>b</sup>	40.94±0.69 <sup>b</sup>	<0.01	0.81

Note: F0: without incubation, F1: 7 days of incubation time, F2: 14 days of incubation time, F3: 21 days of incubation time and F4: 28 days of incubation time. DM: dry matter, OM: organic matter, CP: crude protein, CF, crude fiber, EE: extract ether, NFE: nitrogen-free extract, superscript letter in the same row indicates a significant difference between groups by Duncan's test ( $P < 0.01$ )

The substrate's crude fiber content (Table 1) exhibited an initial increase during a 7-day fermentation period, followed by a progressive decline during a fermentation period of 14-28 days ( $P < 0.01$ ). The study saw a lower reduction in crude fiber compared to the study conducted by Mustabi et al. (2020), i.e., 17.22%. The fungus *P. chrysosporium* extracts the nutrients present in the substrate and breaks them down into simpler forms through catabolism. To ensure its continued existence, the fungus undergoes a biodegradation process, which involves the fungal hyphae making contact with the surface of the substrate and forming resilient colonies. Once reached, the fungus readily decomposes the fiber constituents. During the 7-day long fermentation treatment, the rise in crude fiber content occurred as a result of the fungus cell walls themselves undergoing fermentation. Fesel and Zuccaro (2016) state that chitin is a crucial constituent of the cell walls of most of fungi. Chitin is a polysaccharide that serves as the primary constituent of the exoskeleton found in insects and arthropods.

The dry matter and ash content varied significantly ( $P > 0.05$ ) between treatments. The dry matter content remained slightly stable between treatments, ranging from 88-90% (Table 1). It is anticipated that no evaporation occurs during fermentation when the substrate is placed in a closed polyethylene bag and then opened once the substrate is completely covered with mycelium. The increased dry matter content is most likely due to the limited nutrient availability and the short incubation period (Ruvuga et al. 2022). Degrading enzymes, such as ligninolytic enzymes, do not perform well when degrading other fiber components, such as lignin. This is supported by Kumla et al. (2020), who used only easily fermentable carbohydrate sources during the fermentation process. In contrast, lignin components that are difficult to break down remain in the substrate. The increased lignin content in the substrate in this study was due to ligninolytic enzymes failing to break down lignin molecules optimally. Ligninolytic enzyme activity promotes fungal growth and is dependent on nutrition availability and media type. In this study, we did not give a source of slow-release carbohydrates derived from grains, but rather energy from molasses to kickstart the fermentation process at the start of incubation (Wajizah et al. 2015). Because ash is a component of the cell wall, the fiber content is proportional to it. An increase in crude fiber concentration doesn't directly improve ash content; it can lead to changes in the overall composition of a feedstuff, potentially affecting ash levels indirectly.

The concentrations of ether extract and NFE content showed a substantial reduction ( $P < 0.01$ ) as incubation time progressed (Table 1). This phenomenon occurs because the yeasts use simple carbohydrates as an energy source and the lipase enzyme uses of lipids (Azhar et al. 2017). Amara and El-Baky (2023), demonstrated that fungi consume soluble cell components for their activities throughout the fermentation process. Hidayat et al. (2022) reported that the non-nitrogen extracts from cocoa pod husk fermented with *G. lucidum* decreased in content from 47.8 to 42.1%, and the lipid content reduced from 0.77 to 0.41%.

### Fiber fraction and protein fraction in fiber

The NDF concentration of cocoa pod husk waste was found to be significantly affected ( $P < 0.01$ ) by the administration of *P. chrysosporium* with varying incubation durations, according to an analysis of variance (Table 2). The NDF content fluctuated; treatment F4 (81.20%) showed a higher NDF content than all treatments (Table 2). Comparing this study to Laconi and Jayanegara (2015), the NDF content data is higher, at 80.7%.

