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Research Article
Populations of Rumen Microbes and the *In vitro* Digestibility of Fermented Oil Palm Fronds in Combination with Tithonia (*Tithonia diversifolia*) and Elephant Grass (*Pennisetum purpureum*)

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Abstract

Objective: The aim of this research was to identify the rumen microbial populations and determine the *in vitro* nutrient digestibility of fermented oil palm fronds (FOPF) by *Phanerochaete chrysosporium*, which were supplemented with Ca, P and Mn minerals and combined with tithonia (*Tithonia diversifolia*) and elephant grass (*Pennisetum purpureum*). **Methodology:** This study used a randomized block design consisting of 4 treatment groups: T1 = 20% FOPF+80% (tithonia+elephant grass), T2 = 40% FOPF+60% (tithonia+elephant grass), T3 = 60% FOPF+40% (tithonia+elephant grass) and T4 = 80% FOPF+20% (tithonia+elephant grass). Four replicates were used per treatment. The ratio of tithonia to elephant grass was 1:4. Data were analyzed by analysis of variance (ANOVA) and differences among means were tested using Duncan's multiple range tests (DMRT) with 5 and 1% confidence intervals. The measured variables were total bacteria, total protozoa, dry matter digestibility (DMD), organic matter digestibility (OMD) and crude fiber digestibility (CFD). **Results:** The results showed that total population of bacteria, DMD, OMD and CFD were the highest ($p<0.01$) in the T1 treatment. Total protozoa were lowest in T1 but the difference with the other treatments was not significant ($p>0.05$). **Conclusion:** T1 (combination of 20% FOPF and 80% tithonia+elephant grass) had the best effect on rumen microbial populations and *in vitro* digestibility.

Key words: *In vitro* digestibility, FOPF, *Tithonia diversifolia*, *Pennisetum purpureum*, microbial populations

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

One of the primary determinants affecting the success of a livestock business is the availability of continuous and abundant feed ingredients. Lately, the availability of animal feed has become increasingly limited. Factors affecting the availability of fodder include the increasing prices of raw materials for fodder and the decrease in land used to produce forage due to changes in land use in favor of food production and residential areas. Therefore, the exploration of potential new resources that can be used as alternative feed should be a top priority. Alternative feed is expected to replace some or all of the forage and can also reduce dependence on the commonly used concentrated ingredients.

Palm fronds are a waste product of palm oil plantations that can be used as animal feed. Each palm tree can produce 22 stem/pieces of palm fronds per year and the weight of palm fronds per stem reaches 2.2 kg (after peeling for feed) thus, each hectare can produce approximately 9 t/ha/year of fresh palm fronds for feed, which is equivalent to 1.64 t/ha/year of dry matter[1]. One factor constraining the use of palm fronds as animal feed is the high content of crude fiber and lignin[2,3]. The lignin binds strongly with cellulose and hemicellulose in plant cell walls, making it difficult for rumen microbial enzymes to penetrate cells, which ultimately decreases the digestibility of feed ingredients. A fermentation technique using *Phanerochaete chrysosporium* supplemented with the minerals Ca, Mn and P has successfully degraded lignin in palm fronds, decreasing the lignin content. The decrease in lignin content can facilitate the performance of rumen microbes to use nutrients from feed ingredients, which ultimately increases digestibility[4,5].

Techniques that combine multiple forages can contribute more forage with diverse compositions to increase microbial protein synthesis as a source of protein for host animals and to reduce rumen protozoan populations[6]. A decline in protozoan populations can lead to increases in populations of bacteria, thereby increasing feed degradation, which ultimately increases the digestibility of feed ingredients.

Tithonia (*Tithonia diversifolia*) is a potential feed material for ruminants that can be combined with fermented oil palm fronds (FOPF). Leaves of this plant contain complex amino acids and high protein and the availability is high[6]. Tithonia cultivated in West Sumatra can produce as much as 30 t of fresh material or 6 t of dry matter per year with a land area of approximately 1/5 ha. When grown as a hedge, tithonia can produce 27 kg of dry weight from three harvests in 1 year[7]. The research on tithonia as a ruminant feed material is limited because the leaves contain many anti-nutritional substances that endanger livestock such as phytic acid, tannins, saponins, oxalates, alkaloids and flavonoids[8].

The administration of the correct dose is the key to optimal use of tithonia as a feed ingredient. The correct dose can optimize the nutritional potential of tithonia and minimize the effect of anti-nutritional substances. Jamarun *et al.*[9] obtained the best values for *in vitro* dry matter, organic material and rough fiber digestibility using a 20% dose of tithonia in a combination of tithonia and elephant grass forage. Anti-nutritional content in tithonia is also suspected to affect defaunation, which decreases the number of protozoa in the microbial population leading to increase in the number of bacteria. Increasing the number of bacteria always increases the digestibility of food substances in the rumen.

