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In Vitro Nutrient Digestibility and Ruminal Fermentation Characteristics of Ammoniated and Fermented Treatment of Soaked and Unsoaked *Cymbopogon nardus* Waste

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ABSTRACT

Cymbopogon nardus waste has potential as an ingredients of cattle diet. *C. nardus* waste requires feed processing to increase ruminal fermentation characteristics. This study has aimed to discover the effect of fermentation and ammoniation treatment of soaked and unsoaked *C. nardus* on *in vitro* ruminal fermentation characteristics and nutrient digestibility. Five experimental diets with four replications were evaluated in this study: *Cymbopogon nardus* (CNW) as control, unsoaked fermented CNW (FCNW1), soaked fermented CNW (FCNW2), unsoaked ammoniated CNW (ACNW1), and soaked ammoniated CNW (ACNW2). A randomized block design was used and followed by post hoc Tukey-HSD if there is a significant effect ($P<0.05$). The results showed that there was no significant effect ($P>0.05$) of soaked or unsoaked ammoniated and fermented CNW on *in vitro* dry matter digestibility (DMD) and organic digestibility (OMD). Meanwhile, there was significant effect ($P<0.05$) on *in vitro* digestibility of crude protein (CPD), ADF (ADFD), NDF (ADFD), cellulose (CLD), and hemicellulose (HCLD). There was also no significant effect ($P>0.05$) of soaked or unsoaked ammoniated and fermented CNW on *in vitro* ruminal fermentation characteristics (pH, NH_3, and total VFA level). In conclusion, soaked ammoniated of *C. nardus* waste has potential as cattle diet because it has high value of *in vitro* digestibility of crude protein (56.81%), ADF (47.67%), NDF (48.68%), cellulose (47.19%), and hemicellulose (50.52%).

Key words: Ammoniation, *Cymbopogon nardus* waste, Fermentation, *in vitro* digestibility, Ruminal fermentation, Soaked-unsoaked.

INTRODUCTION

The main problem in livestock feed in Indonesia is limited forage availability either quality or quantity by fluctuate season so that we need feed source that can fulfill livestock need. One of alternatives we can adopt is the utilization of local feed resources. *Cymbopogon nardus* also known as lemongrass or citronella grass is local plant of Indonesia and a perennial plant with long and thin leaves and widely used as the producer of essential oils as its second metabolite. *C. nardus* leaves are extracted to produce essential oil that can be used as raw material for food, pharmaceutical, cosmetics, perfume and even as disinfectant (Manurung et al. 2015). Benefits of *C. nardus* have increased the plantation area into 19,370 hectares and 2,340 ton/years of biomass production in 2017 (Sulaswatty et al. 2019). However, *C. nardus* waste has high crude fiber fraction that impacts on the decrease of nutrient digestibility. It is necessary to adopt the feed processing to decrease crude fiber fraction contained in *C. nardus* waste.

Agricultural industry by products such as *C. nardus* waste is consisted of lower nutritional content. Microbial rumen is still capable to convert this low digestibility into animal product. But due to its high content of crude fiber

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fraction especially lignin, the digestibility of this waste is quite low. Several biological methods have been reported in processing high crude fiber forage in order to increase nutritional content and its digestibility. Sufyan et al. (2022) reported that delegitimated fungi have potential to decrease lignin content in some crop residues and agro-based by-product. They studied the effect of three species of *Pleurotus* in delignification of wheat straw, rice straw and corn cob. They reported that *P. ostreatus* is highly significant fungi in delignification of wheat straw and rice straw, meanwhile *P. florida* has potential to decrease lignin content in corn cob. Some studies also reported that oil palm frond which contains high lignin-cellulose then treated with fermentation and ammoniation could improve nutrient content and its digestibility (Zain et al. 2008; Zain et al. 2014; Jamarun et al. 2017). Jayanegara et al. (2017) found that urea treatment at 1% level (incubated for 4 weeks) and steamed with constant pressure and temperature i.e., 1.4 atm and 121ºC improve nutritional value of rice straw.