In Table 2, it can be seen that there was a decrease in NDF levels but it was not significant, so it cannot be claimed that the use of this fungus is effective in reducing NDF. This statement is supported by the NDF levels in the F4 treatment which show a significant increase in NDF compared to the F0 treatment. This increase is believed to be a result of cellulolytic microbial activity, which initially utilizes the cell contents (NDS) in the substrate, leading to a corresponding increase in cell walls (NDF). The rise in NDF levels signifies that the activity of cellulolytic microorganisms is inadequate in decomposing intricate molecules into simpler ones. According to Gonzalez and Aranda (2023), the growth rate of microorganisms is influenced by a decrease in nutritional availability and the buildup of metabolic byproducts that hinder growth. Inefficient cellulolytic bacteria hinder the activity of the cellulase enzyme in breaking down the cellulose and lignin present in cell walls (NDF). As a result, the conversion of these complex molecules into simpler ones is insufficient, leading to a rise in the proportion of the cell wall (NDF). According to Dewiyanti et al. (2022), cellulolytic bacteria utilize the cellulase enzyme to break down cellulose by hydrolysis. There are dynamics in the NDF figures in this research. On day 0, the NDF value of cocoa pod shells was 74.93%. On day 7 there was an increase to 80.55%, possibly due to degradation of the non-fiber fraction and accumulation of fibrous microbial biomass from *P. chrysosporium*. Furthermore, on the 14<sup>th</sup> and 21<sup>st</sup> days, the NDF value decreased to 73.70 and 72.75% due to the activity of fungal ligninolytic enzymes, which effectively degrade lignocellulose. However, on the 28th day the NDF value rose again to 81.20%, which was thought to be caused by the accumulation of microbial biomass, repolymerization of lignin compounds, and a reduction in easily degradable substrates.

In this study, the cocoa pod husk ADF level increased significantly ( $P < 0.01$ ) from 61.53 to 72.59%, with the highest value occurring in the F4 treatment (28 days of incubation) (Table 2). This information indicates that the ADF rises with the length of the incubation period. The present study's data is less than that of (Lesmana et al. 2020) i.e., 84,07%. The ADF levels increased consistently from treatment F0 to F4, rising from 61.53 to 72.59%. This indicated that during fermentation, there was a relative accumulation of cellulose and lignin components, which are the main constituents of ADF. This increase was not solely due to the formation of more ADF, but rather because most of the non-ADF components, such as sugars, starches, and soluble proteins, had been degraded by the enzymatic activity of the fungus, making the ADF fraction appear to increase proportionally. As an energy source,

lactic acid bacteria activity degrades non-fiber carbohydrates but not ADF, which influences the increase in ADF value on the substrate and results in an increase in ADF percentage. This finding is consistent with the research conducted by Tai et al. (2015), which suggested that the rise in ADF concentrations was due to microorganisms utilizing organic materials that were simple to metabolize during the fermentation process, resulting in an accumulation of unused organic materials like ADF.

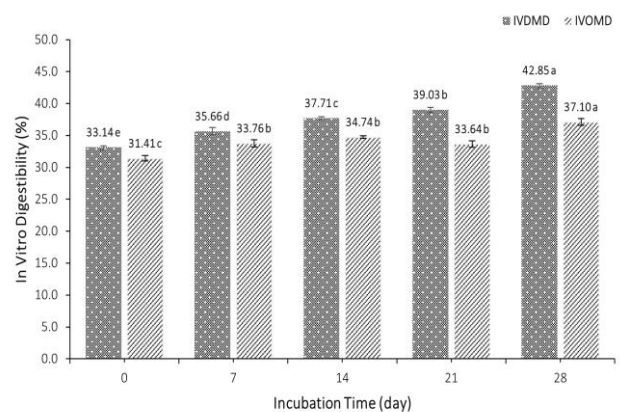
In Table 2, it was observed that the hemicellulose content underwent significant changes, reflecting the dynamics of fiber component degradation during fermentation ( $P < 0.01$ ). The data showed that the highest hemicellulose content was detected in the F1 treatment (Table 2), which was probably caused by the initial decomposition of lignin, so that the hemicellulose fraction became more accessible and was detected at a higher level. According to Hartulistiyoso et al. (2022), the liberation of lignin from cellulose results in an increase in the hemicellulose content. However, in treatments F2 and F3, there was a significant decrease in hemicellulose content, indicating that active degradation of hemicellulose had occurred due to the enzymes produced. Reduction in hemicellulose levels due to the enzymatic action of *P. chrysosporium*, namely the production of ligninase. This enzyme is capable of breaking down lignin, thereby disrupting the linkages of cellulose and hemicellulose (Li et al. 2022; Giri and Sharma 2020). Zhang et al. (2023), suggest that the degradation of lignin will facilitate the breakdown of cellulose and hemicellulose. Meanwhile, in the F4 treatment, the slight increase observed could have been attributed to fluctuations in enzymatic activity or possible substrate limitations and changes in fermentation conditions, which affected the degradation effectiveness. The findings of this study indicate a decrease in F3 treatment compared to the research conducted by Mayangsari et al. (2021), i.e., 4.23%.