The aim of this study was to determine the rumen microbial populations and *in vitro* digestibility of oil palm fronds (OPFs) fermented by *Phanerochaete chrysosporium,* which were supplemented with the minerals Ca, P and Mn and combined with tithonia and elephant grass.

MATERIALS AND METHODS

For this study, the fermentation of OPFs was conducted at the Laboratory of Technology for Feed Industries, Andalas University. Analysis of *in vitro* digestibility and rumen microbial populations was conducted in the Laboratory of Dairy Nutrition of the Bogor Agricultural Institute. Samples of FOPF, tithonia and elephant grass were collected around the town of Padang. All samples were finely ground and mixed according to the treatment combinations. The parameters measured in this study were total protozoa, total bacteria, DMD, DMO and CFD.

Fermented oil palm fronds, tithonia and elephant grass: The palm fronds used as the raw material in this study were taken from the distal two-thirds of the oil palm frond (OPF). The OPF substrates were cut, dried and finely milled. The oil palm frond was fermented using *Phanerochaete chrysosporium,* which was supplemented with 2000 ppm Ca, 2000 ppm P and 150 ppm Mn, according to the procedure of Jamarun *et al.*[5]

The tithonia plants used in this study had soft plant parts (stems, leaves and flowers) with a 6-16 cm long trunk from the top. Tithonia and elephant grass were cut, dried and crushed. The ratio of tithonia and elephant grass in each treatment ratio was 1:4, based on Jamarun *et al.*[9]. The nutritional contents of FOPFs, tithonia and elephant grass are presented in Table 1. The composition of the feed ingredients and the nutrition of the treatment rations are presented in Table 2.

Table 1: Nutritional content of FOPF, tithonia and elephant grass

	Raw material		
Nutritional content (%)	FOPF	Tithonia	Elephant grass
Dry matter	72.01	25.57	21.23
Organic matter	91.34	84.01	89.46
Crude protein	8.89	22.98	10.88
Crude fiber	38.59	18.17	32.77
Lignin	18.35	4.57	6.29

Table 2: Composition of feed ingredients and nutrition of treatment rations

	Treatment rations			
Variables (%)	A	B	C	D
FOPF	20.00	40.00	60.00	80.00
Tithonia	16.00	12.00	8.00	4.00
Elephant grass	64.00	48.00	32.00	16.00
Dry matter	93.58	92.33	90.88	89.73
Organic matter	88.96	89.56	90.15	90.75
Crude protein	12.42	11.54	10.65	9.77
Crude fiber	31.60	33.35	35.09	36.84
Lignin	8.43	10.91	13.39	15.87

***In vitro* procedure:** The *in vitro* procedure in this study followed that of Tilley and Terry[10]. Rumen fluid was taken from a cow fistula at Bogor, LIPI. The fermented tube was filled with 0.5 g of sample and 40 mL of McDougall solution was added. The tube was placed in a shaker bath at 39°C and then filled with 10 mL of rumen fluid and shaken with CO_2 for 30 sec, the pH was checked (6.5-6.9) and then the sample was covered with a ventilated rubber cap and fermented for 48 h. After 48 h, the rubber cap of the fermented tube was removed and 2-3 drops of $HgCl_2$ were added to kill the microbes. The fermented tube was centrifuged at 5,000 rpm for 15 min. The substrate separated into a precipitate layer at the bottom and a clear supernatant at the top. The supernatant was removed, the resulting sediment was centrifuged at 5,000 rpm for 15 min and 50 mL of 0.2% pepsin-HCl solution was added. This mixture was then reincubated for 48 h without a rubber cap. The remaining digested residue was filtered using Whatman filter paper No. 41 (identified by its weight) with the help of a vacuum pump. The precipitate on the filter paper was placed into a porcelain dish, which was placed into an oven at 105°C for 24 h. After 24 h, the porcelain cup+filter paper+residue was removed, inserted into a desiccator and weighed to determine the dry matter content. Furthermore, the ingredients in the cup were placed in a kiln or in an electric furnace for 6 h at 450-600°C and then weighed to determine the amount of organic material. Residue originating from the fermentation without feed ingredients was used as a control. The proximate analysis of materials and residues followed the AOAC[11] procedure.

