ISSN 0853-7380
E-ISSN 2252-696X

Accredited by LIPI
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Jurnal Ilmu Ternak dan Veteriner

IJAVS Indonesian Journal of Animal and Veterinary Sciences

Volume 22
Number 3
September 2017

PUSAT PENELITIAN DAN PENGEMBANGAN PETERNAKAN
BADAN PENELITIAN DAN PENGEMBANGAN PERTANIAN
KEMENTERIAN PERTANIAN

JITV	Volume 22	Number 3	Page 101 -164	Bogor, September 2017	ISSN 0853-7380

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Published by:
Indonesian Center for Animal Research and Development
Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture

Collaborated with:
Indonesian Society of Animal Science

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Website: http://medpub.litbang.pertanian.go.id/index.php/jitv

Indonesian Journal for Animal and Veterinary Sciences is published four times a year in March, June, September and December.

PREFACE

In this edition , volume 22 no 3 September 2017 we proudly present articles from Feed and Nutrition Technology, Forage Technology, Reproduction Technology, and Veterinary Science, those are: "Improvement of nutritional value of cocoa pod husk fermented with *Aspergillus* Spp. and two levels of urea and ammonium sulphate"; "Physical quality and digestibility *in vitro* determination of green pellet concentrate based on *Indigofera zollingeriana*"; "Evaluation of four pasture legumes species as forages and cover crops in oil palm plantation"; "Evaluation on performance of some *Sorghum bicolor* cultivars as forage resources in the dry land with dry climate"; "Primordial germ cells profiles incubated for 24 hours in phosphate buffer saline [-] solution" and "Cytokines profile of mice infected by high and low virulences of Indonesian *T. evansi* isolates".

Hopefully these articles would offer any benefit to readers and the end-users of technological innovation. We extent high appreciation to all authors and peer reviewers those contribute in this edition that would make this journal published. We hope other authors will contribute in the future.

Chief Editor;

Bogor, September 2017

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LIST OF CONTENT

Improvement of Nutritional Value of Cocoa Pod Husk Fermented with *Aspergillus* spp. and Two Levels of Urea and Ammonium Sulphate

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(received 19-06-2017; revised 05-09-2017; accepted 11-09-2017)

ABSTRAK

Rakhmani SIW, Purwadaria T. 2017. Peningkatan nilai gizi kulit buah kakao yang difermentasi menggunakan *Aspergillus* spp. dengan dua tingkat urea dan amonium sulfat. JITV 22(3): 101-113. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1670

Kulit buah kakao melimpah sebagai produk limbah perkebunan kakao dan berpotensi sebagai bahan pakan tetapi memiliki nilai gizi rendah. Untuk meningkatkan nilai gizi kulit buah kakao (CPH), telah dilakukan proses biologis melalui fermentasi substrat padat menggunakan *Aspergillus oryzae* dan *Aspergillus niger* serta penambahan dua dosis (N1 dan N2) nitrogen dalam campuran mineral. Produknya adalah Fermented Cocoa Pod Husk (FCPH).Kandungan protein meningkat dari 50 g/kg sebelum fermentasi menjadi 133,8 g/kg untuk N1 fermentasi menggunakan *A. niger* dan 150 g/kg menggunakan *A. oryzae*. Kandungan protein sejati adalah 99,8 dan 93,5 g/kg untuk perlakuan N1 dan N2 (*A. niger*); 119 dan 104,1 g/kg untuk perlakuan N1 dan N2 (*A. oryzae*). *Aspergillus niger* menunjukkan keunggulan dalam hal produksi enzim bila dibandingkan dengan *Aspergillus oryzae*. Aktivitas mannanase pada produk fermentasi *A. niger* dengan dosis N1 mencapai 2654 U/g dan *A.oryzae* adalah 1122 U/g. Bahan kering dan kecernaan protein masing-masing untuk FCPH *A. niger* adalah 47 dan 57% dan FCPH *A. oryzae* adalah 52 dan 62%. Proses fermentasi kulit buah coklat menghasilkan produk yang sangat potensial sebagai pakan alternatif dengan kadar protein lebih tinggi dan mengandung enzim mannanase.

Kata Kunci: Kulit Buah Coklat, *Aspergillus Niger*, *Aspergillus Oryzae*, Mannanase, Selulase

ABSTRACT

Rakhmani SIW, Purwadaria T. 2017. Improvement of nutritional value of cocoa pod husk fermented with *Aspergillus* spp. and two levels of urea and ammonium sulphate. JITV 22(3): 101-113. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1670

Cocoa pod husk is abundant as a waste product of cocoa plantation and potential as feed ingredient but has low nutritional value. To increase the nutritive value of cocoa pod husk (CPH), biological process through solid substrate fermentation using *Aspergillus oryzae* and *Aspergillus niger* and addition of two doses (N1 and N2) of nitrogen mixture had been done. The product is Fermented Cocoa Pod Husk (FCPH). Protein content increased from 50 g/kg before fermentation to 133.8 g/kg for N1 for *A. niger* and 150 g/kg using *A. oryzae*. True protein were 99.8 and 93.5 g/kg for N1 and N2 treatments (*A. niger*); 119 and 104.1 g/kg for N1 and N2 treatments (*A. oryzae*). *Aspergillus niger* showed a superiority in term of enzymes production when compared to *Aspergillus oryzae*. Mannanase activy in *A. niger* fermentation product with N1 dose reached up to 2654 U/g and *A.oryzae* was 1122 U/g. Dry matter and protein digestibility for *A. niger* FCPH were 47 and 57% and *A. oryzae* FCPH were 52 and 62% repectively. Fermentation processed of CPH yield a product that very potential as an alternative feed with higher in protein content and contain mannanase enzyme.

Key Words: Cocoa Pod Husk, *Aspergillus niger*, *Aspergillus oryzae*, Mannanase, Cellulose

INTRODUCTION

In animal husbandry, the feed comprises the highest expense and appropriate feed quality nutrition is important. Shortage of feedstuff is also one of several factors in animal production in Indonesia, especially since most of feed materials (such as corn and soybean meal) are imported. Therefore, in addition to conventional feed ingredients, uncommon feed ingredients such as of agriculture by-product would be

advantage to solve feed insufficiency. Indonesia's cocoa crop is one of the largest estates after palm oil. Indonesian Cocoa plantation (*Theobroma cacao* L.) area currently is reached 1,745,789 hectares and is largely possessed by smallholders with a total production of 903,092 tons/year (Estate Statistic 2008-2012). It will be 695,467 tons of fresh CPH (equivalent to 893,092 dried material) by-product. Waste and by-products of cocoa fruit itself is more than half of world-wide cocoa production and estimated at 3.53 million tons (World Cocoa Foundation 2010). Indonesia, including the 20 largest cocoa producing countries and was ranked the 3rd after Ghana and Ivory Coast.

Cocoa pod husks has been investigated as an alternative feed and had been fed without toxic effects to cattle in quantities up to 7 kg per day. For dairy cows, pod meal seems to be comparable in value to corncob meal. Rations containing cocoa pod meal have a lower feed efficiency for beef cattle, but this will be compensated by the larger intake (FAO 2002). A digestion study with sheep, was carried out to determine the effect of graded dietary cocoa-pod levels between 0 and 75% in feed, the apparent digestibility of cocoa-pod by sheep was also estimated and reported that sheep digested only 23% dry matter and 51% crude protein of the pod (Smith & Adegbola 1985). Processed-CPH was used as feed material and can replace conventional feed ingredients such as corn and soybean meal and regarded as alternative animal feed (Sobamiwa & Longe 1993; Sobamiwa 1998).

Biological processes can improve the nutritional composition and utilization of CPH and would be more advantage as feed. Fermentation process using cellulolytic fungi could be used to overcome high crude fiber content in CPH, which is as a constraint factor for direct utilization of CPH especially in poultry diet. Processing CPH had been reported such as CPH-silage (North Sumatera and Lampung provinces), and fermentation process using *Aspergillus niger* (South Sulawesi and Bali provinces) has done, but there is no detailed information on the nutritional value such as the digestibility, enzymes activities and true protein of the resulting product. It was reported an increase in protein content of 9% to 12% was achieved after fermentation with *A. niger* (Haryati & Sutikno 1994).

Fungi such as *Aspergillus* spp. produce several enzymes which is important to elevate feed digestibility. When CPH was fermented using this fungi, the final product can be fed to livestock without addition of enzymes. It was reported that minerals and inorganic nitrogen can increase cell growth and correlated on enzymes and protein productions in fermentation of cassava by-product using *Aspergillus* spp (Kompiang 1994). In this study, an experiment had been done to investigate the nutritive value of fermented CPH product by *Aspergillus oryzae* and *Aspergillus niger*. Solid substrate fermentation method was applied to steamed CPH with two levels of urea-ammonium sulphate mixtures (0.5% urea + 1% ammonium sulphate and 1% urea+ 2% ammonium sulphate). Proximate composition, cellulolytic enzymes (mannanase and cellulase) activities, dry matter and protein digestibility (*in vitro*) were also examined in the fermented product.

MATERIALS AND METHODS

Preparation of CPH and *Aspergillus* inoculums

Fresh CPH were obtained from Nusantara Plantation VIII-Public Company (*PTPN VIII*) at Rajamandala District- West Java, and processed at the IRIAP. The husks were cleaned, chopped into smaller pieces, solar-dried to a moisture content of ca. 10% and ground. The dried husk were then stored at room temperature until used. Fungi *Aspergillus oryzae* GS66 was previously isolated from garlic seed) whereas *A. niger* was obtained from IRIAP collection. Dried-spore inoculums of *A. oryzae* and *A. niger* were prepared by growing the fungi in in cooked-rice substrate for 3-4 days, dried at 40°C, ground and stored at 4ºC (Purwadaria et al. 1994).

Substrates and fermentation

Three kind of treatments (C, N1, and N2) were applied. Treatment C as control, substrate + 50 g rice bran/Kg. The composition of minerals mixture for 1 kg dried substrate as shown as in Table 1. Distilled water was first added to the ground dried CPH substrate to make water content of about 60% then steamed for 30 minutes. After cooling to about 40ºC the minerals and 8 g of inoculum were added. The cultures were incubated at room temperature.

Table 1. Mineral content in treatments

Minerals/other materials	Treatments		
	C	N1	N2
Trisuperphosphate (TSP)	0	2.40	2.40
MgSO4.7H2O	0	1.25	1.25
FeSO4. 6H2O	0	0.10	0.10
KCl	0	3.80	3.80
CaCl2.2H2O	0	0.13	0.13
(NH4)2SO4	0	10.00	20.00
Urea	0	5.00	10.00
Rice bran	50	27.32	12.32

Laboratory analysis

All chemical and digestibility analysis were carried out at the Analytical Services and Feed Technology Laboratories, Indonesian Research Institute for Animal Production. Proximate analysis (Crude protein, fat, fiber, ash), Calcium and Phosphor, Energy and van Soest fiber (NDF, ADF, Lignin) were performed in both optimally fermented and unfermented CPH. Optimum fermentation period was determined based on protein content reached in particular time of incubation and was determined using modified Lowry method (Lowry et al. 1951).To obtain total protein concentration, 1 ml of 2*M* NaOH was added to 0.1 g of sample then heated for 30 min at 100ºC. After dilution ten-fold in destilled water, protein concentration was measured by a standard Lowry method using bovine serum albumin (BSA) as a standard.

***In vitro* rumen-pepsin digestibility**

Subsequently, 0.2-0.4 g of experimental samples were weight out in an 100 ml-in vitro tubes, 0.2-0.4 g samples from laboratory standards (grass hay) were added to 2 tubes and 2 tubes were used as blanks for the experiments. In each tube, 25 mL of a Rumen-McDougall buffer mixture was added under purging with CO_2 and caped tightly with a rubber stopper/gas-release port. Samples were incubated for 48 h in a water bath at 39°C, followed by further digestion in an acid–pepsin solution containing 6.6 g pepsin/L 0.1N hydrochloric acid (Catalog P6887-SIGMA) (25 mL of acid–pepsin solution was added to each tube) for 48 h in water bath at 39°C. All tubes were mixed by swirling (Vortex Mixer) them at 2, 4, 8, and 36 h, after adding the rumen-buffer mixture and at 2, 4, and 6 h after adding acid–pepsin. After completion of the digestion, contents were filtered into pre-weighed standard coarse fritted disk Gooch crucibles (G2) under mild vacuum, dried at 105°C for at least 12 h, weighed for determination of DM. Protein content of *in vitro* sediment was determined for protein digestibility calculation (Marais & Evenwell 1983).

True protein analysis

True protein was measured according Marais & Evenwell (1983) by TCA-precipitation with slightly modification. Sample was finely ground and weight out (0.5-1 g) into a 50 ml tube with caped, add 15 ml of water and incubate 10 min in a boiling water-bath, cooled and added 15 ml of 10% TCA and then shake in a medium speed using Griffin Flask Shaker for 20 min and then left for an hour at 4°C. Mixture was centrifuged at 3000 rpm for 10 min. The pellet was rinsed twice with 5 ml 1% TCA and protein content of the precipitate was determined.

Enzymes activity

Mannanase activity was measured using locust gum (0.5 $g.Kg^{-1}$) as substrate and followed the procedure as described by PURWADARIA et al. (2003). Enzymes was extracted from the sample (0.5 - 1 g) with 10 ml of acetate buffer pH 5.8 and mixed for 30 min. The mannanase activity was measured as the amount of reducing sugar released from the substrate. Reducing sugar was determined by using the 3,4-dinitro salicylic acid (DNS) method of Miller (1959) and mannose was used as standard. One unit mannanase activity was defined as the amount of enzyme that required to release 1 μmole of glucose $min^{-1}ml^{-1}$ under assay condition

Cellulase assays are carried out using the carboxy methyl cellulose (CMC) as substrate. Enzymes solution was obtained by extraction of sample (0.5-1 g) in 25 ml of 0.05 M citrate buffer pH 4.8. The amount of released reducing sugar was quantified using the 3,4-dinitro salicylic acid (DNS) method of MILLER (1959) and glucose was used as standard for determining enzyme activity. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmole of glucose $min^{-1}ml^{-1}$ under assay condition (Miller 1959).

Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Growth of *A. niger* and *A. oryzae* on CPH substrate during fermentation period is presented in Table 2. In the first 24 hours mycelium growth was undetected. Mycelia formation was detected in the 2nd day of incubation of both *Aspergillus niger* and *A. oryzae* along with young spores formation scattered for about 10% that can be observed from the substrate surface. For treatment using lower inorganic nitrogen addition (N1), mycelia formation was tend to be slower than higher inorganic nitrogen treatment (N2).

Mycelia became thick and fungal hyphae had been penetrated and bind the substrate was clear in the third day of incubation and a distinction spores color for both species were detected. It was hard to report the difference between inorganic treatments in term of mycelia formation in this stage and in the next day beyond fermentation period. Mycelia thickness was remained in the fourth day and older spore formation

Table 2. Visual observation of fungi growth on CPH substrate during fermentation periods

Incubation period (day)	*Aspergillus niger*		*Aspergillus oryzae*	
	N1	N2	N1	N2
1	*	*	*	*
2	++	++	++	++
3	+++	++++	+++	++++
4	++++	++++	++++	++++
5	***	***	***	***

*Mycelia growth undetected, raised in temperature was observed
++ Thin covering mycelia, tiny spores
+++ Penetration of mycelia
++++ mycelia covering all over substrate
***spores covering all over substrate surface

almost completely found in both species. In the 5th day, spores were found all over the substrate surface.

In every process of fermentation, dry matter loss of the substrate is inevitable. Dry matter loss of CPH during incubation is presented in Figure 1. In the process, substrate was added water up to 60% water content. It was reported that water holding between 60-65% was suitable for solid substrate fermentation (Chalall 1985; Singhania et al. 2006). In this experiment we found out that the maximum capacity of water holding for CPH was in the ratio of CPH-water 100 : 120. The water content was 600 g/kg, however, when it was higher than that ratio, water was not held perfectly and was flowing out from the substrate. It was reported that 70% moisture level in the substrate prevents oxygen penetration and facilitates the contamination, whereas the low moisture level inhibits the growth, enzyme activity and accessibility to nutrients (Mekala et al. 2008). Dry matter loss profiles for both *A. niger* and *A. oryzae* were similar, all below 300 g/kg, and for control group (without addition of minerals), DM loss was detected below 150 g/kg. The moisture level of culture medium affects the physiology of the microorganism.

It was a significant increased ($P<0.05$) in crude protein content of fermented CPH when compared with the control group. The highest protein content in the fermentation product of *A. niger* was shown in the 4th day of incubation (Figure 2). Protein content in N1 treatment was 133.8 g/kg and N2 treatment 131.9 g/kg statistically not significantly different When CPH was fermented using *A. oryzae*, the highest protein content was detected at the 3rd day of incubation (154 g/kg for N1 and 148.8 g/kg for N2). The CP in the *A. oryzae* FCPH statistically not significantly different between N1 and N2. It had been reported that fungal fermentations on several agro-byproduct such as cassava by-product, coffee husk, corn bran and rice bran have also reported similar increases in protein content (Leifa et al. 2001; Iyayi & Aderolu 2004). Solid-state fermentation of CPH using *Rhizopus stolonifer* LAU was reported an increase in CPH protein up to 95% (Lateef et al. 2008). The increased in protein content of CPH fermented by *A. niger* and *A. oryzae* could be due to bioconversion of some of soluble carbohydrates in the substrate into mycelia protein or single cell protein (SCP) by the growing fungus (Iyayi 2004). It was also reported that the growth and sporulation of the fungus *Aspergillus niger* is influenced by the level of nitrogen (from ammonium sulfate and urea) in the culture medium (Swe et al. 2009). However, in this experiment, in the higher of substrate-nitrogen level (N2) CP was not significantly different as of lower nitrogen level (N1). It showed that nitrogen level that supposed to be used by fungi for growing not necessarily high but in some point it will be efficient. Nitrogen level of N1 treatment showed efficiency in using inorganic fertilizer for fermentation process. It will open an opportunity for using more CPH fermentation product as non-ruminant feed ingredient without worried to urea toxic effect.

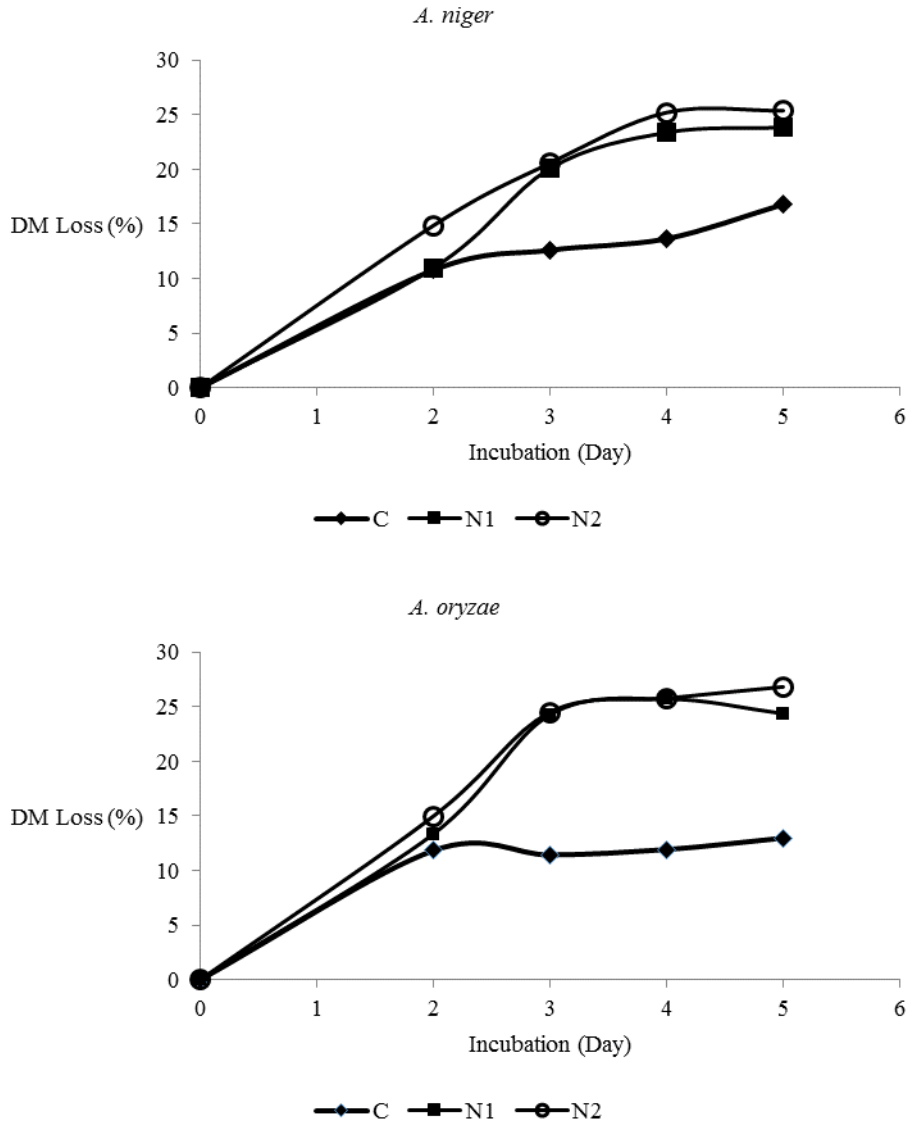

Figure 1. Dry matter loss on CPH substrate fermented with *A. niger* and *A. oryzae.*

Total protein analysis with KJELDAHL digestion measures crude protein, where the total nitrogen content of sample is multiplied by 6.25 to express the results on a protein equivalent basis. The total amount of nitrogen in feed/feed ingredient however, comes from both protein and non-protein nitrogen sources. True protein reflects only the nitrogen associated with protein and does not include the nitrogen from non-protein sources. The true protein was lower than crude protein content and shown in Figure 3. The true protein content in control treatment was between 33.7 and 51.11 g/kg, whereas in fermentation product were 99.8 g/kg and 93.5 g/kg for N1 and N2 treatments respectively (*A. niger*) and 119.0 g/kg and 104.1 g/kg both for N1 and N2 treatments (*A. oryzae*) respectively. The true protein content of fermentation products of *A. oryzae* significantly higher than *A. niger* in both nitrogen levels. It is important to measure the true protein instead of crude protein due to inorganic nitrogen that was added to boost the growth of fungi in the beginning of fermentation will give inaccurate in crude protein calculation. In this experiment, the non-protein fraction in fermented CPH was calculated between 23 and 30%. Residual inorganic nitrogen is included in this fraction. Inorganic nitrogen such as from ammonium sulfate or urea will affect negatively especially in non-ruminant animals, but ruminant can tolerate urea and is used by rumen microbe for protein synthesis.

Proximate analysis of fermented product is presented in Table 3. Control treatment was ground CPH, which was not added any mineral mixture and directly fermented using *A. niger* (AN) and *A. oryzae*

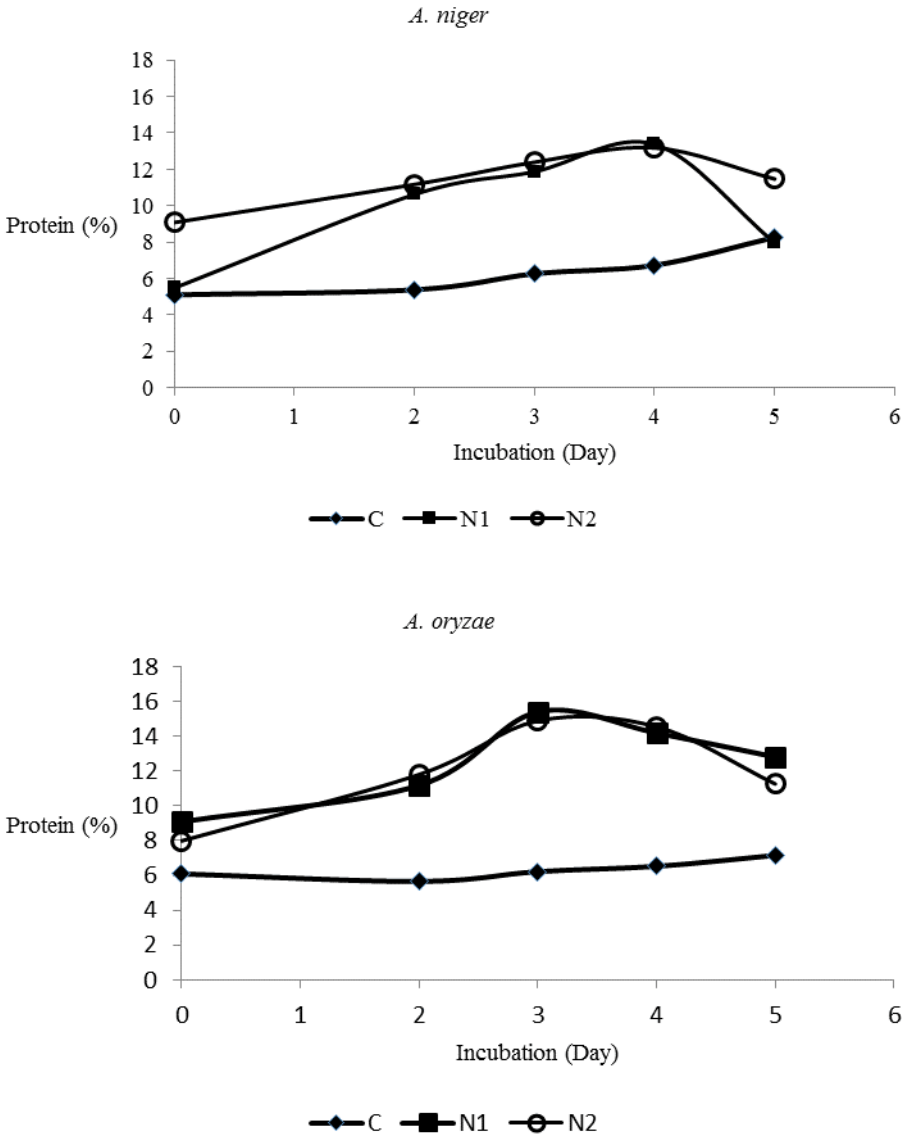

Figure 2. Protein profile of fermentation product (Statistic analysis was done for 3rd and 4th day incubation: the CP was not significantly different between N1 and N2 but significantly higher in *A. oryzae* than *A. niger*).

(AO) inoculums respectively. Growth of fungi in control treatment was detected at the 2nd day of fermentation but for the next day onward the growth was not optimal. The slightly growth of fungi in control batch could explained on increased of crude protein (CP) content of control treatment. The CP content and other data of fermented products from the 3rd and 4th day of incubation period using *A. niger* and *A. oryzae* as fermentation agents were subjected to statistical analysis. Crude protein (CP) content of fermented CPH in N1 treatment was increased up to 140 and 205% when fermented with *A. niger* and *A. oryzae* respectively. However, another treatment (N2) was shown a lower CP increases up to 63 and 187% for *A. niger* and *A. oryzae* FCPH.

Crude fat was decrease significantly for N2 treatment (34 and 35% for *A. niger* and *A. oryzae* fermented CPH) and was higher than in N1 treatment. Neutral Detergent Fiber (NDF) and ADF also decrease 13% for *A.niger* FCPH - N1 treatment, 26% for *A. oryzae* FCPH-N1 treatment, 29% for *A. niger* FCPH N2-treatment and 27% AO FCPH N2 treatment. Crude fiber (CF) also decreased 42% and 55% for *A.niger* FCPH N1 and *A. oryzae* FCPH N1 respectively, and a lower value of decreased was detected on N2 treatment that is 36 and 46% for *A. niger* FCPH and *A. oryzae* FCPH respectively.

Rumen-pepsin dry matter digestibility is presented in Figure 4. It is clearly shown that fermented product of CPH have higher DM digestibility when compared to

control. And *A. oryzae* FCPH both N1 and N2 treatments showed a higher DMD value than *A. niger* FCPH. The same pattern is also shown for rumen-pepsin protein digestibility (Figure 5). Rumen-pepsin protein digestibility of *A. oryzae* FCPH N1 treatment was closed to the protein digestibility of King grass that was run at the same time as a comparison.

Cocoa pod husks utilization for poultry is constrained by the high content of fiber, including lignin (14% w/w) and non-starch polysaccharides (NSPs) such as hemicellulose (110 g/kg), cellulose (350 g/kg) and pectin (60 g/kg) (Alemawor et al. 2009) and need enzyme fortification to increase digestibility. Fermentation using *Aspergillus niger* showed a superiority in term of mannanase and cellulase enzymes production (Figures 6 and 7) when compared to *Aspergillus oryzae*. Mannanase activy in *A. niger* FCPH N1 treatment reached up to 2654 U/gDM and *A. oryzae* FCPH N1 was only 1122 U/gDM. Cellulase activity was lower than mannanase, for AN FCPH N1 treatment reached 1255 U/gDM. Fermentation process increased nutrients composition and digestibility of CPH

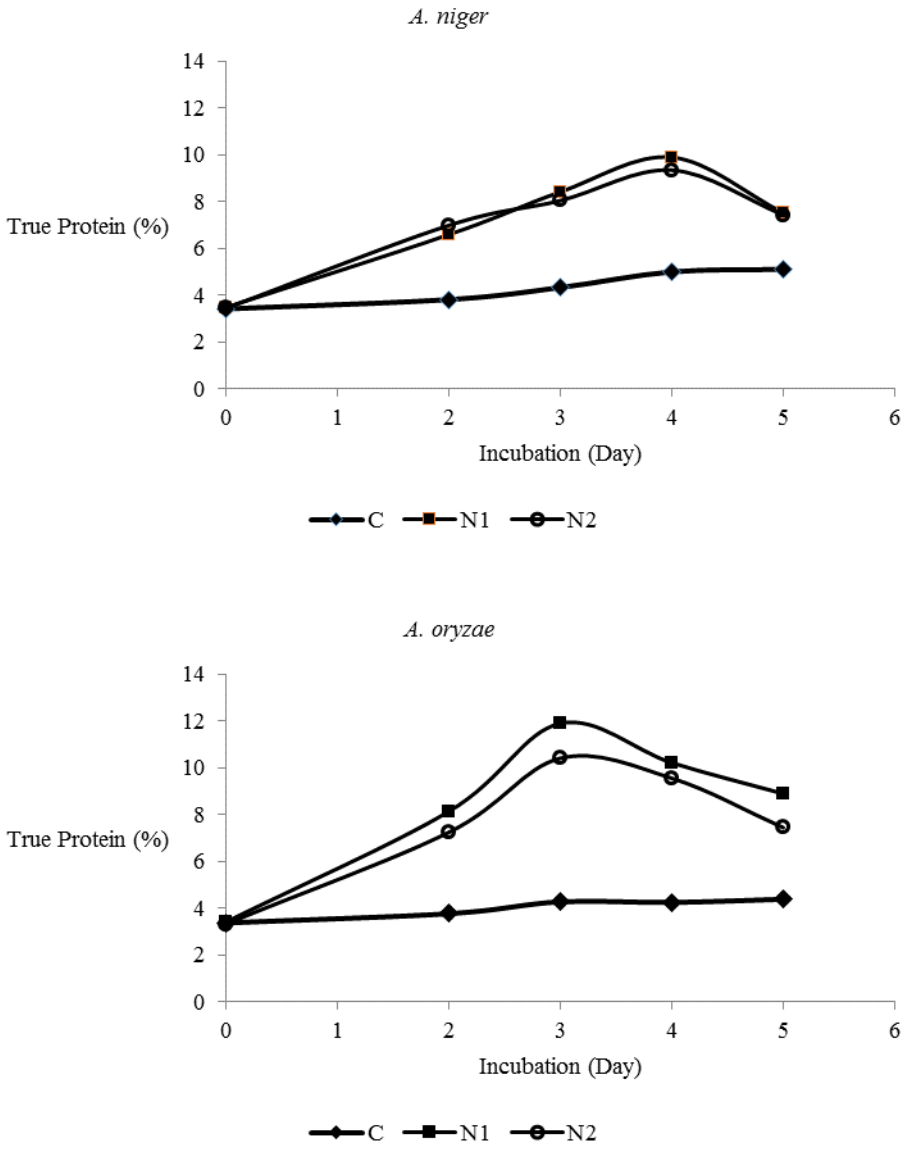

Figure 3. True Protein content of *A. niger* and *A. oryzae* fermentation product.