The Neutral Detergent Insoluble Crude Protein (NDICP) content of cocoa pod husk significantly increased after being incubated for 14 days in the F2 treatment ( $P < 0.01$ ) (Table 2). The F2 treatment, which involved incubation for 14 days, had the highest Acid Detergent Insoluble Crude Protein (ADICP) content. This indicates that the 14-day incubation period had a higher concentration of ADICP compared to other treatments, similar to the concentration observed in NDICP. A fraction of the protein present in plants is located within the cellular structure known as the cell wall (Pratama et al. 2022). Nevertheless, rumen bacteria exhibit a rather restricted use of this protein in comparison to the protein present in the cytoplasm. The feed evaluation approach categorizes protein as NDICP (Neutral Detergent Insoluble Crude Protein) and ADICP (Acid Detergent Insoluble Crude Protein) (Licitra et al. 1996; Samadi et al. 2023). The NDICP and ADICP are protein fractions that exhibit insolubility in neutral detergent and acid detergent, respectively. While NDICP slowly breaks down or remains intact in the rumen, ADICP is considered completely indigestible and does not provide amino acids in the lower

gastrointestinal tract (Jayanegara et al. 2017). Consequently, ADICP contributes to the low protein quality in the diet. The composition includes proteins associated with lignin, complexes of tannins and proteins, and proteins that have been degraded by heat (Licitra et al. 1996). Neutral Detergent Insoluble Crude Protein (NDICP) and Acid Detergent Insoluble Crude Protein (ADICP) are types of crude proteins that are tightly attached to cell walls or resistant to breakdown in the rumen. As a result, they have low digestibility, meaning they are not easily broken down and utilized by the animal's digestive system (Wang et al. 2016). The fiber component is typically employed as a negative indicator of feed quality since it denotes the portion of the feed that is indigestible.

### In vitro digestibility of fermented cocoa pod husk

The results showed that the in vitro dry matter digestibility (IVDMD) and in vitro organic matter digestibility (IVOMD) of fermented cocoa pod husk using *P. chrysosporium* increased significantly in all treatments ( $P < 0.01$ ) (Figure 1). This figure illustrates the range of IVDMD, which is 33.14 to 42.85%. In particular, the findings of this study are inferior to those of Nurhaita et al. (2019), i.e., 55.89%. Despite achieving an increase in IVDMD, the percentage remained below 50%. Consequently, it can be concluded that the dry matter digestibility of cocoa pod husk is comparatively low on the whole. The treatment with a 28-day incubation period exhibited the greatest IVDMD, at 42.85% (Figure 1). The reaction involving F4 (28 days of incubation) generated the crude protein (Table 1), which impacted the substrate's dry matter digestibility. The ability of *P. chrysosporium* to manufacture cellulase and ligninase enzymes demonstrates that it can enhance the quality of cocoa pod husk (Suryadi et al. 2022). IVDMD is affected by the protein content of the nutrition as the solubility and resistance to degradation of proteins vary depending on the source (Wang et al. 2016). As the percentage of digestibility in feed increases, the quality of the feed also improves. The proportion of feed constituents, chemical composition, protein content ratio, and percentage of fat and minerals influence dry matter digestibility values (Gebremariam and Belay 2021).



**Figure 1.** IVDMD and IVOMD values of cocoa pod husks fermented by using *Phanerochaete chrysosporium* at different incubation times

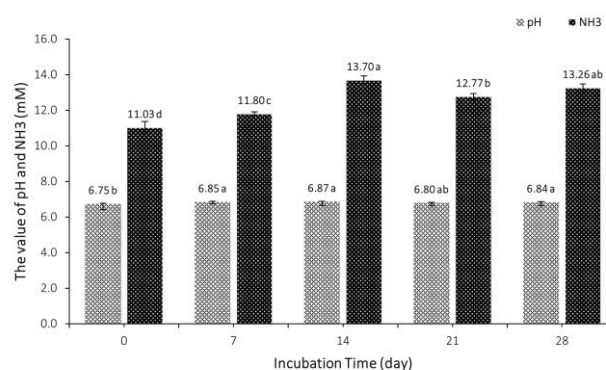
The in vitro organic matter digestibility (IVOMD) values obtained in this study ranged from 31.41% to 39.09% (Figure 1). The F4 treatment (28 days of incubation) exhibited the greatest IVOMD value. The digestibility value of organic matter corresponds to the digestibility value of dry matter. Maranatha et al. (2020) found that the digestibility of organic matter is strongly linked to the digestibility of dry matter, as a portion of dry matter is composed of organic matter. Ndaru et al. (2022) elucidated that the digestibility of dry matter has an impact on the digestion of organic matter. A reduction in dry matter digestibility will lead to a corresponding fall in organic matter digestibility. In this study, the digestibility value of organic matter was found to be greater than the value reported by Hidayat et al. (2022), which was 33.38%. Karimizadeh et al. (2017) stated that the digestibility of feed is a reliable indicator of its quality. In other words, the higher the digestibility of a certain type of feed, the higher its quality.