Rumen protozoa population: Measurements were conducted using a counting chamber. Up to 0.5 mL of rumen solution was fixed with 0.5 mL of methyl green formalin saline (MFS) solution in tubes and thoroughly mixed[12]. Up to 0.1 mL of this sample was placed in a counting chamber (hemocytometer) using a pipette and a coverslip was placed over the sample. The protozoa were quantified in the counter under a microscope at 40X magnification. According to the number of protozoa obtained by the above counting procedure, the number per 1 mL of rumen content was calculated using the following Eq:

$$\text{Protozoa population mL}^{-1} = \frac{1\times1000\times n\times d}{0.1\times0.065\times6\times5}$$

Where:
n = Number of protozoa in the counting chamber
d = Sample dilution

Rumen bacteria population

Sample preparation: First, 1 mL samples of aseptic rumen fluid were pipetted into 9 mL of Butterfield's phosphate buffer diluent to obtain a 1:10 dilution. When homogenized, this sample was a solution at a 10^{-1} dilution.

Analysis of total bacteria: Analysis of total bacteria used the Anoxomat system (MART Microbiology, B.V., Netherlands), for the cultivation of micro-organisms in an oxygen-depleted or CO_2 enriched environment.

For subsequent dilutions (10^{-2}, 10^{-3}, 10^{-4} and others, as required) of the sample, 1 mL of the previous solution (dilution of 10^{-1}) was added to 9 mL of diluent using a sterile pipette. Then, the solutions were homogenized via vortexing. Next, 1 mL of any desired dilution was added to a sterile petri dish, this procedure was replicated twice. Then, 15-20 mL of NA medium (Nutrient Agar) was poured into a petri dish to test the total bacteria in the given sample. The media were homogenized by swirling the petri dish. Afterward, the samples were frozen. The petri dishes were inserted into an Anoxomat jar upside down to create anaerobic conditions according to the instructions for the Anoxomat system. Then, the petri dishes were incubated at 37°C for 48 h with the cup upside down. The number of colonies on a petri dish was counted, typically between 25 and 250 colonies. When the number of colonies was >250 colonies, the dish was considered TMTC (Too Many To Count). Colony calculations were performed.

Experimental design and statistical analysis: The study was conducted using a randomized block design (4×4) with 4 replications (Levels of FOPFs, tithonia and elephant grass as

the treatments). The differences between the treatment means were analyzed using Duncan's multiple range tests with confidence intervals of 5% ($p<0.05$) and 1% ($p = 0.01$).

The treatments were the following:

- T1 = 20% FOPF+80% (tithonia+elephant grass)
- T2 = 40% FOPF+60% (tithonia+elephant grass)
- T3 = 60% FOPF+40% (tithonia+elephant grass)
- T4 = 80% FOPF+20% (tithonia+elephant grass)

RESULTS AND DISCUSSION

Nutritional content of FOPFs: The nutritional contents of OPFs before and after fermentation are presented in Table 3.

Phanerochaete chrysosporium is a white mold that is currently used as a model for many scientists to degrade existing lignin in waste-based feedstocks. Table 3 shows a 40.08% decrease in lignin (from 30.86-18.35%), which was due to the supplementation with mineral (Ca, P and Mn) during fermentation to increase the enzyme activity of ligninase produced by *Phanerochaete chrysosporium*. Gupte *et al.*[13] stated that *P. chrysosporium* reduced the lignin content by 20.5% on the 10th day of incubation and showed maximum enzyme activity in degrading lignin. Supplementation of Ca and Mn minerals to palm fronds fermented by *P. chrysosporium* can reduce lignin by 25.77% and increase *in vitro* digestibility[4]. Fermentation of olive pomace by *P. chrysosporium* decreased lignin by 63.9% after 20 days of fermentation and by 46.44% after a fermentation time of 10 days[14]. These decreases are likely related to the availability of nutrients resulting from the reshuffling of lignocellulose components by the mold for its own growth. The change in lignin content in the substrate occurs because of the reshuffling of the lignin structure into simpler components[15].

Fermentation using *P. chrysosporium* that was supplemented with Ca, P and Mn minerals also increased the protein content of the feed and reduced the content of crude fiber. The amount of substrate protein increased by 144.23% and the amount of crude fiber decreased by 28.72%. The increased percentage of the crude protein content of fermented palm bark is caused by mold growth contributing to the increase in protein. Due to the growth of mold, the more mycelia are there, the more nitrogen is supplied to the body of the mold, which is the factor responsible for the increase of protein observed in the FOPFs. Nelson and Suparjo[15], suggest that an increase in protein content occurs because of the bioconversion of sugar into a mycelium protein or a single cell protein. The decrease in crude fiber content due to enzyme activity by the mold can also increase the percentage of crude protein content. Good protein quality and low levels of crude fiber result in increased digestibility of the feed.