Beside urea and fungi treatment, livestock manure has the ability to improve nutritional value and digestibility of agricultural waste. Livestock manure contains many necessary nutrients such as nitrogen, phosphorus, and potassium to enhance the nutrient value of low-digestibility agricultural waste. Kayombo et al. (2021) reported that chicken manure can be utilized in yeast fermented cassava pulp as a nitrogen source and significantly escalates protein content. Inorganic nitrogen from manure helps to increase nutrition value that will enhance low-nutrient waste utilization. So, this approach is alternatively great to be applied in animal feed industry.

Soaking method is a general and economical way of treating agricultural waste. Soaking straw overnight in water treatment promotes higher intake in line with higher nutrient digestibility. This treatment along with steaming method will have effect on the cell walls delignification and also boost a good environment for the microbial rumen for faster fermentation of nutrients, impact on the increasing of nutrient digestibility (Aquino et al. 2020). The study of Husnaeni et al. (2017) reported that 12 days of sea water soaked rice straw produced the higher dry matter digestibility and organic matter compared to unsoaked. Previous studies have reported that ammoniated and fermented *C. nardus* waste could improve nutrient profile and decrease lignin content compared to untreated *C. nardus* waste (Manurung et al. 2015; Elihasridas et al. 2020). However, the comparative studies about soaked and unsoaked of *C. nardus* pre-treatment with fermentation and ammoniation process are still lacking, especially in Indonesia who produce abundant *C. nardus* waste. Increasing sources of information about pre-treatment of *C. nardus* to enhance its nutrient content and digestibility is very necessary to be observed. For those reasons, the objective of this study was to discover the effect of fermentation and ammoniation treatment of soaked and unsoaked *C. nardus* on *in vitro* ruminal fermentation characteristics and nutrient digestibility.

MATERIALS AND METHODS

Ethical Approval

Ethical approval was not required because this study did not use any live animals.

Study Period and Location

This study was regulated at Ruminant Laboratory in the Faculty of Animal Science, Andalas University, Padang, Indonesia, from November 2020-January 2021.

Sample Preparation and Experimental Diet

The material used in this study was *Cymbopogon nardus* waste (CNW). CNW was acquired from refining citronella oil in *C. nardus* plantations at Limau Manis, Padang, Indonesia. There are five treatments in this study: CNW as control, unsoaked fermented CNW (FCNW1), soaked fermented CNW (FCNW2), unsoaked ammoniated CNW (ACNW1), and soaked ammoniated CNW (ACNW2). This study used Randomized Block Design with five treatments with four replications. Before CNW treated with fermented and ammoniated process, it was chopped into 3-5cm size and then soaked in the water bath for 4h at 60ºC for soaked treatment. The fermentation of CNW process was done by used probiotic starter Starbio with a level of 0.6% DM and urea with a level of 0.6% DM of CNW. FCNW was then stored in anaerobic plastic bag and incubated for 10 days. As for ammonia process, urea was used with a level of 4% DM and chicken manure with a level of 15% DM of CNW. ACNW was then stored in anaerobic plastic bag and incubated for 10 days. After 10 days, the plastic bag of FCNW and ACNW were opened and the substrates were oven-dried at 60ºC for 24h, and then milled into 1mm sieve. After these processes, the sample was ready for the nutrient ingredients analysis and *in vitro* evaluation.

Nutrient Ingredients Analysis

Dry matter, ash, and crude protein was determined using Proximate analysis (AOAC 2005), meanwhile neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose, hemicellulose, and lignin was determined using Van soest analysis (Goering and Van Soest 1970). A total of 2.5g sample was oven dried at 105ºC for 8h to determine dry matter content. Ash content was measured by combusted a total of sample at 600ºC for 4-5h. Organic matter was calculated through this formula: 100%-%ash content. Protein content was measured according to Kjeldahl method consisted of three steps: destruction, distillation and titration. Meanwhile NDF was determined by dissolved sample with Neutral Detergent Soluble (NDS). ADF was determined by dissolved sample with Acid Detergent Soluble (ADS). Cellulose was determined by soaked ADF sample with 72% sulfuric acid (H_2SO_4) for 3h and to be continued to determine lignin content by combusted it in the furnace at 600ºC for 4h. Hemicellulose content was determined by subtracting the NDF and ADF content. The results of nutrient ingredient content are presented in Table 1.