After 7 days of incubation, *P. chrysosporium* began producing ligninolytic enzymes such as lignin peroxidase and manganese peroxidase (Konan et al. 2024), which initiate the breakdown of the complex chemical bonds within lignin. At this stage, a preliminary increase in IVDMD and IVOMD was observed due to the partial degradation of lignin. With continued incubation to 14 days, enzymatic activity intensified, leading to more extensive lignin degradation and the disruption of cellulose and hemicellulose structures previously shielded by lignin. Consequently, the digestibility of the feed substrate increased significantly, a testament to the power of collaboration in scientific research. At 21 days of incubation, lignin degradation progressed deeply, with a substantial reduction in lignin content, thereby producing a further increase in IVDMD and IVOMD. By 28 days, *P. chrysosporium* had achieved near-optimal lignin decomposition, resulting in the highest recorded increases in IVDMD and IVOMD. However, extending the incubation period beyond this point poses a risk of

excessive degradation of other essential nutrients. Biochemically, the improvement in feed quality is attributed to the ligninolytic enzymes' capacity to cleave the aromatic structure of lignin and break the cross-linkages between lignin and polysaccharide fibers (Gharechahi et al. 2023). This enzymatic action enhances microbial access to cellulose and hemicellulose in the cocoa pod husk, thereby facilitating ruminal digestion.

#### pH values and ammonia (NH<sub>3</sub>-N)

Accurate pH values play a very important role in supporting rumen microbes to degrade feed optimally. Fibre will be digested slowly at a pH of <6.2 and will be dangerous for livestock if the pH decreases to <5.4 (Darwin and Blynaut 2019). The pH value in our study was 6.75-6.77 and is in accordance with the ideal pH value as stated by Kitkas et al. (2022), i.e., 6-7 (Figure 2). Although the IVDMD and IVOMD values in the F4 treatment (28 days of incubation) increased, the pH value of the rumen fluid was still in the normal range (Figure 2).



**Figure 2.** pH values and ammonia (NH<sub>3</sub>-N) gas concentration of cocoa pod husks fermented by using *Phanerochaete chrysosporium* at different incubation times

**Table 2.** Fiber fraction and protein fraction in the fiber of cocoa pod husks fermented by using *Phanerochaete chrysosporium* at different incubation times

Parameters	F0	F1	F2	F3	F4	P-value	SEM
Fiber fraction							
NDF (%)	74.93±2.55 <sup>bc</sup>	80.55±1.69 <sup>ab</sup>	73.7±2.29 <sup>c</sup>	72.75±0.61 <sup>c</sup>	81.20±2.08 <sup>a</sup>	0.01	1.05
Hemisellulosa (%)	13.41±3.16 <sup>a</sup>	15.01±1.63 <sup>a</sup>	8.73±2.46 <sup>ab</sup>	2.58±0.70 <sup>b</sup>	8.60±2.02 <sup>ab</sup>	<0.01	1.21
ADF (%)	61.53±0.76 <sup>d</sup>	65.54±0.65 <sup>c</sup>	64.97±0.95 <sup>c</sup>	70.17±0.75 <sup>b</sup>	72.59±0.46 <sup>a</sup>	<0.01	0.79
Protein fraction in fiber							
NDICP (% DM)	9.60±1.06	9.70±0.75	11.12±0.51	9.42±0.15	8.52±0.25	0.10	0.31
NDICP (% CP)	66.19±2.27 <sup>b</sup>	71.62±1.53 <sup>a</sup>	63.36±1.99 <sup>b</sup>	65.24±0.69 <sup>b</sup>	73.25±1.91 <sup>a</sup>	<0.01	1.02
ADICP (% DM)	10.17±0.84 <sup>bc</sup>	9.09±0.37 <sup>c</sup>	11.47±0.23 <sup>ab</sup>	10.00±0.31 <sup>bc</sup>	11.97±0.51 <sup>a</sup>	<0.01	0.28
ADICP (% CP)	54.35±0.66 <sup>d</sup>	58.27±0.56 <sup>c</sup>	55.84±0.77 <sup>d</sup>	62.94±0.89 <sup>b</sup>	65.49±0.43 <sup>a</sup>	<0.01	0.83