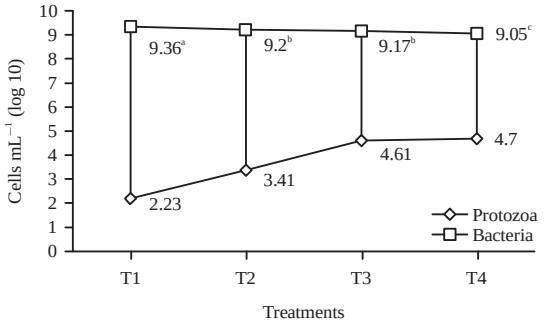

Fig. 1: Rumen microbial populations of various combinations of FOPF levels with tithonia and elephant grass
[a-d]Means in the same color with different letters are highly significantly different ($p<0.01$)

Table 3: Nutritional content of OPFs before and after fermentation

	Raw material	
Nutritional content (%)	OPF	FOPF
Dry matter	83.96	72.01
Organic matter	94.13	91.34
Ash	5.87	8.66
Extract ether	2.42	1.27
Crude protein	3.64	8.89
Crude fiber	49.80	38.59
NDF	89.98	66.52
ADF	73.21	57.85
Cellulose	41.35	37.50
Hemicellulose	16.78	8.67
Lignin	30.63	18.35

NDF: Neutral detergent fiber, ADF: Acid detergent fiber

Population of rumen microbes: Protozoa and bacteria are the most abundant in cattle rumens. In degrading feed ingredients through the production of enzymes, bacteria are very useful, whereas at high densities, protozoa decrease the rumen bacterial population. Rumen protozoan and bacterial populations of the fermented oil palm fronds combined with tithonia and elephant grass are presented in Fig. 1.

Figure 1 shows total protozoan population values that range from 2.23-4.70 cells mL^{-1} (Log 10). These values are lower than the range of protozoa obtained by Jamarun *et al.*[16] [5.312-6.295 cells mL^{-1} (Log 10)] from 100% palm fronds fermented using *Phanerochaete chrysosporium* fungus supplemented with Ca, P and Mn minerals. The values are also lower than the range described by Febrina *et al.*[17] from a 100% substrate of fermented palm fronds by *Phanerochaete chrysosporium* with the addition of minerals (P, S and Mg), which was 5.851-5.864 cells mL^{-1} (Log 10). These differences

are because in this study, tithonia plants were used that contained anti-nutritional tannins and saponins. Tannins and saponins in tithonia are anti-nutrients that can function as defaunation agents[18]. Defaunation is the reduction and elimination of rumen protozoa. With a decrease in protozoan population values, the numbers of bacteria increase because protozoa prey on bacteria. Excessive protozoa kill the bacteria, reducing the population of bacteria in the rumen. The reduction in the bacterial population reduces feed degradation, resulting in low digestibility of the feed. Different levels of FOPFs combined with tithonia and elephant grass did not significantly affect ($p>0.05$) the protozoan populations. However, as shown in Fig. 1, an increasing trend in the protozoan population was observed with reduced doses of tithonia in the treatments. T1 has the lowest protozoan population, which was due to the highest dose of tithonia that was equal to 16% of the feed ingredients. T4 had the highest protozoan population because the dose of tithonia was only 4% of the feed ingredients.

The population of rumen bacteria was inversely proportional to the protozoan population. Figure 1 shows the range of rumen bacteria at 9.05-9.36 cells mL^{-1} (Log 10), which is within the normal limits [9-12 cells mL^{-1} (Log 10)] reported by Stewart[19]. As levels of fermented palm bark increased and those of tithonia decreased, the rumen bacterial population decreased significantly ($p<0.01$). T1 had the highest population of bacteria, whereas the lowest population was in T4. The development and growth of bacteria are influenced by the ease or difficulty of degradation of feed ingredients. The T1 treatment contained lower lignin levels than those of the other treatments (Table 1). Low lignin levels allow more bacteria to degrade feed and feed degradation also produces a source of food for bacteria to grow and multiply. In addition to the influence of low lignin levels, the increasing population of bacteria was also due to the effects of tithonia plants which is containing tannin and saponin anti-nutrients as defaunation agents. Therefore, with reductions in protozoa, the number of bacteria increased.

Digestibility of dry matter, organic matter and crude fiber *in vitro*: Increased levels of FOPFs and decreased levels of tithonia and elephant grass had a highly significant effect on digestibility ($p<0.01$), with decreases in dry matter, organic matter and crude fiber digestibility *in vitro* as shown in Fig. 2. The average digestibility ranged from 38.01-58.17% for the digestibility of dry matter, 38.35-58.23% for the digestibility of organic matter and 33.81-57.35% for the digestibility of crude fiber.