Table 3. Proximate analysis of fermented CPH at specific day of incubation (unit in g/kg except it is stated)

Composition	Control						N1				N2			
	D0		D3		D4		D0		D4	D3	D0		D4	D3
	A.nig	*A.ory*	*A.nig*	*A.ory*	*A.nig*	*A.ory*	*A.nig*	*A.ory*	*A.nig*	*A.ory*	*A.nig*	*A.ory*	*A.nig*	*A.ory*
CP	58.2	51.9	88.0	56.4	92.9	62.7	58.2	51.7	139.4	157.9	58.2	54.2	95.1	155.4
Fat	11.2	11.2	7.6	7.6	7.3	7.3	9.2	9.2	7.8	8.6	11.2	11.4	7.4	7.4
Ash	99.3	99.3	98.4	98.4	102.9	102.9	112.9	112.9	104.4	104.4	114.5	115.7	119.3	119.3
CF	518.4	519.0	517.6	553.7	543.2	542.9	517.8	526.7	302.0	234.6	513.3	542.1	328.0	289.8
GE (Kkal/kg)	3,870	3,857	3,844	3,832	3,848	3,838	3,849	3,837	3,805	3,795	3,756	3,618	3,770	3,770
NDF	652.0	652.0	651.3	641.3	666.2	643.2	650.0	650.2	565.8	482.5	667.8	653.7	473.2	476.8
ADF	621.3	621.3	631.8	631.6	641.5	641.0	611.1	612.0	482.3	497.7	622.8	623.4	416.5	416.5
Ca	3.6	3.8	4.8	4.8	4.5	4.6	7.2	7.7	4.9	5.0	6.6	6.5	6.5	6.5
P	1.8	1.8	1.8	1.8	1.6	1.6	2.3	2.2	1.9	1.9	1.8	1.8	2.3	2.3

A.nig= *Aspergillus niger*; A. ory=*Aspergillus oryzae*; CP= crude protein; CF= crude fiber; GE= Gross energy; NDF= neutral detergent fiber; ADF= acid detergent fiber

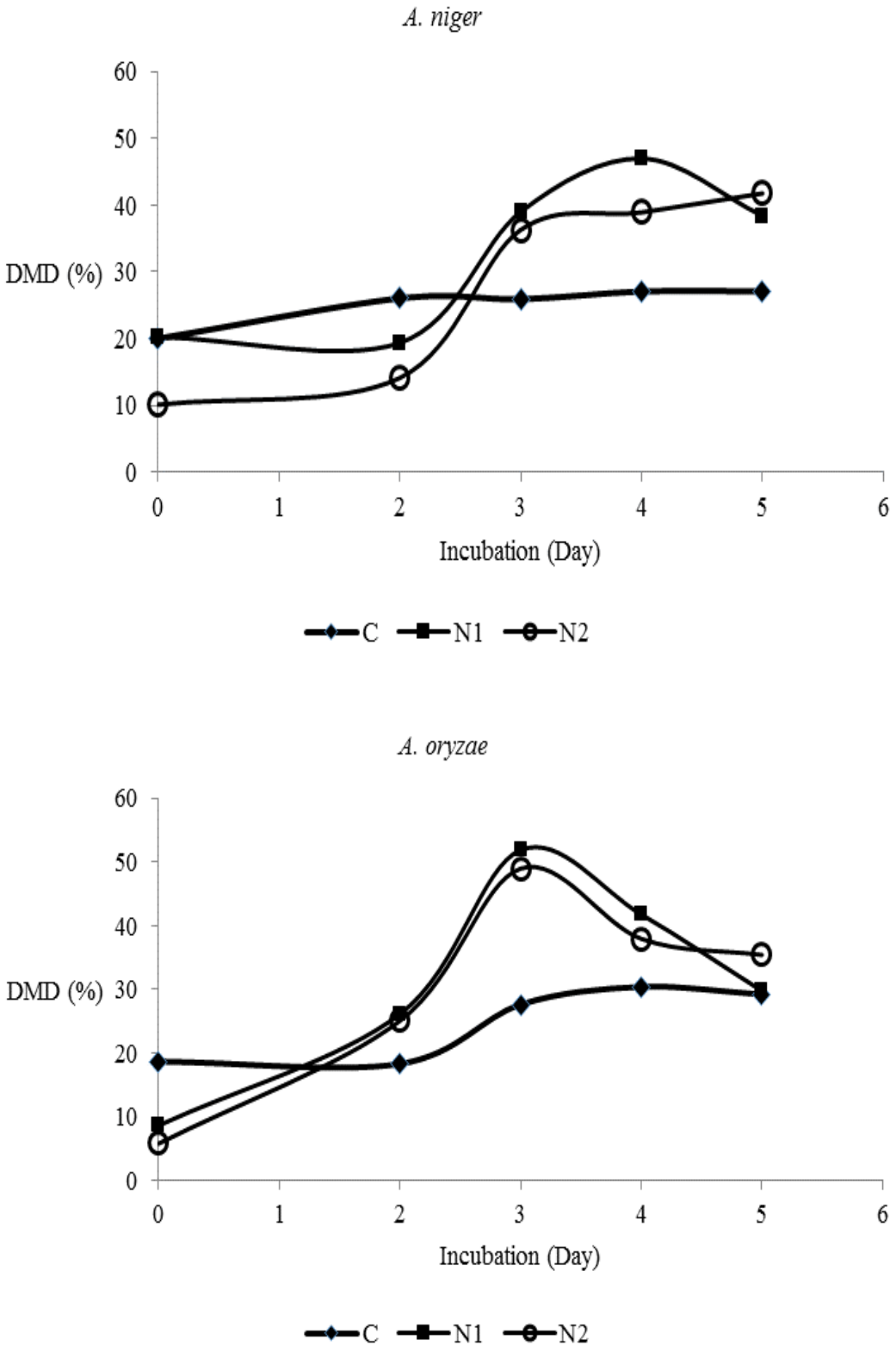

Figure 4. Dry matter digestibility of FCPH after rumen-pepsin *in vitro*.

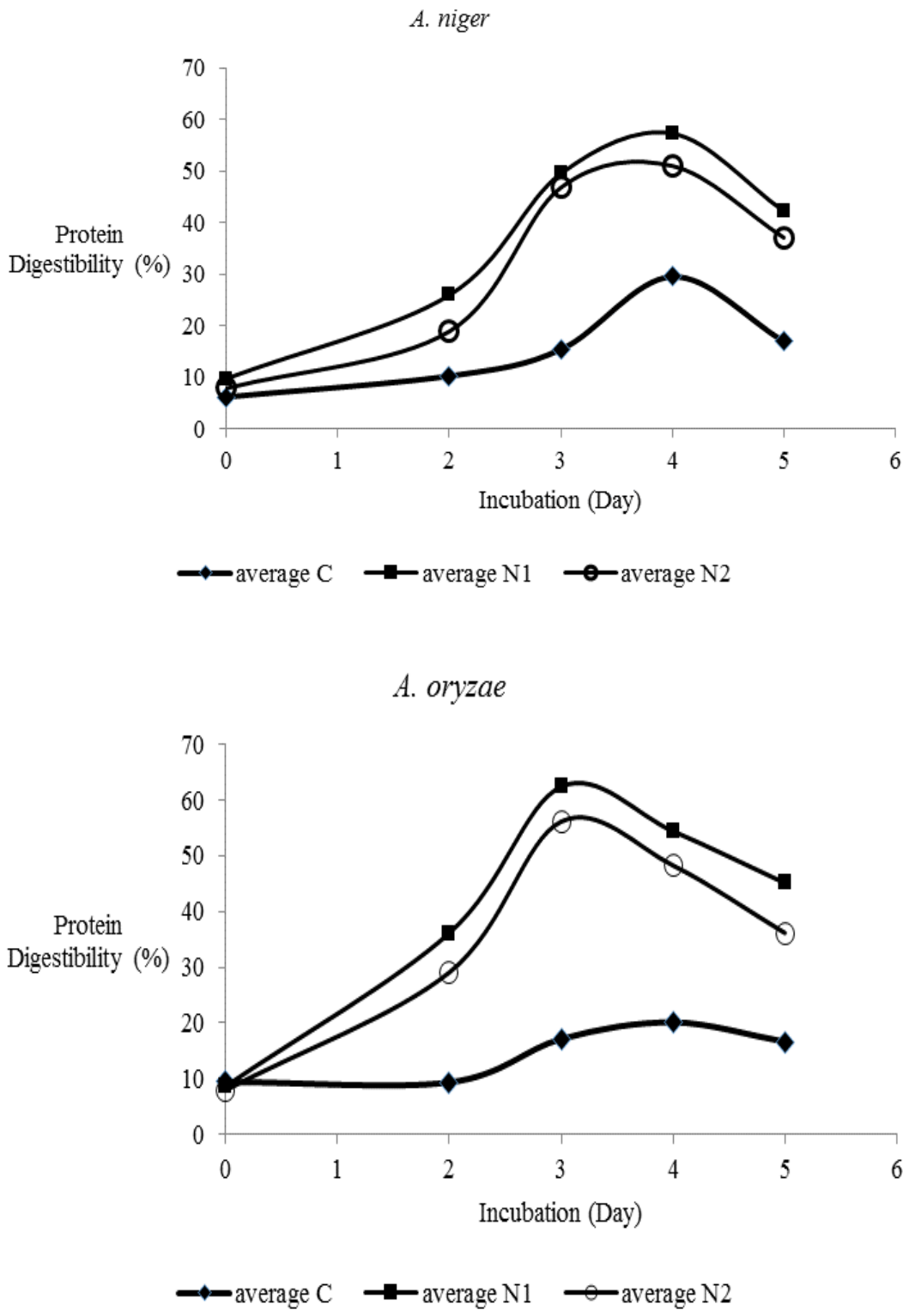

Figure 5. Protein digestibility *in vitro* rumen-pepsin of FCPH.

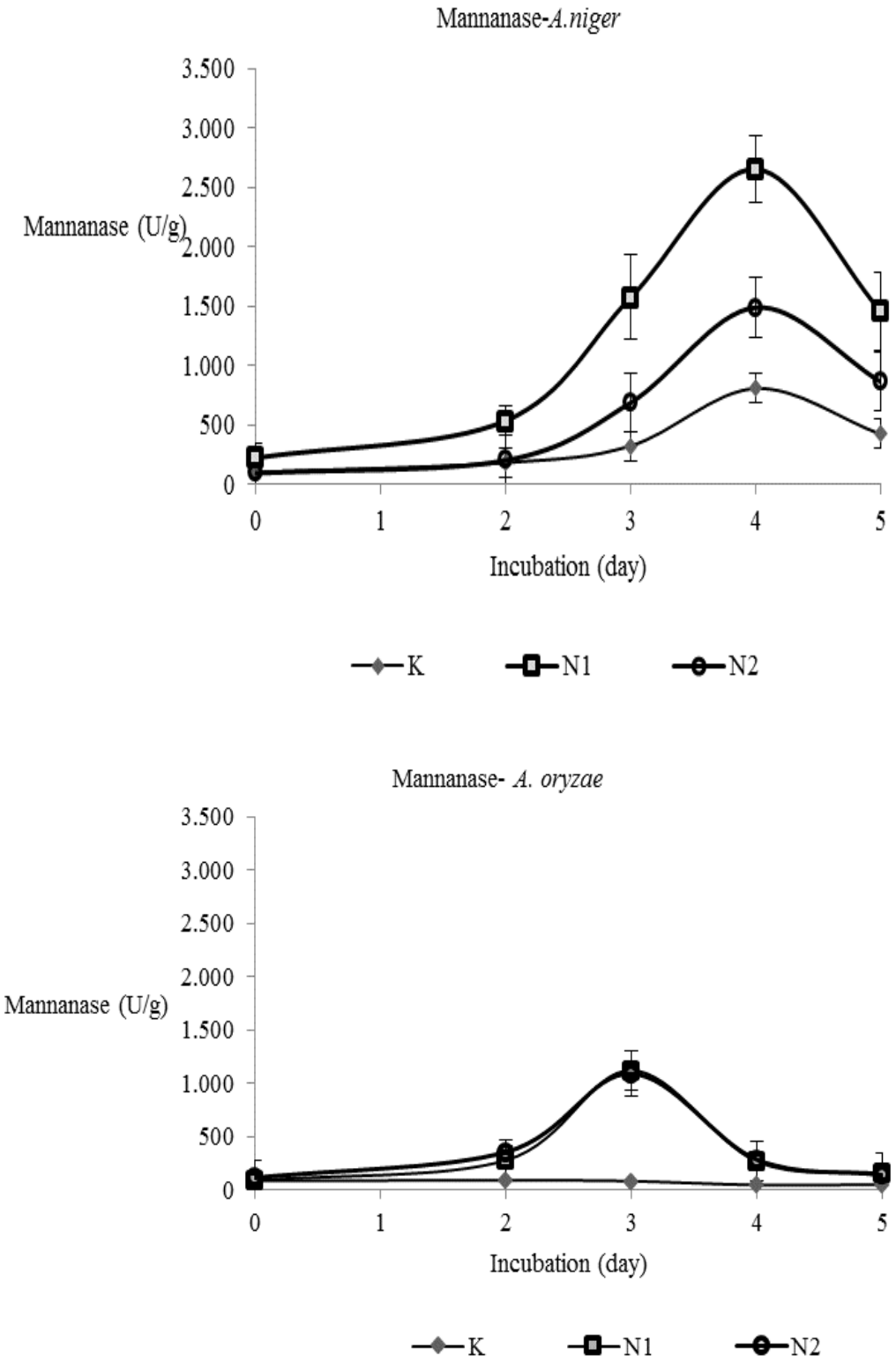

Figure 6. Mannanase activity in fermentation products.

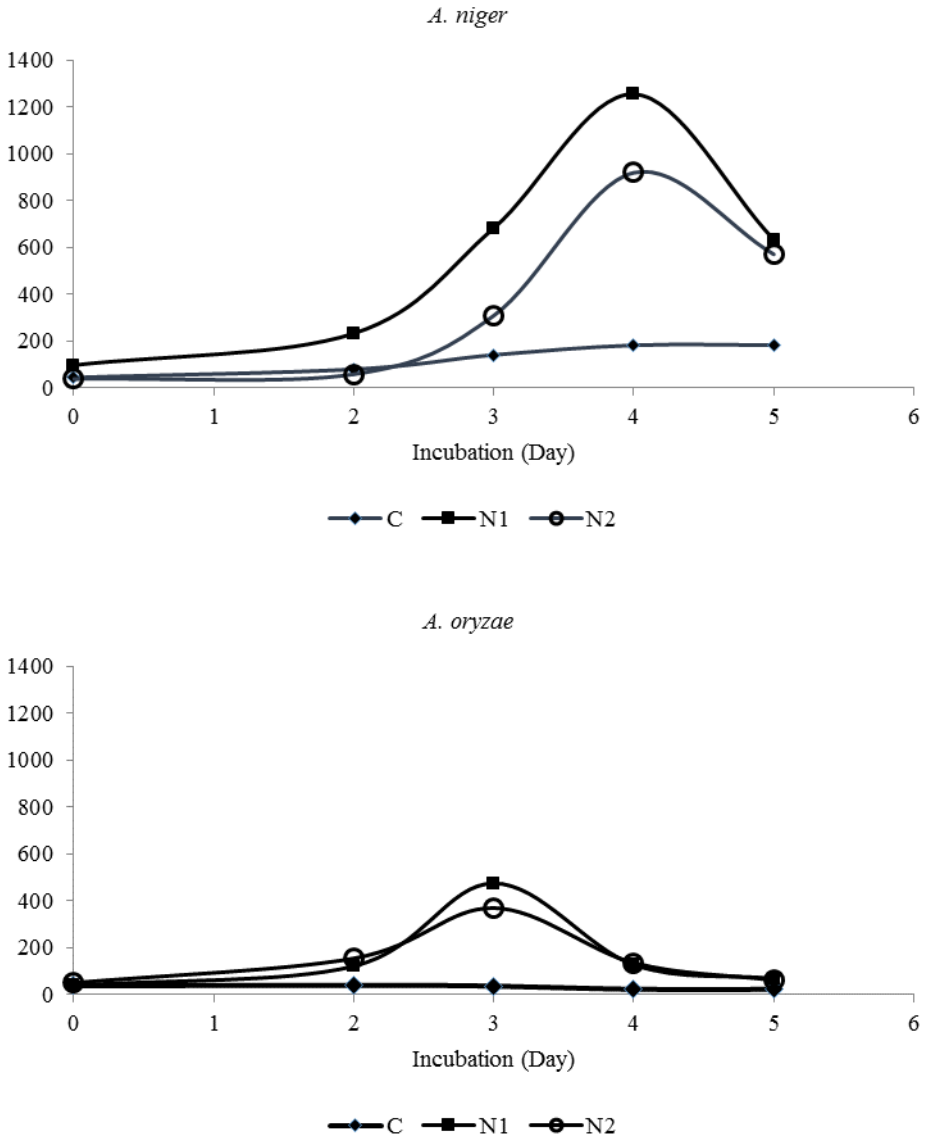

Figure 7. Cellulase activity in fermentation products.

CONCLUSION

The Nutritive value cocoa pod husk can be improved through fermentation using *A. niger* and *A. oryzae*. It was an increased in protein content and decreased in fiber fractions (crude fiber, NDF and ADF). Mannanase and cellulase content also contributed for increasing in digestibility. Fermentation product of CPH is very potential as an alternative feed for mitigation in feed shortage.

ACKNOWLEDGEMENT

This study was funded by the Indonesian government (APBN 2011). The authors thank all the technicians (Mrs. Emma Ludia and Mrs, Emi Sujatmika) who helped in preparation of samples and doing laboratory analyses. We also express our appreciation to Romaulina Yohana Septiani (AKA Bogor Polytechnic) who took part in this research activity.

REFERENCES

Alemawor F, Dzogbefia VB, Emmanuel OK, James HO. 2009. Enzyme cocktail for enhancing poultry utilization of cocoa pod husk. Francis Sci Res Essay**.** 4:555-559.

Chalall DS. 1985. Solid-substrate fermentation with *Trichoderma reseei* for cellulase production. Appl Env Microbiol. 49:205-210.

Elmakeel EA, Titgemeyer EC, Johnson BJ, Armendariz CK, Shirley JE. Fibrolytic enzymes to increase the nutritive value of dairy feedstuffs. J Dairy Sci. 90:5226-5236.

[FAO] Food and Agricultural Organization. 2002. Animal feed resources information system. USA: Food and Agricultural Organization. p. 76-82.

Haryati T, Sutikno I. 1994. Peningkatan nilai nutrisi kulit biji coklat melalui bioproses menggunakan beberapa jenis kapang. Ilmu Peternakan. 8:34-37.

Iyayi EA, Aderolu ZA. 2004. Enhancement of the feeding value of some agro-industrial by-products for laying hens after their solid state fermentation with *Trichoderma viride*. Afr J Biotechnol. 3:182-185.

Iyayi EA. 2004. Changes in the cellulose, sugar and crude protein contents of agro-industrial by-products fermented with *Aspergillus niger, Aspergillus flavus* and *Penicillium* sp. Afr J Biotechnol. 3:186-188.

Kompiang IP. 1994. Cassapro, a promising protein enriched cassava as animal and fish feed. Indones Agric Res Develop J. 16:57-63.

Lateef A, Oloke JK, Gueguim Kana EB, Oyeniyi SO, Onifade OR, Oyeleye AO, Oladosu OC, Oyelami AO. 2008. Improving the quality of agro-wastes by solid-state fermentation: Enhanced antioxidant activities and nutritional qualities. World J Microbiol Biotechnol. 24:2369–2374.

Leifa F, Padey A, Soccol CR. 2001. Production of *Flammulina velutipes* on coffee husk and coffee spend-ground. Braz Arch Biol Tech. 44:205-212.

Lowry OH, Rosebrough NJ, Farrand AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem. 193:265-275.

Marais JP, Evenwell TK. 1983. The use of trichloroacetic acid as precipitant for the determination of 'true protein' in animal feeds. S Afr J Anim Sci. 13:138-139.

Mekala NK, Singhania RR, Sukumaran RK, Pandey A. 2008. Cellulase production under solid state fermentation by Trichoderma reesei RUT C30: Statistical optimization of process parameters. Appl Biochem Biotechnol. 151:122-131.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chemist. 31:426–428.

Purwadaria T, Haryati T, Darma J. 1994. The development of inoculum production of *Aspergillus niger* for cassapro fermentation. Proceeding Seminar on National Science and Technology of Animal Husbandry. Bogor (Indones): Indonesian Research Institute for Animal Production.

Purwadaria T, Nirwana N, Ketaren PK, Iswantini D. 2003. Synergistic activity of enzymes produced by *Eupenicillium javanicum* and *Aspergillus niger* NRRL 337 on palm oil factory wastes, Biotropis. 20:1-10.

Singhania RR, Sukumaran RK, Pillai A, Prema P, Szakacs G, Pandey A. 2006. Solid-state fermentation of lignocellulosic substrates for cellulase production by Trichoderma reesei NRRL 11460. Indian J Biotechnol. 5:332-336.

Smith OB, Adegbola AA. 1985. Studies on the feeding value of agro-industrial by-products. III. Digestibility of cocoa-pod and cocoa-pod-based diets by ruminants. Anim Feed Sci Technol. 13:249-254.

Sobamiwa O, Longe OG, 1993. Utilization of cocoa pod pericarp fractions in broiler chick diets. Anim Feed Sci Technol. 44:237-244.

Sobamiwa O. 1998. Performance and egg quality of laying hens fed cocoa husk based diets. Nigeria J Anim Prod. 25:22-24.

Swe KH, Alimon AR, Ramin M. 2009. Effect delaying sporulation by addition of ammonium sulphate on the fermentation of palm kernel cake based subtrate by *Aspergillus niger*. Am J Agric Biol Sci. 4:262-265.

World Cocoa Foundation. 2010. In: FAOstat, cocoa market update. Published repports and ressources ed. Washington DC (USA): Food and Agricultural Organization.

Physical Quality and Digestibility *In Vitro* Determination of Green Pellet Concentrate Based on *Indigofera zollingeriana*

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(received 19-06-2017; revised 05-09-2017; accepted 11-09-2017)

ABSTRAK

Tarigan A, Ginting SP, Arief II, Astuti DA, Abdullah L. 2017. Identifikasi kualitas fisik dan pelet konsentrat hijau berbasis *Indigofera zollingeriana* secara *in vitro*. JITV 22(3): 114-123. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1651

Penelitian ini bertujuan untuk mengetahui kualitas fisik dan kecernaan pelet konsentrat hijau berbasis *I.zollingeriana* secara *in vitro*. Komposisi pakan berupa pelet konsentrat hijau mengandung *I. zollingeriana* dan *C. calothyrsus* pada berbagai kombinasi sebagai berikut : 1) *I. zollingeriana/C. calothyrsus* (90/0; R0), 2) *I. zollingeriana*/*C. calothyrsus* (75/15; R1), 3) *I. zollingeriana*/*C. calothyrsus* (60/30; R2) dan 4) *I. zollingeriana*/*C. calothyrsus* (45/45; R3). Penelitian kualitas fisik pelet konsentrat hijau dilakukan dengan menggunakan rancangan acak lengkap dengan empat perlakuan pelet konsentrat hijau masing-masing tiga ulangan. Penelitian kecernaan pelet konsentrat hijau dilakukan secara i*n vitro* fermentasi rumen dilakukan menggunakan rancangan acak kelompok dengan empat perlakuan pelet konsentrat hijau dengan tiga kelompok cairan rumen berbeda. Hasil penelitian memperlihatkan bahwa kualitas fisik pakan konsentrat hijau pelet yang mengandung komposisi *I. zollingeriana*/*C. calothyrsus* (60/30; R2) memiliki karakter fisik terbaik (aktivitas air = 0.58; ukuran partikel = 12.69 mm; sudut tumpukan = 21.01^{o}; ketahanan benturan = 99.78%; ketahanan gesekan = 90.42%; kerapatan tumpukan = 590 kg/m^3) dibandingkan dengan pelet mengandung komposisi lainnya. Kualitas pakan berdasarkan kecernaan secara *in vitro*, bahan kering dan bahan organik memperlihatkan pola yang sama yaitu R0 lebih tinggi (P<0.05) dibandingkan dengan perlakuan yang lain, sedangkan kecernaan *In Vitro* protein kasar memperlihatkan R0, R1 dan R2 (P>0.05), namun (P<0.05) lebih tinggi dari perlakuan R3. Tidak terdapat perbedaan pH dan total bakteri pada keempat jenis pakan konsentrat hijau pelet. Perlakuan R3 memiliki konsentrasi NH_3, VFA dan produksi gas total paling rendah (P<0.5) dibandingkan dengan perlakuan R2, R1 dan R0. Dapat disimpulkan bahwa masuknya *Calliandra calothyrsus* tidak mengubah kualitas fisik *green pellet*. Namun, peningkatan kadar *C. calothyrsus* meningkatkan RUP dan mengurangi kecernaan, N-NH3 dan total VFA.

Kata Kunci: *Indigofera zollingeriana*, *Calliandra calothyrsus*, Pelet, Pakan

ABSTRACT

Tarigan A, Ginting SP, Arief II, Astuti DA, Abdullah L. 2017. Physical quality and digestibility *in vitro* determination of green pellet concentrate based on *Indigofera zollingeriana*. JITV 22(3): 114-123. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1651

This study aims to determine the physical quality and digestibility of green pellet concentrate based on *I. zollingeriana*. The composition of green pellet concentrate feed contains *I. zollingeriana* and *C. calothyrsus* in the following combinations: 1) *I. zollingeriana* /*C. calothyrsus* (90/0; R0), 2) *I. zollingeriana*/*C. calothyrsus* (75/15; R1), 3) *I. zollingeriana*/*C. calothyrsus* (60/30; R2) and 4*) I. zollingeriana* / *C. calothyrsus* (45/45; R3). The physical quality study of green pellet concentrate was performed using a completely randomized design with four types of green concentrate as treatments of three replications. Green pellet concentrate studies conducted in vitro rumen fermentation were performed using in a randomized block design with four treatments and three sources of liquid rumen as a block. The results of the experiments conducted with pellet criteria containing *I. zollingeriana/C. calothyrsus* composition (60/30; R2) have the best physical character (water activity = 0.58; particles size = 12.69 mm; degree value angles = 21.01°; collision endurance = 99.78%; endurance friction = 90.42 %; bulk density = 590 kg/m3) compared to the other treatments. The quality of diet based on digestibility in vitro, dry matter and organic material having a pattern equal to R0 is higher (P<0.05) compared with other treatments, while the rough digestibility of In Vitro protein is seen R0, R1, and R2 (P>0.05), but (P<0.05) was higher than the treatment of R3. There was no difference of pH and total bacteria on green pellet concentrate type. The treatment of R3 has the lowest NH3, VFA and total gas concentration (P<0.5) compared with R2, R1 and R0. It can be concluded that inclusion of *Calliandra calothyrsus* did not change physical quality of green pellets. However, increasing level of *C. calothyrsus* increased RUP and reduced digestibility, N-NH_3, and total VFA.

Key Words: *I. zollingeriana, Calliandra calothyrsus*, Pellets, Feed

INTRODUCTION

One of the important inhibiting factors in ruminant production is the scarcity of feed due to its high price and its fluctuation in quantity and nutritional qualities. (Van et al. 2005; Olafadehan & Adewumi 2009). During the last twenty years, it was reported that there was an increase in cost of feeding of ruminant production from 60% of total production cost (1990's) to 64-66% (the 2000's) and 65-75% (the 2010's) because of a tendency to use feed concentrates at any stage of production (Kholif et al. 2015). Leguminous forages have been known widely to have a concentrate feed characteristic due to its high nutrient contents (crude protein, vitamin, and some mineral) and its highly DM digestibility. *Indigofera zollingeriana* is a leguminous tree that contains high crude protein (25-31%) and high (71-86.3%) DM digestibility (Tarigan et al. 2010; Abdullah et al. 2012a). The use of *I. zollingeriana* as a feed supplement (Tarigan & Ginting 2011) or as a sole forage gave a good growth rate of goats (Ginting et al. 2010). In dairy goats, the use of *I. zollingeriana* leaves pellets improved milk production and efficiency of feed and nutrient utilization (Abdullah et al. 2012b). On the other hands, the potential use of *Calliandra calothyrsus* as feed-in ruminant production have been widely reported (Norton 1994; Hess et al. 2008; Jayanegara et al. 2011a). This forage is known for its high tannin content. While the protein-tannin complexes may decrease ruminal protein degradation and may lead to increase the flux of dietary protein for absorption in the intestine (Patra & Saxena 2011; Theodoridou et al. 2010), the high tannin level may also result in reduced feed intake and digestibility. However, the combination of such high-tannin content with highly fermentable and low tannin content will improve microbial protein synthesis and protect the dietary protein without adversely affecting microbial growth efficiency and overall animal productivity (Soltan et al. 2012).

Feed processing such as pelleting may improve the physical (Svihu 2011; Svihu & Zimonja 2011), and nutritional characteristics of the feed ingredient through the increased starch availability by gelatinization (Solanas et al. 2005; Svihu & Zimonja 2011), thus increasing its fermentation rate (Solanas 2008; Bertipaglia et al. 2010). This study aimed to evaluate the physical and in vitro nutritional qualities of green concentrate pellet composed of low tannin forages (*I. zollingeriana*) and high tannin forage (*C. calothyrsus*) of various composition.

MATERIALS AND METHODS

The study was conducted at the Research Institute for Goat Production, Sungai Putih, North Sumatera from July to November 2016. *Indigofera zollingeriana* and *Calliandra calothyrsus* were harvested at 60 day-intervals and were cut 1.0 meter above ground level. The leaves were wilted by air-dried for 3-4 hours under the sun and ground using grinder with 1.0 mm screening size. Green concentrate pellets were formulated as follows: 90% *I. zollingeriana* + 0% *C. calothyrsus* (R0), 75% *I. zollingeriana* + 15% *C. calothyrsus* (R1), 60% *I. zollingeriana* + 30% *C. calothyrsus* (R2) and 45% *I.zollingeriana* + 45% *C. calothyrsus* (R3). The complete composition of the green concentrate is shown in Table 1. Pellet was produced using pelleting machine to yield green concentrate pellet of 5 mm diameter.

Chemical analysis

Dry matter, Kjeldahl N, crude fiber, and ash was analyzed according to AOAC (2005). The content NDF and ADF) were analyzed according to the method Van Soest (1991) and total tannin was analysed according to the folin-ciocateu method (Makkar 2003).

Physical qualities

Activity water (Aw) was measured using Aw meter that has been calibrated. Particles size was determined using vibrator ball mill with mesh/sieve of 4, 8, 16, 30,

Table 1. The composition of green concentrate pellets

Ingredients	Ratio (%)			
	R0	R1	R2	R3
I. zollingeriana meal	90	75	60	45
C. calothyrsus meal	0	15	30	45
Cassava meal	5	5	5	5
Molasses	3	3	3	3
Premix	1	1	1	1
Salt	1	1	1	1

50, 100, 400 according to the method of Syarief & Halid (1993). The determination of angles of heap was performed according to Khalil (1999b). The endurance of green concentrate pellets was tested using shatter test by throwing pellets of known weights over a plates iron according the method of Balagopolan et al. (1988). The endurance pellets to friction (durability) were determined using the tumbling method as described by Fairfield (1994). The density was measured by pouring out samples of known weights into measuring glasses, and density was calculated as the ratio sample weight to the volume it occupies in the glass meter (kg/m^3) according to Khalil (1999a).

In vitro ruminal fermentation

In vitro fermentation was done according to the method Tilley & Terry (1963). Rumen fluid was obtained from goats in the slaughterhouse. Rumen liquid was put into a flask surrounding hot water to maintain a temperature in flask between 39-40°C. Samples (0.5 g) of each feed treatment was put into fermentor tube into which 18.0 ml artificial saliva solution (McDougall) was added (temperature 39-40°C and pH 6.5-6.9), and then inoculated with 12 ml of rumen liquid. CO_2 added to the flask for ±30 seconds in order to maintain the anaerobic condition and the tube was covered with rubber with one direction out of ventilation. Tube fermentor then inserted into a shaker water bath at a temperature of 39°C and incubated for four hours. NH_3 concentration was determined according to the Conway micro diffusion method (Department of Dairy Science 1966), the VFA total was determined by a steam distillation method (Department of Dairy Science 1966), and pH was measured using a pH meter.

After 48 hours of incubation, the rubber cover was opened and 0.2 ml saturated $HgCl_2$ was dropped (3-2 drops). The tubes were centrifuged at 5000 rpm for 20 minutes. The solution was taken and filtered with paper Whatman N.41 with the help of vacuum pump, and the residue was then transferred to the cup and dried. The residues were analysed for N by the KJELDAHL method, and considered as the rumen undegradable protein (Licitra et al. 1995).

After the supernatant was disposed of, the residues were centrifuged in a tube fermentor then dissolved in 30 ml solution a pepsin-HCl (0.2%) and incubated for 48 hours in the aerobic condition at 39-40°C. Then solution was filtered with Whatman N.41 paper with the help of vacuum pump. The residues was then dried in an oven at 105°C for 24 hours and weighed to calculate the dry matter degradable (IVDMD). The sample then dried in the electric furnace at a 600°C for 24 hours to determine the degradable organic matter (IVDOM). The residue was used to analyzed the N content for the calculating of degradable crude protein (IVCPD). IVCPD was calculated as the difference between the crude protein content of the samples before being incubated and after being incubated.

***In Vitro* gas production**

Total gas production was measured according to the procedures of Close & Menke (1986). Syringes (50 ml) were filled with 0.2 g sample and then added 30 ml of rumen liquid that has been mixed with a buffer solution (1 : 2). The syringes are then inserted into a shaker water bath at 39°C. Gas production was measured at 2, 4, 6, 8, 12, 24, and 48 hours of fermentation by noting the volume of gas formed during the process of fermentation.

Statistical analysis

The physical characteristic evaluation was arranged in a completely randomized design of four treatment (composition of green concentrate pellet) with three replicates. The in vitro study was arranged in a randomized block design with rumen liquid sampling time the block consisting of four treatments with three replicates. Data were analysed using the method of e Analysis of Variance (ANOVA). Uses software SAS version 9.1 (2002). The means were compared using the Duncan test (Steel & Torrie 1995).

RESULTS AND DISCUSSION

Chemical compositions

The nutrient compositions of the green concentrates were shown Table 2. The dry matter (DM) and organic matter (OM) content were relatively comparable among all treatments. The DM and OM contents ranged from 93.87 to 95.62% and from 89.65-91.11%, respectively.

The crude protein (CP) contents were numerically higher when the inclusion level of *I. zollingeriana* increased which is due to the higher crude protein content of *I. zollingeriana* compared to *C. calothyrsus* (Tarigan et al. 2010). The crude protein content of the green concentrates pellets ranged from 18.82 to 21.44%. The high crude protein content of green concentrates pellet based on the mixtures *I.zollingeriana* and *C. calothyrsus*, is expected to increase productivity of ruminants through increasing of the flux of dietary protein for absorption in the intestines, and improving the amount of amino acid absorbed from intestinal digestion (Bach et al. 2005; Patra & Saxena 2011). The content of crude fibers is relatively low and ranged from 8.95 to 12.04 %.

Table 2. Chemical compositions of green concentrate pellets (% DM)

Parameter	Experimental forage		Experimental diets			
	I. zollingeriana	*C. calothyrsus*	R0	R1	R2	R3
Dry matter (DM)	20.40	33.30	95.62	95.15	93.87	94.05
Organic matter (OM)	90.85	93.40	89.79	89.65	90.77	91.11
Crude protein (CP)	25.78	18.83	21.44	20.49	19.17	18.82
Crude fiber (CF)	14.25	11.70	12.04	10.25	8.95	9.35
NDF	35.64	47.70	42.18	40.20	38.30	33.13
ADF	24.64	33.90	27.70	21.10	17.29	18.02
Total tannin	0.85	12.76	0.85	2.75	3.79	5.56

The NDF and ADF content range from 33.13 to 42.18%, and from 17.29 to 27.70%, respectively. These level of NDF and ADF contents seem not cause restrict feed consumption when fed to ruminant animals. (Van Soest et al.1991; De Boever et al. 2005).