Note: F0: without incubation, F1: 7 days of incubation time, F2: 14 days of incubation time, F3: 21 days of incubation time and F4: 28 days of incubation time. NDF: neutral detergent fiber, ADF: acid detergent fiber, NDICP: neutral detergent insoluble crude protein ADICP: acid detergent insoluble crude protein, superscript letter in the same row indicates a significant difference between groups by Duncan's test (P<0.01)

Organic acids play a crucial role in regulating the pH value of the rumen and maintaining the balance of bacterial populations within it. Based on the findings of Suiryanrayna and Ramana (2015), organic acids can accelerate the reduction in pH. Kara et al. (2018) demonstrated that organic acids, specifically formic and propionic acids, affect the breakdown of proteins, causing them to be converted from complicated structures into simpler and more soluble forms. In order to ensure the proliferation of microorganisms, pH of the rumen must be maintained within the range of 6-7.

The concentration of ammonia/NH<sub>3</sub>-N is directly proportional to the number of microbes in the rumen. Ammonia is a product resulting from fermentation activity in the rumen, the result of protein degradation from feed and is a source of nitrogen which is quite important for rumen microbial synthesis (Tahuk et al. 2016). The results showed that the NH<sub>3</sub>-N value of fermented cocoa pod husk using *P. chrysosporium* had a significant effect (P<0.01). In this study, there was a linear increase in NH<sub>3</sub>-N concentration along with the incubation time up to 14 days, but there was a decrease at an incubation time of 21 days, then increased again at an incubation time of 28 days (Figure 2). The NH<sub>3</sub>-N value in this study was 11.03-13.70 mM, in accordance with that reported by McDonald et al. (2002), which ranges from 6-21 mM. Ruminants need a balanced NH<sub>3</sub>-N value to digest fiber optimally. The high NH<sub>3</sub>-N value in the F4 treatment may be due the fast rate of protein degradation, causing NH<sub>3</sub>-N production to increase. The high or low digestibility of protein can increase the degradability and fermentability of feed ingredients in the rumen so that the resulting NH<sub>3</sub>-N concentrations are high (Permana et al. 2022). After incubation for 7 days, the activity of proteolytic and lignolytic enzymes produced by *P. chrysosporium* began to break down the complex bonds between protein and lignin, increasing in NH<sub>3</sub>-N levels. This increase continued significantly on day 14, as enzymatic activity increased and organic matter decomposition released more nitrogen in the form of ammonia. The decrease in NH<sub>3</sub>-N production in the F3 treatment (21 days of incubation) is thought to be due to limited nutrition, disrupted mycelium growth, and the laccase enzyme not working optimally (Zhang et al. 2022). After the incubation time reaches 28 days in F4 treatment, the crucial role of enzyme activity in degrading cell walls

increases and releasing monosaccharides to be used by microorganisms as substrates during metabolic activities (Rho et al. 2020) is highlighted. The NH<sub>3</sub>-N value in this research is lower than the research reported by Iflijar et al. (2021) namely 14.70 mM. Putri et al. (2021) explained that the factors influencing the increase in NH<sub>3</sub>-N production were protein sources in ratios that were easily degraded by rumen microbes and high feed energy, and high growth of rumen microbes.

#### Total VFA and VFA partial concentration

The average VFA production between *P. chrysosporium* treatments with different fermentation times can be seen in Table 3. The results of the analysis of variance show that the fermentation time has a very significant effect (P<0.01) on the concentration of total VFA and partial VFA of cocoa pod husk. VFA production in this study ranged from 74.90 to 125.70 mM (Table 3). According to McDonald et al. (2002), the range of total VFA that supports rumen microbial growth is 70-150 mM. The rumen fluid VFA concentration value of the research feed ingredients meets these parameters. Rumen microbial activity alters the rumen metabolic profile, particularly VFA produced by carbohydrate breakdown.

The total concentration of volatile fatty acids (VFA) showed a significant increase with prolonged incubation time. The lowest concentration was observed in the F0 treatment (control) at 74.90 mM, while the highest was recorded in the F4 treatment at 125.70 mM. This increase indicates that fermentation by *P. chrysosporium* progressively enhanced total VFA production. The efficiency of substrate decomposition by fungus into VFA appeared to improve with extended fermentation duration (Li et al. 2017); the *P. chrysosporium* fungus produces lignolytic and hydrolytic enzymes which gradually break down the complex components of the substrate into simple molecules. Microbes then ferment these molecules to become VFA. With increasing fermentation time, enzymatic activity and microbial fermentation take place more optimally, so that VFA accumulation increases significantly. The statistical significance among treatments suggests that the changes in total VFA concentration were not random, but rather a result of the different fermentation treatments applied.