In vitro Method

This study followed Tilley and Terry method (Tilley and Terry 1963) to conduct rumen *in vitro* incubation. A total of 2.5g experimental diet was incubated with 50ml rumen fluid and 200ml buffer solution in a fermenter tube. Rumen fluid was obtained from a slaughter house of Kacang goat with an average BW ± 20kg fed a diet of elephant grass, legume, and concentrate. Buffer solution was prepared according to McDougall method

(McDougall, 1947) by dissolving 9.8g $NaHCO_3$, 3.68g Na_2HPO_4, 0.57g KCl, 0.12g $MgSO_4.7H_2O$, 0.47g NaCl, and 0.05g $CaCl_2$ with per liter of distilled water. The mixture of 50ml rumen fluid and 200ml buffer solution without experimental diet sample was prepared as blank treatment. Each tube was injected with CO_2 gas for 30s, sealed using rubber cap, and then placed in the shaker incubator with a rotational speed of 100rpm at a temperature of 39ºC for 48h. After incubation process, each tube was immersed in iced water to stop the microbial fermentation, after which the pH was measured using Eutech Instruments pH 700 device. Then, supernatant and residue were separated using centrifuge machine at 3000rpm for 5m at 4ºC. Supernatant was stored at freezer (-18ºC) before it was used to NH_3 and total VFA analysis. NH_3 level was determined by following the Conway and O'Malley method (Conway and Malley 1942) and total VFA level was determined through steam distillation (Abdurachman and Askar 2000). Meanwhile, residue was filtered with Whatman No. 41 filter paper and then oven-dried at 60ºC for 24h. Nutrient content of dried residue was determined following proximate (AOAC 2005) and Van Soest (Goering and Van Soest 1970) analysis. *In vitro* digestibility was calculated using these formulas:

$$\text{DMD} = \frac{DM\ samples-(DM\ residue-DM\ blanks)}{DM\ sample} \text{ x } 100\%$$

$$\text{OMD} = \frac{OM\ samples-(OM\ residue-OM\ blanks)}{OM\ sample} \text{ x } 100\%$$

$$\text{CPD} = \frac{CP\ samples-(CP\ residue-CP\ blanks)}{CP\ sample} \text{ x } 100\%$$

$$\text{ADFD} = \frac{ADF\ samples-(ADF\ residue-ADF\ blanks)}{ADF\ sample} \text{ x } 100\%$$

$$\text{NDFD} = \frac{NDF\ samples-(NDF\ residue-NDF\ blanks)}{NDF\ sample} \text{ x } 100\%$$

$$\text{CLD} = \frac{CLD\ samples-(DM\ residue-DM\ blanks)}{DM\ sample} \text{ x } 100\%$$

$$\text{HCLD} = \frac{HCL\ samples-(HCL\ residue-HCL\ blanks)}{HCL\ sample} \text{ x } 100\%$$

Where:

DMD: dry matter digestibility, OMD: organic matter digestibility, CPD: crude protein digestibility, ADFD: acid detergent fiber digestibility, NDFD: neutral detergent fiber digestibility, CLD: cellulose digestibility, HCLD: hemicellulose digestibility, DM: dry matter, OM: organic matter, CP: crude protein, ADF: acid detergent fiber, NDF: neutral detergent fiber, CL: cellulose, HCL: hemicellulose.

Statistical Analysis

This study used randomized block design which consisted of five treatments with four replications. Data obtained from this study was analyzed using statistical software namely Statistical Package for the Social Sciences (SPSS) software. The significant difference in treatments ($P<0.05$) was followed by posthoc Tukey-HSD test.

RESULTS

In vitro Nutrient Digestibility

The effect of fermentation and ammoniation treatment of soaked and unsoaked *Cymbopogon nardus* waste had no significant difference ($P>0.05$) on *in vitro* dry matter and organic matter digestibility. Meanwhile the effect of fermentation and ammoniation treatment of soaked and unsoaked *C. nardus* waste had significant difference ($P<0.05$) on *in vitro* crude protein, ADF, NDF, cellulose, and hemicellulose. The highest nutrient digestibility was observed in soaked ammoniated *C. nardus* waste with 4% urea+15% chicken manure (ACNW2). *In vitro* nutrient digestibility of this study can be seen in Table 2.