The content of the total tannin in R0 (8.50 g/kg DM) is classified as very low, far below the threshold level (50 g/kg DM) that could express its role as anti of nutrient (Min et al. 2003; Al-Dobaib 2009; Piluzza et al. 2013). In line with the increasing inclusion level of *C. calothyrsus* the total tannin content increased in R1, R2, and R3 (2.75, 3.79, 5,56%, respectively). Tannin was related to the protection of dietary protein against increasing the flux of dietary protein for absorption in the intestines and may result improving the amount of amino acid absorbed from intestinal digestion (Jayanegara et al. 2009; Szumacher-Strabel & Cieslak 2010; Goel & Makkar 2012). The use of tannin in rations feed on certain concentration can improve efficiency nutrients, increase the productivity of ruminants. To increase the use of protein feed, especially feed with level degradation rumen high required protection. Protection protein intended to reduce the level of degradation protein in the rumen but digested by an enzyme post-ruminally so that directly can use through a process absorption post-ruminally in the small intestines. One way protection protein with a coating is to the tannin (Smith et al. 2005; Patra & Saxena 2011).

Physical qualities

The value of water activity (Aw) on green pellet feed concentrates based *I. zollingeriana* combined with *C. calothyrsus* (treatment) shown in Table 3. It is shown that the value of the water activity (Aw) green concentrate pellets with composition of R3 (45% *I. zollingeriana* and 45% *C. calothyrsus*) were lower ($P<0.05$) compared to those with composition of 90% or 60 % *I. zollingeriana*, but similar to those with composition of 75% *I.zollingeriana* and 15% *C. calothyrsus*. The value of water activity obtained in this study are low ranging from 0.49 to 0.60. It has been known that microbes can only grow in materials with specific water activity and materials having water activity of 0.7 is considered to be sufficient for lasting storage (Winarno 1991). The relatively low water activity values of green concentrate pellets found in this study is indicative of its potential for long lasting storage. The pelleting process could be defined as the agglomeration of small particles using a mechanical process in combination with moisture, heat, and pressure (Abdollahi et al. 2013). The particles size of green concentrate pellets is shown in Table 3. The particle size of the green concentrate pellets was lowest in that with composition of 45% *I. zollingeriana* and 45% *C. calothyrsus* and there were no differences ($P>0.05$) among other treatments. The particle size of green concentrate pellets in all treatments ranged from 12.12 to 13.24 mm. It was reported that the particle size of pellets was influenced by the particle size of its ingredients, and the smaller the particle size of its ingredients was the smaller the particle size of the pellets (Saenab et al. 2010). Amerah et al. (2007) reported that pelleting reduced the relative proportion of materials with particle size of > 1.0 mm and increased the proportion of those with particle size < 0.075 mm in coarse diets.

The degree of angles of repose of green concentrate pellets was not different ($P>0.05$) among the treatments and ranged from 18.06 to 21.01. Angulo (1995) confirm that materials that can easily flow will have degree of angles of repose ranging from 20 to 30°. When compared to these values the green concentrate pellets of various composition of *I. zollingeriana* and *C. calothyrsus* would easily flow across the compartments within the whole feed processing system. This trait is important in improving the efficiency of feed processing.

Table 3. Physical qualities green concentrates pellets based *I. zollingeriana* in combination with *C. calothyrsus*

Parameter	Treatment			
	R0	R1	R2	R3
Water activity (Aw)	0.60±0.08[a]	0.54±0.01[ba]	0.58±0.01[a]	0.49±0.02[b]
Particles size (mm)	13.16±0.14[ba]	13.24±0.13[a]	12.69±0.71[ba]	12.12±0.81[b]
Degree value angles (°)	18.84±1.11	18.06±0.45	21.01±1.40	19.04±3.59
Collision endurance (%)	99.84±0.06[ba]	99.90±0.01[a]	99.78±0.05[b]	99.65±0.06[c]
Friction endurance (%)	92.53±0.70[a]	92.92±1.46[a]	90.42±0.41[a]	87.19±2.50[b]
Bulk density (kg/m^3)	590±0.03	640±0.02	590±0.02	570±0.00

[a,b,c] means within a row without a common superscript letter differ significantly

Collision endurance is variable that is used to test endurance of pellets to collision. The collision endurance of green concentrate pellets R3 composed of 45% *I. zollingeriana* and 45% *C. calothyrsus* was lowest (P<0.05), but it was not different among other treatments (Table 3). The range of collision endurance values of the green concentrate pellets was 99.65 to 99.90% which are slightly higher compared to the study of Saenab et al. (2010) in complete pellet feed containing hydrolysates of shrimp by-products having collision endurance ranging from 92.25 to 99.34 %. The gelatinizing process may help to form a string ties among the particles that resulted in improving a compact pellet feed (Cheeke 1999).

Friction endurance value (durability) of green concentrate pellets significantly (P<0.05) increased as the proportion of *I. zollingeriana* in the green concentrate increased. The friction endurance value (durability) of green concentrate pellet ranged from 87.19 to 92.92%. These values are numerically greater than the minimum friction endurance value (durability) of 80 % reported by Dozier (2001). The relatively high friction endurance values of green concentrate pellets in the present study could be affected by low the moisture content and small particle size of materials used to produce the green concentrate pellet so that they were compact and not easily destroyed by friction. The smaller the size of particles of materials the larger are the surface area for particles to contact and to form a strong bound among the particles which then cause the pellets are not easily destroyed (Saenab et al. 2010).

The bulk density was not affected (P>0.05) by the composition of the green concentrate pellet (Table 3). The bulk density of the pellet was in the range of 570 to 640 kg/m3. Numerically, the bulk density was highest in green concentrate pellet composed of 75% *I. zollingeriana* (640 kg/m3) and was lowest in that composed of 45% *I. zollingeriana* (570 kg/ m3). Kahlil (1999a) stated that feed having low bulk density (<450 kg/m3) require longer flowing time flows (vertical direction) and vice versa with other feed which have the bulky density >500 kg/m3). Materials in the form of pellets or tube having bulky density of 600-800 kg/m^3 are easier to handle, delivery, storage and to use (Kaliyan & Morey 2009). The bulky density of green concentrates pellets in the present study is considered to be relatively high indicating that handling of these pellet feed will be easy and less space is needed to store. Khalil (1999a) reported that feeds with high bulk density need a period of falling and flowing more concise than those with lower bulk density.

In Vitro rumen degradability

The in vitro rumen degradability of green concentrate pellets based on mixtures of *I. zollingeriana* and *C. calothyrsus* is shown in Table 4. IVDMD and IVOMD of pellet composed of 90% *I. zollingeriana* were significantly higher (P<0.05) compared to other treatment. The IVDMD and IVOMD increased in line with the increasing proportion of *I. zollingeriana* and decreasing proportion of *C. calothyrsus*. The higher content of tannin in *C. calothyrsus* may contributed to this phenomenon as indicated by the highest tannin content (55.6 g/kg) of those green concentrate pellet with the highest proportion of *C. calothyrsus* (Table 2). This tannin level is beyond the threshold of 50 g/kg DM that has a decreasing effect on the rate of protein degradation (Min et al. 2003; Al-Dobaib 2009; Piluzza et al. 2013). Thus, an increase in the tannin content in any green concentrates pellets composition is tending to cause decreases in IVDMD and IVOMD. Suharlina et al. (2016) reported that feed concentrates composing of 20% *I. zollingeriana* has a high in vitro degradability due to its high content of crude protein (CP) and low content of crude fibers. Increasing nitrogen content in forage mixtures by adding legumes have been shown to improve ruminal digestion of feed dry matter (Dal Pizzol et al. 2017). In the present study, the IVDMD and IVOMD of green concentrate pellets may have been positively influenced by high CP content of *I. zollingeriana*.

IVCPD of green concentrates pellet were no significantly different (P>0.05) among treatments, except that IVCPD was lowest (P<0.05) in that composed of 45% *I. zollingeriana* and 45% *C. calothyrsus* (Table 4). IVCPD values ranged from 77.65 to 86.78% in all treatments with the highest values obtained in the pellet composed of 90% *I. zollingeriana* (86.78%). The increasing level of *I. zollingeriana* in the green concentrate pellets linearly increased the crude protein content of the pellets (Table 2).This increase of the crude protein in diet may trigger the activity of microbial fermentation in the rumen (Kiran & Mustsvangwa 2009; Sauve et al. 2009). This indicates that the green concentrate may have provided sufficient N to favor the growth rumen microbes that serves as a source of protein for of ruminants. IVCPD obtained in the present study are higher than those reported by Suharlina et al. (2016) ranging from 79.1 to 83.38% on diets composed of 0.20 and 40% *I. zollingeriana* fed to goats. The relatively high digestibility of these green concentrate pellets could also be affected by the process (grinding, pressuring, and heating) that has been put on ingredients in producing the pellets. The application of heat and pressure during pelleting might promote starch gelatinisation which increased the fermentation rate (Solanas et al. 2008).

RDP of green concentrate pellets was affected by the composition of the pellet (P<0.05). The greatest RDP was observed in pellet with highest level proportion of *I. zollingeriana* and decrease in line with increasing proportion of *C. calothyrsus.* This indicates that solubility of the protein in green concentrate tend to rise as the proportion of *I. zollingeriana* is increasing. This is in agreement with the fact that *I. zollingeriana* has high protein content with high degradability in the rumen and low fiber and tannin contents (Tarigan et al. 2010; Suharlina et al. 2016).

The RUP of green concentrates composed of 45% *I. zollingeriana* and 45% *C. calothyrsus* was higher compared to that composed of 60% *I. zollingeriana* and 30% *C. calothyrsus* or 90% *I. zollingeriana* and 0% *C. calothyrsus*, but was not different to that composed of 75% *I. zollingeriana* and 15% *C. calothyrsus*. The ranking order of the green concentrate pellet in term of their RUP values was R3>R2≥R1>R0. RUP value increase in line with the increased proportion of *C. calothyrsus* in the mixture with *I. zollingeriana*. Combination of high-tannin forages with other highly fermentable forages will improve microbial protein synthesis and microbial growth efficiency (Soltan et al. 2012). Tannin is beneficial to form a complex with protein in the rumen for supplying protein to the digestive tract after rumen, to increase productivities of ruminants (Al-Dobaib 2009; Jayanegara et al. 2011b; Piluzza et al. 2013). This indicates the potential of green pellets concentrates for supplying protein needs of ruminants after rumen.

Characteristic of fermentation rumen

The pH values of the media (in vitro) were not affected (P>0.05) by the composition of *I. zollingeriana* and *C. calothyrsus* of the green concentrate pellets (Table 5). The lowest pH (P>0.05) was observed in media where substrate used was the mixture of *I. zollingeriana* (45%) and *C. calothyrsus* 45%. Nevertheless, there was a trend of decreasing pH of in vitro media when the proportion of *C. calothyrsus* in substrates increase. The pH levels obtained in the present study ranged from 6.73 to 6.75 and is relatively lower compared to those reported by Syahrir et al. (2009), i.e., 6.9-7.0 when using flour mulberry as substrates. This pH levels are at least in the range of 6.2-7.2 reported by Van Houtert (1993) and Van Soest (1994) to ensure optimal ruminal digestion.

The ammonia-N (NH3-N) concentration (9.32 mM) was highest (P<0.05) when 90% *I. zollingeriana* was used as substrate, but it was not different (P>0.05) to that when 75% *I. zollingeriana* and 15% *C. calothyrsus* was used as substrates (8.21 mM). The concentrations

Table 4. *In vitro* rumen degradability green pellets concentrates based *I. zollingeriana* in the combination with *C. calothyrsus* (% DM)

Parameter	Treatment			
	R0	R1	R2	R3
IVDMD (%)	73.62±1.30^{a}	66.58±2.65^{b}	60.09±0.91^{c}	53.47±1.63^{d}
IVOMD (%)	71.84±1.80^{a}	64.29±1.08^{b}	57.58±1.11^{c}	50.43±2.01^{d}
IVCPD (%)	86.78±0.96^{a}	85.59±5.87^{a}	84.07±2.81^{ba}	77.65±2.41^{b}
RDP (%)	54.35±0.54^{a}	47.00±0.92^{b}	48.08±2.5^{b}	42.47±3.27^{c}
RUP (%)	45.65±0.54^{c}	53.00±0.92^{b}	51.92±2.5^{b}	57.54±3.27^{a}

^{a,b,c,d} Means within a row without a common superscript letter differ significantly
RDP - rumen degradable protein; RUP-rumen undegradable protein

Table 5. Characteristic fermentation rumen concentrate green pellet based *I. zollingeriana* in the the combination with *C. calothyrsus*

Parameter	Treatment			
	R0	R1	R2	R3
pH	6.75±0.05	6.75±0.05	6.73±0.06	6.73±0.03
N-NH3 (mM)	9.32±1.25[a]	8.21±0.50[ba]	7.51±0.66[b]	6.30±0.67[c]
Total VFA (mM)	138.12±7.57[a]	117.45±7.09[b]	95.50±3.21[c]	78.31±5.57[d]
Total bacteria (LogCFU/ml)	6.69±0.31	6.24±0.06	6.45±0.34	6.51±0.45

[a,b,c,d] Means within a row without a common superscript letter differ significantly

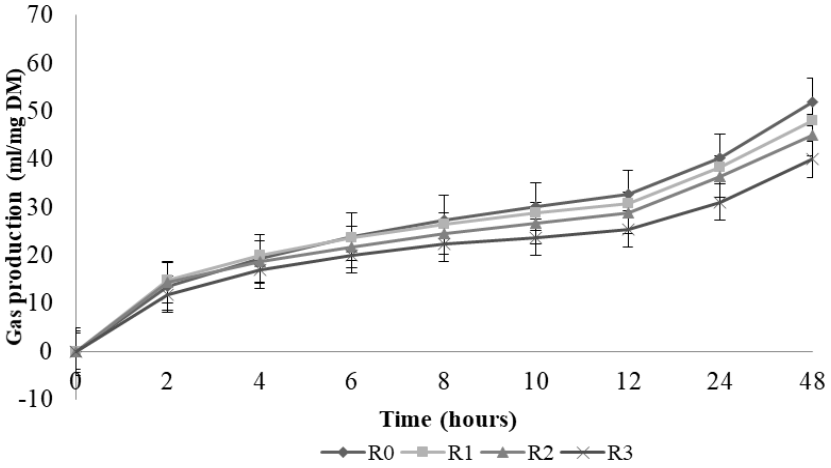

Figure 1. Total gas production of feed green concentrate pellets in different combination *I. zollingeriana*/*C. calothyrsus* (90/0; R0), *I. zollingeriana*/*C. calothyrsus* (75/15; R1), *I. zollingeriana*/*C. calothyrsus* (60/30; R2) and *I. zollingeriana*/*C. calothyrsus* (45/45; R3).

ruminal NH_3-N of all treatments ranged from 6.30 to 9.32 mM. McDonald et al. (2010) stated that the concentration NH_3-N required to support protein of rumen microbes ranged from 85 to 300 mg/l or 6-12 mM. The increased proportion of *I. zollingeriana* tends to increase the concentrates of NH_3-N of the rumen liquid which may related to the increased protein level of the green concentrate. The NH_3-N concentration of the rumen liquid observed in this study is higher compared to 3.87-5.23 mM reported by Suharlina et al. (2016).

The concentration of total VFA in the rumen liquid was highest when 90% *I. zollingeriana* was used as substrate. As the proportion of *I. zollingeriana* in the green concentrate pellet increased the VFA concentration tent to increase. The VFA concentration ranged from 78.3 to 138.12 mM and in parallel with the normal range 78.81 to 138.2 mM reported by Wanapat & Pimpa (1999). There was a decrease of 33% in the VFA concentration of the rumen liquid when the proportion of *I. zollingeriana* deceased from 90 to 45% in the mixtures of *I. zollingeriana* and *C. calothyrsus*. Total bacteria number was not different ($P<0.05$) among treatments and ranged from 6.24-6.69 log CFU/ml. Numerically, total rumen bacteria numbers were higher when 90 % *I. zollingeriana* was used as substrate.

In Vitro gas production

Total gas production for 48 hour incubation period of green concentrate pellets is presented in Figure 1. Total gas production obtained at all treatments ranged from 38 to 52 ml which are numerically higher compared to 20.31-24.83 ml reported by Suherlina et al. (2016) worked on feed concentrates based on *I. zollingeriana* in vitro. The gas production was highest ($P<0.05$) when green concentrate pellet composed of 90% *I. zollingeriana* was incubated. The gas production tent to increase as the proportion of *I. zollingeriana* in the green concentrate pellets increased and which may related to the it's higher crude protein and lower fiber contents that could trigger increased feed digestion. The study of Tschernig et al. (2006) showed that addition of

I. zollingeriana increases the rate of the production of gas and degradation N more than *C. calothyrsus*. The gas production curve of all treatments showed a linear fashion over the 48 hour-incubation periods. This is indicative of the presence of substrates that are readily fermented provided that incubation period was prolonged over 48 hours period.

CONCLUSION

It can be concluded that inclusion of *Calliandra calothyrsus* did not change physical quality of green pellets. However, increasing level of *C. calothyrsus* increased RUP and reduced digestibility, N-NH_3, and total VFA.

REFERENCES

Abdullah L, Tarigan A, Suharlina, Budhi D, Jovintry I, Apdini TA. 2012a. *Indigofera zollingeriana*: A promising forage and shrubby legume crop for Indonesia. Astuti DA, editor. Proceeding the 2nd International Seminar on Animal Industry. Jakarta (Indones): Bogor Agricultural University. p. 149-153.

Abdullah L, Apdini T, Astuti DA. 2012b. Use of *Indigofera zollingeriana* as a forage protein source in dairy goat rations. Abdullah R, Omar MA, Makkar H, Otte J, Rajion MA, Alimon AR, Boo LJ, Kam HA. Li CW, editors. Proceeding of the 1st Asia Dairy Goat Conference. Kuala Lumpur (Malays): Universiti Putra Malaysia and The Food and Agricultural Organization of the United Nations. p. 72-74.

Abdollahi MR, Ravindran V, Shihus B. 2013. Pelleting of broiler diets: An overview with emphasis on pellet quality and nutritional value. Anim Feed Sci Tech 179:1-23.

Al-Dobaib SN. 2009. Effect of different levels of quebracho tannin on nitrogen utilization and growth performance of Najdi sheep fed alfalfa (*Medicago sativa*) hay as a sole diet. J Anim Sci. 80:532–541.

Amerah AM, Ravindran V, Lentle RG, Thomas DG. 2007. Influence of feed particle size and feed form on the performance, energy untilisation, digestive tract development and digesta parameters of broiler starters. J Poult Sci. 86:2615-2623.

Angulo EJ, Brufu EE. Garcia. 1995. Effect of sepoilite on pellet durability in feeds differing in fat and fiber content. J Anim Feed Technol. 53:233-241.

[AOAC] Association of Official Analytical Chemists. 2005. Official methods of analysis. 2005. 18th ed. Washington DC (USA): Association of Official Analytical Chemists.

Bach A, Calsamiglia S, Stern MD. 2005. Nitrogen metabolism in the rumen. J Dairy Sci. 88:E9-E21.

Balagopolan C, Padmaja G, Nanda SK, Moorthhy SN. 1988. Cassava in food: Feed Industry. Florida (USA): IRC Press.

Bertipaglia LMA, Fondevila M, van Laar H, Castrillo C. 2010. Effect of pelleting and pellet size of a concentrate for intensively reared beef cattle on *in vitro* fermentation by two different approaches. Anim Feed Sci Technol. 159:88-95.

Cheeke PR. 1999. Applied animal nutrition: Feeds and feeding. 2nd ed. New Jersey (USA): Prentice Hall.

Close W, Menke KH. 1986. Manual selected tropics in animal nutrition. 2nd ed. Stuttgart (Germany): The Institute of Animal Nutrition, University of Hohenhelm.

Dal Pizzol JG, Ribeiro-Filho HMN, Quereuil A, Le Morvan A, Niderkorn V. 2017. Complementarities between grasses and forage legumes from temperate and subtropical areas on in vitro rumen fermentation characteristics. Anim Feed Sci Technol. 228:178-185.

De Boever JL, Aerts JM, Vnacker JM, De Brabander DL. 2005. Evaluation of the nutritive value of maize silages using a gas production technique. Anim Feed Sci Technol. 123-124:255-265.

Department of Dairy Science. 1966. General laboratory procedures. Medison (USA): University of Wisconsin.

Dozier WA. 2001. Pellet quality for more economical poultry meat. J Feed Int. 52:40-42.

Fairfield D. 1994. Pelleting cost center. In: McEllhiney R, editor. Feed Manufacturing Technology IV. Arlington, VA (USA): American Feed Industry Association. p. 121-122.

Ginting SP, Krisnan R, Sirait J. 2010. The utilization of Indigofera sp as the sole foliage in goat diets supplemented with high carbohydrate or high protein concentrates. JITV. 15:261-268.

Goel G, Makkar HPS. 2012. Methane mitigation from ruminants using tannins and saponins, a status review. J Trop Anim Health Prod. 44:729-739.

Hess HD, Mera ML, Tiemann TT. 2008. In Vitro assessment of the suitability of replacing the low-tannin legume *Vigna unguiculata* with the Tanniniferous legumes *Leucaena leucocephala*, *Flemingia macrophylla* or *Calliandra calothyrsus* in a tropical grass diet. Anim Feed Sci Technol. 147:105-115.

Jayanegara A, Togtokhbayar N, Makar HPS, Becker K. 2009. Tannins determined by various methods as predictors of methane production reduction potential of plants by an in vitro rumen fermentation system. J Feed Sci Technol. 150:230-237.

Jayanegara A, Wina E, Soliva CR, Marquardt S, Kreuzer M, Leiber F. 2011a. Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis. Anim Feed Sci Technol. 163: 231-243.

Jayanegara A, Leiber F, Kreuzer M.2011b. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. J Anim Phsiol Anim Nutr. 96:265-375.

Kaliyan N, Morey RV. 2009. Factors affecting strength and durability of densified biomass products. J Biomass Bioenergy. 33:337-359.

Khalil. 1999a. Pengaruh kandungan air dan ukuran partikel terhadap sifat fisik pakan lokal, kerapatan tumpukan, pemadatan tumpukan, dan berat jenis. Media Peternakan. 22:1-11.

Khalil. 1999b. Pengaruh kandungan air dan ukuran partikel terhadap perubahan perilaku fisik bahan pakan lokal, sudut tumpukan, daya ambang dan faktor higroskopis. Media Peternakan. 22:33-42.

Kholif AE, Gauda GA, Morsy TA, Salem AZM, Lopez S, Kholif AM. 2015. Moringa Oleifera meal as a protein source in lactating goat's diets: Feed intake, digestibility, ruminal fermentation, milk yield and composition, and its fatty acids profile. Small Rumin Res. 129:129-137.

Kiran D, Mutsvangwa T. 2009. Nitrogen utilization in growing lambs fed oscillating dietary protein concentration. Anim Feed Sci Technol. 152:33-41.

Licitra G, Hernandez TM, Van Soest PJ. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim Feed Sci Technol. 57:347-358.

Makkar HPS. 2003. Quantification of tannin in tree an shrub foliage. A laboratory manual. Dordrecht (Netherlands): Kluwer Academic Publisher.

McDonald P, Edward RA, Greenhalgh JFD, Morgan CA, Sinclair LA, Wilkinson RG. 2010. Animal nutrition. 7th ed. New York (USA): Scientific and Tech John Willey & Sons, Inc.

Min BR, Barry TN, Attwood GT, McNabb WC.2003.The effect of condensed tannin on the nutrition and health of ruminants fed fresh temperate forage: A review. Anim Feed Sci Technol. 106:3-19.

Norton BW. 1994. Tree legumes as dietary supplements for ruminants. In: Gutteridge RC, Sheiton HM, editors. Forage tree legumes in tropical agriculture. Wallingford (UK): CAB International. p. 15-30.

Olafadehan OA, Adewumi MK. 2009. Productive and reproductive performance of strategically supplemented free grazing prepartum Bunaji cows in the agropastoral farming system. Trop Anim Health Prod. 41:1275-1281.

Patra AK, Saxena J. 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J Sci Food Agric. 91:24-37.

Piluzza G, Sulas L, Bullitta S. 2013. Tannis in forage plants and their role in animal husbandry and environmental sustainability: a review. J Grass Forage Sci. 69:32-48.

Saenab A, Laconi EB, Retnani Y, Mas'ud MS. 2010. Evaluasi kualitas pelet ransum komplit yang mengandung produk samping udang. JITV. 15:31-39.

[SAS] Statistical Analysis System Institute. 2002. Statistical analysis system. Version 9.1.3. Cary NC (USA): SAS Institute Inc.

Sauve AK, Huntington GB, Burns JC. 2009. Effects of total nonstructural carbohydrates and nitrogen balance on voluntary intake of goats and digestibility of gamma grass hay harvested at sunrise and sunset. Anim Feed Sci Technol. 148:93-106.

Smith AH, Zoetendal E, Mackie RI. 2005. Bacterial mechanisms to overcome the inhibitory effect of dietary tannins. J Microb Ecol. 50:197-205.

Solanas E, Castrillo C, Fondevila M, Ruiz Narvaez QO, Guada JA. 2005. Effect of concentrate extrusion and castration on diet digestion and performance of intensively reared male calves. Livest Prod Sci. 94: 225-236.

Solanas E, Castrillo C, Fondevila M, Ruiz Narvaez QO, Guada JA. 2008. Effects of cereals and/or protein supplement extrusion on diet utilisation and performance of intensively reared cattle. Livest Sci. 117:203-214.

Soltan YA, Morsy AS, Louvandini H, Abdalla AL. 2012. Comparative *in vitro* evaluation of forage legumes (Prosopis, Acacia, Atriplex, and Leucaena) on ruminal fermentation and methanogenesis. J Anim Feed Sci. 21:759-772.

Steel RGD, Torrie JH. 1995. Prinsip dan prosedur statistika. 2nd ed. Sumantri B, translator. Translate from: The Principle and Procedure of Statistics. Jakarta (Indones): Gramedia Pustaka Utama.

Suharlina, Astuti DA, Jayanegara A, Abdullah L. 2016 Nutritional evaluation of dairy goat rations containing *Indigofera zollingeriana* by using *in vitro* rumen fermentation technique (RUSITEC). Int J Dairy Sci. 11:100-105.

Svihu B, Zimonja O. 2011. Chemical alterations with nutritional consequences due to pelleting animal feeds: A review. Anim Prod Sci. 51:590-596.

Svihu B. 2011. The gizzard: Function, Influence of diet structure and effects on nutrient availability. World's Poult Sci J. 67:207-223.

Syarief R, Halid H. 1993. Teknologi penyimpanan pangan. Jakarta (Indones): Penerbit ARCAN.

Szumacher-Strabel M, Cieslak A. 2010. Potential of phytofactors to mitigate rumen ammonia and methane production. J Anim Feed Sci. 19:319-337.

Syahrir S, Wiryawan KG, Parakkasi A. 2009. Efektivitas daun murbei sebagai pengganti konsentrat dalam sistem rumen *in vitro*. Media Peternakan. 32:112-119.

Tarigan A, Ginting SP. 2011. Pengaruh taraf pemberiaan *Indigofera Sp*, terhadap konsumsi dan kecernaan pakan

serta pertumbuhan bobot hidup kambing yang diberi rumput *Brachiaria ruziziensis*. JITV. 163:231-243.

Tarigan A, Abdullah L, Ginting SP, Permana IG. 2010. Produksi dan komposisi nutrisi serta kecernaan *in vitro Indigofera sp* pada interval dan tinggi pemotongan berbeda. JITV. 15:188-195.

Theodoridou K, et al. 2010. Effects of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on *in vivo* and *in situ* digestion in sheep. Anim Feed Sci Tech. 160:23-38.

Tilley JMA, Terry RA. 1963. A two-stage technique for the in vitro digestion of forage crops. J Grass Forage Sci. 18:104-111.

Tschernig L, Lascano K, Barrios E, Schultze-Kraft, Peters M. 2006. The effect of mixing prunings of two tropical shrub legumes (*Calliandra houstoniana* and *I. zollingeriana*) with contrasting quality on N release in the soil and apparent N degradation in the rumen. J Plant Soil. 280:357-368.

Van Houtert M. 1993. The production and metabolism of volatile fatty acids by ruminants fed roughages: A review. Anim Feed Sci Technol. 43:189-225.

Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci. 74:3583-3597.

Van DTT, Nguyen TM, Inger Ledin. 2005. Tropical foliages: Effect of presentation method and species on intake by goats. Anim Feed Sci Technol. 118:1-17.

Wanapat M, Pimpa O. 1999. Effect of ruminal NH_3-N level on ruminal fermentation, purine derivative, digestibility and rice straw intake in swamp buffaloes. Asian-Aust J Anim Sci. 12:904-907.

Winarno FG. 1991. Kimia pangan dan gizi. Cetakan ke-5. Jakarta (Indones): PT. Gramedia.

Evaluation of Four Pasture Legumes Species as Forages and Cover Crops in Oil Palm Plantation

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(received 28-07-2017; revised 16-08-2017; accepted 31-08-2017*)*

ABSTRAK

Hutasoit R, Juniar S, Tarigan A, Ratih DH. 2017. Evaluasi empat spesies legume herba sebagai sumber hijauan dan *cover crop* pada perkebunan kelapa sawit. JITV 22(3): 124-134. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1801

Tanaman leguminosa adalah jenis pakan yang sangat berkualitas sebagai pakan ternak. Terbatasnya lahan merupakan masalah dalam pengembangannya. Integrasi dengan perkebunan sawit merupakan salah satu potensi dalam pengembangannya. Penelitian bertujuan untuk mengevaluasi produktivitas beberapa tanaman leguminosa (*Arachis glabrata*, *Stylosanthes guianensis*, *Clitoria ternatea*, and *Chamaecrista rotundifolia*) sebagai sumber pakan dan *cover crop*. Uji potensi dilakukan di kebun sawit seluas 4608 m^2 dalam rancangan acak lengkap dengan empat perlakuan (spesies legum) dan tiga ulangan. Parameter yang diamati antara lain: Produksi legum, rasio daun/batang, komposisi kimiawi legum, konsentrasi N, P dalam tanah, kandungan mikroba dalam tanah, kecernaan legum dan produksi buah sawit. Hasil penelitian menunjukkan produksi legum BK tertinggi (P<0,05) pada spesies *Clitoria ternatea* (16,15 ton/$ha^{-1}$$tahun^{-1}$), Rasio daun/batang tertinggi (P<0,05) pada *Arachis glabrata* (2,09). Komposisi kimiawi BK yang dihasilkan tidak berbeda (P>0,05) berkisar antara 33,75-35,75%, kandungan BO (P<0,05) tertinggi dimiliki *Clitoria ternatea*. Protein kasar sangat bervariasi (P<0,05) tertinggi pada *Clitoria ternatea* 17,84%. kandungan NDF tidak berbeda (P>0,05). Kandungan ADF paling rendah (P<0,05) pada *Chamaecrista rotundifolia*. Kandungan N pada tanah awal kegiatan relatif sama (rata-rata 0.10%), namun pada akhir kegiatan meningkat (P<0,05) tertinggi 0,16% pada perlakuan *Stylosanthes guianensis*. Populasi bakteri penambat N tertinggi 1,76 x 10^9 dan bakteri pelarut fosfat 9,8x10^5 terdapat pada perlakuan *Clitoria ternatea*. Produksi buah kelapa sawit relatif sama (P>0,05) berkisar antara 16,52-19,21 ton $ha^{-1}$$tahun^{-1}$. Disimpulkan bahwa jenis *Clitoria ternatea* merupakan tanaman leguminosa yang paling baik untuk digunakan sebagai pakan ternak dan *cover crop* pada tanaman kelapa sawit.

Kata Kunci: Leguminosa, *Cover Crop*, Tanaman Pakan, Kelapa Sawit

ABSTRACT

Hutasoit R, Juniar S, Tarigan A, Ratih DH. 2017. Evaluation of four pasture legumes species as forages and cover crops in oil palm plantation. JITV 22(3): 124-134. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1801

Pasture legumes is a very high quality of forage. The limited land is the problem of its development. Integration with oil palm plantations is one of the potentials for its development. This study was aimed to investigate the productivity of several legumes (*Arachis glabrata*, *Stylosanthes guianensis*, *Clitoria ternatea*, and *Chamaecrista rotundifolia*) as forages and cover crop. The potential tests were conducted in oil palm area of 4608 m^2, in a complete block design with four treatments (legume species) and three replications. Parameters observed were: Legum production, leaf/stem ratio, chemical composition of legume, concentration of N, P in the soil, microbes in the soil, leguminous digestibility and palm fruit production. Results showed that the highest legume production (DM) was (P<0.05) in the species of *Clitoria ternatea* (16.15 tons $ha^{-1}$$year^{-1}$), the highest leaf/stem ratio (P<0.05) was in the *Arachis glabrata* (2.09). The chemical composition (DM) did not differ (P>0.05) ranged from 33.75 to 35.75%, the organic matter (OM) varied greatly (P<0.05) the highets was in *Clitoria ternatea*. The highest Crude protein (P<0.05) was in *Clitoria ternatea* 17.84%. NDF concentrations did not differ (P>0.05). The lowest ADF concentration (P<0.05) was in *Chamaecrista rotundifolia*. The concentration of N in the soil indicated that early year of activity was similar (average 0.10%), at the end of activity increased (P<0.05) in treatment *Stylosanthes guianensis* (0.16%). The highest population of N-fixation bacteria of 1.76x10^9 and phosphate solvent of 9.8x10^5 were in the treatment of *Clitoria ternatea*. Production of fresh fruit bunches of the palm was relatively similar (P>0.05) ranged from 16.52-19.21 tons $ha^{-1}$$year^{-1}$. It is concluded that *Clitoria ternatea* is the best species of legume tested as forage and cover crop in oil palm plantations.

Key Words: Legume, Forage, Oil Palm

INTRODUCTION

An increasing livestock, particularly ruminants population should be followed by the availability of feeds, however in Indonesia land availability for forage crop is limited. This condition is also occurred in many countries (Jalaludin 1997; Harahap et al. 2017) which caused by increasing human population forced

conversion of pasture land for housing or industrial purpose (Mapiye et al. 2007). Alternative area for planting forage crop should be explored, this including land area under plantation including oil palm, rubber, and coffee plantation. Integration of oil palm plantation with forage crop such as herbaceous legume as cover crop is an option as feed source for ruminants.