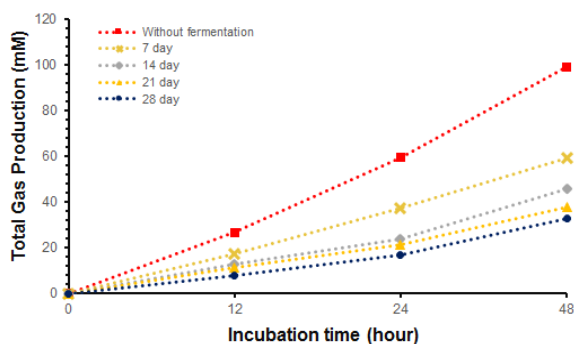
**Table 3.** VFA total and VFA partial concentrations of cocoa pod husks fermented by using *Phanerochaete chrysosporium* at different incubation times

VFA contents	F0	F1	F2	F3	F4	P-value	SEM
VFA total (mM)	74.90±3.23 <sup>d</sup>	95.23±2.45 <sup>c</sup>	110.17±2.16 <sup>b</sup>	113.09±2.67 <sup>b</sup>	125.70±1.96 <sup>a</sup>	<0.01	3.40
VFA Partial							
C <sub>2</sub> (mM)	43.12±6.10 <sup>a</sup>	18.9±3.72 <sup>c</sup>	18.66±3.37 <sup>c</sup>	32.71±3.28 <sup>ab</sup>	26.28±1.72 <sup>bc</sup>	<0.01	2.36
C <sub>3</sub> (mM)	8.79±1.25 <sup>a</sup>	3.89±0.79 <sup>c</sup>	4.10±0.73 <sup>c</sup>	7.53±0.90 <sup>ab</sup>	5.69±0.49 <sup>bc</sup>	<0.01	0.51
iC <sub>4</sub> (mM)	0.74±0.11 <sup>a</sup>	0.65±0.12 <sup>a</sup>	0.59±0.15 <sup>ab</sup>	0.34±0.05 <sup>bc</sup>	0.24±0.02 <sup>c</sup>	0.01	0.05
nC <sub>4</sub> (mM)	4.48±0.56 <sup>a</sup>	2.36±0.49 <sup>b</sup>	2.33±0.44 <sup>b</sup>	3.25±0.40 <sup>ab</sup>	2.13±0.18 <sup>b</sup>	<0.01	0.24
iC <sub>5</sub> (mM)	1.28±0.19 <sup>a</sup>	0.96±0.20 <sup>ab</sup>	0.86±0.24 <sup>abc</sup>	0.60±0.08 <sup>bc</sup>	0.38±0.02 <sup>c</sup>	0.01	0.09
nC <sub>5</sub> (mM)	0.59±0.08 <sup>a</sup>	0.36±0.08 <sup>b</sup>	0.34±0.09 <sup>b</sup>	0.36±0.04 <sup>b</sup>	0.23±0.01 <sup>b</sup>	0.02	0.04

Note: F0: without incubation, F1: 7 days of incubation time, F2: 14 days of incubation time, F3: 21 days of incubation time, and F4: 28 days of incubation time. C<sub>2</sub>: acetic acid, C<sub>3</sub>: propionic acid, iC<sub>4</sub>: isobutyric acid, nC<sub>4</sub>: n-butyric acid, iC<sub>5</sub>: iso-valeric acid, nC<sub>5</sub>: n-valeric acid, superscript letter in the same row indicates a significant difference between groups by Duncan's test (P<0.01)

VFA partial showed a more complex pattern (Table 3), where C2 (acetate) experienced a sharp decrease from F0 to F1 and F2, then increased again at F3 and decreased slightly at F4, while C3 (propionate) also showed a similar trend; the initial sharp decrease in C2 and C3 could be caused by the dominance of lignin and cellulose degradation into unstable compounds at the start of fermentation, indicating that although total VFA increases, certain fractions such as butyric acid (iC4 and nC4) and valeric acid (iC5 and nC5) tend to decrease with fermentation time, suggesting transformation into other compounds—this dynamic pattern is important to consider because, according to Cheng et al. (2022), acetic acid is the most abundant product in rumen fermentation and its proportion, along with that of propionate and butyrate, reflects the type of fermentable substrate, where acetate is dominant in high-fiber diets and propionate in glucogenic substrates (Hua et al. 2022), and to achieve optimal energy efficiency, the molar balance between ketogenic (acetate and butyrate) and glucogenic (propionate) VFAs must be adjusted accordingly (Mullik et al. 2024). The findings of this study, as presented in Table 3, indicate that the quantity of butyrate in terms of moles is quite modest, with a range of just 2.13 to 4.48 mM in total VFA generation.

Overall, although the total VFA increased, most of the partial components such as C2, C3, and branched chain VFA (iC4, iC5, nC4, nC5) showed fluctuations or decreases. This suggests that the increase in total VFA is likely contributed to the accumulation of other fractions or subsequent metabolic reactions that alter the overall volatile fatty acid profile. A negative correlation can be seen between the increase in total VFA and the decrease in iC4 and iC5, which may be due to changes in microbial composition during fermentation or more selective enzymatic activity towards certain compounds. Thus, fermentation not only increases the quantity, but also changes the quality or type of volatile fatty acids formed, which is important to understand in the context of the nutritional value and use of fermented feed ingredients.

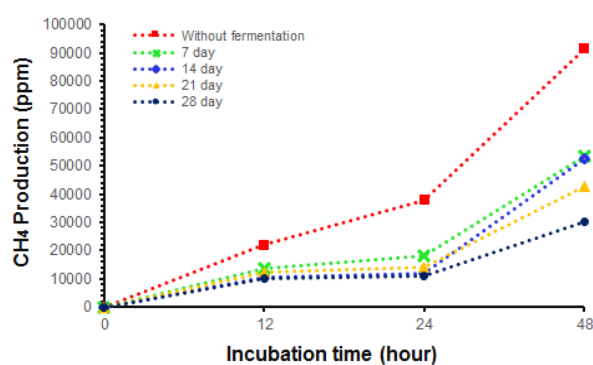


**Figure 3.** Total gas production formed over 48 hours of cocoa pod husk fermented using *Phanerochaete chrysosporium* at different incubation times

### Total gas production

Figure 3 displays the production data for cocoa pod husk gas production for each treatment. The gas analysis results indicated a positive correlation between the concentration of feed materials and the amount of gas produced. This suggests that the capacity of rumen bacteria to break down and process feed items is growing. The gas production rate exhibited a sluggish pace from hour 0 to hour 12. This indicates that the rumen microorganisms are still in the process of acclimating to the new environment, resulting in a somewhat sluggish rate of gas generation. An appreciable surge becomes apparent during the 24 to 48-hour period, as rumen microbes in this phase exhibit optimal gas production due to their transition into the exponential growth phase and successful adaptation to the new environmental conditions. Additionally, the substrate in their surroundings remains abundantly available. According to Paul et al. (2023), gas production data nearly doubled when the observation time interval was extended to 12 and 24 hours.

Rumen fermentation produces not only VFA but also gases such as CO<sub>2</sub>, CH<sub>4</sub>, NO<sub>2</sub>, NO, N<sub>2</sub> and approximately 3.5% O<sub>2</sub> (Mccauley et al. 2020). The amount of gas produced largely depends on the nutritional composition of the diet. Treatment F0 exhibited the highest gas production (Figure 3), which is attributed to its elevated levels of organic matter and NFE compared to other treatments (Table 1). The high NFE content reflects abundant non-structural carbohydrates such as starch and sugars that are readily fermented by rumen microbes, resulting in substantial gas production. However, the high gas production observed in F0 did not correspond to the highest digestibility. This is likely due to its low CP content (Table 1), which may limit fermentation efficiency despite the high fermentation of non-structural carbohydrates generating more gas. Conversely, treatments F1 through F4, characterized by increased CP and variable CF contents (Table 1), showed reduced gas production but improved digestibility.