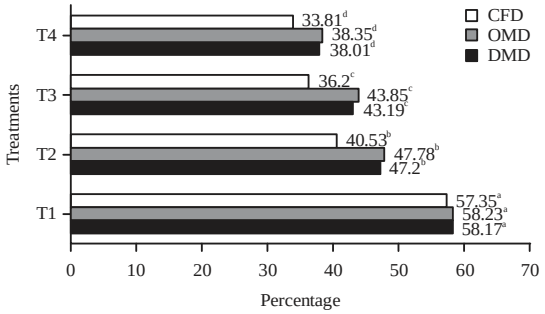

Fig. 2: *In vitro* digestibility of dry matter, organic matter and crude fiber of various combinations of FOPF levels with tithonia and elephant grass

[a-d]Means in the same color with different letters are highly significantly different ($p<0.01$)

Figure 2 shows a trend of decreased digestibility of dry matter, organic matter and crude fiber from treatment T1 to T4. The decrease in dry matter digestibility, organic matter and crude fiber was caused by the differences in crude fiber content and lignin content of each treatment. Imsya *et al.*[20] stated that the digestibility of dry matter and organic matter decreases because of high levels of crude fiber content in the ration. T4 showed the highest crude fiber and lignin contents (36.84% crude fiber and 15.87% lignin) and the lowest dry matter, organic matter and crude fiber digestibility. These results suggested that although OPFs were fermented using *P. chrysosporium* supplemented with Ca, Mn and P minerals, which decreased the lignin content, the nutritional value produced by the fermentation process was not able to achieve the nutritional value with tithonia and elephant grass (Table 1).

Fermentation of OPFs with *P. chrysosporium* supplemented with Ca, P and Mn minerals for 20 days showed a good lignin degradation rate. The content of lignin of OPFs without fermentation was 30.63%, whereas lignin content of fermented OPFs was 18.35% (Table 3). This result showed that although the lignin content could be reduced by the fermentation process, the lignin content remained higher than the lignin contents in tithonia and elephant grass, which were 4.57 and 6.29%, respectively (Table 1). Thus, at the high levels of FOPFs used in the treatment rations, the levels of digestibility remained low.

The high digestibility of T1 suggested that this combination of forages was more easily degraded by rumen microbes than the other treatments. Low lignin content is very helpful for rumen microbial breakdown of cellulose and hemicellulose feed. The contribution of tithonia to this

treatment, in addition to donating proteins, was a reduction in the protozoan population (Fig. 1) such that the number of bacteria significantly increased.

The pattern of digestibility of organic materials follows that of dry matter digestibility, i.e., the low digestibility of the dry matter causes the low digestibility of organic materials and vice versa. Thus, an increase in the digestibility of dry substrate materials is followed by an increase in the digestibility of organic substrate materials[21,22]. Jamarun *et al.*[9] also observed the same trend combining tithonia with elephant grass.

The values of DMD, OMD and CFD in this study are higher than those in the treatment of 100% *in vitro* FOPFs used by Jamarun *et al.*[21], Mariani [23], Rahayu *et al.*[24] and Febrina *et al.*[4,17]. This is because this treatment combines several feed ingredients such that the composition of nutrient substances donated, particularly the mineral content and amino acids, was more complete. Minerals and more complex amino acids optimize the metabolic activities in the rumen. Rumen microbes also optimally degrade feed ingredients, resulting in increased dry matter, organic matter and crude fiber feed digestibility.

In contrast, Febrina[25] and Febrina *et al.*[26] reported that fermented OPFs by *Phanerochaete chrysosporium* supplemented with Ca and Mn minerals matched the digestibility and consumption of elephant grass rations. The difference in these results was likely due to the influence of the addition of concentrate to the ration.

CONCLUSION

The combination of 20% FOPF and 80% (tithonia+elephant grass) (T1) yielded the best *in vitro* nutrient digestibility values and microbial populations.

SIGNIFICANCE STATEMENT

The effect of FOPFs combined with tithonia and elephant grass on microbial populations and *in vitro* nutrient digestibility was evaluated in this study. The results indicated that the combination of 20% FOPF with 80% (tithonia and elephant grass) was highly effective in rations derived from plantation waste products, such as oil palm fronds. This study help researchers to realize that the combination of FOPFs with tithonia and elephant grass is important to support growth and microbial activity and therefore to improve the digestibility of nutrients. Thus, this research could lead to a new theory on the importance of combination feed materials in rations derived from plantation waste products.

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