Previous research has reported the benefits of legume cover crops used to increase soil fertility (Zhou et al. 2012; Gomes et al. 2009; Mazzoncini et al. 2011) and to conserve land conservation (Plieninger & Gaertner 2011). However, information on double function of cover crop to increase soil fertility and as feed source is limited. Previous study reported that herbaceous legume in ruminant feeding supplied rapidly degradable protein for microbial protein synthesis and contribute to the pool of amino acids available for the synthesis of milk protein and retention in the body tissue (Ramos-Morales et al. 2010). Juma et al. (2006) reported that Clitoria, Mucuna, and Gliricidia can be used as nitrogen supplement to the lactating of Jersey cows. Kemp et al. (2010) feeding chicory (*Cichorium intybus*), plantain (*Plantago lanceolata*), red clover (*Trifolium pratense*) and white clover (*Trifolium repens*) produced live weight gains in lambs 70% greater than feeding perennial ryegrass (*Lolium perenne*) based pastures. Legume as cover crop is known to increase soil organic matter quantity and the soil fertility (Franzluebbers & Stuedemann 2007; Mohamad et al. 2010) this due to legume symbiosis with Rhizobium which able to fixing nitrogen from the air resulted in reduction the cost of fertilizers such as Urea and TSP up to 40-50% (Saraswati et al. 1996). The characteristics and morphology of herbaceous legume plants in that they have many root nodules (Oldroyd & Downie 2008) this make them potential to provide a multitude of benefits to soil conservation and nutrient cycling efficiency while reducing economic risk and increasing profitability.

According to Valbuena et al. (2012); Bell et al. (2014) planting legumes is potential to improve the sustainability and productivity of the smallholder mixed farming systems by providing a high-quality feed which can increased crop and livestock production. Legume forages play an important role in feeding ruminant systems in many tropical and subtropical countries in the world. The information on legume species as cover crop has been reported, the species of herbaceous legumes commonly planted in oil palm plantation are *Pueraria phaseloides*, *Centrosema pubescens*, *Calopogonium mucunoides*, *Calopogonium caeruleum* and of late *Mucuna bracteata* (Chiu & Madsun 2006), but these plants have not been used as animal feed for the production, might be due to its palatability is very low. There are many types of legumes that are familiar as livestock feed. For example, *Arachis glabrata*, *Stylosanthes guianensis*, *Clitoria ternatea*, and *Chamaecrista rotundifolia*. Each of them has an advantage. According to Constanza et al. (2015) *Arachis glabrata* have been studied for their potential as a high quality forage plant and the high nutritional value of the forage close to alfalfa. *Arachis glabrata* as a high quality legume is used for hay in most of the Southern USA. Ghosh & Bera (2000) reported that the high quality of *Arachis glabrata* was developed in India as a possible source of legume fodder.

Stylosanthes guianensis has good nutrition, palatable, and high digestibility (Thang et al. 2009; Muamba et al. 2014). It has been the best feed for Hainan black goats because of its high yield and nutritive value (Guodao et al. 1997) Moreover this forage was reported able to increase the performance of west African dwarf sheep (Ogunbode & Akinlade 2012) and have suitable nutritive values for livestock in tropical regions of China (Li et al. 2014). Phengsavanh & Ledin (2003) indicated that the diet of goats containing 30–40% dry matter (DM) of stylo could improve growth rate to 64–70 g day^{-1}.

Clitoria ternatea is commonly known as butterfly pea, potential as high quality forage legume (Abreu et al. 2014). Other than as animal feed, clitoria also has potential as medicinal plants used in various traditional systems such as antidiabetic, antioxidant and anti-inflammatory activity (Ponnuswamy & Wesely Jebasingh Devairrakam 2011; Zingare et al. 2013; Suganya et al. 2014), it is useful in ophthalmopathy, bronchitis, asthma tubercular glands, hemicrania, burning sensation, starngury, helminthiasis, inflammation, vitiated condition of pitta vicerormegaly, and fever (Chauhan et al. 2012).

Chamaecrista rotundifolia had high DM digestibility, potential to provide greater quantities of high quality forage and still have high production in dry season (Larbi et al. 1999) and used as feeding stock during seasonal gap in smallholder mixed farms in the West African derived savanna. The utilization of *C. rotundifolia* hay powder to replace 40% of wheat bran in the traditional substrate formula for mushroom production increase 76.92% of the biological efficiency and increase 29.14% of total yield of mushroom (Weng et al. 2013)

There is few information on those type of legume as a forage and cover crop planted in plantation. Therefore, the purpose of this research was to evaluate the productivity of the four species of legumes as a feed source and cover crop in oil palm plantations, and to evaluate the effect of planting these legumes on soil biophysic condition and fruit bunch production.

MATERIALS AND METHODS

Location

The study was carried out in Asahan District, North Sumatera Province, Indonesia. It is one of the centers of oil palm plantations with a total area of 74.83.70 ha, and number of cattle was 666.496, located at 2°03'-3°26 'North latitude, 99°1'-100°0' East Longitude and elevation of ± 100 m above sea level. The mean annual temperature was 250°C, humidity 70% with an average rainfall of 1500 mm/year. This study used 5 years old oil palm owned by smallholder farmers covering an area of 4608 m^2, with a light intensity of 50%. The oil palm spacing is 8 x 8 m. Therefore, there are 72 palm trees on a whole area of research. The type and structure of the soil which is attached in this research area is podsolic with sandy soil structure 52.32%, dust 13.70%, clay 33.98%, pH 5.05, N Total 0.10%, C-Organic 1.01%, P_2O_5-total of 0.53 mg/100 g, P-Available 4.77 ppm. This condition indicates that the soil is very poor (infertile).

Land and seed preparation

The field was spray to eradicate all green vegetation by using the herbicides Roundup (Glyphosate 360 g/l). Then the soil in the inter-rows was plowed to a depth of approximately 10 cm to prepare the seedbeds. Four species of legume were: *Stylosanthes guianensis*, *Arachis glabrata*, *Clitoria ternatea*, and *Chamaecrista rotundifolia*. Seeds were planted into polybags of size 15 × 25 cm. Polybags were filled with two parts top soil + sand + 1 part of compost. Insecticide treatment was given after seedling to avoid ants and another insect.

Experimental design and treatments

Fourth treatments were arranged in a randomized complete design with three replications. There were 12 plots, each plot size was 16 x 24 m = 384 m^2. The seedlings were water every day before transferred to the plots area. Polybags were kept in the nursery for eight weeks. For better germination, polybags were kept in 50% shade for two weeks, then exposed to direct sunlight afterward. All plots were manually weeded and kept without weeds during the experiment. Plots fertilized with 20000 kg/ha of goat manure (972 kg/plot), limestone 1500 kg/ha (72.9 kg/plot), K_2O 100 kg/ha (4.86 kg/plot), and P_2O_5 as much 50 kg/ha (2.43 kg/plot). Goat manure and the limestone applied before tillage. While K_2O and P_2O_5 were applied to cover crops one month when the forth seedlings were transplanting from nursery to the field. Legumes were grown throughout the oil palm trees with Alley-cropping system (about 1 m) so that the area spacing between legumes 0.5 x 0.5 m area of oil palm were used for the study.

Variable observed

Dry matter yield

The biomass of each legume species was harvested from all area in the plot at early flowering (10%), at 90 days after planted and at 60 days average interval cutting. Observation of harvesting was done of six times. All legumes were harvested at 20 cm above ground. The data from each plot and harvesting time was calculated to estimate production (ton/ha/year).

Leaf/steam ratio

The proportion of leaves and stems on legumes taken every harvesting were measured. A 500 g/plot samples biomass production of legumes were taken to determine the ratio of leaves and stems. The separation was done, then the weighing was also done in each fraction to calculate leaf and stem ratio. Observation was done every harvest, then all data were averaged at the end of experiment. After separation of leave from stem, the width of leave was measured. Another 500g/plot each time harvesting were taken then dried in air forced oven at 65°C for 48 h for dry matter (DM) determination and to analyze nutrient content of legumes. Chemical composition

All samples were bulked according to treatments and replications as a material to determine the chemical content. Samples were ground using hummer mill with a screen 1.0 mm. N content was analyzed according to AOAC (2005). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to the method of Van Soest et al. (1991)

Consumption and digestibility

Feed consumption and dry matter digestibility pasture legumes were performed on 20 cross breed Boer goat x kacang goat (Boerka) with an average weight of 20 kg placed in the metabolism cage. Goats were divided into four groups and randomly assigned each group to one of four legume species as a single feed ad libitum in the morning and evening. Drinking water was available at all the times. The adaptation period was allowed for ten days, then followed by measuring feed consumption, total feces excretion and urine secretion performed for five consecutive days. Feed samples were taken daily at feeding and composited and dried at 60°C in the oven for three days. Total feces were collected in the morning before feeding. After weighing, 10% of the feces were subsample and dry in oven at 100°C for 24 hours.

Nitrogen and phosphorus (P) available in the soil

Analysis of nitrogen and phosphorus content in the soil was done twice, before and after experiment. Before the legume was grown every plot of soil samples collected using soil drill at depth of 20 cm under the ground. Drilling done in 5 points of each plot at random, then each soil sample of the plots were bulked then taken 500g/plot. Drilling was conducted with a distance of 10 cm from the legume plant. Soil was analysis for Nitrogen (N) content according to AOAC (2005), phosphorus (P) available using extraction method HCL 25%, according to AOAC (2005).

Bacterial colonies

Counting of Nitrogen-fixing (N-fixing) bacteria and Phosphate solubilizing (P-solubilizing) bacteria in this study was done twice at the beginning and the end of the study. Soil samples were taken at depth of 20 cm around the research area of 100g at random (Suganda et al. 2006). Calculates the amount of N-fixing bacteria was counted using plate count method (Somasegaran & Hoben 1985). While P-solubilizing bacteria counted using pikovskaya media (Saraswati et al. 2007). The colonies of N-fixing bacteria were characterized by pink color, round and convex, whereas P-solubilizing bacteria were characterized by a clear color. Counting the number of colonies was done using equation:

$$\text{Total population (CFU) g-1 dry soil} = \frac{\text{(number of colonies) x (df)}}{\text{dm soil}}$$

df = dilution factor in petri dishes where colonies are counted

dm = dry matter of soil sample (g) = wet weight x (1 - water content)

Fresh fruit bunches production

To determine the production of fresh fruit bunches (FFB) of oil palm was done after legume grown in all areas of research. harvesting done by cutting the bunches of ripe fruit red-orange marked, FFB production was obtained with harvest rotation 14 days using a chisel tool of 10-12.5 cm with the handle of an iron pipe. To facilitate harvesting, the leaf midrib should be cut first, then the fruit bunches of ripe are cut as close to the fruit base maximum of 2 cm. Fruit that falls directly weighed and production recorded in each plot. The data for each harvesting time were collected and calculated for total production in one year.

Statistical analysis

The study was conducted in a completely randomized design (Gomez & Gomez 1984) with four treatments (species of legume) and three replications. All data obtained except soil biology were analyzed with a linear model using SAS software (2009). Significant differences among treatment means were tested using Duncan's multiple range test (DMRT) at the 5% level of. Soil biology (N fixing bacteria and P solubilizing bacteria were analyzed descriptively.

RESULTS AND DISCUSSION

Plant characteristics and nutrient composition

Dry mater yield

Plant characteristics and nutrition composition of four species herbaceous legumes is presented in Table 1. The highest of dry matter yield ($P<0.05$) was *C. ternatea* 16.15 t/ha/y. The high of *C. ternatea* production is most likely due to its rapid propagation ability compared to the three others of species, as indicated by the whole plant was able to cover the soil surface and produced high biomass. Previous study reported that *C. ternatea* production was higher than present study. Ratnawaty & Fernandes (2009) reported production of C ternatea was 22 t ha^{-1} y^{-1}, while Gomez & Kamani (2003) was 30 t ha^{-1} y^{-1}. The high production of the previous study due to *C. ternatea* was planted in the open field, while in the present study this legume were planted in the shaded a under oil palm plantation. The amount of decreased yield in the shaded area is in line with decreasing light intensity from the sun (Fanindi et al. (2010). The lack of sun light resulted in disruption of the metabolism, causing a reduction in the rate photosynthesis and synthesis of carbohydrates. (Alvarenga et al. 2003; Sopandie et al. 2003; Kirschbaum 2011). A tendency of concentrations of chlorophyll and photosynthesis decreased rate with increasing level of shade in plants Croton urucurana Baill. The optimum light intensity differs according to the type of plant (Alvarenga et al. 2003; Sopandie et al. 2003; Kirschbaum 2011). There are plants that grow very well in the shady place, there are also plants that require high light intensity. Plants of this last type are called "sunplants", while the likes shade called "shade plants".

Table 1. Plant characteristics and nutrition composition of four species of herbaceous legumes grown under oil palm plantation

Species	Dry matter yield (t/h/y)	Leaf of width (mm)	Leaf/stem ratio	Nutrion composition			
				DM (%)	CP (%)	NDF (%)	ADF (%)
A. glabrata	7.2[b]	16.50[b]	2.09[a]	35.31[a]	14.57[b]	46.30[a]	44.03[a]
S. guianensis	8.16[b]	14.03[b]	1.19[b]	35.75[a]	13.85[b]	45.06[a]	43.94[a]
C. ternatea	16.15[a]	36.37[a]	1.53[b]	35.06[a]	17.84[a]	45.84[a]	42.00[a]
C. rotundifolia	2.65[c]	19.05[b]	1.69[b]	33.75[a]	14.33[b]	43.17[a]	39.66[b]

Different superscripts in the same column indicate significant different (P<0.05)
DM = Dry matter
CP = Crude protein
NDF= Neutral detergent fiber
ADF= Acid detergent fiber

Legume *C. ternatea* indicates more tolerant to shading than three others legumes in this study. Solar intensity was 50% in oil palm can be accommodated and utilized efficiently by this legume. This is most likely because of the leaf of *C. ternatea* widthest among the three other species. Sunlight acquired by *C. ternatea* sufficient for photosynthesis process, the formation of carbohydrates from CO_2 and H_2O in the green leaves, resulting in the availability of energy in the form of carbohydrates and protein for the growth and development of plants and therefore contributes to the dry matter production (Hatfield et al. 2011).

Leaf/stem ratio. The average of leaf/stem ratio in this research was 1.62. The highest leaf/stem ratio was in *A. glabrata* (2.09). This ratio was moderate, and almost comparable with *Indigofera sp* cutting at 60 days was 1.70 reported by Tarigan et al. (2010). Although *S. guianensis* had the lowest ratio (1.19) but still higher than the mulberry plants which was 0.73 (Ginting et al. 2014) Similarly, the lower leaf/stem ratio was also reported on Clitoria (0.94), mucuna (1.0) and lablab (0.58) (Macedo et al. 2015). The high leaf ratio in this study is likely influenced by the shade from oil palm, the shade caused widened the leaves and try to find and accommodate the sunlight, the expansion of leaf area is one of the mechanisms of tolerance on shade to obtain a higher light or the optimization of light reception by the plant. Therefore, widen leaves resulted in the increase proportion of leaf. The higher leaves proportion of forages might an indication that these feed have good quality (Shehu et al. 2001; Gustavsson & Martinsson 2004) and could increase feed consumption and nutrient intake due to some of the nutrients were higher the leaves than stems.

Nutrition composition

The nutrition composition fourth species of legumes shows that the average content of dry matter (DM) produced were relatively similar and was not significantly different (P>0.05) among legume species. Overall, DM content was high ranging between 33.75 to 35.75%, which was higher than DM mulberry plants (16%) reported by Ginting et al. (2014). Crude protein (CP) obtained ranged from 13.85 to 17.84% the highest (P<0.05) CP content was in *C. ternatea* (17.84%). CP content in *C. ternatea* in this study is comparable to the report by Gomez & Kamani (2003) between 14-20%, but was higher than previous studies reported by Macedo et al. (2015) which was in range 21.3-22.01%. The fiber content was relatively moderate, NDF content was between 43.17 to 46.30% was not significantly different (P>0.05) among legumes studied. Whereas ADF content was slightly varied in ranged between 39.66 to 44.03%, and was significantly different (P<0.05) in which *C. rotundifolia* contained the lowest ADF (39.66%). This was likely due to the softer structure of the plant. These results were similar to the study by Pholsen et al. (2014) 39.46% ADF. However higher than (study by Clem et al. (1996) with averaged ADF content 36.62% (leaves 28.85, and stems 44.39%), the lower ADF content could indicate that the lignin level is low and the fiber content is good, this is often used to calculate digestibility (Van Saun & Herdt 2013). Although ADF and NDF *C. rotundifolia* were low in this study, but it digestibility was not significantly different from the three other of legumes (Table 2).

Consumption and digestibility

Feed consumption and dry matter digestibility of four legumes species on goats are shown in Table 2. Feed consumption of sheep fed on *A. glabrata* and *S. guianensis* were similar and was significantly higher (P<0.05) than *C. ternatea* and *C. rotundifolia* . Feed consumption in %DM/BW of *A. glabrata* and *S. guianensis* were 3.1 and 3.3% DM/body weight respectively. While DM consumption of *C. ternatea* and *C. rotundifolia* was lower (2.6-2.8% body weight)

Table 2. Consumption and digestibility of dry matter four species of legumes given ad libitum on goats

Species of legumes	Consumption (DM)		Digestibility (%)
	g/d	g/kg BW	
A. glabrata	805[a]	33.17[a]	56.44[a]
S. guianensis	775[a]	31.87[a]	54.16[a]
C. ternatea	611[b]	26.87[b]	61.50[a]
C. rotundifolia	654[b]	28.02[b]	56.16[a]

Different superscripts in the same column indicate significant differences (P<0.05)

respectively. The high consumption of *A. glabrata* was probably due to the high proportion of leaves of this legume (Table 1). While *S. guianensis* have smaller leaf size (14.03 mm), so easy to eat and good preference for goat. However, the high consumption of *A. glabrata* and *S. guianensis* was not followed by high levels of digestibility. DM digestibility was not significantly different among four herbaceous legumes with average of 57.06%. This result is comparable to the study reported by Macedo-Barragán et al. (2011) who observed DM digestibility of *C. ternatea* was 59.37%, while Juma et al. (2006) observed when Clitoria used as nitrogen supplements to the grass basal Napier diet, they found that diet digestibility was 58.9%.

Soil biophysical condition and fruit bunch production

Biophysic of soil before and fruit bunch production is presented in Table 3. The average nitrogen (N) concentration in the soil at the beginning of the study was similar (P>0.05) ranged from 0.9-0.10%. This condition indicates that the concentration of N contained in these plantation areas is very low. At the end of the study, N content in the soil increased, the highest increased was in land grown by *S. guianensis* (0.16%). The increased of N content indicates that legumes as cover crop was able to increase N concentration in the soil. The increase in N content is caused by the presence of Rhizobium in root nodule which fixed nitrogen from air. The high N content in soil grown by *S. guianensis* treatment was probably because *S. guianensis* produces more root nodules compared to the other three species. Despite the increase N in the soil in this study, this concentration has still below the minimum threshold. Hardjowigeno (2003) suggested the normal N concentration in soil is approximately 0.2-0.3%. The low N content acquired was likely due to shorter duration of this study (one year). (Carlsson et al. 2009) reported that the significant effects of cover crop on soil condition estimated about 3-4 years to reach a normal level of N concentration. So it might be still takes another 2 years to observe the increase of N concentration due to cover crop by legume. The highest increased of N content in soil due to cover crop was obtained by *S. guianensis* plant (0.06%). When it was calculated as economic returns from one hectare of land under oil palm, inter-cropping as well as fodder cultivation *S. guianensis* can be accumulated with N content x soil/ha (2,000,000 kg) equal to 0.06% x 2,000,000 = 1200 kg N /ha/year, equivalent to 2667 kg of urea. By converting the price of 5000 IDR per kg urea, *S. guianensis* as a cover crop has to benefit of 13,335,000 IDR /ha^{-1}/yr^{-1}. While, the three others have a profit of 6,667,000 IDR, (*A. glabrata*), 4445000 IDR (*C. rotundifolia*), and 2,223,000 IDR /ha/year (*C. ternatea*).

Biochemical test to observe concentration of P-available shows that at the start of the research had an average of 2.92 ppm ranged from 1.51 (*A. glabrata*) to 5.76 (*C. ternatea*) (Table 3). At the end of the study, the average of P. Available increases to 5.15 ppm. The highest increased was in *S. guianensis* (7.75 ppm), this indicates that the microbes have ability to increase of P in the soil, without affecting legume biomass production. This most likely caused by *S. guianensis* plants is intolerant to shade under oil palm, resulted in smaller leaf size as caused by light received was not maximal for plant biomass to grow. On the other hand, P-available on the treatment *C. ternatea* numerically decreased from 5.76 to 4.09 ppm, while phosphate-solubilizing was higher (9.8×10^5) in this plant. This might be associated with the higher of dry matter yield production in *C. ternatea*. The biomass plant harvested of this was quite big (16.15 ton ha^{-1} yr^{-1} (Table 2)). The high biomass production of *C. ternatea* as a result of higher population of P solubilizing bacteria, wich related to availability P in the soil. Phosphate in the soil directly absorbed by the roots and used for the legume production (Baligar & Fageria 2007; Karti & Setiadi 2011). Legume as a cover crop in this study positively increased the P-available in the soil. Type of bacteria that caused P-available increase are Bacillus sp, and Pseudomonas sp, this microbes was detected effectively in dissolving phosphate in the shade of oil palm. Interactions between plants and microorganisms can cause significant effect on increasing the availability of phosphate to 7.75 ppm. The activity of

Table 3. Concentration of Nitrogen, Phosphate, nitrogen fixing bacteria and phosphor solubilizing bacteria population, and fresh fruit bunches production on land grown by four species of herbaceous legumes under oil palm plantation

Species	N. Content (%)		P. Available (ppm)		Bacteria colonies CFU/g		Fresh fruit bunches (FFB) t/ha
	Start	End	Start	End	N-fixing	P-solubilizing	
A. glabrata	0.10^{a}	0.13^{b}	1.51^{b}	4.98^{b}	3.4×10^{6}	9.0×10^{1}	16.52^{a}
S. guianensis	0.10^{a}	0.16^{a}	2.53^{b}	7.75^{a}	1.28×10^{6}	5.0×10^{2}	17.52^{a}
C. ternatea	0.10^{a}	0.11^{b}	5.76^{a}	4.09^{b}	1.76×10^{9}	9.8×10^{5}	17.31^{a}
C. rotundifolia	0.9^{a}	0.11^{b}	1.89^{b}	3.79^{b}	5.0×10^{8}	1.6×10^{3}	19.21^{a}

Different superscripts in the same column indicate significant different ($P<0.05$)

the microorganisms found in legumes can secrete organic acids such as oxalate, succinate, fumarate, and malate.

Organic acids react with the binder phosphates such as Al^{3+}, Fe^{3+}, Ca^{2+}, or Mg^{2+} to form a stable organic chelate, so can release the phosphate ions from binding. Therefore P supplied from fertilizer can be absorbed by plants (Singh & Reddy 2011). Phosphate-solubilizing bacteria produce phytohormones such as Indole Acetic Acid (IAA) and Gibberellic Acid (Ga3), as well siderophores which is antagonists against the pathogen (Nenwani et al. 2010; Parani & Saha 2012), Therefore phosphate-solubilizing bacteria a potential biofertilizer to support plant growth and absorbed phosphate ions, especially H_2PO_{4-} and -$HPO4_2$ contained in the soil solution. (Kumalasari & Setyorini 2008) reported the presence of phosphate-solubilizing bacteria in *Calopogonium mucunoides* grown in soil post-mine tailings gold gave resulted in the best growth response to this plant. The increase of P. Available was also reported by Agisti et al. (2014) using Legume Cover Crop (LCC) in the area of the former coal mines give real effect on the increase of C-organic, and N-total content, the soil pH , and increased P available from 24-25 ppm to 55-64 ppm.

Population of N-fixing bacteria was highest in *C. ternatea* (1.7×10^{9} cfu/g) followed by *C. rotundifolia* (5.0×10^{8} cfu/g) and (Table 3). According to Indonesian Government regulation (Permentan 2011), the standard number of N-fixing bacteria in the soil is $\geq 10^{7}$ CFU/g. This indicates that cover crop *C. ternatea* and *C. rotundifolia* was able to produce good quality soil that meet standard by Permentan (2011) for growing crop. Purwaningsih (2009) reported that arable land contains more than 100 million microbes per gram of soil, while Alexander (1977), reported the population of bacteria was 10^{9} cell/g soil.

The high population of bacteria in *C. rotundifolia* and *C. ternatea* was due to environmental such as temperature, humidity, and light under oil palm growth by these two legumes species was suitable for the growth of microbes (Widawati 2006). Suitable environement condition can support the survive of soil microbial which in turn improve soil fertility through fixing nitrogen from the air then convert it into a compound that can be absorbed by the plants (Agisti et al. 2014). Soil biology in present study shows that the high N-fixing bacteria in legume *C. rotundifolia* and *C. ternatea* planted in the area under oil palm plantation has potential as biofertilizer to support plant growth. This is caused that organic material such as leaf and flower produced by *C. rotundifolia* and *C. ternatea* that fall to the ground in large quantities, so that sufficient to support the growth and development microbes. On the other hand, N-fixing microbial populations in *A. glabrata* and *S. guianensis* plants were lower (3.4×10^{6} cfu/g) and (1.28×10^{6} cfu/g) respectively. The low organic material contained in the media around the roots of both legume species caused the low growth of microbes. Rhizobium bacteria living in the rhizosphere must compete with other soil microbes to obtain exudates and specific compounds may affect the growth of microorganisms adapt to environmental conditions (Nikiyan et al. 2010).

The population of phosphate-solubilizing bacteria (PSB) in the present study was varied (Table 3). Numericaly, the highest population was in treatment *C. ternatea* (9.8×10^{5} CFU/g), this result was in line with the high dry matter yield obtained in *C. ternatea* (Table 1). The high population *C. ternatea* could be due to the ability of phosphate solubilizing bacteria growing in this legume to provide P to be absorbed by the plant. Bacteria Pseudomonas, Bacillus, Escherichia, Actinomycetes a group of soil microorganisms are capable of dissolving phosphorus (P) which is fixed in the soil and turn it into a form that is available can be absorbed by plants that affect to the biomass production (Khen 2006). According to Jalaludin et al. (2010) these microbes secrete of kinds organic acids such as formic acid, acetic, propionic, lactic, glycolic, fumaric, and succinic. They chelate the ions with the cation Al, Fe or Ca binding P, so that the ion $H_2P0_4^{-}$ can be released from the bond becomes available for plants to be absorbed.

The PSB populations is relatively low in plans *A. glabrata*, *S. guianensis*, and *C. rotudifolia* (9.0×10^1, 5.0×10^2, and 1.6×10^3 cfu/g) respectively this population affect to the effectiveness of phosphate dissolution in the soil subsequently effect on production dried matter yield of legumes (Table 1). This result of the present study indicates that there were differences in the mechanisms by P-bound on a wide variety of species of legume. Therefore the low PSB population around the roots in all three legume species caused low biomass production. Schipanski et al. (2014) stated that the low population of bacteria is most likely caused by environmental conditions which are less than optimal caused by the lack of organic material around the roots of legumes that affect the activity of living microorganisms. Therefore the low PSB population around the roots in all three plant species mentioned above.

Fresh fruit brunches (FFB) production

The average of FFB production obtained in this study (Table 3) was 17.64 t ha^{-1} yr^{-1}, ranged from 16.52-19.21 t ha^{-1} yr^{-1}. There was no significant difference FFB production in each treatment. While Hafif et al. (2014) reported lower FFB production at oil palm age 4.5 year in Tuba Distrik with cover crop grown under oil palm plantation was 15.36 t ha^{-1} yr^{-1}, whereas production without cover crop was 12.36 t ha^{-1} yr^{-1}.

Some legumes in this study showed no effect on fruit bunches production. This is probably the absence of any apparent effect of some legume crop species since the time observations is only one year. The Possibility N-fixing (Rhizobium) and phosphate solvent bacteria secreting coenzymes, nucleic acids, and other metabolic compounds have not spread to all roots, and exploited by of oil palm (Samedani et al. 2014). Numerically *C. rotundifolia* plots began to show higher FFB production yield than the three others of treatments. This is closely related to the high N-fixing bacteria population on *C. rotundifolia* plants (Table 3) can bind free nitrogen from the air and convert it into a compound began absorbed by plants, indicated in the treatment of metabolic compounds ranging role of soil biology to increase FFB production. Although FFB production in the treatment of *C. ternatea* is lower than *C. rotundifolia*, but the legume biomass produced was higher (Table 1). This is most likely nutrients of N and P produced by legume crops more absorbed for legume biomass compared to FFB production.

CONCLUSION

Clitoria ternatea is potential legume as fodder due to better nutrient quality and potential as cover crop in oil palm plantation as indicated by highest biomass production due to higher number of bacteria N-fixing and phosphate-solubilizing bacteria in the soil of *Clitoria ternatea* increase available N and P.

REFERENCES

Abreu MLC, Vieira RAM, Rocha NS, Araujo RP, Glória LS, Fernandes AM, De Lacerda PD, Gesualdi A. 2014. *Clitoria ternatea* L. as a potential high quality forage legume. Asian-Australas J Anim Sci. 27:169–178.

Agisti A, Alami NH, Hidayati TN. 2014. Isolasi dan dentifiasi bakteri penambat nitrogen non simbiotik pada lahan restorasi dengan metode Legume Cover Crop (LCC) di Daerah Pasirian Lumajang Jawa Timur. J Sains Seni Pomits. 3:2301–9271.

Alexander M. 1977. Soil microbiology. 2nd ed. New York (USA): John Wiley and Sons Inc. p. 472.

Alvarenga AA de, Castro EM de, Lima Junior É de C, Magalhães MM. 2003. Effects of different light levels on the initial growth and photosynthesis of Croton urucurana Baill. in southeastern Brazil. Rev Árvore. 27:53–57.

[AOAC] Association of Official Analytical Chemists. 2005. Official methods of analysis of AOAC international. 18th ed. Gathersburg, MD (USA): Association of Official Analytical Chemists.

Baligar VC, Fageria NK. 2007. Agronomy and physiology of tropical cover crops. J Plant Nutr. 30:1287–1339.

Bell LW, Moore AD, Kirkegaard JA. 2014. Evolution in crop-livestock integration systems that improve farm productivity and environmental performance in Australia. Eur J Agron. 57:10–20.

Carlsson G, Palmborg C, Jumpponen A, Scherer-Lorenzen M, Högberg P, Huss-Danell K. 2009. N_2 fixation in three perennial Trifolium species in experimental grasslands of varied plant species richness and composition. Plant Ecol. 205:87–104.

Chauhan N, Rajvaidhya S, Dubey BK. 2012. Pharmacognostical, phytochemical and pharmacological review on *Clitoria ternatea* for antiasthmatic activity. Int J Pharm Sci Res. 3:398–404.

Chiu SB, Madsun B. 2006. Mucuna bracteata - biomass, litter and nutrient production. Planter. 82:247–254.

Clem RL, Compton JF, Cook BG, English BH, Ford G, Gilbert M, Hilder TB, Hopkinson JM, James TA, Middleton CH, et al. 1996. Interim Final Report on Project DAQ. 083 Backup Legumes for Sty los Technical Collaborators :

Constanza K, Tallury S, Whaley J, Sanders T, Dean L. 2015. Chemical composition of the essential oils from leaves of edible (*Arachis hypogaea* L.) and Perennial (*Arachis glabrata* Benth.) peanut plants. J Essent Oil Bear Plants. 18:605–612.

Fanindi A, Prawiradiputra BR, Abdullah L. 2010. Pengaruh intensitas cahaya terhadap produksi hijauan dan benih

Kalopo (*Calopogonium mucunoides*). JITV. 15:205–214.

Franzluebbers AJ, Stuedemann JA. 2007. Crop and cattle responses to tillage systems for integrated crop–livestock production in the Southern Piedmont, USA. Renew Agric Food Syst. 22:168–180.

Ghosh PK, Bera SK. 2000. Wild Arachis species: a possible source of legume fodder in India. Int Arachis Newsl. 20:88–89.

Ginting SP, Tarigan A, Hutasoit R, Yulistiani D. 2014. Preferensi, kecernaan dan karakteristik fermentasi rumen beberapa spesies murbei pada kambing. JITV. 19:176–183.

Gomes J, Bayer C, de Souza Costa F, de Cássia Piccolo M, Zanatta JA, Vieira FCB, Six J. 2009. Soil nitrous oxide emissions in long-term cover crops-based rotations under subtropical climate. Soil Tillage Res. 106:36–44.

Gomez KA, Gomez AA. 1984. Statistical procedures for agricultural research. New York (USA): John Wiley Sons Inc. p. 206-212.

Gomez SM, Kamani A. 2003. Butterfly Pea (*Clitoria ternatea*): A nutritive multipurpose forage legume for the Tropics- An Overview. Pak J Nutr. 2:374-379.

Guodao L, Phaikaew C, Stur WW. 1997. Status of Stylosanthes development in other countries. II. Stylosanthes development and utilisation in China and south-east Asia. Trop Grassl. 31:460–466.

Gustavsson AM, Martinsson K. 2004. Seasonal variation in biochemical composition of cell walls, digestibility, morphology, growth and phenology in timothy. Eur J Agron. 20:293–312.

Hafif B, Ernawati R, Pujiarti Y. 2014. Peluang peningkatan produktivitas kelapa sawit rakyat di Provinsi Lampung. J Littri. 20: 100-108.

Hardjowigeno S. 2003. Ilmu tanah. Jakarta (Indones): Akademika Pressindo.

Harahap F, Silveira S, Khatiwada D. 2017. Land allocation to meet sectoral goals in Indonesia - An analysis of policy coherence. Land Use Policy. 61:451–465.

Hatfield JL, Boote KJ, Kimball BA, Ziska LH, Izaurralde RC, Ort D, Thomson AM, Wolfe D. 2011. Climate impacts on agriculture: Implications for crop production. Agron J. 103:351–370.