**Figure 4.** Methane (CH<sub>4</sub>) gas concentrations formed over 48 hours of cocoa pod husks fermented using *Phanerochaete chrysosporium* at different incubation time

The increase in CP from F1 to F4 is hypothesized to provide a better nitrogen supply for rumen microbes, enhancing fermentative activity and supporting substrate degradation. This aligns with the understanding that protein fermentation produces less gas than carbohydrate fermentation. Furthermore, the elevated CF content in F1 and F2, likely derived from less fermentable fiber fractions such as lignin, contributed to the reduced gas production. The presence of crude fiber and anti-nutritional factors, such as tannins, has been reported to inhibit rumen enzymes and microbial activity, thus lowering total gas production (Sari et al. 2022). This phenomenon is supported by Sui (2018), who demonstrated a direct correlation between degradation rate values and total gas production (Figure 3). Additionally, treatments F1 to F4 exhibited an inverse relationship between incubation time and total gas output; longer incubation periods resulted in decreased gas production. This suggests that incubation treatments increasing fiber and protein content to a balanced level enable more efficient fermentation, resulting in significantly improved digestibility despite lower gas production than F0.

#### Methane (CH<sub>4</sub>) gas concentrations

The generation of CO<sub>2</sub> gas and two different forms of VFA, butyric and acetic acid, are strongly associated with the synthesis of methane gas. In the F0 treatment (no incubation), there was a rise in methane gas (Figure 4). This results from the waste from cocoa pod husk having a high acetic acid content (Table 3). A stoichiometric reaction occurs when a large amount of hydrogen gas is produced during the process of generating acetic acid. Methane gas is created first, using hydrogen gas and carbon dioxide as precursors (Zabranska and Pokorna 2018). Aside from that, the link between protozoa and methanogenic bacteria in the synthesis of methane results in an increase in methane output. Methanogenic bacteria produce enzymes that catalyze the reduction of CO<sub>2</sub> and H<sub>2</sub> in the rumen, which is the basic process by which methane is formed. Microorganisms known as methanogens actively contribute to the production of methane (Volmer et al. 2023). While between 63 and 80% of methanogens still function freely in the rumen, as many as 20 to 37% of methanogens are in symbiosis with protozoa (Volmer et al. 2023).

The treatment with the shortest methane gas production after 28 days of incubation was F4 (Figure 4). This result is attributed to the lower concentration of butyric acid in F4 compared to the other treatments (Table 3). The inoculation with *P. chrysosporium* reduced the butyric acid content, which likely contributed to the decreased methane production. *P. chrysosporium* has the ability to generate polyunsaturated fatty acids (PUFA), which may influence microbial community structure and metabolic activity during fermentation (Whiteford et al. 2021). It is hypothesized that PUFA can affect alterations in the partial VFA proportion profile within the rumen. Upon entering the rumen fluid, a lipolysis and biohydrogenation process is initiated in the fat (Huda et al. 2023). According to Tran et al. (2017), the relatively brief half-life of PUFA in rumen fluid can be attributed to their immediate biohydrogenation

process. Saturated fatty acids, which are produced as a byproduct of acetic acid and butyric acid synthesis, undergo this process (Talan et al. 2022). Cellulolytic microorganisms are the primary agents involved in the biohydrogenation of unsaturated fatty acids (Zhang et al. 2020). Hydrogenation, the process by which the hydrogen used to saturate the fat, can impede the methane gas synthesis reaction, resulting in a reduction in methane gas production.

Methane production was highest at 0 days, followed by a step-by-step decrease at 7, 14, 21, and the lowest value at 28 days of incubation. Cocoa pod husk was at 0 days incubation rich in lignin and other intricate compounds that not only hindered digestibility but also led to fermentable substrates that promoted hydrogen (H<sub>2</sub>) production. Methanogenic archaea utilize this hydrogen to produce methane. After 7 days of incubation with *P. chrysosporium*, the initial lignin degradation started through ligninolytic enzymes like lignin peroxidase and manganese peroxidase, altering the chemical composition and reducing the availability of methanogenic substrates. With longer incubation (14, 21, and 28 days), lignin degradation was more effective, further limiting the substrate available for the synthesis of methane. Degradation of lignin can also produce phenolic compounds, some of which are antimicrobial characteristic and could potentially suppress the growth and activity of methanogenic microorganisms. Methanogenesis was thus progressively suppressed with increased incubation periods.

In conclusion, fermentation treatment using *P. chrysosporium* fungus was able to improve the chemical quality of cocoa pod husk such as increasing crude protein even though there was an increase in the NDF and ADF values. The increase in dry matter digestibility and organic matter digestibility is still relatively low even though the pH, NH<sub>3</sub> and VFA Total values are in the normal range. The treatment was able to reduce the value of total gas production and methane gas production from cocoa pod husk. Based on the data that has been collected, it was found that the F4 treatment is the best treatment of all those tested. To maximize the potential of *P. chrysosporium* fungus, it is recommended to carry out physical and chemical treatment first to increase the digestibility value.

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