In vitro Ruminal Fermentation Characteristics

The effect of fermentation and ammoniation treatment of soaked and unsoaked *C. nardus* waste had no significant difference ($P>0.05$) on *in vitro* ruminal fermentation characteristics. Meanwhile pH, NH_3, and total VFA level are in the normal range to improve ruminal fermentation and nutrient digestibility. *In vitro* ruminal fermentation characteristics of this study can be seen in Table 3.

DISCUSSION

In vitro Nutrient Digestibility

Ammoniated and fermented treatment of soaked and unsoaked *Cymbopogon nardus* waste on *in vitro* nutrient digestibility had significant effect except dry matter (DMD) and organic matter (OMD). Meanwhile, *in vitro* digestibility of crude protein (CPD), ADF (ADFD), NDF (NDFD), cellulose (CLD), and hemicellulose (HCLD) gave significant effect in this study. The highest CPD was observed in ACNW2 (56.81%). The lowest was observed in control diet (39.87%). From Table 1, we can see that the highest crude protein content was in ACNW2 (13.99%) and the lowest was in CNW (7.72%). High crude protein contents in ACNW2 improve microbe's growth and lead to increase nutrient digestibility. ACNW generated high protein compared to FCNW and ACNW. Similarly with Huyen et al. (2018) who stated that there is an increasing trend of protein content of urea-treated rice straw compared to untreated rice straw. Linearly, urea-treated rice straw increased nutrient intake and digestibility of male Phan Rang sheep. They claimed that urea-treated rice straw consists of 129.37g/kg DM of crude protein, meanwhile untreated rice straw consists of 117.50g/kg DM of crude protein. Besides that, crude protein intake and *in vivo* digestibility of Phan Rang sheep fed with urea-treated rice straw showed higher content (71.58g/d and 601.69g/kg, respectively) compared to Phan Rang sheep fed with untreated rice straw (59.40g/d and 570.69g/kg, respectively). Feed processing such as fermentation and ammoniation could increase the digestibility of nutrient (Pazla et al. 2022). Ammoniated treatment not only enhances nutrient digestibility in the rumen but also adds amount of nitrogen (Jayanegara et al. 2017). High nutrient digestibility reflected the activity of microbial rumen in feed degradation, which means that microbial rumen had high activity in the present study.

Huyen et al. (2018) claimed that rice straw treated with *Pleurotus eryngii* have greater crude protein content compared to untreated rice straw. Crude protein content of rice straw treated with *P. eryngii* was higher (131.08g/kg DM) than untreated rice straw (117.50g/kg DM). The same pattern was also observed in crude protein digestibility where the higher crude protein was in rice straw treated with *P. eryngii* (687.07g/kg DM) than untreated rice straw (570.69g/kg DM). Fermentation feed processing with fungal will improve protein content of the substrate due to the addition on body cell protein from fungal itself. The

Table 1: Nutrient Ingredients of Experimental Diets (%DM)

Nutrient ingredients	Experiment diet				
	CNW	FCNW1	FCNW2	ACNW1	ACNW2
Dry matter	61.86	95.18	94.29	95.05	94.22
Organic matter	84.45	96.31	96.75	95.41	95.79
Crude protein	7.72	12.7	12.75	13.76	13.99
Ash	15.55	3.68	3.24	4.59	4.2
Neutral detergent fiber	69.93	58.7	64.44	61.78	65.27
Acid detergent fiber	44.45	40.74	41.93	41.07	42.08
Cellulose	30.39	28.88	29.41	30.13	31.07
Hemicellulose	25.48	17.96	22.52	20.71	23.19
Lignin	10.38	9.98	9.00	9.37	8.75

CNW = *Cymbopogon nardus* waste, FCNW1=Unsoaked fermented *C. nardus* waste with 0.6% probiotic starter+0.6% urea; FCNW2=Soaked fermented *C. nardus* waste with 0.6% probiotic starter+0.6% urea, ACNW1=Unsoaked ammoniated *C. nardus* waste with 4% urea+15% chicken manure, ACNW2=Soaked ammoniated *C. nardus* waste with 4% urea+15% chicken manure.

Table 2: *In vitro* Nutrient Digestibility of Experimental Diet (%)

In vitro Digestibility	Experimental diet					SEM
	CNW	FCNW1	FCNW2	ACNW1	ACNW2	
DMD	49.62	50.71	53.43	51.24	54.43	1.38
OMD	53.37	54.72	56.60	56.53	58.34	1.31
CPD	39.87^{b}	42.13^{b}	50.96^{ab}	44.37^{b}	56.81^{a}	2.81
ADFD	40.22^{b}	41.80^{b}	47.33^{a}	44.44^{ab}	47.67^{a}	1.26
NDFD	40.56^{b}	41.81^{b}	47.54^{a}	44.50^{ab}	48.68^{a}	1.36
CLD	40.67^{b}	41.37^{b}	46.93^{a}	44.30^{b}	47.19^{a}	1.35
HCLD	40.24^{b}	41.82^{b}	48.11^{a}	44.62^{ab}	50.52^{a}	1.79

DMD=Dry matter digestibility, OMD=Organic matter digestibility, CPD=Crude protein digestibility, ADFD=Acid detergent fiber digestibility, NDFD=Neutral detergent fiber digestibility, CLD=Cellulose digestibility, HCLD=Hemicellulose digestibility, CNW=*Cymbopogon nardus* waste, FCNW1=Unsoaked fermented *C. nardus* waste with 0.6% probiotic starter+0.6% urea; FCNW2=Soaked fermented *C. nardus* waste with 0.6% probiotic starter+0.6% urea, ACNW1=Unsoaked ammoniated *C. nardus* waste with 4% urea+15% chicken manure, ACNW2=Soaked ammoniated *C. nardus* waste with 4% urea+15% chicken manure.

Table 3: *In vitro* Ruminal Fermentation Characteristics of Experimental Diet (%)

Parameters	Experimental diet					
	CNW	FCNW1	FCNW2	ACNW1	ACNW2	SEM
pH	7.05	7.09	7.02	7.07	6.88	0.05
NH_3 concentration (mM)	6.51	6.82	7.10	7.06	7.21	0.07
Total VFA (mM)	75.4	77.5	82.5	81.3	87.5	2.13

NH_3=Ammonia, VFA=Volatile fatty acid, CNW=*Cymbopogon nardus* waste, FCNW1=Unsoaked fermented *C. nardus* waste with 0.6% probiotic starter+0.6% urea; FCNW2=Soaked fermented *C. nardus* waste with 0.6% probiotic starter+0.6% urea, ACNW1=Unsoaked ammoniated *C. nardus* waste with 4% urea+15% chicken manure, ACNW2=Soaked ammoniated *C. nardus* waste with 4% urea+15% chicken manure.

present study is in agreement with Khonkhaeng and Cherdthong (2020) who reported rice straw treated with *P. ostreatus* had greater crude protein 4.5% compared to untreated rice straw (3.0% of crude protein content). Khonkhaeng and Cherdthong (2020) also reported the greater crude protein and crude fiber fraction content in purple corn stover and purple corn cob treated with *P. ostreatus* to untreated one. The addition of *Saccharomyces cerevisiae* in the low-quality of oil palm frond ammoniated-based ration has potential to increase dry matter, organic matter, and crude fiber fraction *in vitro* and *in vivo* digestibility (Zain et al. 2016; Ningrat et al. 2020).

The highest ADFD and NDFD were observed in ACNW2 (47.67 and 48.68%, respectively), meanwhile the lowest were observed in CNW (40.22 and 40.56%, respectively). The alike pattern was observed in CLD and HCLD with the highest in ACNW2 (47.19 and 50.52%, respectively) and the lowest in CNW (40.67 and 40.24%, respectively). Among the treatments we can hypothesize that soaked ammoniated of *C. nardus* waste has potential to be used as livestock feed because of its high nutrient digestibility. Feed processing of agricultural waste will increase its potential as feed source for livestock. Soaking treatment also a potential method to enhance nutrient content and digestibility of high-lignin agricultural waste. The present study showed that soaked treatment of *C. nardus* had greater nutrient content and *in vitro* digestibility compared to unsoaked *C. nardus* waste. The main purpose of soaking treatment is to remove or dilute lignin-cellulose bond that decreases nutrient digestibility. In line with Zayed (2018) who reported that the combination of inoculant and soaking methods during 24h increase crude protein content and decrease NDF and ADF of rice straw High.

In vitro Ruminal Fermentation Characteristics

Fermented and ammoniated of soaked and unsoaked *Cymbopogon nardus* waste on *in vitro* ruminal fermentation characteristics had no significant effect (P>0.05) that can be seen in Table 3. The experimental diets did not affect pH, NH_3, and total VFA. The present study reported that the range of pH in this study is 6.88-7.09. Ruminal pH takes the crucial role for microbial growth and fermentation process in the rumen. In line with present study, Bach et al. (2005) stated that microbial ability in degradation depends on ruminal pH level. High ruminal pH level that reach into 7.5-8.01, will cause a alkalosis environment in rumen that can lead in microbial mortality (Kumbhar et al. 2018; Darwin and Blignaut

2019). The normal range of pH level to optimize microbial rumen's activity is 5.5-7.0, supported by 10-13% of rumen's dry matter, and 38-41ºC of rumen's temperature (Puniya et al. 2015). Ruminal pH level in this study is in line with Elihasridas et al. (2020) who obtained stable pH value (6.68-6.77) of fermentation and ammoniation treatment of *C. nardus* waste as a substitution of grass. Another study reported that rice straw, purple corn cob, and purple corn stover treated with the combination of inoculant and urea treatments did not alter pH of *in vitro* fermentation (Khonkhaeng and Cherdthong, 2020). The same pattern was observed from the study of Kayombo et al. (2021) who reported there was no alteration in pH *in vitro* evaluation in cassava pulp treated with the combination of *Saccharomyces cerevisiae* and chicken manure.

NH_3 is important in the rumen due to its function as ammonia source for microbial protein synthesis. When protein source is digested in the rumen, NH_3 is produced as the metabolite of rumen microbial activity (Sari et al. 2022). NH_3 level in this study was not significant difference among the experimental diets which was within the range 6.51-7.21mM. NH_3 is the product of protein fermentation in the rumen, so NH_3 level is influenced by the crude protein of feed. High protein content of feed will increase ammonia availability required by the microbial rumen for its growth (Putri et al. 2019, 2021; Sari et al. 2022). We hypothesized these unsignificant levels were due to equal protein content in the diet treatments. We observed that this range of NH_3 concentration in the present study can support microbial protein synthesis in the rumen. McDonald et al. (2010) claimed that the optimal range of NH_3 level to increase microbial protein synthesis is 6-21 mM. The results of present study was in disagreement with the study from Khonkhaeng and Cherdthong (2020) who reported that rice straw fermented with *P. ostreatus* generated significantly higher NH_3 (12.26mg/dL) concentration compared to untreated rice straw (3.60mg/dL). The increase of NH_3 level indicate high degradable protein in the feed. In addition, protein of *P. ostreatus* also increased NH_3 level in that study. Contrastly, Vorlaphim et al. (2021) reported that there was a obvious effect on NH_3 concentration of urea-treated rice stubble and urea-fungi-treated rice stubble compared to untreated rice stubble. Previous study also reported that addition of chicken manure altered NH_3 accumulation in the rumen. They hypothesized that chicken manure acted as an absorber of ammonia and affected on the fluctuating NH_3 level.

NH_3 level with the availability of energy will increase the growth of microbial rumen. Total VFA indicated the amount of energy for ruminant also for microbial rumen. Synchronization of protein and energy in the rumen will enhance microbial protein synthesis activity. High fermentable energy feed rapidly converted into volatile fatty acid (VFA). In the current study, total VFA was stable in 75.4-87.5mM. It can be seen that the value of total VFA in this study was not significant different. It can be understood that the fiber fraction in the experimental diets were not slightly different. This study has the same pattern with Dewi et al. (2018) who found insignificant effect on total VFA concentration of several agricultural waste treated with the combination of high temperature, high pressure, and urea treatment. They assumed that the insignificant total VFA was related to the insignificant methane gas production in that study since the methane value is obtained from stoichiometrically estimate from the profile of acetate, propionate, and butyrate concentration. The present study also in agreement with Vorlaphim et al. (2021) who claimed that there was no significant effect on VFA concentration of urea-treated rice stubble, and urea-fungi-treated rice stubble compared to untreated rice stubble.

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Author's Contribution

M. Zain and Elihasridas supervised the experiment and wrote original manuscript. E.M. Putri conducted the experiment in the laboratory. M. Makmur analyzed data and finalized draft. The final version of the manuscript was read and approved by all authors.

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