Jalaludin A, Ngim J, Bakar BHJ, Alias Z. 2010. Preliminary findings of potentially resistant goosegrass (*Eleusine indica*) to glufosinate-ammonium in Malaysia. Weed Biol Manag. 10:256–260.

Jalaludin S. 1997. Integrated animal production in the oil palm plantation. Livest Feed Resour within Integr Farming Syst Second FAO Electron Conf Trop Feed. p. 37–43.

Juma HK, Abdulrazak SA, Muinga RW, Ambula MK. 2006. Evaluation of Clitoria, Gliricidia and Mucuna as nitrogen supplements to Napier grass basal diet in relation to the performance of lactating Jersey cows. Livest Sci. 103:23–29.

Karti PDMH, Setiadi Y. 2011. Respon pertumbuhan, produksi dan kualitas rumput terhadap penambahan fungi mikoriza arbuskula dan asam humat pada tanah masam dengan aluminium tinggi. JITV. 16:105–112.

Karti PDMH, Kumalasari NR, Setyorini D. 2008. Peranan fungi Mikoriza arbuskula, mikroorganisme pelarut fosfat, rhizobium sp dan asam humik untuk meningkatkan pertumbuhan dan produktivitas legum Calopogonium mucunoides pada tanah latosol dan tailing tambang emas di PT. Aneka Tambang. Pastura. 3:44–47.

Kemp P, Kenyon P, Morris S. 2010. The use of legume and herb forage species to create high performance pastures for sheep and cattle grazing systems. Rev Bras Zootec. 39:169–174.

Khen CV. 2006. Impacts of forest conversion on biodiversity as indicated by moths. Malayan Nat J. 57:383–418.

Kirschbaum MUF. 2011. Does enhanced photosynthesis enhance growth? Lessons learned from CO2 enrichment studies. Plant Physiol. 155:117–124.

Larbi A, Adekunle IO, Awojide A, Akinlade J. 1999. Identifying *Chamaecrista rotundifolia* accessions and Centrosema species for bridging seasonal feed gaps in smallholder mixed farms in the West African derived savanna. Trop Grassl. 33:91–97.

Li M, Zi X, Zhou H, Hou G, Cai Y. 2014. Chemical composition and in vitro digestibility of *Stylosanthes guianensis* varieties. Grassl Sci. 60:125–129.

Macedo-Barragán R, Isela López-Poblete N, Arredondo-Ruiz V, Rodríguez-Ramírez R. 2011. Precision of an equation to estimate dry matter degradability of *Clitoria ternatea*. Trop Subtrop Agroecosystems. 14:943–947.

Macedo RJ, Arredondo V, Garcia N, Ramirez R, Prado O, Garcia LJ. 2015. Productive performance of three tropical legumes for protein banks in the dry tropics of Colima, Mexico. Trop Grassl-Forrajes Trop. 3:104–111.

Mapiye C, Mwale M, Mupangwa JF, Mugabe PH, Poshiwa X, Chikumba N. 2007. Utilisation of ley legumes as livestock feed in Zimbabwe. Trop Grassl. 41:84–91.

Mazzoncini M, Sapkota TB, Bàrberi P, Antichi D, Risaliti R. 2011. Long-term effect of tillage, nitrogen fertilization and cover crops on soil organic carbon and total nitrogen content. Soil Tillage Res. 114:165–174.

Mohamad RB, Wibawa W, Mohayidin MG, Puteh AB, Juraimi AS, Awang Y, Lassim MBM. 2010. Management of mixed weeds in young oil-palm plantation with selected broad-spectrum herbicides. Pertanika J Trop Agric Sci. 33:193–203.

Muamba IT, Ignatius VN, Mangeye HK, Hornick J-L. 2014. Nutritive value of Adenodolichos rhomboideus leaves compared with Leucaena leucocephala and *Stylosanthes guianensis* forages in indigenous goats in Lubumbashi

(DR Congo). Biotechnol Agron Soc Environ. 18:165–173.

Nenwani V, Doshi P, Saha T, Rajkumar S. 2010. Isolation and characterization of a fungal isolate for phosphate solubilization and plant growth promoting activity. Yeast. 1:9–14.

Nikiyan H, Vasilchenko A, Deryabin D. 2010. Humidity-dependent bacterial cells functional morphometry investigations using atomic force microscope. Int J Microbiol. 2010:704170.

Ogunbode S, Akinlade J. 2012. Effect of Three Species of Stylosanthes on The Performance of West African Dwarf Sheep. Fountain J Nat Appl Sci. 1:36–40.

Oldroyd GED, Downie JA. 2008. Coordinating Nodule Morphogenesis with Rhizobial Infection in Legumes. Annu Rev Plant Biol. 59:519–546.

Parani K, Saha BK. 2012. Prospects of using phosphate solubilizing Pseudomonas as bio fertilizer. Eur J Biol Sci. 4:40–44.

Permentan. 2011. Peraturan Menteri Pertanian Nomor 70/Peraturan Menteri Pertanian Republik Indonesia/SR.140/10/2011 Tentang Pupuk Organik, Pupuk Hayati Dan Pembenah Tanah. Permentan. p. 16.

Phengsavanh P, Ledin I. 2003. Effect of Stylo 184 (*Stylosanthes guianensis* CIAT 184) and Gamba grass (Andropogon gayanus cv. Kent) in diets for growing goats. Livest Res Rural Dev. 15:1–11.

Pholsen S, Rodchum P, Higgs DEB. 2014. Dry matter yields and quality of forages derived from grass species and organic production methods (Year lll). Pak J Biol Sci. 17:898–904.

Plieninger T, Gaertner M. 2011. Harnessing degraded lands for biodiversity conservation. J Nat Conserv. 19:18–23.

Ponnuswamy S, Wesely Jebasingh Devairrakam EG. 2011. Comparative study of primary metabolites in different plant parts of *Clitoria ternatea* Linn. J Chem Pharm Res. 3:614–617.

Purwaningsih S. 2009. Populasi Bakteri Rhizobium di Tanah pada beberapa Tanaman dari Pulau Buton , Kabupaten Muna , Propinsi Sulawesi Tenggara. J Tanah Trop. 14:65–70.

Ramos-Morales E, Sanz-Sampelayo MR, Molina-Alcaide E. 2010. Nutritive evaluation of legume seeds for ruminant feeding. J Anim Physiol Anim Nutr (Berl). 94:55–64.

Ratnawaty S, Fernandes PTH. 2009. Perbaiakan kualitas pakan sapi melalui introduksi leguminosa herba dalam menunjang program kecukupan daging nasional di Kabupaten Timor Tengah Selatan. Prosiding Seminar Nasional Teknologi Peternakan dan Veteriner. Bogor (Indones): Pusat Penelitian dan Pengembangan Peternakan. p. 107–112.

Samedani B, Juraimi AS, Abdullah SAS, Rafii MY, Rahim AA, Anwar MP. 2014. Effect of cover crops on weed community and oil palm yield. Int J Agric Biol. 16:23–31.

Saraswati R, Husen E, Simanungkalit RD. 2007. Metode analisis biologi tanah. Bogor (Indones) Balai Penelitian Bioteknologi Tanaman Pangan.

Saraswati R, Hastuti RD, Sunarlin N, Hutamu. 1996. Penggunaan Rhizoplus Generasi 1 untuk meningkatkan Produksi Tanaman Kedelai. Bogor (Indones) Balai Penelitian Bioteknologi Tanaman Pangan.

SAS Institute Inc. 2009. SAS/STAT 9.2 User's Guide. User's Guide. Cary, NC (USA): SAS Inst Inc.

Van Saun RJ, Herdt T. 2013. Nutritional Assessment. In: Llama Alpaca Care Med Surgery, Reprod Nutr Herd Heal First Ed. p. 100–123.

Schipanski ME, Barbercheck M, Douglas MR, Finney DM, Haider K, Kaye JP, Kemanian AR, Mortensen DA, Ryan MR, Tooker J, White C. 2014. A framework for evaluating ecosystem services provided by cover crops in agroecosystems. Agric Syst. 125:12–22.

Shehu Y, Alhassan WS, Pal UR, Phillips CJC. 2001. Yield and chemical composition responses of *Lablab purpureus* to nitrogen, phosphorus and potassium fertilisers. Trop Grassl. 35:180–185.

Singh H, Reddy MS. 2011. Effect of inoculation with phosphate solubilizing fungus on growth and nutrient uptake of wheat and maize plants fertilized with rock phosphate in alkaline soils. Eur J Soil Biol. 47:30–34.

Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci. 74:3583–3597.

Somasegaran P, Hoben HJ. 1985. Methods in legume-rhizobium technology. Trop Agric. p. 1–510.

Sopandie D, Chozin MA, Tjitrosemito S, Sahardi. 2003. Keefektifan uji cepat ruang gelap untuk seleksi ketenggangan terhadap naungan pada padi Gogo. Hayati. 10:91–95.

Suganda H, Rachman A, Sutono S. 2006. Petunjuk pengambilan contoh tanah. In: Sifat Fisik Tanah dan Metode Analisis. p. 3–24.

Suganya G, Sampath Kumar P, Dheepa B, Sivakumar R. 2014. In vitro antidiabetic, antioxidant and anti-inflammatory activity of *Clitoria ternatea* L. Int J Pharm Pharm Sci. 6:342–347.

Tarigan A, Abdullah L, Ginting SP, Permana IG. 2010. Produksi dan komposisi kimia serta kecernaan invitro Indigofera sp pada interval dan tinggi pemotongan berbeda. JITV. 15:188–195.

Armiadi. 2009. Penambatan nitrogen secara biologis pada tanaman leguminosa. Wartazoa. 19:23–30.

Thang CM, Ledin I, Bertilsson J. 2009. Effect of feeding cassava and/or Stylosanthes foliage on the performance of crossbred growing cattle. Trop Anim Health Prod. 42:1–11.

Valbuena D, Erenstein O, Homann-Kee Tui S, Abdoulaye T, Claessens L, Duncan AJ, G??rard B, Rufino MC, Teufel N, van Rooyen A, van Wijk MT. 2012. Conservation Agriculture in mixed crop-livestock systems: Scoping crop residue trade-offs in Sub-Saharan Africa and South Asia. F Crop Res. 132:175–184.

Weng B, Lei J, Jiang Z, Zhong Z, Xu G, Ye J. 2013. Substituting wheat bran with *Chamaecrista rotundifolia* hay powder in the substrate of tricholoma lobayense culture: Substrate weight loss dynamics and mass transformation ratios. Sci Hortic. 155:105–110.

Widawati S. 2006. Augmentation of potential phosphate solubilizing bacteria (PSB) stimulate growth of green mustard (Brasica caventis Oed.) in marginal soil. Biodiversitas, J Biol Divers. 7:10–14.

Zhou X, Chen C, Wu H, Xu Z. 2012. Dynamics of soil extractable carbon and nitrogen under different cover crop residues. J Soils Sediments. 12:844–853.

Zingare ML, Zingare PL, Dubey AK, Ansari MA. 2013. *Clitoria ternatea* (APARAJITA): A Review of the antioxidant, antidiabetic and hepatoprotective potentials. Int J Pharm Biol Sci. 3:203–213.

Evaluation on Performance of Some *Sorghum bicolor* Cultivars as Forage Resources in the Dry Land with Dry Climate

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(received 19-06-2017; revised 28-08-2017; accepted 05-09-2017)

ABSTRAK

Sajimin, Purwantari ND, Sarijan, Sihono. 2017. Evaluasi performa beberapa kultivar *Sorghum bicolor* sebagai sumber hijauan pakan di lahan kering iklim kering. JITV 22(3): 135-143. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1611

Penelitian bertujuan mengevaluasi beberapa kultivar Sorghum bicolor sebagai pakan ternak di lahan kering iklim kering dengan pH tanah 5,4, N 0,08% C/N 9%, P 0,06% dan K 0,01%. Sembilan kultivar S. bicolor (Super 1, Super 2, Numbu, Kawali, G2, G5, PAC 537, PAC 593 dan PAC 501) ditanam dengan jarak tanam 15 x 75 cm. Pupuk yang digunakan kompos 4 kg/plot, SP-36 160 g/plot, KCl- 144 g/plot dan urea 240 g/plot. Rancangan percobaan acak kelompok dengan 3 ulangan. Pengamatan meliputi tinggi tanaman, umur berbunga, produksi hijauan tanaman primer dan ratun, serta kualitas hijauan. Hasil penelitian menunjukkan pertumbuhan tanaman primer tidak beda antar kultivar. Pada tanaman primer umur 65 hari, sorgum Super 2, PAC 537 dan Kawali belum berbunga. Produksi biomas bervariasi antar kultivar pada tanaman primer dengan kisaran 11,35 – 26,17 kg/16 m^2. Produksi biomasa tertinggi dicapai oleh kultivar PAC 537 (26,17 kg/16 m^2 setara dengan 16,34 ton/ha), berbeda nyata dengan G2 (11,35 kg/16 m^2) dan tidak berbeda nyata dengan kultivar yang lain. Pada tanaman ratun 1 umur 45 hari, kultivar Super 2, G5 dan Super 1 menunjukkan pertumbuhan yang lebih cepat. Produksi biomasa meningkat pada ratun 1 berkisar 19,88 kg/16 m^2. Kultivar PAC 537 menghasilkan biomasa tertinggi (30,14 kg/16 m^2) dan tidak berbeda nyata dengan kultivar yang lain kecuali galur G2. Produksi biomasa ratun II menurun berkisar 1,83 – 4,77 kg/16 m^2 dan meningkat pada ratun III, yang berkisar 15,72 – 26,05 kg/16 m^2. Kualitas hijauan ratun 1 lebih baik dibanding tanaman utama dan terendah ratun II. Disimpulkan bahwa kultivar sorgum Super 1, Super 2 dan PAC 537 dapat direkomendasikan sebagai sumber hijauan pakan ternak yang paling potensial.

Kata Kunci: Sorghum, Kultivar, Produksi Hijauan, Kualitas

ABSTRACT

Sajimin, Purwantari ND, Sarijan, Sihono. 2017. Evaluation on performance of some *Sorghum bicolor* cultivars as forage resources in the dry land with dry climate. JITV 22(3): 135-143. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1611

The aim of this study was to evaluate the performance of several Sorghum bicolor cultivars as forage on a dry land with pH of 5.4, N of 0.08%; C/N of 9%, P of 0.06% and K of 0.01%. Nine cultivars of S. bicolor (Super 1, Super 2, Numbu, Kawali, G2, G5, PAC 537, PAC 593 and PAC 501) were evaluated. Plot size was 16 m2 with space planting of 15 x 75 cm. The experimental design used was randomized block design with three replications. Parameters observed were plant height, time of flowering, forage production and quality. The result showed that the primary plant growth was not different in all cultivars. In the 65 days old primary plant, the Super 2, PAC 537 and Kawali had no flower yet. Biomass production varied in primary plant between cultivars of 11.35 - 26.17 kg/16 m^2. The highest biomass production was obtained in PAC 537 of 26.17 kg/16 m^2 (16.34 t/ha) which were significantly higher than G2 of 11.35 kg/16 m^2 (7.09 ton/ha) and was not significantly different with other cultivars. In the 45 days ratoon I, Super 2, G5 and Super 1 showed faster growth. Biomass production increased in the ratoon I around 19.88 kg/16 m^2 (12.42 ton/ha). PAC 537 produced the highest biomass of 30.14 kg/16 m^2 (18.84 ton/ha) and was not significantly different with other cultivars, except with the G2. Biomass production of ratoon II decreased around 1.83 kg/16 m^2 (1.14 t/ha) – 4.77 kg/16 m^2 (2.98 t/ha) and increased in the ratoon III of 15.72 kg/16 m^2 (9.82 t/ha) – 26.05 kg/16 m^2 (16.28 t/ha). The quality of forage ratoon I was better compared to the primary plant with the lowest one was in ratoon II. It could be concluded that Super 1, Super 2 and PAC 537 cultivars might be recommended as potential forage.

Key Words: Sorghum, Cultivar, Biomass Production, Quality

INTRODUCTION

Feed availability for ruminant, especially forage in dry land is highly influenced by season. The forage is available abundantly in the rain season, but rare in the dry season. So that in quantity, quality and continuity the forage is not guaranteed throughout the year leading to the not optimal production (Nugraha et al. 2013).

A problem commonly faced in ruminant rearing is the unavailability of adequate forage especially in the dry season (Aswar 2005). Besides, during the dry season, the quality of forage commonly low with low productivity in the dry land area. The productivity of the elephant grass (*Pennisetum purpureum*) in wet climates area is able to reach 300 tonnes/ha/year of fresh weight on a fertile land (Prawiradiputra et al. 2012). However, in a dry area with dry climate or in the areas with a relatively long dry season, this grass provides a much lower fresh weight production as about 48-70 tonnes/ha/year. To overcome the forage availability issue, it is necessary to find a multifunctional and easy to adapt to a dry land with dry climate forage.

The crop is the cheapest forage resource which is an economic production input in the livestock industry. One of the crops potential to be developed in a dry land with dry climate is Sorghum spp. Sorghum is one of the crops that can be used as a source of forage for ruminants, the seeds can be used for food and feed materials. Sorghum grows well in a dry land with dry climate or in the land with limited irrigation (Marsalis 2011). Study of sorghum is widely conducted in Indonesia, especially as a food resource (Chavana et al. 2009) as well as the study of bioenergy obtained by processing its stem as ethanol material resource (Lestari & Dewi 2015).

There are much sweet sorghum cultivars available so far which its productivity has been improved to be cultivated both as food and feed resource (Deb et al. 2004; Efendi et al. 2013). Sorghum breeding has been widely applied for both as feed and bioenergy through hybridization and irradiation, namely sorghum pahat (pangan sehat); sorghum samurai 1 and 2 (sorghum from radiation mutation) (Surya & Soeranto 2006; Soeranto et al 2011). Sweet sorghum produces higher bioethanol than a cane (80 vs 50 L/ha/yr) and cassava (45 L/ha/yr) (Indonesian Bioethanol Entrepreneurs Association 2010).

Many sorghum cultivars have bifunction as food and feed resource for both chopped and hay and silage. Sorghum waste (fresh leave and stem) can be used as forage. Sweet sorghum leaves production is around 14-16% of the fresh weight of stem or about 3 ton of fresh leave pe hectare of the total production of 20 ton/ha. Sorghum leave contains crude protein (7.82%) higher compared to elephant grass (6%) and cane ratoon (5.33%) (Sirappa 2003). This study was aimed to evaluate the most optimal sorghum cultivar in producing biomass in a dry land with dry climate as forage.

MATERIALS AND METHODS

The study was conducted in a dry land with dry climate in Gunung Kidul district, Special Region of Yogyakarta. It had rainfall of 3230.5 mm/year with dry season period more than six months and wet season period of 4 months classifies as type D (Oldeman 1975). Rainfall and rainy day observations were performed during this research using OBS rain scraper. The data were shown in Table 1.

The land used was processed perfectly of weeds cleaning and soil loosing. Soil samples were analyzed for its nutrient of pH, organic material (C/N ratio), P and K. This is performed to confirm nutrient amount should be added in the fertilization. Fertilizer added followed the standard of sorghum cultivation (Suminar et al. 2017) consisted of commercial compose fertilizer by 4 kg/pot (2.5 ton/ha) and chemical fertilizer by 160 g/plot (100 kg/ha), KCl- 144 g/plot (90 kg/ha) and urea by 240 g/plot (150 kg/ha). Those materials addition assumed to rich the soil nutrient required by sorghum: 120 kg/ha N, 36 kg/ha P2O5 and 90 kg/ha K2O (Suminar et al. 2017). The fertilization was performed at the beginning of planting with no re-fertilizing during the research until the harvesting return III.

Nine Sorghum spp cultivars derived from a breeding program of Indonesian Cereal Research Institute (Super 1, Super 2, Numbu, Kawali), National Nuclear Energy Agency of Indonesia (mutant G2 and G5) also from Australia (PAC 537, PAC 593 and PAC 501). Each cultivar was planted in a 4 x 4 m plot with a spacing of 15 x 75 cm. Each hole was filled with 3 seeds which were only one seedling being maintained then. A randomized block design with 9 treatments and 3 repetitions.

The observation was conducted to the primary and ratoon plant. The primary plant was a first plant of the seed planting, while the ratoons were the new bud growing on the felled stem. The primary plant was harvested at 65 days and the ratoon was re-harvested at 45 days for three times (I, II and III). The variables observed were heigh, the time of first flowering, forage production, and the quality of forage. The height was measured from the top of the soil to the highest leaf tip using meter measuring instrument (3 m). The primary plant was harvested at 65 days (approaching flowering) by cutting of 10 cm soil surface. Then, the harvesting of the I, II, and III ratoons was done 45 days. The fresh biomass was weighed immediately using a two-digit scale. A 2.5 kg were taken composite, chopped and dried in the 60°C oven for 48 hours, then milled to flour. A 500 g meal samples were analyzed at the laboratory of Indonesian Research Institute for Animal Production, Ciawi-Bogor. Data were tabulated in the Excel program and statistically analyzed using SAS.

Table 1. Rainfall for 17 months of research in the dry land with dry climate of Gunung Kidul district, Yogyakarta

No/Month	Rainfall (mm)	Rainy day/ month
Year: 2011		
January	399	16
February	347	18
March	160	11
April	162	4
May	160	4
June	65	3
July	31	3
Augst	0	0
September	0	0
October	0	0
November	95	2
December	385.5	20
Year: 2012		
January	295	14
February	302.5	14
March	384	18
April	342.5	15
May	102	6

RESULTS AND DISCUSSION

Chemical characteristic of soil in this study consisted pH 5.4; organic material with the low ratio of C/N (9%); very low in N (0.08%); C (0.08%) and K (0.08%); but very high in P (116 ppm). It can be concluded that the soil was less fertile with low organic material and sandy clay texture. Purwowidodo (1993) and Rahmi & Biantary (2014) described that fertile soil consists CN ratio of >10% and PH>6. A high ratio of C/N is able to provide abundant energy for the soil organisms. The anorganic N compound available in the soil is converted into organic N in the soil organism body. In this stage, the decomposition rate of organic material is at the lowest point.

Sorghum plant height

The height of 65 days sorghum plant was varied between the cultivars for about 99.67 – 118.33 cm. While the height of 45 days ratoons I, II and III was around 79.86 – 110.15%; 7.76 – 63.61%; and 30.96 – 81.97% respectively. Statistical analysis showed no significant differences in all cultivars both the primary and ratoon I, II and III plants (Table 1).

The sorghum cultivars are very diverse, both in terms of production, harvest age, seed color, taste, and quality of its processed seed. This study results differed from those reported by Purnomohadi (2006), which reported that four sweet sorghum cultivars, namely Rio, Cawley, Wray, and Keller showed the same vegetative growth in both 50 and 100 days after planting cutting age (primary plant). Heigh range of 50 and 100 days sorghums each was 51.61-58.85 cm and 63.03-67.53 cm. This indicates that those four cultivars have the same response to the environment condition (climate and nutrients content in the soil). The primary plant in this study had the shortest (99.67 cm) height in the G5 cultivar and the highest was in the Super 2 cultivar (118.33 cm) in the same climate condition. No significant difference of all the nine cultivars in this study indicates a same response to the climate condition. Different height of 65 days sorghum in this study with the 100 days sorghum in the Purnomohadi (2006) might be caused by different place of planting. Purnomohadi (2006) planted in the polybag, while in this study the sorghum was planted on the ground.

The growth of 45 days ratoons showed faster than the primary plants (Table 2). Ratoons I, II and III, Super 2, G5 and Super 1 cultivars showed faster growth than other cultivars. The factors that may influence the growth of the ratoons plants are the quality of the first plant: the genotype on height, number of leaves and stem diameter (Efendi et al. 2013; Meliala et al. 2017). A relative similar mass and height indicate that the nine cultivars have the same quality.

Flowering phase

From total 240 trees of 65 days after planting sorghum, five cultivars have not flowered, namely: Super 2, G5, Kawali, PAC 593, and PAC 537 (Table 3). The Super 2 and PAC 593 were the slowest flowering compared to other cultivars. It was only 4.8% pregnancy in the Super 2 cultivar without flowering and only 5.6% of pregnancy without flowering in the PAC cultivar. Only one cultivar that had a short flowering period (67-70 days) with 9 flowering plants (4.5% of total population) in the 65 days after planting (Table3).

Efendi et al. (2013) said that ratoon plant had a faster production which is closely related to the carbohydrate supply from photosynthesis process of the primary plant stored in the roots and stems which that translocated for bud initiation. A cultivar with the most flowering in 65 days after planting was Super 1 (Table 3). This flowering period was faster than the previous study conducted by Revy et al. (2014) that reported 90-92 days of flowering period.

Table 2. Average heigh of primary and ratoons plant of nine sorghum cultivars

Cultivars	Average heigh of primary plant (65 days)	Average heigh of ratoons I plant (45 days)	Average heigh of ratoons II plant (45 days)	Average heigh of ratoons III plant (45 days)
PAC 501	102.67±15.06	190.40±9.71	136.27±14.74	138.80±24.94
Super 2	118.33±25.89	248.67±45.09	193.60±29.91	198.93±31.00
G5	99.67±34.05	234.80±13.72	168.87±14.70	215.33±29.32
Kawali	105.33±17.57	179.27±7.58	115.40±21.07	154.33±20.82
PAC 593	106.33±42.22	234.87±37.07	147.33±24.57	169.00±44.73
Numbu	109.67±49.58	220.20±32.69	131.07±22.11	173.73±39.31
G2	107.00±34.70	200.40±37.75	107.40±27.31	150.27±38.62
Super 1	102.00±39.84	242.20±23.09	165.07±23.77	213.87±19.42
PAC 537	116.33±26.24	183.53±7.97	141.00±11.66	130.53±9.39

This difference of flowering period is highly influenced by the different of planting location, especially the climate (rainfall, temperature, and nutrient content). The research of Revy et al. (2014) was conducted in Riau, while this study was conducted in Yogyakarta, where there was no rain in August-October which did not affect the flowering period. A cultivar with the slower flowering period would have longer vegetative phase. With this characteristic, this cultivar is potential as a forage resource.

Maturation phase of forage influences its quality (Ball et al. 2001; Ayub et al 2012). Therefore, forage with longer vegetative phase would keep forage quality longer. Flowering age of a plant is influenced by genetic and environment (Widyastuti et al (2012). Super 1 cultivar could be concluded to have the fastest flower age. In this study, all nine cultivars were planted in the same climate and soil condition, so that it was likely, the flowering age was influenced by genetic. This is in accordance with the report of Darjanto & Satifah (1987) in Pasaribu et al. (2015) who said that the transition from vegetative period to generative is mostly determined by genetic and the rest of it is temperature, light, water and nutrient.

Table 3 showed that in the ratoon I all cultivars have been flowered in 45 days after harvesting of the primary plant, so it might start flowering before 45 days. The PAC 537 cultivar had the fewest booting and flowering (<50%). The appearance of flowering in 45 days in the Ratoon I was at the same month of low rainfall (31-65 mm) with the rainy day of 3 days. That condition spurs the flowering (Ibrahim et al. 2011).

Fresh forage production

Primary plant harvest was done in 65 days after planting before flowering. However, in the 65 days, PAC 501, Numbu, G2 and Super 1 cultivars had been flowered (Table 3). The production of upper part of a plant consisting of leaves and stems varied between all cultivars of 7.09 – 16.36 ton/ha (Table 4). The highest production of 16.36 ton/ha was in PAC 537 cultivar followed by Super 1 cultivar of 14.58 ton/ha. It was not significantly different ($P<0.05$) with other cultivars, except the G2 of 7.09 ton/ha.

Forage production of the primary plant was lower than previous study report. Super 1 and 2 cultivars are an inbred cultivar of sorghum with 30-40 ton/ha of biomass potential as a renewable energy source. Those two cultivars could reach 3 to 4 meters with seed production of 5-6 ton/ha which is potential to be used as silage (Indonesian Cereal Research Institute 2011).

Table 3. The number of booting and flowering of nine sorghum cultivars of the primary plant in 65 days after planting and ratoons plants in 45 days after planting

Cultivar	Primary plant in 65 days after planting		Ratoon I plant in 45 days after planting	
	Booting	Flowering	Booting	Flowering
PAC 501	134	35	164	99
Super 2	6	0	76	105
G5	36	0	219	160
Kawali	32	0	194	36
PAC 593	7	0	203	158
Numbu	58	9	124	78
G2	51	7	131	92
Super 1	81	148	185	286
PAC 537	63	0	38	21

Table 4. Fresh forage production of primary and ratoon plant in dry land with dry climate in Kunung Kidul District, Yogyakarta

Cultivars	Production of primary plant		Production of primary plant					
			Ratoon I		Ratoon II		Ratoon III	
	kg/16 m^2	ton/ha	kg/16 m^2	ton/ha	kg/16 m^2	ton/ha	kg/16 m^2	ton/ha
PAC 501	19.26	12.04^{ab}	2.43	1.52^{ab}	22.35	13.97^{ab}	16.70	10.44^{cd}
Super 2	14.17	8.86^{ab}	2.97	1.86^{ab}	22.76	14.22^{ab}	24.56	15.35^{abc}
G5	17.53	10.96^{ab}	3.23	2.02^{ab}	27.77	17.36^{ab}	15.72	9.85^{d}
Kawali	20.23	13.43^{ab}	2.30	1.44^{b}	25.28	15.80^{ab}	20.65	12.91^{abcd}
PAC 593	16.54	10.34^{ab}	2.63	1.64^{ab}	24.41	15.26^{ab}	20.16	12.60^{abcd}
Numbu	13.77	8.61^{ab}	2.22	1.39^{b}	22.21	13.88^{ab}	18.13	11.33^{abcd}
G2	11.35	7.09^{b}	1.83	1.14^{b}	19.88	12.42^{b}	17.62	11.01^{bcd}
Super 1	23.33	14.58^{ab}	2.67	1.67^{ab}	27.86	17.41^{ab}	26.05	16.28^{a}
PAC 537	26.17	16.36^{a}	4.77	2.98^{a}	30.14	18.84^{a}	25.76	16.10^{a}

The same superscript in the same column shows not significant difference (P<0.05)

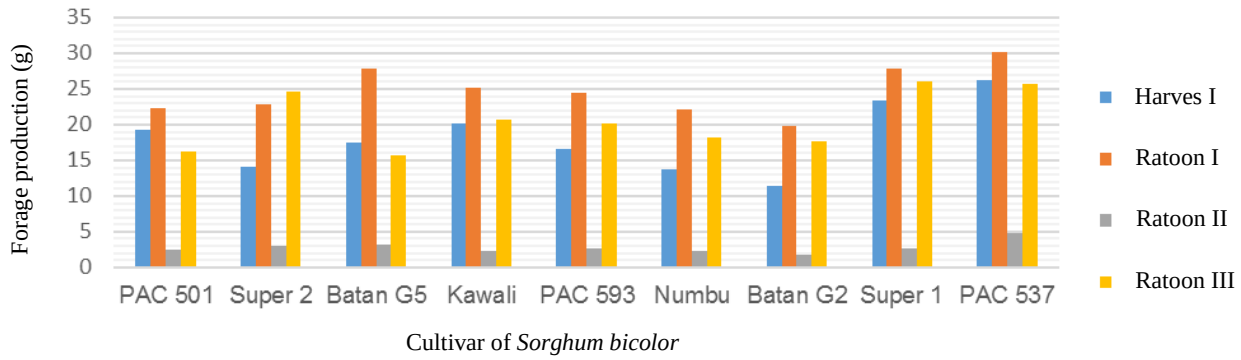

Figure 1. Forage production of nine cultivars of *Sorghum bicolor* in primary, ratoon I, ratoon II and ratoon III planted in the dry land with dry climate in Gunung Kidung, Yogyakarta.

The lower result in this study was suspected to be influenced by climate and rainfall in Gunung Kidul and low quality of the organic material of the soil. Subagio & Aqil (2014) reported that Super 1 and 2 cultivars from Sumba-East Nusa Tenggara had a height stems of 2.16-2.3 m and biomass production of 38.7–39.3 ton/ha from two ratoon harvesting in 105-115 days planted in Indonesian Cereal Research Institute.

In the ratoon I phase, forage production pattern was similar to the primary plant. The PAC 537 produced the highest forage of 18.42 ton/ha and significantly different with the G2 (12.42 ton/ha). In the ratoon II phase, it was at the time of the long dry season, where there was no rain at all for 3 months before the harvesting (Table 1), so that the plant only depended on the residual moisture of soil for several months before. Even though the production of forage decrease up to 84.2-90.9% than the ratoon I (Table 4), in the condition of no rain at all, the farmers still could provide forage of 2.98 ton/ha which is equal to 993 heads cattle with the body weight of 300 kg.

The forage production of ratoon III significantly increased than the ratoon II. According to the data, November was the early rainfall with two days of rainy day and 95 mm of rainfall. In December, the rainfall was relatively high of 385 mm with the rainy day of 20 days, while in January 2015, the rainfall still high (295 mm) with the rainy day of 14 days (Table 4). This indicates that water availability highly influences the growth of sorghum. All this time, Sorghum bicolor is cultivated only up to ratoon I and II (Efendi et al. 2013). However, in this study sorghum cultivar could be harvested up to ratoon III. This is in accordance with a research result of Tsuchihashi & Goto (2008) that sorghum could result in ratoon III both in dry and wet seasons so that it could be 2-3.

Table 5. Analysis of forage nutrient of some cultivars of Sorghum bicolor of primary and ratoon planted in dry land with dry climate in Gunung Kidul, Yogyakarta

Cultivar		DM (%)	CP (%)	CF (%)	Energy Kcal/ kg	Ash	NDF (%)	ADF (%)	Ca (%)	P (%)
PAC 501	Primary	23.64	8.66	3.51	4021	10.15	67.07	44.43	0.56	0.34
	Ratoon I	19.26	11.19	2.80	3747	12.68	65.40	42.74	0.63	0.37
	Ratoon II	23.01	6.23	5.31	4160	5.46	69.32	41.00	0.14	0.09
Super 2	Primary	22.57	9.32	2.81	3963	9.77	65.81	42.10	0.52	0.35
	Ratoon I	23.55	10.36	2.49	3722	11.34	66.28	43.78	0.73	0.27
	Ratoon II	22.88	4.56	3.94	4096	6.78	72.49	44.62	0.14	0.07
G5	Primary	23.76	10.31	3.12	3863	9.99	61.83	40.10	0.84	0.22
	Ratoon I	20.27	10.32	2.68	3857	12.21	67.40	46.04	0.86	0.23
	Ratoon II	22.72	8.37	4.38	4139	6.12	65.89	38.54	0.29	0.12
Kawali	Primary	26.57	10.78	3.23	3931	9.79	66.32	42.99	0.65	0.26
	Ratoon I	19.34	9.78	2.63	3755	11.34	64.28	42.19	0.86	0.25
	Ratoon II	26.90	8.14	4.83	3857	7.85	69.50	41.43	0.47	0.18
PAC 593	Primary	27.19	7.32	2.53	3841	9.30	67.32	40.83	0.65	0.23
	Ratoon I	22.11	8.14	2.49	3661	14.26	67.82	45.88	0.91	0.32
	Ratoon II	26.74	5.64	4.70	4040	6.51	69.07	43.06	0.33	0.15
Numbu	Primary	20.35	9.07	2.95	3805	8.96	62.12	36.39	0.56	0.20
	Ratoon I	20.65	11.59	2.70	3843	11.42	64.84	42.36	0.97	0.37
	Ratoon II	20.96	6.49	5.14	4134	5.98	67.82	45.92	0.18	0.09
G2	Primary	25.98	11.53	3.22	4002	9.00	67.79	37.09	0.59	0.33
	Ratoon I	15.87	13.65	3.37	4007	11.42	66.92	42.62	0.77	0.34
	Ratoon II	25.82	6.05	3.93	4088	5.89	68.25	41.03	0.24	0.08
Super 1	Primary	26.03	11.58	2.72	3939	8.41	68.52	42.29	0.51	0.31
	Ratoon I	19.50	9.84	3.00	3862	12.81	68.78	44.96	0.99	0.21
	Ratoon II	24.72	5.10	4.31	4068	6.33	69.98	42.65	0.24	0.08
PAC 537	Primary	24.18	6.73	3.05	3947	8.78	66.15	40.75	0.65	0.29
	Ratoon I	22.69	8.72	2.93	3683	11.86	67.92	44.62	0.86	0.20
	Ratoon II	23.56	5.87	3.92	4035	6.77	67.40	44.73	0.25	0.14

Description: DM(dry material). CP (crude protein).CF (crude fat). NDF (neutral detergent fiber). ADF (acid detergent fiber). Ca (calsium) P (phosphor)

Forage production in Table 4 shows even though the Super 1 produces the highest biomass but it is not significantly different with the PAC 537, Numbu, PAC 593, Kawali, and Super 2. The lowest biomass production significantly produced by the G5 followed by PAC 501 compared the other cultivars. This decrease is suspected to be influenced by the lack of nutrients in the soil, so that it requires more nitrogen (N) administration to overcome the difference in production of the primary and ratoon plant (Efendi et al. 2013).

Forage 1 shows forage production of all cultivars decreases in the ratoon II phase. The PAC 537, consistently has the highest production both in the first harvest, ratoon I and ratoon II.

Table 6. Dry material and organic material digestibility of cultivars of Sorghum bicolor of primary and ratoon I planted in dry land with dry climate in Gunung Kidul, Yogyakarta

Cultivar		DMD (%)	OMD (%)
PAC 501	Primary	74.67	73.07
	Ratoon I	71.18	70.72
Super 2	Primary	75.95	74.29
	Ratoon I	73.25	71.31
G5	Primary	75.31	74.01
	Ratoon I	65.74	63.06
Kawali	Primary	56.11	53.20
	Ratoon I	66.85	64.19
PAC 593	Primary	70.37	69.23
	Ratoon I	51.92	47.97
Numbu	Primary	78.31	76.35
	Ratoon I	61.55	59.21
G2	Primary	54.55	51.54
	Ratoon I	41.19	36.57
Super 1	Primary	60.51	58.18
	Ratoon I	53.91	51.17
PAC 537	Primary	60.88	58.94
	Ratoon I	62.06	59.57

The decrease in biomass production from the first harvest to the ratoon II was caused by the decrease in its growth percentage. Effendi et al. (2013) also reported that primary plant of 15021A of Sorghum bicolor had highest biomass production of 63.4 ton/ha and decreased drastically into 24.6 ton/ha in the ratoon I and 20.6 ton/ha in ratoon II. The drastic decrease of the ratoon I to ratoon II was caused by the decrease in growth percentage of the ratoon I (44.2%) to ratoon II (33.3%).

Growth percentage of the ratoon would determine the number of the plant would be harvested per area unit which affected fresh biomass production. The genotype 15011A has a high enough potential ratoon with the growth percentage of the ratoon I of 73.0% and ratoon II of 54.2%, so that fresh biomass production of the primary plant, ratoon I and ratoon II. The difference in biomass production in this study of the primary and ratoon I because the forage production was measured before the flowering period which is intented to maintain the quality of forage. High production in ratoon I and the production decrease in ratoon II were influnced by the season. The rainfall in ratoon I was 31 mm with the rainy day of 3 days, while the rainfall in ratoon II was 0 mm (Table 1). Besides, it was also influenced by age of the plants, where the ratoon plants were more mature compared to the primary plants and the period of photosyntate acumulation to the biomass became lower (Efendi et al. 2013).

Nutrient in the forage

Chemical analyzes were performed on the upper part of the plant on the primary, ratoon I and ratoon II. The primary forage consisted protein of 6.37 - 11.58% of DM, NDF of 61.68 - 68.52%, ADF of 36.39 - 44.43%, Ca of 0.51 - 0.84% and P 0.20 - 0.35% (Table 5). Crude protein in this study was higher than that reported by Sirappa (2003) that the leaves and stems contained crude protein of 7.82% and 4.4% respectively and crude fiber of 28.94% and 32.30% respectively. Higher crude protein content in the primary, ratoon I and ratoon II was because the crops harvested as they approached the flowering period at 45 days of age. This is the right age to be used as feed because it contains optimum nutrient. Atis et al (2012) also reported that the right time to harvest sorghum for feed was before the seed physiology mature where it can be obtained the highest productivity and quality.

Dry matter and organic matter digestibility ranged from 54.55 - 78.31% and 51.54 - 76.35%, respectively (Table 6). This value was higher compared to the digestibility of straw of other cultivars reported by Praptiwi et al (2016), where three sorghum cultivars tested had dry material digestibility ranging from 45.80-48.93 and organic material digestibility ranged from 46.89 to 50.11%. In the ratoon I, the protein content of forage was higher than the first harvest and the ratoon II was about 8.14 - 13.65% (Table 5). Ca and P content of ratoon I was higher than the primary and II plants. Crude protein (ranged from 4.56 - 8.37%) content of ratoon II forage was lower than those of primary and ratoon I (Table 5).

The increase in stem and leaf growth and crude protein level (Escalada & Pluchnett 1977) was as a result of the absorption of the N of urea and organic fertilizers or the one existed in the soil by the roots which then was delivered to stems and leaves. Bogdan (1977) stated that cultivars and species of different genetic properties will affect their response to the formation of crude protein. Besides the genetic factors and crude protein content, crude fiber content of a plant is also influenced by climate, soil fertility where they grow and the age of plant (Atis et al. 2012; Sher et al. 2016).

Sorghum is adaptive in the tropics and drought tolerant, so that it has a good opportunity to be developed as forage. The development is a viable alternative. It should be done to meet forage demand in the dry areas in Indonesia increase which continues to

increase. The results showed that the nutrient content of sorghum was not much different from corn, sugar cane bud and elephant grass, so that it has the same dry and organic materials digestibility.

CONCLUSION

Sorghum could grow and adapt well in a dry land with dry climates and long dry season. Forage production in primary plants and the three ratoons varied between cultivars and harvest time. Forage production of PAC 537, Super 1 and Super 2 were consistently highest in both primary and ratoon I, II and III. The highest forage quality was achieved in the ratoon I and the lowest was in the ratoon II. Biomass production was 18.84 ton/ha/harvest with the crude protein content of 5.87 - 8.72%, dry matter digestibility of 62.06% and organic matter digestibility of 59.57%. These three cultivars were most potential as forage resources in a dry land with dry climate.

REFERENCES

Aswar R. 2005. Peran tanaman pakan ternak sebagai tanaman konservasi dan penutup tanah di perkebunan. Prosiding Lokakarya Tanaman Pakan Ternak. Bogor (Indones): Pusat Penelitian dan Pengembangan Peternakan. p. 18-24.

Atis A, Konuskan O, Duru M, Gozubenli H, Yilmez S. 2012. Effect of harvesting time on yield composition and forage quality of some forage Sorghum cultivars. Int J Agric Biol. 14:879-886.

Ayub M, Khalid M, Tariq M, Elahi M, Nadeem MA. 2012. Comparison of sorghum genotypes for forage production and quality. J Anim Plant Sci. 22:733-737.

Indonesian Cereal Research Institute. 2011. Pemanfaatan batang sorghum untuk produksi etanol. Maros (Indones): Balai Penelitian Tanaman Serealia.

Ball DM. Collins M. Lacefield GD. Martin NP. Mertens DA. Olson KE. Putnam DH. Undersander DJ. Wolf MW. 2001. Understanding forage quality. American Farm Bureau Federation Publication 1-01. Park Ridge. www.uky.edu/Ag/forage (2/8/2015)

Bogdan AV. 1977. Nutritive value. tropical pasture and fodder plans. London and New York: Longman Inc. p. 14-18.

Chavana UD, Patil JV, Shinde MS. 2009. Nutritional and roti quality of sorghum genotypes. Indones J Agric Sci. 10:80-88.

Deb UK, Bantilan MCS, Reddy BVS, Bramel PJ, Kameswara Rao N. 2004. Impact of improved sorghum cultivars on genetic diversity and yield stability. In: Bantilan MCS, Deb UK, Gowda CLL, Reddy BVS, Obilana AB, Evenson RE, editors. Sorghum genetic enhancement: Research process, dissemination, and impacts. Patancheru 502 324. Andhra Pradeshn (India): International Crops Research Institute for the Semi-Arid Tropics. p. 225-234

Efendi R, Aqil M, Pabendon M. 2013. Evaluasi genotipe Sorghum manis (*Sorghum bicolor* (L.) Moench) produksi biomas dan daya ratun tinggi. Penelitian Pertanian Tanaman Pangan. 32:116-125.

Escalada RG, Pluchnet DL. 1977. Ratoon cropping of Sorghum. origin. Time of Appearance and fate of Tillers. Agron J. 69:341-346.

Ibrahim AA, Ati FO, Adebayo AA. 2011. Effect of climate on the growth and yield of sorghum *(Sorghum bicolor*) in Wailo. Ganjuwa Local Government Area. Bauchi State. Res J Environ Earth Sci. 3:469-472.

Lestari EG, Dewi IS. 2015. Evaluasi dan seleksi galur mutan sorgum manis varietas numbu hasil mutasi. Seminar Nasional Biologi II.

Marsalis MA. 2011. Sorghum Forage Production in New Mexico. Guide A-332. New Mexico State University and the US Department of Agriculture Cooperating. p. 1-12.

Meliala MG, Trikoesoemaningtyas, Sopandie D. 2017. Keragaan dan Kemampuan meratun lima genotype Sorgum. J Agronomi Indonesia. 45:154–161.

Nugraha BD, Handayanta E, Rahayu ET. 2013. Analisis daya tampung (carrying capacity) ternak ruminansia pada musim penghujan di daerah pertanian lahan kering Kecamatan Semin Kabupaten Gunungkidul. Trop Anim Husb. 2:34-40.

Oldeman LR. 1975. An agroclimatic map of Java. contributions No.17. Central Research Institute for Agriculture. Bogor.

Pasaribu RP, Yetti H, Nurbaiti. 2015. The Effect of main branch pruning and giving liquid organic fertilizer complementary on the growth and yield of Tomato (*Lycopersicum esculentum* Mill). JOM FAPERTA. 2:14P.

Prawiradiputra BR, Sutedi E, Sajimin, Fanindi A. 2012. Hijauan pakan ternak untuk lahan sub-optimal. Bogor (Indones): Pusat Penelitian dan Pengembangan Peternakan, Badan Penelitian dan Pengembangan Pertanian. 63 p.

Purnomohadi M. 2006. Potensi penggunaan beberapa varietas sorghum manis (*Sorghum bicolor* (l.) moench) sebagai tanaman pakan. Berk Penel Hayati. 12:41–44.

Purwowidodo. 1993. Telaah Kesuburan Tanah. Bandung (Indones): Angkasa Bandung.

Rahmi A, Biantary MP. 2014. Karakteristik sifat tanah dan status kesuburan tanah lahan pekarangan dan lahan usaha tani beberapa kampong di Kabupaten Kutai Barat. ZRAA'AH. 39:30-36.

Revy AP, Zuhry E, Nurbaiti. 2014. Pertumbuhan dan produksi berbagai varietas sorgum (*Sorghum bicolor* L.) dengan pemberian pupuk urea. JOM Fak Pertanian. 1.

Sher A, Ansar M, Ijaz M, Sattar A. 2016. Proximate analisis of forage sorghum cultivar with different doses of nitrogen and seed rate. Fields Crops Turk J. 21:276-285.

Sirappa MP. 2003. Prospek pengembangan sorgum di Indonesia sebagai komoditas alternative untuk pangan. pakan. dan industry. Jurnal Litbang Pertanian. 22:133-140.

Soeranto H, Sihono, Parno. 2011. Application of mutation techniques in sorghum breeding for improved drought tolerance. J Agron Indon.

Subagio H, Aqil M. 2014. Perakitan dan pengembangan varietas unggul sorgum untuk pangan. pakan dan bioenergy. IPTEK Tanaman Pangan. 9:39-50.

Suminar R, Suwarto, Purnamawati H. 2017. Penentuan dosis optimum pemupukan N, P, dan K pada sorgum (*Sorghum bicolor* [L.] Moench). J Ilmu Pertanian Indonesia. 22:6-12.

Surya IM. Soeranto H. 2006. Pengaruh iradiasi sinar gamma terhadap pertumbuhan sorghum manis (*Sorghum bicolor* L.). Risalah Seminar Ilmiah Aplikasi lsofop dan Radiasi. p. 209-215.

Tsuchihashi N, Goto Y. 2008. Year-round cultivation of sweet sorghum (*Sorghum bicolor* (L.) Moench) through a combination of seed and ratoon cropping in Indonesia savanna. Plant Prod Sci. 11:377-384.

Widyastuti Y, Rumarti IA, Satoto. 2012. Perilaku pembungaan galur-galur tetua padi hibrida. IPTEK Tanaman Pangan. 7:67-78.

Primordial Germ Cell Profile Incubated for 24 Hours in the Phosphate Buffer Saline [-] Solution

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(received 03-07- 2017; revised 25-08-2017; accepted 04-09-2017)

ABSTRAK

Kostaman T, Sopiyana S. 2017. Gambaran primordial germ cell yang diinkubasi selama 24 jam dalam larutan phosphat bufer saline [-]. JITV 22(3): 144-150. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1802

Perkembangan gonad adalah sebuah rangkaian proses terdiri dari tiga peristiwa besar, yaitu migrasi PGC, penentuan jenis kelamin, dan diferensiasi gonad. Penelitian ini bertujuan untuk melihat perkembangan PGC yang diisolasi dari gonad embrio, diinkubasi selama 7 hari dan kemudian diinkubasi dalam larutan Phosphat Bufer Saline (PBS) [-]. Gonad yang sedang berkembang dapat di isolasi dari embrio ayam umur 7 hari dan dapat di inkubasi pada temperatur 37,8^{o}C dalam larutan PBS [-], yaitu tanpa Ca2+ dan Mg2+. Pelepasan PGC dari gonad diamati dalam waktu 1, 8, 16, dan 24 jam setelah gonad embrio dimasukkan ke dalam larutan PBS [-]. Hasil penelitian menunjukkan bahwa PGC dapat lepas dari jaringan gonad dan layak untuk dikoleksi dengan cara memasukkan gonad yang sedang berkembang ke dalam larutan PBS [-]. Jumlah PGC dan persentase survival rate tertinggi diperoleh setelah gonad diinkubasi selama 1 jam, namun tidak berbeda dengan 8 jam (P>0,05), sedangkan dengan 16 dan 24 jam setelah inkubasi berbeda nyata (P<0,05). Sebaliknya untuk persentase tingkat kemurnian yang tertinggi adalah yang diinkubasi selama 8 jam, akan tetapi tidak menunjukkan perbedaan yang nyata dengan 1 dan 16 jam (P>0,05). Persentase tingkat kemurnian berbeda nyata setelah gonad diinkubasi selama 24 jam (P<0,05). Dapat disimpulkan bahwa waktu inkubasi yang paling tepat untuk mendapatkan PGC dari jaringan gonad embrio ayam KUB adalah pada waktu tidak lebih dari 8 jam.

Kata Kunci: Gonad, PGC, Ayam Lokal

ABSTRACT

Kostaman T, Sopiyana S. 2017. Primordial germ cells profiles incubated for 24 hours in phosphate buffer saline [-] solution. JITV 22(3): 144-150. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1802

Gonadal development is a sequential process that can be divided into three major events: the PGCs migration, sex determination and gonadal differentiation. This study was aimed to see the development of PGCs isolated from the gonads of embryos after being incubated for 7 days and then was incubated using a solution of Phosphate Buffer Saline (PBS) [-]. The developing gonad can be isolated from 7 days old chick and can be incubated at a temperature of 37.8^{o}C in a solution of PBS [-]: without Ca2+ and Mg2+. The release of gonadal PGC was observed within 1, 8, 16, and 24 hours after the embryonic gonad was placed in a PBS solution [-]. The results showed that PGCs can be separated from gonadal tissues and can be collected by entering the developing gonad to the PBS [-] solution. The highest percentage of PGCs and survival rate was obtained after gonad was incubated for 1 hour and was not different with 8 hours (P>0.05). Those result was significantly different (P<0.05) with the 16 and 24 hours incubation. The highest purity rate percentage was in the 8 hours incubation, but did not show a significant difference (P>0.05) with the 1 and 16 hours incubation. The percentage of the purity differed (P<0.05) after the 24 hours incubation. It can be concluded that the most appropriate incubation time to obtain PGCs from the KUB chicken embryonic gonad is no more than 8 hours.

Key Words: Gonad, PGCs, Native Chicken

INTRODUCTION

Primordial germ cell (PGC) are the precursors of the progenitors of the oocytes and spermatocytes, or according to D'Costa et al. (2001), PGC are the precursors of mature ovum and spermatozoa of the mature poultry and an intergenerational genetic linkage. Germ cells play a very important role in the species identification as they transmit genetic information to the next generation. In many animal species, including birds, germ cells emerge from a small population of cells known as PGC (Bednarczyk 2014).

Chicken PGCs were collected from the germinal crescent (Naito et al. 2001), the embryonic blood vessel (Naito et al. 1994) and from the embryonic gonads (Park et al. 2003). Although no phenotypic changes between the different sources of PGcs were observed, and the expression patterns of specific markers were

identical (Park & Han 2012), however the advantage of using gonadal PGCs compared with another sources of these cells is, that a greater number of PGCs can be retrieved from one embryo. (Bednarczyk 2014)

Characterization and detail description of the differentiation of the germinal cell gen will result in better understanding of fertility, germ cell tumor and contraception method (Kakegawa et al. 2008).

A unique character of poultry PGC during the initial development provides a chance to manipulate the poultry germplasm. Two purpose of PGC use widely reported is for the conservation of genetic resources especially the endanger poultry germplasm (Glover & McGrew 2012) and the production of transgenic poultry (Nakamura et al. 2013).

The primordial germ on poultry has a significant potential to be used in cell-based studies since it can be collected from the blastoderm, embryo blood and embryo gonad (Chojnacka-Puchta et al. 2012). Although there is no phenotypic change between the various PGC sources observed and the expression pattern of the certain marker, most PGC can be collected only from one embryo which is an advantage of the gonad PGC (Park & Han 2012). Considering the difficulty of the collection process from the tissue, the isolation and culture of the chicken PGC from embryo gonad may provide many sources for the chicken genetic manipulation (Shiue et al. 2009).

Isolation method of the gPGC using Phosphate-Buffered Saline (PBS) [-] solution is a simple method developed for chicken PGC isolation derived from the gonad tissue. The advantage of the gPGC isolate using the PBS [-] is easy to be collected in the short time and does not require expensive equipment (Nakajima et al. 2011). PBS is a media which is very useful and convenient for various animal cell cultures. Besides, the PBS is rich in nutrient (dos Santos et al. 2002). PBS is available commercially in the different formulation: PBS with calcium and magnesium (PBS[+]) and without calcium and magnesium (PBS[-]). Therefore, it was conducted a study to isolate PGC derived from gonad tissue with high purity by incubating the embryo gonad in the PBS [-] for 24 hours

MATERIALS AND METHODS

Location

This study was conducted at the Laboratory of Non-Ruminant Reproduction, Indonesian Research Institute for Animal Production (IRIAP), Ciawi-Bogor.

Fertile egg preparation

The eggs used in this study was 60 fertile eggs of Kampung Unggul Balitbangtan (KUB) chicken from the IRIAP, Ciawi-Bogor. Those eggs were cleaned using cotton moistened with 70% alcohol, then were incubated in a portable incubator (P-008B Biotype; Showa Furanki, Saitama, Japan) with 37.8°C of temperature for 7 days.

Procedure of gonad collection

The 7 days old eggs were taken from the incubator and placed in an egg tray. One by one egg was cracked using tweezers on the plunt part to create a small hole. The small hole was cracked more slowly to create a bigger hole to see the embryo (Figure 1).

The embryo was carefully separated and put into a petri dish (90x15mm, LBS60001PT, BIOLAB) filled with PBS [-] solution. This process was repeated for all embryo. One by one embryo was taken and placed into a charcoal plate filled with the PBS [-] where its abdomen part was upward. The head and legs were pinned using a pin to lock the embryo during the gonad collection (Figure 2).

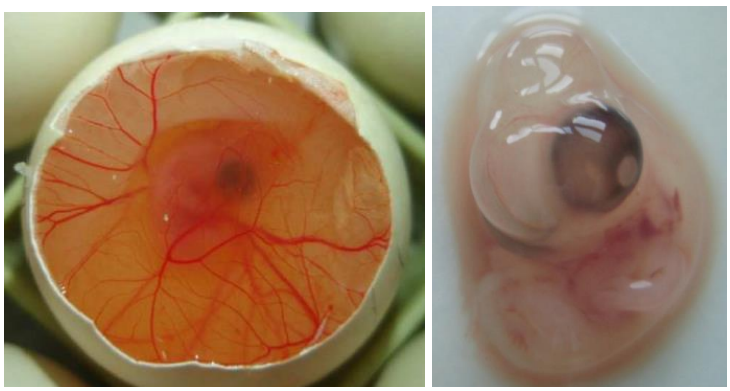

Figure 1. The normal development of KUB chicken embryo incubated for 7 days.

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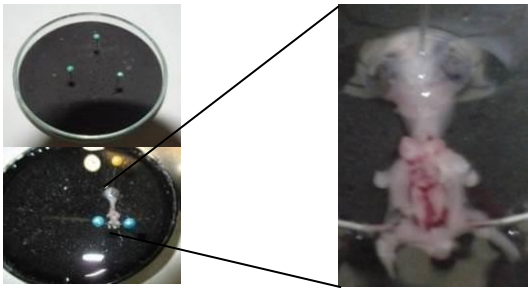

Figure 2. Embryo of KUB chicken incubated for 7 days placed on the charcoal plate.

Figure 3. Gonad position (the white arrows) of KUB embryo incubated for 7 days observed under Microsoft with 400x magnification attaching to the right and left mesonephros (the black arrows).

The abdomen and the innards were removed using tweezer carefully to see the mesonephros. The gonad is attached to the left and right mesonephros (Figure 3). Gonad was taken carefully using a tweezer, verify that the gonad can be taken with no cut off and there no other part included.

Isolation and collection of Gonadal Primordial Germ Cell

After the 7 days incubation, both left and right gonads successfully isolated were placed in the 1.5 ml Eppendorf tube filled by 500 µl PBS [-] and then incubated for 1, 8, 16, and 24 hours in the CO2 incubator (Thermoline) at 37.8°C, then, the purity of PGC was performed following Nakajima et al. (2011).

Variables

Variables visually observed was morphological characteristic which was identic with the PGC circulation as a huge round cell with large nucleus that was observed under contrast phase Microsoft (Olympus CKX41, Japan), the number of PGC, survival rate (measured using trypan blue staining of Nakajima et al. 2011) and level of purity (defined as ratio of PGC amount released and total cell amount removed that expressed in percentage)

Statistical analysis

Data of morphological characteristic were analyzed descriptively. Average PGC amount, survival rate, and purity level were analyzed in the ANOVA. It was

continued by Duncan test when there was a difference using SPSS Ver 10 program.

RESULTS AND DISCUSSION

The basic principle of PGC isolation using PBS [-] solution is storing the gonad has been removed from the mesonephros and then incubated in the PBS [-]. The isolated PGC (Figure 4) under the contrast phase Microsoft showed a unique characteristic of PGC and almost similar with circulation-PGC (cPGC) which having a large nucleus. Also, it showed several membrane microphilies that is important for in vivo migration (Raucci et al. 2015). Microscopic observation with larger magnification showed a large nucleus with a diameter of about 9 µm, which was eccentric. Several cPGC had pseudopodia and irregular shape showing the character of this cells is in the development stage (Kuwana & Rogulska 1999).

Gonad embryo within 7 days of incubation chicken contained a little (if there was any) somatic cells and rarely or no connective tissue between PGC (Nakajima & Tajima 2013). This is common that the gonad of somatic cells promotes the maturation and differentiation of germinal cells in the gonad (Johnson 2000; Kirby & Froman 2000). Although the mechanisms underlying PGC release from gonadal development in PBS [-] are not understood, the fast and simple method described in this study will be very convenient for the production of germline chimera.

The releasing or migrating the PGC from gonad tissue can be performed by incubating the gonad in the PBS [-] solution (Figure 5). The PBS [-] solution played the main role to create an ideal micro environment for PGC interact, maintaining the pluripotency or differentiating into functional germinal. This structure showed that the current incubation condition was able to maintain the appropriate physiology of cell (Raucci et al. 2015). The other reason for the use of PBS [-] is because in the PGC release from gonadal tissue does not need a help from cation (Sopiyana et al. 2017).

Temporal change in the amount, survival rate and purity of the PGC isolated from gonad embryo incubated in the PBS [-] solution for 24 hours showed in Table 1. The increase of PGC amount which was not continuing collected after 16 and 24 hours incubation was compared to the 1 and 8 hours of incubation. This can be caused by the release of somatic cell continue to increase along with longer incubation time. A similar result was also reported by Nakajima et. al. (2014).
Average survival rate percentage of PGC was more than 80% at the first 8 hours of gonad incubation. It then decreased to 65% on the 24 hours incubation. This significant decrease in the survival rate of PGC after 8 hours of incubation can be correlated to the depletion of essential nutrients to maintain cell metabolism of PGC. This may because increased cellular metabolism has accelerated the consumption of nutrient media and beyond the PBS buffer power. Consequently, it results in longer incubation time which dos Santos et al. (2002) described that the composition of media is a crucial factor to survive.

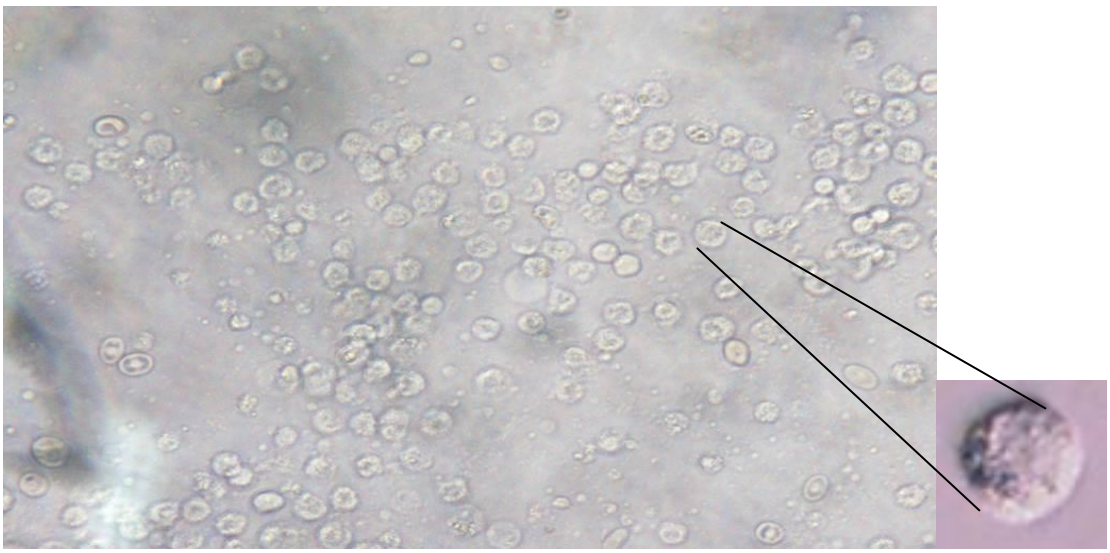

Figure 4. Primordial germ cell isolated from 7 days old gonad with 400x of magnification.

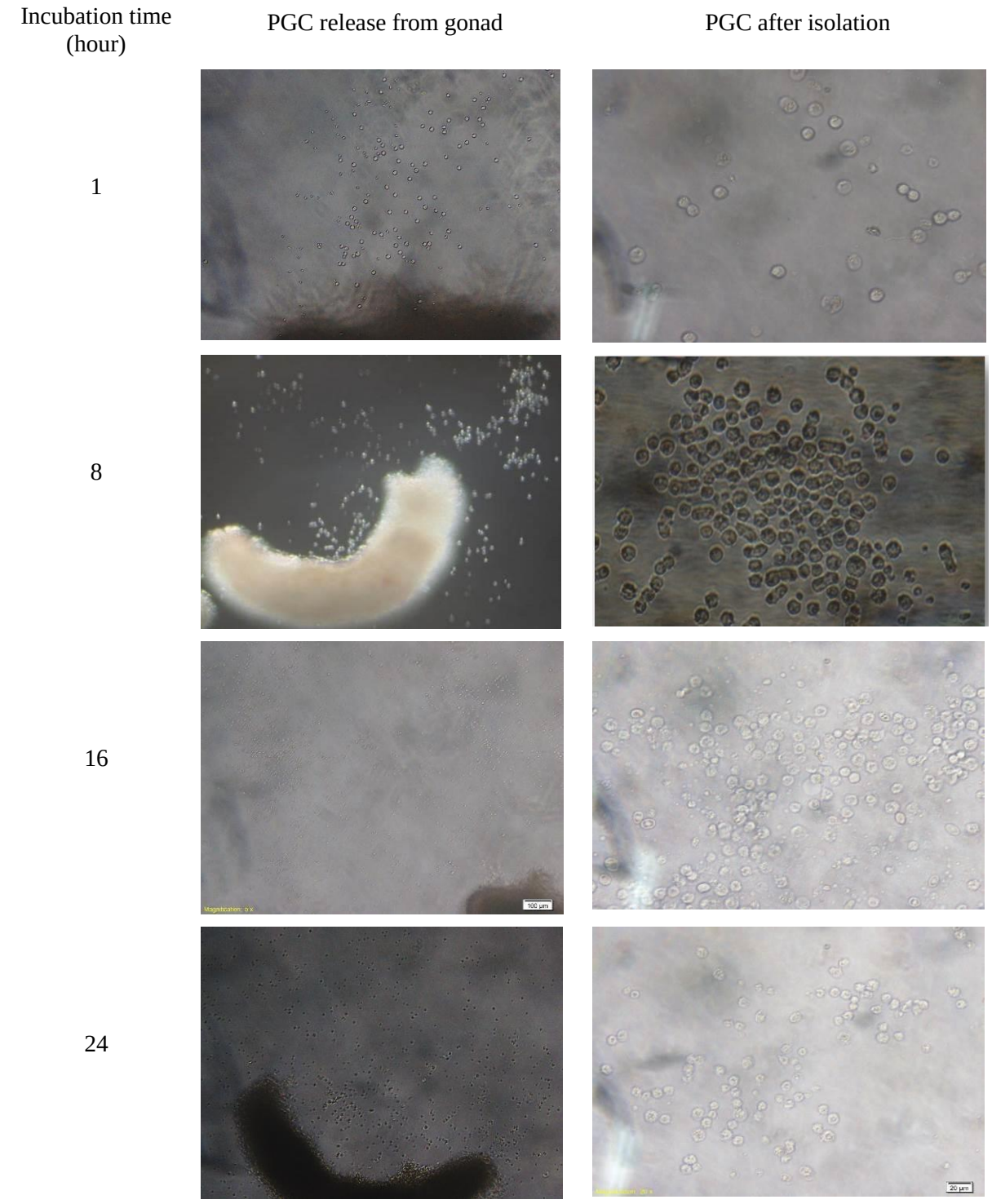

Figure 3. The average score of udder parenchymal (PAR) and mammary fat pads (MFP) of each quarter of buffalo calve udder. Score: <1=very small PAR size/thin MFP, 1-<2=small PAR/medium MFP, ≥2= medium PAR/thick MFP.

Table 1. The Amount, survival rate and purity level of primordial germ cell (pgc) isolated from kub chicken embryos

Variable	Incubation Time (hour)			
	1	8	16	24
gPGC amount (cell/embryo)	151.47[a]±16.12	140.25 [a]±7.77	122.40[b]±5.75	130.60[b]±3.65
Survival rate (%)	90[a]	82[a]	70[b]	65[b]
Purity level (%)	48[a]	50[a]	45[a]	40[b]

Description: different superscript on the same rows shows a significant difference (P<0.05)

As well as the percentage of PGC purity level that decreased after 8 hours incubation. This may be caused by spontaneous dissociation of gonad was accelerated after 8 hours of incubation in the PBS [-] (Nakajima et al. 2014). Therefore, spontaneous PGC release into the PBS [-] solution from gonad embryo observed in this study may have been affected by the existence of protein adhesion cell such as cadherin and integrin controlled by the concentration of Ca2+ and Mg2+ (Gumbiner 2005; Lien et al. 2006).

The same result was also reported by Nakajima et al. (2011) on White Leghorn (WL) and Rhode Island Red (RIR) that showed longer incubation of gonad, the more decrease in survival rate and purity percentage of PGC. This decrease may also be correlated to the incubation time of the cell in the CO2 room. Storing cell more than 8 hours without media or calcium and magnesium compound may have contributed to high PGC mortality. It then Li et al. (2005) proved that more decrease in PGC amount when incubation caused by the cessation of aggregation factor by PGC, so that the PGC was back into phenotype derived from blood.

Currently, the fastest method for isolating PGC from gonad embryo of chicken is PBS [-] solution. This method provides a possibility to collect the most PGC amount purified and feasible to be transferred in a short time (Nakamura et al. 2013). It also has been proved that cells isolated from gonad and injected into X and 17 stage-embryo also experienced normal development and had normal gamete (Mozdziak et al. 2006).

It was expected from this study that isolation method of gPGC can be used in reproduction management system forming of specific-species for the success of Indonesian native chicken genetic resource. Also, it was expected that this new technology can help in reducing the maintenance cost and protecting the bird from an unexpected incident such as the highly pathogenic avian influenza or tsunami.

CONCLUSION

The most appropriate incubation time to obtain PCG isolated from KUB chicken embryo was from 1-8 hours. The Viability of PCG until 8 hours of incubation showed high PGC amount, the percentage of survival rate and purity compared to the 16 and 24 hours of incubation. To obtain the high PGC amount, survival rate and purity level, Ca2+ and Mg2+ should be added after 8 hours incubation.

ACKNOWLEDGEMENT

Authors wish to thank Prof. Dr. Argono R. Setioko for the advises, Tati Kurniasih and Agus Suhendri for the technical support in this study.

REFERENCES

Bednarczyk M. 2014. Avian Primordial Germ Cells and their application. The 2nd International Scientific Conference "Animal Biotechnology". Slovak J Anim Sci. 47:185-187.

Chojnacka-Puchta L, Kasperczyk K, Plucienniczak G, Sawicka D, Bednarczyk. 2012. Primordial germ cells (PGCs) as a tool for creating transgenic chickens. Polish J Vet Sci. 15:181-188.

D'Costa S, Pardue SL, Petitte JN. 2001. Comparative development of avian primordial germ cells and production of germ line chimeras. Avian Poult Biol Rev. 12:151-168.

dos Santos RR, Silva JRV, Costa SHF, Rodrigues APR, Lobo RNB, de Figueiredo JR. 2002. Effect of 0.9% saline solution and phosphate buffer saline at different temperatures and incubation times on the morphology of goat preantral follicles. Braz J Vet Res Anim Sci. 39:254-259.

Glover JD, McGrew MJ. 2012. Primordial germ cell technologies for avian germplasm cryopreservation and investigating germ cell development. J Poult Sci. 49:155-162.

Gumbiner BM. 2005. Regulation of cadherin-mediated adhesion in morphogenesis. Nat Rev Mol Cell Biol. 6:622-634.

Johnson AL. 2000. Reproduction in the female. In: Whittow GC, editor. Sturkie's avian physiology. 5th ed. London (UK): Academic Press. p. 569-596.

Kakegawa R, Teramura T, Takehara T, Anzai M, Mitani T, Matsumoto K, Saeki K, Sagawa N, Fukuda K, Hosoi Y.

2008. Isolation and culture of rabbit primordial germ cells. J Reprod Dev. 54:352-357.

Kirby JD, Froman DP. 2000. Reproduction in the female. In: Whittow GC, editor. Sturkie's avian physiology. 5th ed. London (UK): Academic Press. p. 597-616.

Kuwana T, Rogulska T. 1999. Migratory mechanisms of chick primordial germ cells toward gonadal anlage. Cell Mol Biol. 45:725-736.

Li BC, Chen GH, Qin J, Wu XS, Wu SL, Cai ZT. 2005. Suitable stages for isolation and culture PGCs from chicken embryos. Int J Poult Sci. 4:885-890.

Lien WH, Klezovitch O, Vasioukhin V. 2006. Cadherin-catenin proteins in vertebrate development. Curr Opinion Cell Biol. 18:499-506.

Mozdziak PE, Wysocki R, Angerman-Stewart J, Pardue SL, Petitte JN. 2006. Production of chick germline chimeras from fluorescence activated cell-sorted gonocytes. Poult Sci. 85:1764-1768.

Naito M, Sano A, Matsubara Y, Harumi T, Tagami T, Sakurai M, Kuwana T. 2001. Localization of primordial germ cells or their precursors in stage X blastoderm of chickens and their ability to differentiate into functional gametes in opposite-sex recipient gonads. Reproduction. 121:547-552.

Naito M, Tajima A, Yasuda Y, Kuwana T. 1994. Production of germline chimeric chickens, with high transmission rate of donor-derived gametes, produced by transfer of primordial germ cells. Mol Reprod Develop. 39:153-162.

Nakajima Y, Asano A, Shikawa N, Tajima A. 2014. Factors involved in spontaneous discharge of gonadal germ gells from developing gonad of 7-day-old chick embryos. J Poult Sci. 51:416-423.

Nakajima Y, Minematsu T, Naito M, Tajima A. 2011. A new method for isolating viable gonadal germ cells from 7-day old chick embryos. J Poult Sci. 48:106-111.

Nakajima Y, Tajima A. 2013. Development of a novel method for isolating gonadal germ cells from early chick embryos. J Dev Sustainable Agric. 8:75-78.

Nakamura Y, Tasai M, Takeda K, Nirasawa K, Tagami T. 2013. Production of functional gametes from cryopreserved primordial germ cells of the Japanese quail. J Rep Dev. 59:580-587.

Park TS, Han JY. 2012. Piggybac transposition into primordial germ cells is an efficient tool for transgenesis in chickens. Proc Natl Acad Sci. USA 109: 9337-9341.

Park TS, Hong YH, Kwon SC et al. 2003. Birth of germline chimeras by transfer of chicken embryonic germ (EG) cells into recipient embryos. Mol Reprod Dev. 65:389-395.

Raucci F, Fuet A, Pain B. 2015. In vitro generation and characterization of chicken long-term germ cells from different embryonic origins. Theriogenology. 84:732-742.

Shiue YL, Tailiu JJ, Liou JF, Lu HT, Tai C, Shiau JW, Chen LR. 2009. Establishment of the long-term in vitro culture system for chicken primordial germ cells. Reprod Dom Anim. 44:55-61.

Sopiyana S, Supriatna I, Setiadi MA, Fahrudin M. 2017. Efisiensi isolasi primordial germ cell (PGC) dari sistem sirkulasi dan gonad serta produksi germline chimera pada ayam KUB (Disertation). [Bogor (Indones)]: Institut Pertanian Bogor.

Cytokines Profile of Mice Infected by High and Low Virulences of Indonesian *T. evansi* Isolates

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(received 19-06-2017; revised 05-09-2017; accepted 11-09-2017)

ABSTRAK

Sawitri DH, Wardhana AH, Wibowo H. 2017. Profil sitokin tikus yang terinfeksi dengan virulensi tinggi dan rendah isolat *T. evansi* Indonesia. JITV 22(3):151-164. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1666

Surra pada ternak disebabkan oleh *Trypanosoma evansi*, yaitu protozoa darah homoflagella yang bersirkulasi secara ekstraseluler. Penyakit ini tersebar luas dikawasan Asia, Afrika, Amerika Selatan dan Tengah. Ditinjau dari aspek imunologis, tingkat keparahan surra pada hewan ternak (sapi) dan mencit yang diinfeksi trypanosoma berhubungan dengan respon inflamasi. Disisi lain lama hidup mencit tergantung pada regulasi sintesis Th1 dan sitokin proinflamasi seperti IFN-γ dan TNF-α. Penelitian ini bertujuan untuk melihat respon sitokin pro inflamasi IFN γ, TNF-α dan anti inflamasi IL-10 yang terjadi akibat adanya interaksi dengan parasit pada hewan. Informasi ini dibutuhkan untuk perbaikan tatalaksana penanggulangan surra pada hewan. Sebanyak 30 ekor mencit dibagi dalam 3 kelompok, yang secara berurutan diinfeksi dengan *T. evansi* virulensi rendah (Pml287); virulensi tinggi (Bang87) dan tidak diinfeksi sebagai kelompok Kontrol. Serum mencit dikoleksi pada setiap 4 hari untuk pemeriksaan sitokinnya dengan ELISA. Hasil penelitian menunjukkan terdapat perbedaan profil sitokin proinflamasi dan antiinflamasi antara mencit yang diinfeksi isolat Bang 87 dan Pml 287. Kematian dini pada mencit yang diinfeksi isolat Bang 87 diduga akibat terjadinya sindrom respon inflamasi sistemik yang ditandai dengan peningkatan kadar IFN-γ yang tidak dikompensasi secara cukup oleh antiinflamasi. Terjadinya anemia berkontribusi sebagai penyebab kematian pada mencit yang mendukung terjadinya kegagalan multi organ (*multiple organ disfunction*).

Kata Kunci: Surra, *Trypanosoma evansi*, Sitokin, IFN γ, TNF-α, IL-10

ABSTRACT

Sawitri DH, Wardhana AH, Wibowo H. 2017. Cytokines profile of mice infected by high and low virulences of Indonesian *T. evansi* isolates. JITV 22(3): 151-164. DOI: http://dx.doi.org/10.14334/jitv.v22i3.1666

Surra in livestock is caused by *Trypanosoma evansi*, a homoflagella blood protozoa that circulate in extracellular. This disease is widespread in Asia, Africa, South and Central America. According to the immunological aspect, the severity of surra in livestock and mice which infected by trypanosoma is associated with an inflammatory response. On the other hand, the survival time of mice depends on the regulation of Th1 synthesis and pro inflammatory cytokines such as IFN-γ and TNF-α. The aim of this study was to observe the responses of pro inflammatory (IFN γ, TNF-α) and anti-inflammatory (IL-10) cytokines which result from interaction with parasites. This information is needed for improvements in the management of prevention of Surra in animals. A total of 30 mice were divided into 3 groups. The group was infected with a low virulence (Pml287) and high virulence (Bang87) T. evansi respectively and one group was not infected as control. Mice sera were collected in every 4 days for cytokine measurement using an Enzyme Link-Immunosorbent Assay (ELISA). The result showed a difference response of pro inflammatory and anti-inflammatory cytokine profile between mice infected by Bang 87 and Pml 287 isolate. Early deaths in mice infected by Bang 87 isolate were suspected as a result of the response of systemic inflammation syndromes characterized by elevated IFN-γ levels that were not adequately compensated by anti-inflammatory. Anemia contributes to the cause of death in mice that support multiple organ failures (multiple organ dysfunction).

Key Words: Surra, *Trypanosoma evansi*, Cytokine, IFN γ, TNF-α, IL-10

INTRODUCTION

Surra is a disease leading to high economy losses in the livestock farming business due to causing abortus, estrus cycle disorder of the female, body weight decrease, immune system depression and death (Payne et al. 1993; Payne et al. 1994; Jittapalapong et al. 2009).

The previous reports of many Diseases Investigation Centre (DIC) in Indonesia : Wates, Subang, Bukittinggi and Denpasar during the year of 2010-2014 indicated that the prevalent of surra in cattle, horse and buffalo was around 0.28%- 69.3%. From the point of view of immunology aspect, the severity level of surra on cattle and mice infected by trypanosome is related to the

inflammation response (Maina et al. 2004; Mekata et al. 2013). While, the survival time of mice is depended on synthesis regulation of Th1 and cytokine proinflammation like IFN-γ and TNF-α (Darji et al. 1996) and profile change of feedback negative of anti-inflammatory cytokine and Th2 pattern (Namangala et al. 2001a). This is related to the expression of variant surface glycoprotein (VSG) of *T. evansi* which changes periodically. Magez et al. (1993) said that VSG released by *T. evansi* was identified as the main factor that induces the TNF-α and also known playing a role in excessive activating of macrophage. The study of the interaction between the host with infection of *T. congolense, T. brucei* and *T. evansi* is always associated to the species of the host with sensitivity difference, but until present there is no report how the response of the similar host due to the infection of *T. evansi* with different virulence so far. This information is needed to describe the different clinical manifestation which is not only caused by the species difference, but also due to different virulence level. Therefore, the effect of the divergence of virulence of *T. evansi* against its immune response on the same host is required.

Within the bloodstream system, trypanosome will be faced with natural immune response as the early defense of the host against the infection. Nature immune system consists of physical and chemical defense to the infection that is represented by the epidermis, cilial ephitel of respiration system, blood vessel endotel and mucosal surfaces by the antimicrobial secretion (Basset et al. 2003). Nature immune system is responsible to recognize and restrict the microbe/paracyte on the beginning of infection by activating the complement, phagocytosis and immune activation by different families from the pattern recognition receptors (PRRs). The nature immune response of the host focus on the introduction of conserved structure which is evolved on the microbe called pathogen-associated molecular pattern (PAMPs) coded as PRRs. One of PRRs families has been widely studied is Toll-like receptors (TLRs) (Akira et al. 2006; Medzhitov & Janeway 2000). The most PAMPs identified on protozoa is glycosylphosphatidiylinositol (GPI) link activating the TLR 2 and 4 (Almeida & Gazzinelli 2001) and the antimetilation DNA activating the TLR 9 (Debierre-Grockiego et al. 2003; Gazzinelli & Denkers 2006) (Figure 2.9). Almeida et al. (2000) and Coller et al. (2003) described that the GPI molecule from the VSG will interact with macrophage which then inducing the proinflammation cytokine. Therefore, the first immune response of the host immune system is the pro inflammatory cytokine (IFN-γ, IL-1, IL-6) and NO excreted by the macrophage activity through classic path (Ca MΦs). Furthermore, the DNA of trypanosome released when the parasite dies, is also known to activate the macrophage through the same path to secrete the other proinflammation cytokine such as TNF-α, IL-12 dan NO (Shoda et al. 2001; Harris et al. 2006).

Severity of the disease occurred at the initial stadium of trypanosome infection on mice is signed by an increase in synthesis of pro inflammatory cytokines such as IFN-γ, TNF-α and the Th-1 pattern (Namangala et al. 2001a), while the survival time of mice depends on the expression of anti-inflammatory cytokines to do negative regulation and the Th-2 pattern (Darji et al. 1996). Mabbott et al. (1998) and Paulnock & Coller (2001) described that IFN-γ is the first pro inflammatory cytokine which has a crucial role in activating macrophage after being stimulated by parasite antigen. The activated macrophage will induce the production of pro inflammatory cytokine such as IL-1, IL-6 and TNF-α paling a role in parasitemia and host immune response (Paulnock & Coller 2001; Gao et al. 2002; Magez et al. 2007). The increase in IFN-γ level in the serum is useful to control parasitemia by activating the macrophage to clean the paracyte from the blood (Silva et al. 1992; Hertz & Filutowicz 1998; Magez et al. 2006). Cytokine IFN-γ produced by Th-1 and T CD8+ cells also have a role to stimulate the production of antibody IgG2a and IgG3 (component opsonization and microbial phagocytosis). An experimental study showed that high level of IFN-γ and low parasitemia related to the animal resistance infected by T. brucei and T. evansi (Hertz & Filutowicz 1998; Paim et al. 2011). Then it is described by Shi et al. (2003) that early motility on BALB/c mice sensitive to the infection of T. congolense related to the increase in IFN-γ synthesis leading to systemic inflammatory response syndrome (SIRS) (Figure 2.10).

The sensitivity to the infection was reported in mice deficiency to TNF-α infected by *T. congolense* (Naessens et al. 2004). Another study also reported that TNF-α produced by macrophage played a role in directly and undirectly eliminating the parasite by inducing the nitric oxide (NO). Nitric oxide is a free radical produced by macrophage synthesized from L-arginine by NO synthase (NOS). This substantion has the effect of killing the parasite and destroying tissue function (spleen, liver, peritoneum, and central nervous system) (Eckersall et al. 2001; Reed et al. 1989; Kitani et al. 2002). The existence of selective inhibition by Th-1 cell causes negative effect to NO. Specific antigen CD4+ Tr1 is possibly IL-10 to do negative regulation. The macrophage activity, especialy the kupffer cell in the liver increase significantly. The excisive SIRS lead to a death (Shi et al. 2003; Shi et al. 2004). Macrophage activation is marked by at least three factors: trypanosoma agocytosis, excessive production of IFNγ and IL-10 production (Shi et al. 2005).

The IL-10 is a Th-2 cytokine related to the sensitivity of the host to protozoa. It can decrease the

response of T cell by regulating the APC to decrease the secretion of TH-1 cell (IFN-γ and IL-2), activation of IFN-γ and suppressing the release of TNF-α, IL-1 and IL-6 by macrophage (Ghalib et al. 1993; Ralph et al. 1992). The IL-10 is needed to maintain a balance of protective and pathogenic immune responses during trypanosomiasis. No TL-10 is related to the decrease in lifetime and increase in inflammation in mice infected by *T. brucei* (Quan et al. 1999).

In addition, macrophage also produces IL-1. It is described by Sileghem et al. (1991) that IL-1 release by macrophage increase during acute phase on mice infected by T. brucei. This cytokine also induces acute phase of protein and stimulate the immune response mediated by lymphocyte. Other cytokine produced by macrophage and dendritic cell is IL-12 inducing Th-1 cell. In trypanosome infection, the production of IFN-γ and IL-12 is related to the response of Th-1 and correlated to the resistence of host (Uzonna et al. 1998).

MATERIALS AND METHODS

Ethic statement

The protocol for the animal experiments was approved by Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia (Approval number 124/H2.F1/ETIK/2013).

T. evansi isolates

T. evansi isolates used in this study were Bangkalan 87 (high virulence) and Pemalang 287 (low virulence) isolates.

Experimental animal

As much as 30 DDY mice were divided into three groups and treated with: group 1 (control, without infection), group 2 (infected by *T. evansi* Pml 287 isolate-low virulence) and group 3 (infected by *T. evansi* Bang 87 isolate-high virulence). The infection of both *T. evansi* isolates was performed on the $1x10^4$ paracytes/head of dose each intraperitoneal. The number of parasitemia was observed quantitatively in every two days of interval. Blood collection for the serum and PCV (hematocrit) examination was performed in every four days for 24 days.

PCV test

Sixty microlites of blood was taken by using microhematocrit (heparinized) through mice tail vein every four days interval. Both microhematocrit tips were closed by micro seal and then centrifuged for five minutes with 13,500 g speed until the blood and plasma were separated. Then, the hematicryte level was measured by microhematocrit reader (Hawsksley) and expressed in percent.

Preparation of mice serum for ELISA

Serum separation was performed by collecting blood from tail vena for about 90 µl in the 0.6 ml microtube without EDTA. The bleeding was stopped by wiping it with alcohol. The serum was isolated from mice blood which was incubated beforehand at room temperature for 30 minutes. After the bloodshed and clear liquid were formed, it then incubated for 30 minutes at 4°C. The blood was centrifuged with 3.000 g of speed for 5 minutes at 4°C. All the serums were transferred into the new tube using a pipette. The second centrifugation was performed with the same speed and the pure serum was transferred to the new tube and stored at -20°C until used.

ELISA

The proinflammation and antiinflammation cytokines were qualified using ELISA against antimouse IFN-γ, TNF-α, and IL-10 (eBioscience, USA). The ELISA 96-well plate (Corning, USA) was coated using solution of 100µl of 0,5-4µg/ml antimouse IFN-γ (0.5 µg/mL), TNF-α (1µg/mL), IL-1β (1µg/mL), IL-10 (1µg/mL), and IL-12 (1µg/mL) which was separately done by dissolving each cytokine on the dapar carbonate pH 9.6 then incubated at 4°C for 24 hours. The plates were washed once using phosphate buffer saline (PBS) and added by the blocking buffer (PBS-0.5% tween 20) as much as 200 µl/well and incubated for 60 minutes at 37°C. The blocking buffer was removed and the serum (mice serum) was diluted by 1:25 on the blocking buffer. It then was inserted into ELISA plate and duplicated. After incubated for 60 minutes at 37°C, the plates then were washed six times using PBS 0.5% Tween 20 (Sigma Aldrich). Total of 100 µL of Biotinilated antimouse conjugate with the concentration of 0.25 – 4 mg/mL: IFN-γ (0.5 µg/mL), TNF-α (0.5 µg/mL), IL-1β (1µg/mL), IL-10 (1 µg/mL), and IL-12 (1 µg/mL) which was diluted separately on the blocking buffer and added by ELISA plate and incubated for 60 minutes at 37°C. After the 5-6 times of washing with PBS- 0.5% tween 20, it was added by 100 mL Avidin-HRP (Horseradish peroxidase) that was diluted on the blocking buffer (1:1500) and incubated for 60 minutes at 37°C. After 8-10 times of washing with PBS 0.5% tween 20, it then was added to 100 mL of substrate/chromogen which was TMB (Tetramethylbenzidine). The plates were shaken and incubated for five minutes. When the color changed, the reaction was stopped by adding 100 mL 2N H2SO4 and

incubated for 10 minutes. Then, the ELISA plate was read using ELISA reader on the wavelength of 450 nm.

Statistical analysis

The ELISA result was descriptively and statistically analyzed. The determination of the difference of the response of cytokine of each group infected by high virulence (bang87), low virulence (Pml287) isolates and control were used one-way analysis of variance (ANOVA) and non-parametric test of Mann Withney. Spearman Correlation test was used to determine the association between the parameters.

RESULT AND DISCUSSION

Immunopathogenity of the *T. evansi*

Normal PCV value on the mice used as a reference in this study was normal PCV value range from all mice before infection (0 dpi) and the control group which is around 43-54%. The PCV value under 43% was categorized as an anemia. Average value of PCV decrease in every experimental interval showed in Figure 1.

Group of mice infected by high virulence isolate (Bang87) the PCV value decreased by average 27.1% at 4 dpi, while the low virulence (Pml287) group by average 31.2-50.7% at the 8-24 dpi respectively. This indicated that mice infected with *T. evansi* both high and low virulence had anemia. The control group had an average normal value of PCV until the end of the study (24 days).

The Bang87 group experienced simultaneous death at 5 dpi, so the next observation was performed only on the PML 287 and the control groups. Statistical analysis result showed that PCV level at 4 dpi was significantly difference ($P<0.05$) between the three groups (Bang87, PMl287 and control). The T-test result also showed a significant difference ($P<0.05$) between the Pml287 group and control at 8-24 dpi.

This study result showed no correlation between the level of parasitemia with hematocrit value ($P>0.05$). The level of parasitemia showed a fluctuation in every interval of experimental of mice infected by Pml287 (Figure 2), while the hematocrit value tended to decrease from the beginning to the end of the study (Figure 1). As well as between the hematocrit value

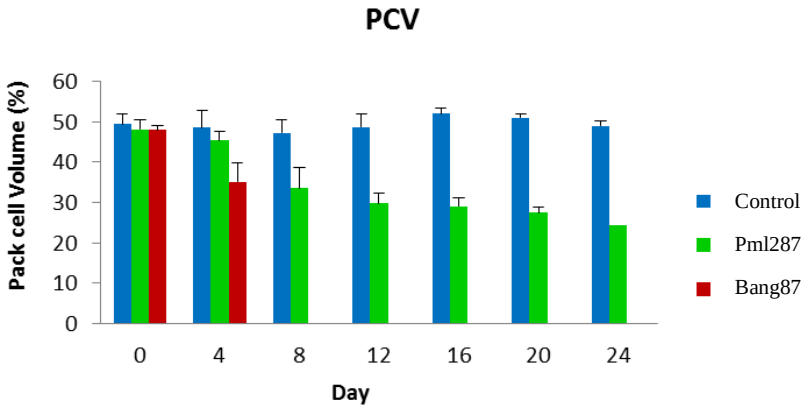

Figure 1. Packed Cell volume of high virulence (Bang 87) and Low virulence (Pml287) *T. evansi* infection in DDY mice at 24 days observation with 4 days intervals. * represent statistical difference between infected and control groups ($P<0.05$, n= 5/each group).

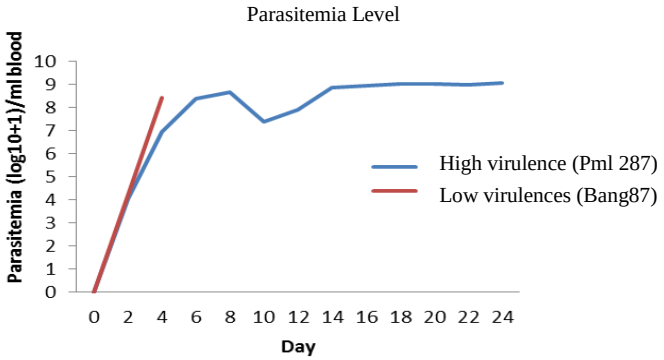

Figure 2. Parasitemia level of high virulence (Bang 87) and low virulence (Pml 287) *T. evansi* isolates.

with the survival time did not show a correlation. The sensitive mice strain (DDY) infected by *T. evansi* low virulence isolate (Pml287) showed lower hematocrit value then the mice infected with *T. evansi* high virulence isolate (Bang87). However, the mice infected with Pml287 isolate survived up to 24 dpi.

Profile of pro imflammatory (TNF-α, IFN-γ) and anti implammatory (IL-10) cytokine level

The result of ANOVA analysis showed that TNF-α level at 4 dpi was significantly different on those three treatments (P<0.05). The profile of TNF-α level on the Bang87 group incisively increased on the peak of parasitemia (4 dpi) sevenfold of the control (Figure 3). Then, this group show simultaneous death on 5 dpi so that the TNF-α level was not available for further observation.

The result of Mann Whitney analysis showed that the increase in TNF-α level during of 4-20 dpi was significantly different between the Pml287 group and the control (P<0.05). The Pml287 group showed a fluctuative profile of TNF-α level following its parasitemia pattern (figure 3) by increasing on 4 and 8 dpi by 2 and 3 fold each of the control. The decrease was at the 12 dpi. Then, there was an incisive increase on 16 dpi (about 4 fold of the control). The decrease in the TNF-α level are similar to the control occur at 20-24 dpi until dead at 26 dpi.

ANOVA analysis result at IFN-γ level at 4 dpi also showed significant difference (P<0.05) between those three groups. The profile of IFN-γ level on the Bang87 similar to TNF-α: the incisive increase in IFN-γ at 4 dpi (sevenfold of the average level of the control) followed by the simultaneous mice death at 5 dpi.

According to the result of Mann Whitney analysis, it also showed that IFN-γ level significantly different (P<0.05) between Pml287 and control at 8-20 dpi. Increase pattern of IFN-γ of Pml287 group was fluctuative which was occure at 4-24 dpi. The increase of almost twofold of the control occured at 4 dpi then continued to increase into fourfold at 8 dpi. The IFN-γ level decreased at 12 dpi and then increasing fivefold at 16 dpi followed by a decrease to near the normal level at 24 dpi (Figure 4)

The ANOVA analysis showed that the IL-10 level at 4 dpi of Bang87 and Pml287 groups was significantly different (P<0.05) with the control. The IL-10 level on Bang87 showed an incisive increase at 4 dpi by fourfold of the average level of the control followed by simultaneous death at 5 dpi.

The result of Mann Whitney analysis of the IL-10 level of Pml287 showed significant different (P<0.05) with the control during 8-20 dpi. The increase of the IL-10 level of Pml287 began at 4 dpi by threefold of the control. This increase was maintained until reaching the peak at 16 dpi, then decreased at 20 dpi. After the decrease, the IL-10 level was not significantly different (P>0.05) with the control at 24 dpi (Figure 5).

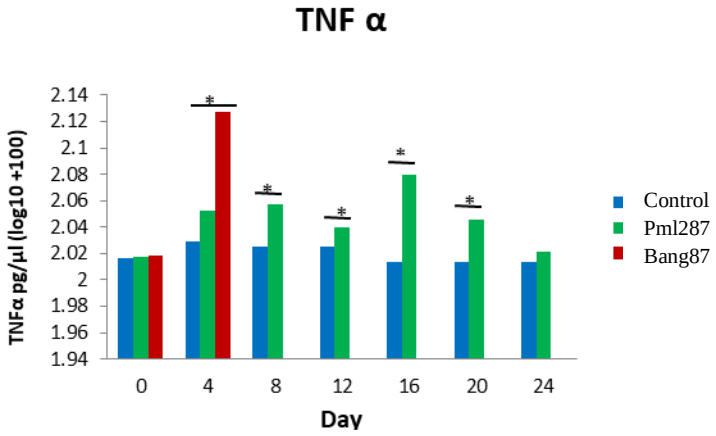

Figure 3. Serum level of pro inflamatory cytokines (TNF-α) in DDY mice infected by high virulence (Bang 87) and low virulence (Pml 287) *T. evansi* isolate compared with uninfected controls. *represent statistical difference between infected and control groups (P<0.05, n= 5/each group).

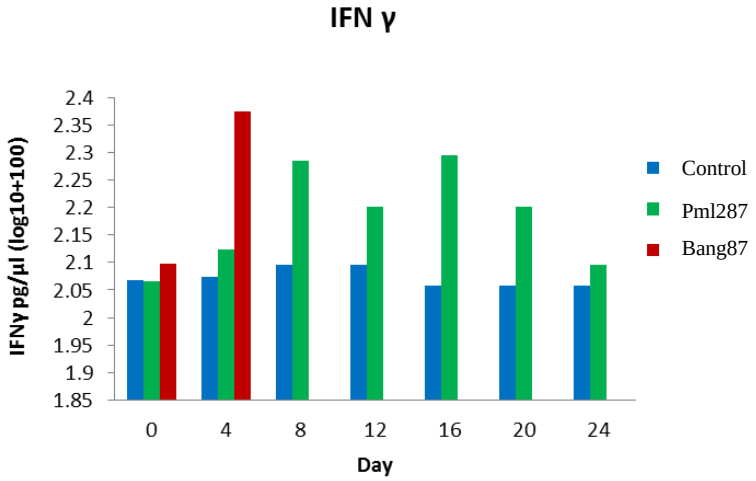

Figure 4. Serum level of proinflamatory cytokines (IFN-γ) in DDY mice infected by high virulence (Bang 87) and low virulence (Pml 287) *T. evansi* isolates compared with uninfected controls. * represent statistical difference between infected and control groups (P<0.05, n= 5/each group).

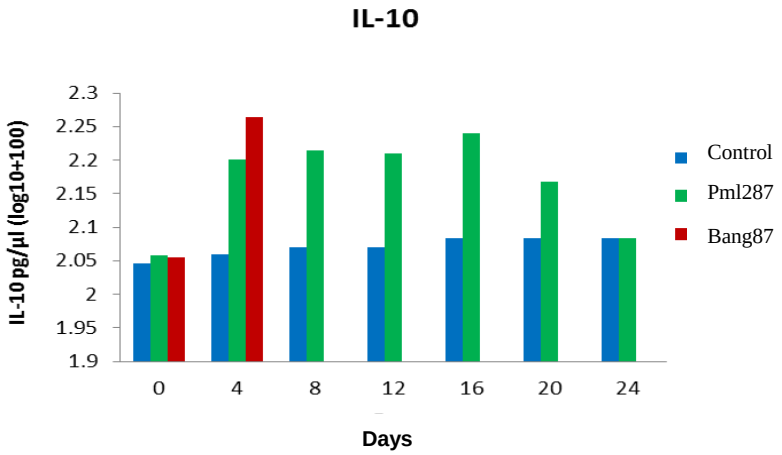

Figure 5. Median of IFN-γ level on mice serum infected by *T. evansi* with high virulence (Bang87), low virulence (Pml287) and control (without infection of *T. evansi*). The *sign shows a significant difference between treatments (P<0.05).

The relationship of IFN-γ level to the IL-10

The correlation of the IFN-γ and IL-10 showed that in the group infected by *T. evansi* isolate Pml287 and control showed an identic result with the balance of the IFN-γ with IL-10. However, there was a slight increase in Il-10 level. In contrast to the Bang 87 group, the IL-10 tended to decrease (Figure 6). The analysis result of spearman correlation between the IFN-γ and IL-10 at 4 dpi of the mice infected by Bang87 isolate showed a significant negative correlation (P<0.05). Analysis result showed that the increase of IFN-γ level was not followed with the increase of IL-10. In the other word, higher IFN-γ, the more IL-10 level decrease. The analysis result on the Pml287 group showed a not significant positive correlation (P>0.05). This indicated that there was an increase in IFN-γ level followed by an increase in IL-10 level (Figure 7).

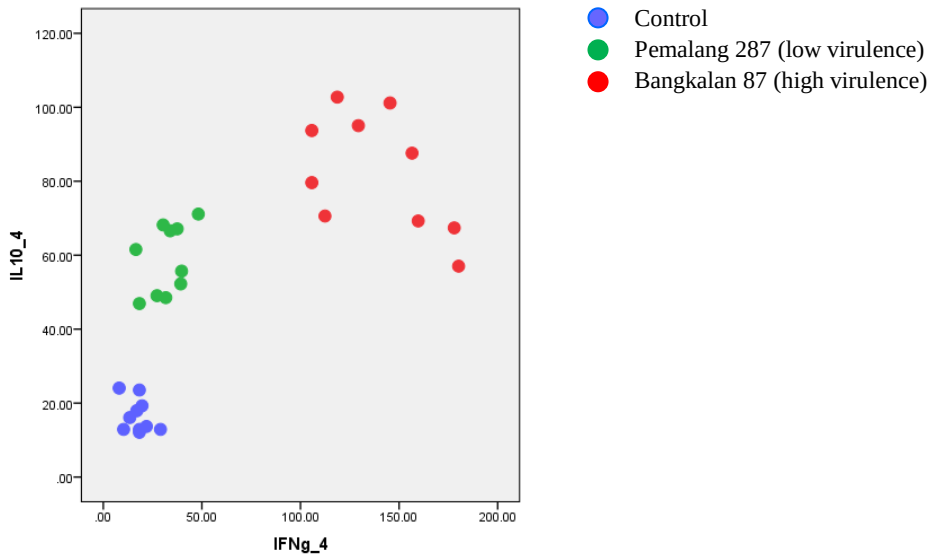

Figure 7. Scatter diagram showing distribution pattern of control group and mice infected by *T. evansi* isolate Bang87 and Pml287 to the expression correlation of the IFN-γ and IL-10.

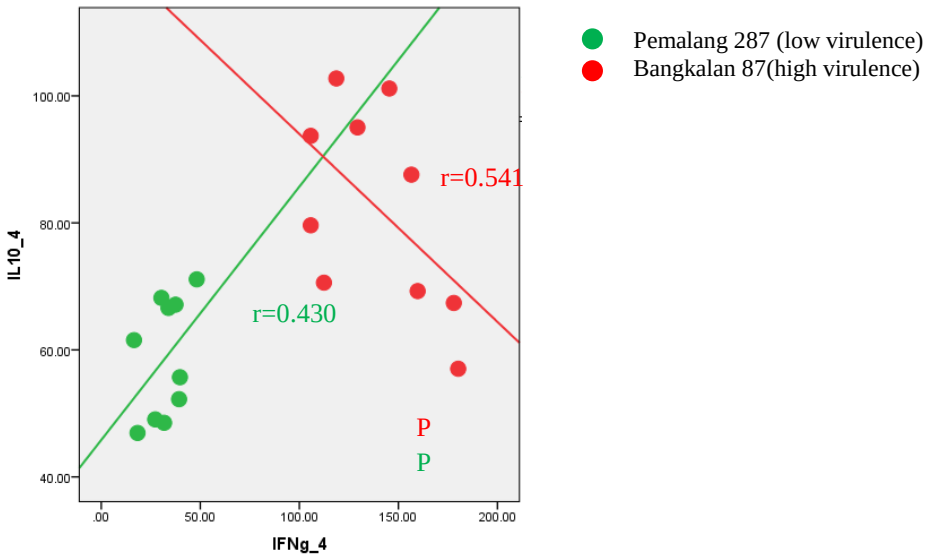

Figure 8. Scatter diagram showing distribution pattern of mice infected by *T. evansi* isolate Bang87 and Pml287 according to the analysis of expression correlation of IFN-γ and IL-10.

Discussion

The difference of PCV profile between isolate T. evansi with high virulence (Bang 87) and low virulence (Pml 287)

Verdillo et al. (2012) reported that all the mice infected by *T. evansi* will showing a significant decrease in PCV followed by anemia progressive. However, the decrease in PCV by 27.1% on mice infected by Bang87 isolate is expected not as the main cause of the early death but contributed to the multiple organ dysfunction (MOD). This can be explained by the observation result of the mice infected by Pml287 isolate . The decrease in PCV value of mice due to the infection was 31.2-50.7%, but some mice were able to survive up to 28 days. However, Groenveld et al. (2008) stated that although the most cases of anemia are mild, the decrease of red blood cells amount will decrease the ability to absorb oxygen from lungs which in a long term it can cause the damage or multiple organ dysfunction including aritmia and heart failure. Differ with the surra, anemia related to the immunopathology is reported as the main cause of death in the Nagana case caused by *T. vivax*.

According to the parasitemia level, mice infected by Bang87 isolate experienced a decrease in PCV value at the peak of parasitemia. This incident was not found in mice infected with Pml287 isolate. The PCV value tended to decrease, but its parasitemia level was fluctuative until the mice dead at 28 dpi. This phenomenon is identic with the African trypanosomiasis case caused by *T. congolense*. The anemia was detected at the beginning of infection when the parasite was found in the blood circulation. Furthermore, anemia was maintained even until the parasite amount in the blood was low. This condition proves that anemia is not directly induced by the number of parasite in the blood, but is mediated by the cytokine. In many studies, it is stated that there is a correlation between anemia and inflammation (Naessens et al. 2005). This state is supported by Magez et al. (2004) who reported that anemia in a mouse infected by *T. brucei* is not correlated to the parasite amount and the survival time of the host.

One of the cytokines played a role in anemia process is tumor necrosis factor-a (TNF-α). The anemia in a mouse infected by *T. brucei* rhodesiense is mediated by the TNF-α (Naessens et al. 2004). A study on the C57BL/6 mice strain which resistant to *T. evansi* and *T. brucei* proves that the development of anemia through the TNF-α mediated by TNF-R2 signal. This is supported by a study result of Magez et al. (1999) who proved that TNF-α is a key moderator involved in parasitemia control process and a pathology damage on mice infected by *T. brucei*. This result is in accordance with the statement that the peak of parasitemia of mice infected by Bang87 isolate at 4 dpi was increased of TNF-α level, otherwise, this causes the decrease of PCV value (anemia). Even though statistically did not show any significant correlation ($P>0.05$) between the TNF-α level and parasitemia amount and the decrease of PCV value, there was a tendency PCV value and TNF-α level to be inversely proportional. An increase in the TNF-α level in mice infected by Bang87 isolate was up to sevenfold compared to the control. In contrast with this result, the mice infected by *T. congolense* shows that the TNF-α did not play an important role in the anemia (Naessens et al. 2005).

A role of TNF-α in the anemia process also occurred in the mice infected by Pml287 isolate. This Profile pattern of the TNF-α on mice was different to the Bang87 isolate. An increase in the TNF-α level in mice infected by Pml287 isolate was fluctuative by around twofold to fourfold compared to the control following its parasitemia pattern (Figure 1). However, the PCV value tended to decrease since the first peak of parasitemia until the mice dead (at 26 dpi). The TNF-α contributes in the anemia process at different level due to its activity on hemopoiesis by decreasing erythropoietin and inhibiting proliferation of the precursor cell (Jelkmann 1998) or playing a role in hyperactivation of macrophage system (Larroche & Mouthon 2004).

Pro-inflammatory cytokine profile (IFN-γ, TNF-α,) and anti-inflammatory (IL-10) in mice infected with high virulence (Bang 87) and low virulence (Pml287)

Response pattern of cytokine during parasite infection is able to describe interaction between the parasite agent and host sensitivity (Sher & Coffman 1992). The cytokine is an immune system regulator which works by keeping up the balance between the type 1 (pro-inflammatory cytokine) and type 2 (anti-inflammatory cytokine) in influencing a manifestation of a disease. On trypanosomiasis infection, lymphocyte produces IFN-γ as a response to the parasite antigen which then will activate macrophage to increase its ability in destroying organism through phagocytosis. The activated macrophage will induce the production of TNF-α and IL-10 cytokines. Those cytokines have an important role in replicating the parasite of the host (Magez et al. 2007).

Isolate Bang 87 (high virulence)

This study results showed that mice infected by Bang87 and Pml287 isolate increased regulation of IFN-γ and TNF-α during infection period. The mice infected with isolate Bang87 showed extreme production of IFN-γ and TNF-α (by sevenfold of the

control respectively) on the peak of parasitemia (4 dpi). Magez et al. (1997) said that pro-inflammatory cytokine such as IFN-γ and TNF-α contributes to the defense mechanism of the host against an infection. On the infection of *T. brucei* and *T. congolense*, IFN-γ and TNF-α cytokine are reported having an important role on the parasitemia control and survival time of the host (Hertz & Filutowicz 1998; Magez et al. 2007; Naessens et al. 2004). However, an excessive cytokine response can be harmful the host even causing death (Magez et al. 1999; Magez et al. 2004).

The increase of pro-inflammatory cytokine level was also followed by an increase in anti-inflammatory (IL-10) cytokine. The IL-10 level increased fivefold from the control at 4 dpi. However, the IL-10 increase is allegedly unable to offer sufficient negative control due to the incisive increase of the pro-inflammatory cytokine (IFN-γ). This condition is allegedly as the main cause of mice death in a short time (5 dpi) due to a cytokine storm.

Shi et al. (2003) said that early mortality of sensitive mice (BALB/c) infected by *T. congolense* is triggered by the excessive synthesis of IFN-γ leading to a Systemic Inflammatory Response Syndrome (SIRS). This phenomenon is initiated by the fagositosis trypanosome by kupffer cell which ultimately causes swelling and apoptosis of the cell kupffer, lesion focal of liver parenchyma cell, capiler swelling, hypotension, body temperature decrease and the host becomes limp (Shi et al. 2003; Shi et al. 2006). Then it is stated that the SIRS is related to the excessive production of the pro-inflammatory cytokine such as TNF-α, IL-6, IL-12 and IFN-γ (Kaushik et al. 2000; Stijlemans et al. 2010; Uzonna et al. 1998). It is reinforced by Abbas & Litchman (2009) who said that TNF-α, IL-1 and chemokine are the main cytokine involved in monocyte and neutrophil to the location of the infection. The high concentration of the TNF-α can cause a thrombus on the surface of endothelial, increasing blood pressure due to the decrease of heart muscle contraction, dilatation and blood vessel puncture and body metabolism failure.

The SIRS phenomenon is also reported on the severe infection case of gram-negative bacteria leading a deadly clinical syndrome called "septic shock" (Abbas & Litchman 2009). Coldewey et al. (2013) said that sepsis is a complex clinical symptom aggregate caused by the systematic response of the individual in facing the infection which manifests on various clinical symptoms. This syndrome is a cause of the MOD or severe sepsis leading to the death. The mechanism causing the sepsis is cardiovascular dysfunction including the microvascular disfunction, the activation of leucocyte and the effect of excessive production of a pro inflamatory cytokine such as TNF-α and IL-1β (Krishnagopalan et al. 2002).

The cytokine storm is also proved by Guilliams et al. (2009) by knocking out the IL-10 gen on the C57BL/6 resistant mice infected by *T. brucei brucei* which causing an early death. Therefore, the IL-10 is a very important cytokine to control the emergence of pro inflammatory cytokine storm causing the early death due to the effect of SIRS.

In terms of inflammation signal activation by nature immune system cell, the failure of compensation of the IL-10 may be caused by the blocade activity of IFN-γ against the certain protein on the activation process of IL-10. Saraiva & O'Garra (2010) described that IL-10 expression of macrophage and dendritic cells can be induced through TLR or non-TLR pattern (Figure 9.a). Activation of TLR and its molecule adaptor, the myeloid differentiation primary-response protein 88 (MYD88) and TIR-domain-containing adaptor protein inducing IFN-β (TRIF) activate the extracellular path of signal-regulated kinase 1 (ERK1) and ERK2, p38 and nuclear factor-κB (NF-κB) (Saraiva & O'Garra 2010). The activation of this path results in an induction of the expression of the pro-inflammatory (IFN-γ) and anti-inflammatory (IL-10) cytokine (Saraiva & O'Garra 2010). While, the activation path of the non-TLR through dendritic cell (DC) specific ICAM3-grabbing non-integrin (DC-SIGN) and RAF1 that play a role to increase the induction of TLR2 for then producing IL 10. On the macrophage, the role of nucleotide-binding oligomerization domain 2 (NOD2) signal on the IL-10 induction is similar with the TLR2.

This study results showed the IL-10 production did not compensate the production of IFN-γ in mice infected by Bang87 isolate at 4 dpi which was a day before the dead at 5 dpi. That is allegedly to be led by two factors (Figure 9). The first factor (Figure 9a) is the presence of positive and negative feedback interference of IL-10 on macrophage the expression of dual-specificity protein phosphatase 1(DUSP1) which will reduce IL-10 production is not compensated by positive feedback increased expression regulation of locus 2 a progression tumour (TPL2) to increase the production of TL-10. The second factor is the high level of IFNγ will disturb the path of phosphoinositide 3-kinase (PI3K)–AKT which will release glycogen synthase kinase 3 (GSK3). Normally, the GSK3 will block the expression of IL-10 with its action on the transcription factor of cAMP response element-binding protein (CREB) and activator protein 1 (AP1) where the Il-10 production is inhibited by IFN-γ through its effect on PI3K (Saraiva & O'Garra 2010).

Pml 287 *T. evansi* isolate (low virulence)

The mice infected with Pml287 had longer survival time (more than 18 days). This condition also occurs in resistance mice (C57BL/6) infected with *T. brucei*

(Namangala et al. 2001b). The IFN-γ and TNF-α level slowly increased and fluctuated following the level of its parasitemia. At the peak of parasitemia, the level of IFN-γ increased, and then decreased where there is a relapse. That pattern indicates a regulation of cytokine through negative feedback so that the cytokines do not harm the physiology of the host which is able to extend the life of mice. An increase in IL-10 level was estimated to able to maintain the balance of the pro inflammatory cytokine (IFN-γ). This mechanism is able to extend the average survival time of mice. The decrease in IL-10 level followed by death started from 20 dpi. Namangala et al. (2009) and Noël et al. (2002) said that the response of type-1 cytokine (IFN-γ, TNF-α) which then turned into type-2 cytokine (IL-10) related to the characteristic of resistance phenotype of mice. An important role of IL-10 on trypanotolerant has been proved well using mouse deficient to gen IL-10 on the *T. brucei* model (Namangala et al. 2001b). That statement has been confirmed by Shi et al. (2003) who provided anti IL-10 receptor therapy on mice C57BL/6 infected by *T. congolense*. This therapy is able to stimulate a neutralization of the IFN-γ with the antibody anti IFN-γ, so that the mice can survive longer. A similar result is also reported by Guilliams et al. (2009) who proved a fact that the mice deficient to IL-10 shows immunopathology and reduce its survival time drastically because mice can not control the production of very strong immune response of the type-1 (pro-inflammatory) cytokine at the beginning of infection.

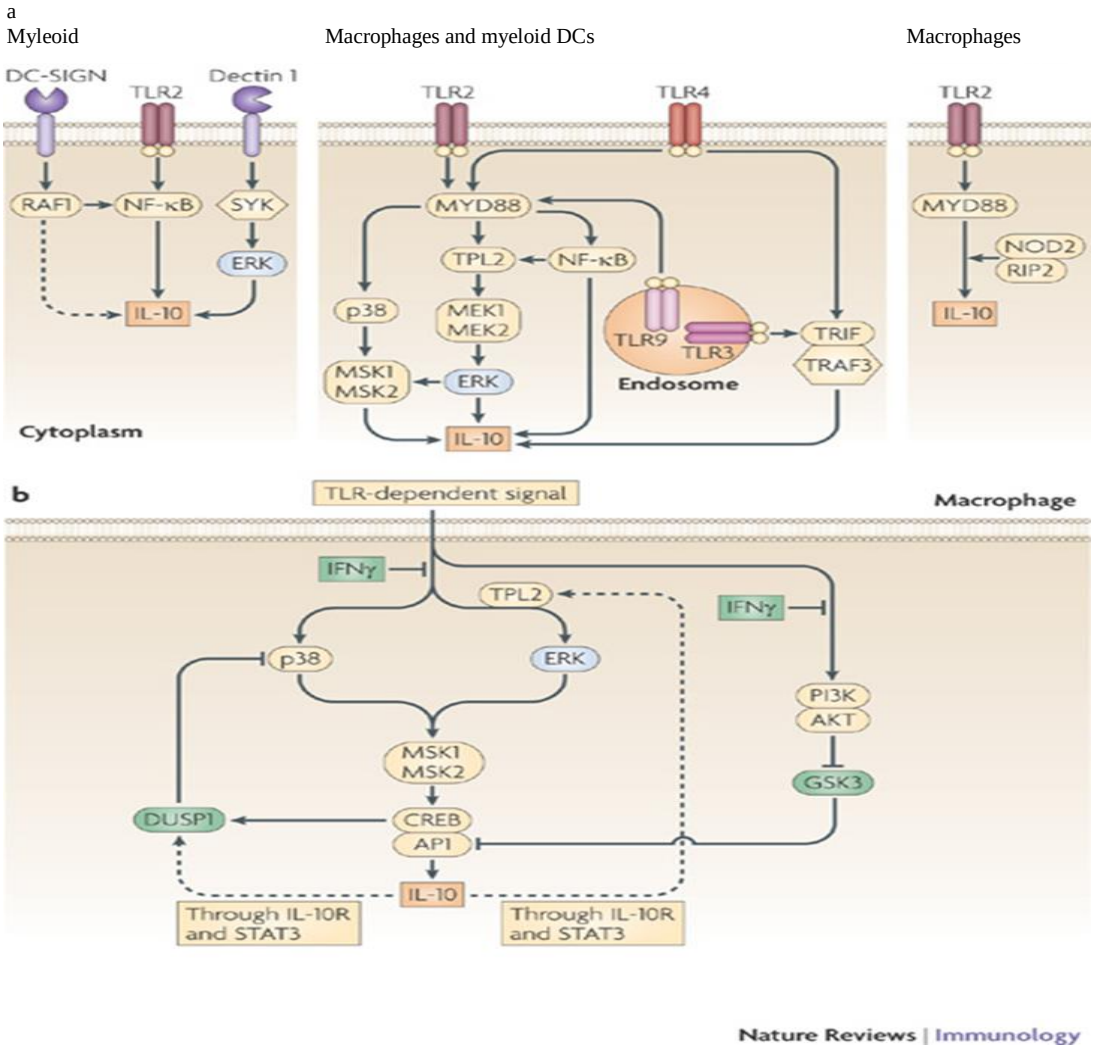

Figure 9. (a) Expression of IL-10 on macrophage and dendritic through TLR ao non TLR path. (b) Positive and negative regulation of IL-10 (Saraiva & O'Garra 2010).

Titus et al. (1991) stated that the response of the first host on the infection of protozoa is a secretion of TNF-α, IL-1β and IL-6 cytokines. The combination of the activity of those three cytokines can cause leukocytosis, fever and the production of acute phase proteins. This can be explained on a study conducted by Sawitri et al. (2016) on a description of the DDY mice leucocyte infected by high virulence *T. evansi* with a leukocytosis on the first peak of parasitemia. While mice infected by low virulence *T. evansi*, the leukocytosis occurred at 8 dpi which was 2 days after the first peak of parasitemia with increasing numbers of leukocytes up to 24 dpi.

The early death of mice in this study can be described with many reasons. First, the multiplication rate of parasite on blood and central nervous system of the host can harm the host due to the excessive exploitation of the host causing severe disease and death (Biswas et al. 2001; Habila et al. 2011). Secondly, an imbalance of cytokine production of pro inflammatory cytokine such as TNF-α, IL-6, IL-12 and IFN-γ (Kaushik et al. 2000; Shi et al. 2003). The uncontrolled IFN-γ production causes an imbalance in IFN-γ and IL-10 response leading to early death (Shi et al. 2006). Third, an acute anemia related to parasitemia wave (the trypanosome in the blood) and the severity of disease (Adamu et al. 2008).

CONCLUSION

There is a difference response of pro inflammatory and anti-inflammatory cytokine between mice which infected by Bang 87 and Pml 287 isolates. Early deaths in mice infected by Bang 87 isolate were suspected as a result of the response of systemic inflammation syndromes characterized by elevated IFN-γ levels that were not adequately compensated by anti-inflammatory. Anemia contributes to the cause of death in mice that support multiple organ failures (multiple organ dysfunction).

ACKNOWLEDGMENT

Acknowledgements are addressed to the Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture-Republic of Indonesia for the finansial support allocated in the APBN T.A 2013 where this project could be conducted. The authors acknowledge to Prof. dr. Muhammad Sadikin DSc for the discussion and Mr. Eko Setyo Purwanto, Mr. Farlin Nefho, and Mr. Edi Satria for their laboratory works.

REFERENCES

Abbas A, Litchman A. 2009. Basic immunology. Philadelphia (USA): Saunders, Elsevier.

Adamu S, Ibrahim NDG, Nok AJ, Esievo KAN. 2008. Sialyltransferase activity probably counteracts that of sialidase as one of the possible mechanisms of natural recovery or stabilization of erythrocyte mass in trypanosome-infected animals - A perspective. Afr J Biotechnol. 7:4992–5001.

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. Cell. 124:783–801.

Almeida I, Camargo M, Proc´opio D. 2000. Highly purified glycosylphosphatidylinositols from Trypanosoma cruzi are potent proinflammatory agents. EMBO Journal. 19:1476–1485.

Almeida I, Gazzinelli R. 2001. Proinflammatory activity of glycosylphosphatidylinositol anchors derived from *Trypanosoma cruzi*: Structural and functional analyses. J Leukoc Biol. 70:467–477.

Barkhuizen M, Magez S, Atkinson RA, Brombacher F. 2007. Interleukin-12p70-dependent interferon- gamma production is crucial for resistance in African trypanosomiasis. J Infect Dis. 196:1253–1260.

Basset C, Holton J, O'Mahony R, Roitt I. 2003. Innate immunity and pathogen-host interaction. Vaccine. 21:512–513.

Biswas D, Choudhury A, Misra KK. 2001. Histopathology of *Trypanosoma* (Trypanozoon) *evansi* infection in Bandicoot Rat.I.Visceral organs. Exp Parasitol. 99:148–159.

Coldewey SM, Rogazzo M, Collino M, Patel NS, Thiemermann C. 2013. Inhibition of IκB kinase reduces the multiple organ dysfunction caused by sepsis in the mouse. Dis Model Mech. 6:1031-1042.

Coller S, Mansfield J, Paulnock D. 2003. Glycosylinositolphosphate soluble variant surface glycoprotein inhibits IFN-γ-induced nitric oxide production via reduction in STAT1 phosphorylation in African trypanosomiasis. J Immunol. 171:1466–1472.

Darji A, Beschin A, Sileghem M, Heremans H, Brys L, De Baetselier P. 1996. In vitro simulation of immunosuppression caused by Trypanosoma brucei: active involvement of gamma interferon and tumor necrosis factor in the pathway of suppression. Infect Immun. 64:1937-1943.

Debierre-Grockiego F, Azzouz N, Schmidt J, Dubremetz JF, Geyer H, Geyer R, Weingart R, Schmidt RR, Schwarz RT. 2003. Roles of glycosylphosphatidylinositols of Toxoplasma gondii. Induction of tumor necrosis factor-alpha production in macrophages. J Biol Chem. 278:32978–32993.

Eckersall PD, Gow JW, McComb C, Bradley B, Rodgers J, Murray M, Kennedy PG. 2001. Cytokines and the acute phase response in post-treatment reactive encephalopathy of Trypanosoma brucei brucei infected mice. Parasitol Int. 50:15-26.

Gao W, Wortis HH, Pereira MA. 2002. The Trypanosoma cruzi trans-sialidase is a T cell-independent B cell mitogen and an inducer of non-specific Ig secretion. Int Immunol. 14:299-308

Gazzinelli R, Denkers E. 2006. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nat Rev Immunol. 6:895–906.

Ghalib HW, Ahmed M, Steven G. 1993. Interleukin 10 Production correlates with pathology in human leishmania donovani infections. J Clin Invest. 92:324–329.

Groenveld HF, Januzzi JL, Damman K, van Wijngaarden J, Hillege HL, van Veldhuisen DJ, van der Meer P. 2008. Anemia and mortality in heart failure patients a systematic review and meta-analysis. J Am Coll Cardiol. 52:818–827.

Guilliams M, Movahedi K, Bosschaerts T, VandenDriessche T, Chuah MK, Hérin M, Acosta-Sanchez A, Ma L, Moser M, Van Ginderachter JA, Brys L, De Baetselier P, Beschin A. 2009. IL-10 dampens TNF/inducible nitric oxide synthase-producing dendritic cell-mediated pathogenicity during parasitic infection. J Immunol. 182:1107–1118.

Habila N, Inuwa MH, Aimola IA, Udeh MU, Haruna E. 2011. Mechanisms of *Trypanosoma evansi* infections. Res Vet Sci. 93:13-17.

Harris TH, Cooney NM, Mansfield JM, Paulnock DM. 2006. Signal transduction, gene transcription, and cytokine production triggered in macrophages by exposure to trypanosome DNA. Infect Immun. 74:4530-4537.

Hertz CJ, Filutowicz H. 1998. Resistance to the African trypanosomes is IFN- gamma dependent. J Immunol. 161:6775–6783.

Jelkmann W. 1998. Proinflammatory cytokines lowering erythropoietin production. J Interferon Cytokine Res. 18:555–559.

Jittapalapong S, Pinyopanuwat N, Inpankaew T, Sangvaranond A, Phasuk C, Chimnoi W, Kengradomkij C, Kamyingkird K, Sarataphan N, Desquesnes M, Arunvipas P. 2009. Prevalence of *Trypanosoma evansi* infection causing abortion in dairy cows in Central Thailand. Kasetsart J Nat Sci. 43:53-57.

Kaushik RS, Uzonna JE, Zhang Y, Gordon JR, Tabel H. 2000. Innate resistance to experimental African trypanosomiasis: differences in cytokine (TNF-alpha, IL-6, IL-10 and IL-12) production by bone marrow-derived macrophages from resistant and susceptible mice. Cytokine. 12:1024–1034.

Kitani H, Black SJ, Nakamura Y, Naessens J, Murphy NB, Yokomizo Y, Gibson J, Iraqi F. 2002. Recombinant tumor necrosis factor alpha does not inhibit the growth of African trypanosomes in axenic cultures. Infect Immun. 70:2210-2214.

Krishnagopalan S, Kumar A, Parrillo JE, Kumar A. 2002. Myocardial dysfunction in the patient with sepsis. Curr Opin Crit Care. 8:376–388.

Larroche C, Mouthon L. 2004. Pathogenesis of hemophagocytic syndrome (HPS). Autoimmun Rev. 3:69-75.

Mabbott NA, Coulson PS, Smythies LE, Wilson RA, Sternberg JM. 1998. African trypanosomose infections in mice that lack the interferon-c receptor gene: nitric oxide-dependent and independent suppression of T-cell proliferative responses and the development of anaemia. Immunol. 94:476–480.

Magez S, Radwanska M, Drennan M, Fick L, Baral TN, Brombacher F, De Baetselier P. 2006. Interferon-gamma and nitric oxide in combination with antibodies are key protective host immune factors during *Trypanosoma congolense* Tc13 infections. J Infect Dis. 193:1575–1583.

Magez S, Stijlemans B, Caljon G, Eugster HP, De Baetselier P. 1993. Murine tumour necrosis factor plays a protective role during the initial phase of the experimental infection with *Trypanosoma brucei* brucei. Parasite Immunol. 15:635–641.

Magez S, Truyens C, Merimi M, Radwanska M, Stijlemans B, Brouckaert P, Brombacher F, Pays E, De Baetselier P., 2004. P75 tumor necrosis factor-receptor shedding occurs as a protective host response during African trypanosomiasis. J Infect Dis. 189:527-539.

Magez S, Radwanska M, Drennan M, Fick L, Baral TN, Allie N, Jacobs M, Nedospasov S, Brombacher F, Ryffel B, De Baetselier P. 2007. Tumor necrosis factor (TNF) receptor-1 (TNFp55) signal transduction and macrophage-derived soluble TNF are crucial for nitric oxide-mediated Trypanosoma congolense parasite killing. J Infect Dis. 196:954-962.

Magez S, Radwanska M, Beschin A, Sekikawa K, De Baetselier P. 1999. Tumor necrosis factor alpha is a key mediator in the regulation of experimental *Trypanosoma brucei* infections Infections. Infect Immun. 67:3128–3132.

Magez S, Geuskens M, Beschin A. 1997. Specific uptake of tumor necrosis factor-α is involved in growth control of Trypanosoma brucei. J Cell Biol. 137:715–727.

Maina N, Ngotho JM, Were T, Thuita JK, Mwangangi DM, Kagira JM, Ndung'u JM, Sternberg J. 2004. Proinflammatory cytokine expression in the early phase of Trypanosoma brucei rhodesiense infection in vervet monkeys (*Cercopithecus aethiops*). Infect Immun. 72:3063-3065.

Medzhitov R, Janeway CJ. 2000. Innate immunity. N Engl J Med. 343:338–344.

Mekata H, Konnai S, Mingala CN, Abes NS, Gutierrez CA, Dargantes AP, Witola WH, Inoue N, Onuma M, Murata S, Ohashi K. 2013. Isolation, cloning, and pathologic

analysis of Trypanosoma evansi field isolates. Parasitol Res. 112:1513–1521.

Murray RK, Granner DK, Mayes PA, Rodwell. 2003. Harper's illustrated biochemistry. 26th ed. USA: Appleton & Lange.

Naessens J, Kitani H, Momotani E, Sekikawa K, Nthalea JM, Iraqi F. 2004. Susceptibility of TNF-α-deficient mice to *Trypanosoma congolense* is not due to a defective antibody response. Acta Trop. 92:193–203.

Naessens J, Kitani H, Nakamura Y. 2005. TNF- a mediates the development of anaemia in a murine *Trypanosoma brucei* rhodesiense infection, but not the anaemia associated with a murine *Trypanosoma congolense* infection. Clin Exp Immunol. 139:405–410.

Namangala B, De Baetselier P, Noël W, Brys L, Beschin A. 2001a. Alternative versus classical macrophage activation during experimental African trypanosomosis. J Leuko Biol. 69:387–396.

Namangala B, Noël W, De Baetselier P, Brys L, Beschin A. 2001b. Relative contribution of interferon-gamma and interleukin-10 to resistance to murine African trypanosomosis. J Infect Dis. 183:1794-1800.

Namangala B, Baetselier PD, Beschin A. 2009. Both type- I and type-II responses contribute to murine trypanotolerance. J Vet Med Sci. 71:313–318.

Noël W, Hassanzadeh G, Raes G, Namangala B, Daems I, Brys L, Brombacher F, Baetselier PD, Beschin A. 2002. Infection stage-dependent modulation of macrophage activation in *Trypanosoma congolense*-resistant and -susceptible mice. Infect Immun. 70:6180–6187.

Paulnock DM, Coller SP. 2001. Analysis of macrophage activation in African trypanosomiasis. J Leukoc Biol. 69:685-690.

Payne RC, Sukanto IP, Partoutomo S, Jones TW. 1994. Efficacy of cymelarsan treatment of suramin resistant *Trypanosoma evansi* in cattle. Trop Anim Health Prod 26:92-94.

Payne RC, Sukanto IP, Bazeley K, Jones TW. 1993. The effect of *Trypanosoma evansi* infection on the oestrous cycle of Friesian Holstein heifers. Vet Parasitol. 51:1–11.

Quan N, Mhlanga JD, Whiteside MB, McCoy AN, Kristensson K, Herkenham M. 1999. Chronic overexpression of proinflammatory cytokines and histopathology in the brains of rats infected with *Trypanosoma brucei*. J Comp Neurol. 414:114-130.

Ralph P, Nakoinz I, Sampson-Johannes A, Fong S, Lowe D, Min HY, Lin L. 1992. IL-10, T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. J Immunol. 148:808–814.

Reed S, Pihl D, Grabstein KH. 1989. Immune deficiency chronic *Trypanosoma cruzi* infection recombinant IL-1 restores Th function for antibody production. J Immunol. 142:2067-2071.

Saraiva M, O'Garra A. 2010. The regulation of IL-10 production by immune cells. Nat Rev Immunol. 10:170–181.

Sawitri DH. 2016. Virulence study of *Trypanosoma evansi* isolates from Indonesia and identification of molecular marker based on microsatellite DNA and cytokine profile analyses in mice (*Mus musculus*) (Thesis). [Depok (Indones)]: University of Indonesia.

Sher A, Coffman RL. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. Ann Rev Immunol. 10:385–409.

Shi M, Wei G, Pan W, Tabel H. 2006. Experimental African trypanosomiasis: a subset of pathogenic, IFN-gammaproducing, MHC class II-restricted CD4+ T cells mediates early mortality in highly susceptible mice. J Immunol. 176:1724–1732.

Shi M, Wei G, Pan W, Tabel H. 2005. Impaired Kupffer cells in highly susceptible mice infected with *Trypanosoma congolense*. Infect Immun. 73:8393–8396.

Shi M, Wei G, Pan W, Tabel H. 2004. Trypanosoma congolense infections: antibody-mediated phagocytosis by Kupffer cells. J Leuko Biol. 76:399–405.

Shi M, Pan W, Tabel H. 2003. Experimental African trypanosomiasis: IFN-gamma mediates early mortality. Eur J Immunol. 33:108–118.

Shoda L, Kegerreis K, Suarez C. 2001. DNA from protozoan parasites *Babesia bovis*, *Trypanosoma cruzi*, and *T. brucei* is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor alpha, and nitric oxide. Infect Immun. 69:2162–2171.

Sileghem M, Darji A, Hamers R, De Baetselier P. 1989. Modulation of IL-1 production and IL-1 release during experimental trypanosome infections. Immunol. 68:137–139.

Sileghem M, Darji A, Instituut PDEB. 1991. *In vitro* simulation of immunosuppression caused by *Trypanosoma brucei*. Immunol. 73:246–248.

Silva JS, Morrissey PJ, Grabstein KH, Mohler KM, Anderson D, Reed SG. 1992. Interleukin 10 and interferon g regulation of experimental Trypanosoma cruzi infection. J Exp Med. 175:169-174.

Stijlemans B, Vankrunkelsven A, Caljon G, Bockstal V, Guilliams M, Bosschaerts T, Beschin A, Raes G, Magez S, De Baetselier P. 2010. The central role of macrophages in trypanosomiasis-associated anemia: rationale for therapeutical approaches. Endocr Metab Immune Disord Drug Targets. 10:71-82.

Titus RG, Sherry B, Cerami A. 1991. The involvement of TNF, IL-1 and IL-6 in the immune response to protozoan parasites. Trends Immunol. 12:A13–A16.

Trinchieri G. 1995. Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Ann Rev Immunol. 13:251–276.

Uzonna JE, Kaushik RS, Zhang Y, Gordon JR, Tabel H. 1998. Experimental murine *Trypanosoma congolense* infections. II. Role of splenic adherent CD3+Thy1.2+ TCR-alpha beta- gamma delta- CD4+8- and CD3+Thy1.2+ TCR-alpha beta- gamma delta- CD4-8- cells in the production of IL-4, IL-10, and IFN-gamma and in trypanosome-elicited immunosuppression J Immunol. 161:6189-6197.

Verdillo JC, Lazaro JV, Abes NS, Mingala CN. 2012. Comparative virulence of three Trypanosoma evansi isolates from water buffaloes in the Philippines. Exp Parasitol. 130:130–134.

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Acknowledgement

Editorial board and executive editor of Indonesian Journal for Animal and Veterinary Science (IJAVS) extent high appreciation to the expertises of peer reviewer of IJAVS (Volume 22 No. 3 2017).

1.	Dr. Nurhayati D. Purwantari	:	Cultivation - IRIAP
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We hope this good collaboration would be continued in the future in improving IJAVS quality.

Jurnal Ilmu Ternak dan Veteriner

IJAVS *Indonesian Journal of Animal and Veterinary Sciences*

Volume 22, Number 3, September 2017 ISSN 0853-7380 E-ISSN 2252-696X

LIST OF CONTENT

Registered in: