Effect of Condensed Tannin of Leucaena and Calliandra Leaves in Protein Trash Fish Silage on *In Vitro* Ruminal Fermentation, Microbial Protein Synthesis and Digestibility

Efka Aris Rimbawanto[1)*], Lies Mira Yusiati[2)], Endang Baliarti[2)] and Ristianto Utomo[2)]

[1)]Faculty of Animal Science, Jenderal Soedirman University Jl. Dr. Soeparno No. 60, Purwokerto, Indonesia
[2)]Faculty of Animal Science, Gadjah Mada University, Jl. Fauna 3, Yogyakarta, Indonesia
[*]Corresponding author email: fk.aris.r@gmail.com

Abstract. Two plants as condensed tannin (CT) sources were supplemented to protein trash fish silage (TFS) to observe their effect on *in vitro* ruminal fermentation product, micobial protein synthesis, and digestibility. CT supplementation on protein trash fish silage was on control proportion (0), under optimum level (2.0%), at optimum level (4.0%), and above optimum level (6.0%) of g TFS); of protein precipitation of bovine serum albumin (BSA) with CT from *L. leucocephala;* and under optimum level (1.5%), at optimum level (3.0%) and above optimum level (4.5% of g TFS), BSA protein precipitation with CT from *C. calothyrsus*. The effect on degradation in ruminal fluid and ruminal fluid followed by incubation in HCl-pepsin was evaluated using a modified two-step *in vitro* method. The CT level of *L. leucocephala* and *C. calothyrsus* was 4 and 6%, respectively with protein precipitation BSA was 26.25 and 31.77 g BSA/g CT, respectively. CT supplementation to trash fish silage, ruminal fermentation product (NH_3-N, C_2, C_3, and total VFAs) and digestibility (DM and OM) decreased and increased total CP digestibility (in HCl-pepsin). The difference was attributed to CT source without affecting ruminal microbial protein synthesis. CT of *L. leucocephala* was better in increasing total protein digestibility (70%) than that of *C. calothyrsus* (15%). It indicated that CT of *C. calothyrsus* was less effective in protected TSF protein degradation in rumen compared to that of *L. leucocephala.*

Key words: condensed tannin, Leucaena, Calliandra leaves, rumen fermentation, trash fish silage

Abstrak. Dua tanaman sebagai sumber tanin kondensasi ditambahkan pada protein silase ikan rucah (SIR) untuk mengetahui pengaruhnya terhadap produk fermentasi dan sintesis protein mikrobia rumen, dan kecernaan secara *in vitro*. Penambahan tanin kondensasi pada protein silase ikan rucah pada proporsi kontrol (0), di bawah level optimum (2,0%), level optimum (4,0%), dan di atas level optimum (6,0% of g SIR) presipitasi protein *bovine serum albumin* (BSA) dengan tanin kondensasi *L. leucocephala* dan di bawah level optimum (1,5%), level optimum (3,0%), dan di atas level optimum (4,5% of g SIR) presipitasi protein BSA dengan tanin kondensasi *C. calothyrsus*. Pengaruh degradasi di dalam cairan rumen dan inkubasi dalam HCl-pepsin dievaluasi menggunakan metode *in vitro* dua tahap. Kadar tanin kondensasi *L. leucocephala* dan *C. calothyrsus* berturut-turut 4, 6% dengan presipitasi protein BSA 26.25, 31.77 g BSA/g tanin kondensasi. Penambahan tanin kondensasi pada silase ikan rucah, produk fermentasi rumen (NH_3-N, C_2, C_3, and VFAs total) dan kecernaan (BK and BO) di dalam rumen menurun dan meningkatkan kecernaan PK total (dalam HCl-pepsin/paska rumen). Perbedaan tersebut dipengaruhi oleh sumber tanin kondensasi tanpa mempengaruhi sintesis protein mikrobia di dalam rumen. Penggunaan tanin kondensasi dari *L. leucocephala* lebih baik dalam meningkatkan kecernaan protein total SIR 70% dibanding *C. calothyrsus* 15%. Ini menunjukkan bahwa tanin kondensasi dari *C. calothyrsus* kurang efektif melindungi degradasi protein SIR di dalam rumen dibanding tanin kondensasi dari *L. leucocephala.*

Kata kunci: tanin kondensasi, daun Leucaena, daun Calliandra, fermentasi rumen, silase ikan runcah

Introduction

Some species from Leguminoseae family like *Leucaena leucocephala* and *Calliandra calothyrsus* are cultivated on either low or medium fertility soil. Indonesian farmers are used to utilizing both legume to improve the protein quality in ruminant feed due to high crude protein content. Supplementation *C. calothyrsus* as cattle feed is limited by high-condensed tannins (CT) up to 24% dry matter (DM) (Hess et al., 2008). CT medium content of

L. leucocephala (5-7% DM) results in low effect on rumen (Tieman et al., 2008b). *C. calothyrsus* and *L. leucocephala* are rich in phenolic compound mainly CT with high affinity in protein, carbohydrate and other plant elements. In animal nutrition, tannin is potential to improve digestive utilization from protein ruminant feed. This feature may reduce nutrient digestibility, particularly where tannin binds feed protein and protects it against microbial degradation. Tannin-protein complex binding is likely to occur in pH 3.5-7.0 and will break in pH <3.5 (Min et al., 2003), as in rumen and abomasum pH. The detached complex bound of acidic tannin-protein in abomasum can increase the amount of protein in intestine (Makkar, 2003). Protein digestive process depends on the separation of tannin-protein complex in abomasum, because complex tannin-protein cannot fully detach respective to post rumen pH and the formation of complex tannin-protein in rumen; accordingly, it is related to different tannin and protein structure (Muller-Harvey, 2006).

CT supplementation in protein enriched feed could decrease *in vitro* protein degradation in rumen and decrease ammonia concentration (Bento et al., 2005; Hess et al., 2008). Local and hybrid *L. leucocephala* protect protein in rumen differently (Huang et al., 2010), so as with *C. calothyrsus* cultivar (Tiemann et al., 2008b). The difference is shown in equal CT concentration in purified *C. calothyrsus* and *L. leucocephala,* demonstrating different characteristic regarding *in vitro* ruminal nutrient degradation (Tiemann et al., 2008a; Cortes et al., 2009).

The present research evaluated CT of *C. calothyrsus* and *L. leucocephala* in binding bovine serum albumin protein and the effect of different CT level of *C. calothyrsus* and *L. leucocephala* on trash fish silage as protein source in ruminal fermentation, based on *in vitro* ruminal fermentation product, protein microbe and total digestibility.

Materials and Method

Legume sample (*L. leucocephala* and *C. calothyrsus*) was grown in Baturraden (Purwokerto, Indonesia) at 650m elevation with 3250 mm/yr average rainfall and 24^{o}C average temperature in yellowish brown andosol soil and latosol association and brown regosol. Upon harvest, leaves were sun-dried and finely ground (88% DM) with 200 mesh sieve. Tannin extraction was conducted using Soxhlet method (Markom et al., 2007), then 5g DM of each *C. calothyrsus* and *L. leucocephala* leaf powder was put in timble (Whatman 25x100 mm) with 150ml dissolvent which was 70% acetone containing 1% HCL (Chavan et al., 2001; Shahidi and Naczk, 2004). Heating temperature was adjusted to 6-time dissolution in 1h and extraction was conducted for 3h. Crude extract solution was thickened with rotary evaporator at ≤ 80°C to optimize component degradation. Measuring total phenol, total tannin and CT was based on method by Makkar et al. (2007). CT precipitation of *C. calothyrsus* and *L. leucocephala* with BSA protein was according to Makkar et al. (2007).

Testing the optimum precipitation of CT of *C. calothyrsus* and *L. leucocephala* leaves with BSA protein was conducted as the basis of determining the concentrate level of CT in trash fish silage protein in *in vitro* test. The evaluated variable *in vitro* was 250 mg DM trash fish silage (corresponding to 113 mg of CT) placed in 100 ml glass tube. CT was supplemented under optimum level, at optimum level and above optimum level of CT precipitation of *C. calothyrsus* and *L. leucocephala* leaves in BSA protein and without CT as control. Each treatment and incubation time was repeated 5 times. Glass tube containing experimental sample was incubated at 39ºC in two sets, 48h for *in vitro* ruminal degradation and 96h for total in vitro degradation.

In vitro technique following procedure by Tilley and Terry (1963) modified by Utomo (2010) was applied to determine degradation of

rumen microbe and HCl/pepsin. Contrary to Tilley and Terry procedure (1963), the first set of this method filtered the content of glass tube after 48h incubation to estimate fermentation product (ammonia and volatile fatty acids), DM, OM and ruminal microbe protein from the obtained supernatant. On the second set, upon 48h incubation, the glass tube was added with HCl/pepsin and incubated up to 96h to estimate total CP digestibility.

Rumen fluid was extracted from Bali cattle attached with rumen fistula, fed with 3% body weight DM consisted of 70% *Pennisetum purpureum* and 30% concentrate given at 08.00 and 15.00 *ad libitum*. Rumen fluid was taken before morning feeding, then filtered and added with McDougall buffer solution at 39°C with ratio 1:4 (v/v) as inoculum source. The inoculum (25 ml) was added to glass tube containing experimental sample then anaerobic incubated at 39°C.

In the first glass tube set, fermentation was ceased after 48h by adding 1ml mercuric chloride (50mg/ml), while the second set was added with 6 ml HCl 20% (v/v) and 2 ml pepsin 5% (w/v, 10 FIP-U/mg, EC 3.4.23.1, Merck) followed by 48h anaerobic incubation. After incubation, the content of the first glass tube was filtered using glass wool-coated crucible, and supernatant was centrifuged at 10.000g for 15 minutes to separate supernatant and microbial sediment (Yusiati et al., 2007). Supernatant was analyzed for ammonia level with phenol-hypochlorite reaction (Weatherburn, 1967) and volatile fatty acids (acetic, propionic, butyric) with gas chromatography. Microbial cell protein was analyzed from the microbial sediment using Lowry method (Plummer, 1987). After incubation, the content of second set was filtered using filter paper (no. 10300012, S&S Whatman), and the residue was oven-dried (12h, 105^{o}C) to obtain constant weight (AOAC, 2005) and to analyze the crude protein using Kjeldahl method (AOAC, 2005).

The obtained data was subject to analysis of variance with completely randomized design, followed by Duncan's new multiple range test upon significant effect between treatment averages. CT supplementation on protein trash fish silage was on control proportion, under optimum level, at optimum level and above optimum level or 0, 2.0, 4.0 and 6.0% of g TFS, respectively, of protein precipitation of bovine serum albumin (BSA) with CT from *L. leucocephala*. And under optimum level, at optimum level and above optimum level or 1.5, 3.0 and 4.5% of g TFS, BSA protein precipitation with CT from *C. calothyrsus*. Data were processed with IBM SPSS Statistics (2013).

Results and Discussion

Tannin of *L. leucocephala* and *C. calothyrsus*

The mean of proximate analysis tannin of *C. calothyrsus* dan *L. leucocephala* leaves is presented in Table 1. Ash content of *C. calothyrsus* dan *L. leucocephala* leaves was < 80 g/kg DM and the crude protein was >200 g/kg DM. Crude protein of *C. calothyrsus* dan *L. leucocephala* leaves was 83.2 g/kg DM dan 189.5 g/kg DM, respectively, and the ether extract was 96.2 g/kg DM and 110.6 g/kg DM, respectively. Protein and crude fiber was lower than > 280 g/kg DM and > 205 g/kg BK, respectively (Susanti and Marheiniyanto, 2014), but similar to ≤ 180 g/kg DM and 226 g/kg BK, respectively (Utomo, 1997). Crude protein (>200 g/kg DM) of *C. calothyrsus* dan *L. leucocephala* leaves was within tropical legume range.

C. calothyrsus leaves contained higher total phenol, total tannin and CT than those of *L. leucocephala* leaves. Total tannin and total phenol of either *C. calothyrsus* or *L. leucocephala* leaves were common with Jayanegara et al. (2011). CT of *L. leucocephala* leaves (40g/kg DM) and *C. calothyrsus* leaves (69.6 g/kg DM) in this research was higher than values of 18 and 22 g/kg DM (Jayanegara et al., 2011), 28 and 21 g/kg DM, respectively

Table 1. Dry matter, ash, crude protein, crude fiber, crude fat, nitrogen free extract (NFE), total phenol, total tannin, condensed tannin, protein-phenol precipitation, and protein-tannin of *L. leucocephala* and *C. calothyrsus*.

Item	Leaf	
	L. leucocephala	*C. calothyrsus*
Dry matter, %	38.07±0.36	33.13±0.28
Ash, %	7.89±0.01	6.06±0.03
Crude protein , %	22.16±1.03	20.54±0.32
Crude fiber, %	8.32±0.10	18.95±0.36
Ether extract, %	9.62±0.62	11.06±0.02
Nitrogen free extract, %	52.03±1.73	43.41±0.10
Total phenols, %	7.59±1.87	10.93±1.28
Total tannins, %	6.21±1.65	8.70±1.29
CTs, %	4.00±0.30	6.96±0.63
Protein BSA precipitated with phenols (mg BSA/mg DM leaf)	7.94±0.37	11.84±0.66
Protein BSA precipitated with CTs (g BSA/g CTs)	26.25	31.77

(Santoso et al., 2013). Concentration of CT in *C. calothyrsus* and *L. leucocephala* leaves was lower than values of 72 – 83 g/kg DM and 189 – 230 g/kg DM, respectively (Tiemann et al., 2010).

Varied chemical composition of *C. calothyrsus* and *L. leucocephala* leaves of previous research was due to environmental factor (temperature, rainfall and soil fertility) and plant species. Environmental factors corresponding to tannin production are plants grown in high temperature region (tropical), dry land and acidic that cause higher tannin concentration compared to the opposite condition (Tiemann et al., 2010).

Effects on cencentration and properties of condesed tannins

Precipitation protein by phenol compound served as the basis to determine the optimization of precipitation tannin of *C. calothyrsus* and *L. leucocephala* leaves with BSA protein. The amount of phenol for optimum BSA protein bound was 7.94 ± 0.37 mg BSA/100mg DM in *L. leucocephala* leaves and 11.84 ± 0.66 mg BSA/100mg DM in *C. Calothyrsus* leaves.

Measuring tanin-bound protein BSA was by measuring the tanin-unbound protein using Lowry method. Result showed that 1 g CT of *C. calothyrsus* and *L. leucocephala* leaves could bind 31.77 g and 26.25 g BSA protein, respectively. Tannin condensability of *C. calothyrsus* and *L. leucocephala* leaves was not significantly different from that of other plant. Sasongko et al. (2010) reported that 1 g CT of jackfruit leaves bound 28.89 g BSA protein. CT bound with BSA protein was different across the source of CT due to different prodelphinidin and procyanidin ratio, molecule weight (Huang et al., 2010). Tiemann et al. (2010) reported that CT profile of *C. calothyrsus* was dominated by prodelphinidin, followed by procyandin and pelargonidin, while *L. leucocephala* was dominated by procyandin followed by prodelphinidin and pelargonidin. In CT whose monomer was dominated by prodelphinidin would bind protein more significantly (Jones et al., 1976: Tiemann et al., 2010).

Huang et al. (2010), compared various molecule weights of *Leucaena spp*., demonstrated that CT with low molecule weight had higher affinity to bind BSA protein. Besides binding with protein, CT also formed complex bound with mineral, carbohydrate and lipid, thereby lowering affinity to bind protein (Smith et al., 2005).

Effects on *in vitro* ruminal fermentation product and microbial protein synthesis

Protecting trash fish silage protein with CT of *C. calothyrsus* and *L. leucocephala* leaves significantly affected (P<0.01) the decrease of ammonia level (Table 2). Ammonia level of unprotected trash fish silage fermentation was higher than the protected one with CT. The increase of CT level could not lower ammonia level in rumen (P<0.05). Ammonia level of all treatments was above optimum level for bacteria growth. Satter and Slyter (1974), the optimum need for ruminal bacteria growth was 5 - 8 mg/dl rumen fluid. Result demonstrated that protected fish protein silage was partly bound with CT.

The decreasing amonia concentration compared to control in trash fish silage protected with CT of *C. calothyrsus* and *L. leucocephala* leaves after 48h fermentation indicated the decrease of proteolysis bacteria activity in rumen. In line with Getachew et al. (2008a,b) and Temann et al. (2008a,b), CT of *C. calothyrsus* and *L. leucocephala* leaves could hinder protein enriched feed degradation in rumen *in vitro* and decrease ammonia concentration (Bento et al., 2005; Hess et al., 2008; Cortés et al., 2009). Similarly, Mohammadabadi and Chaji (2012) stated that supplementing CT from oak fruit could lower amonia concentration of sunflower seed waste whose protein was highly soluble in rumen. The difference in lowering amonia concentration during rumen fermentation between trash fish silage added with CT of *C. calothyrsus* and *L. leucocephala* leaves was due to different tannin source. The source of tannin or types of tannin had different effect on ruminal bacteria species (Sivakumaran et al., 2004).

Table 2. Effect of CT of *L. Leucocephala* and *C. calothyrsus* on *in vitro* ruminal fermentation product, rumen microbe synthesis and digestibility

Item	Trash fish silages (TFS)							SEM	P-*value*
		CT of Leucaena (% of g protein TFS, DM basis)			CT of Calliandra (% of g protein TFS, DM basis)				
	0	2.0	4.0	6.0	1.5	3.0	4.5		
NH_3-N[2] (mg/dL)	27,53^{c}	18,74^{a}	18.38^{a}	15.97^{a}	20.24^{b}	20.85^{b}	19.04^{ab}	2.122	0.000
VFAs[3] (m*M*)									
C_2	47.83^{c}	45.46^{bc}	45.53^{bc}	34.98^{a}	37.46^{ab}	33.45^{a}	33.78^{a}	2.928	0.008
C_3	16.27^{cd}	17.40^{d}	15.49^{bcd}	12.84^{abc}	13.15^{abc}	11.74^{a}	11.95^{ab}	1.041	0.007
C_4	6.73	7.46	5.87	6.29	6.40	4.99	7.08	0.617	0.207
Total	78.87^{d}	75.08^{cd}	70.45^{bcd}	57.08^{abc}	51.36^{ab}	45.59^{a}	47.06^{a}	1.062	0.003
C_2:C_3	3.35	2.84	3.09	3.01	3.04	3.08	2.79	0.341	0.950
Microbial protein (mg/dL)	21.785	15.702	18.169	14.011	18.340	13.831	17.904	2.064	0.147
IVDMD[4] (% DM)	66.29^{d}	44.47^{ab}	42.53^{a}	41.60^{a}	53.74^{c}	53.90^{c}	50.76^{bc}	2.139	0.000
IVOMD[5] (% DM)	75.81^{c}	54.25^{a}	53.11^{a}	51.25^{a}	64.31^{b}	63.40^{b}	60.37^{b}	1.772	0.000
IVCPD[6] (% DM)	46.94^{a}	86.32^{d}	79.27^{c}	74.50^{c}	57.94^{b}	55.27^{b}	49.40^{a}	1.519	0.000

^{abcd}Means withinn a row without common superscript letter differ (P<0.05).

[1]0, 2.0, 4.0, 6.0, 1.5, 3.0, 4,5 = level of CT; 4.0 = optimum precipitation level of CT of *L. Leucocephala* with bovine serum albumin (BSA) protein ;3.0 = optimum precipitation level of CT of *C. calothyrsus* with bovine serum albumin (BSA) protein.

[2]N-NH_3 = nitrogen ammonia

[3]VFAs = Volatile Fatty Acids after 48h fermentation, C_2 =acetic acid; C_3 = propionic acid; C_4 = butyric acid.

[4]IVDMD = in vitro dry matter digestibility after 48 h

[5]IVOMD = in vitro organic matter digestibility after 48 h.

[6]IVCPD = in vitro crude protein digestibility after 96 h (in HCl/pepsin).

Volatile fatty acids derived from the fermentation of protected trash fish silage showed lower (P<0.01) acetic, propionic and butyric acid level, and total VFAs than those of control by increasing the level of CT. Butyric acid level or acetic and propionic ratio was not different (P>0.05) in protected trash fish silage. The increased CT level either of *C. calothyrsus* or *L. leucocephala* leaves did not affect (P>0.05) the decrease of acetic and propionic acid level of trash fish silage.

Similar ratio of acetic and propionic acid level was because the decreasing acetic acid was not followed by the increase of propionic acid, even propionic acid decreased as CT level increased. Hess et al. (2008) and Tiemann et al. (2008b) reported that the increasing legume CT in fermentation grass subtrate *in vitro* reduce the activity of fibrolytic microbe, thereby decreasing acetic acid concentration and increased propionic acid proportion. This research result showed that CT of *C. calothyrsus* and *L. leucocephala* leaves did not optimally reduce fibrolytic rumen microbe as seen from the non-increasing propionic acid concentration, and no different acetic and propionic ratio. However, the increased CT concentrate in this research lowered total volatile fatty acids and consistently lowered ruminal degradation of nitrogen fraction.

The effect of CT protection of *C. calothyrsus* and *L. leucocephala* leaves in trash fish silage fermentation on biomass synthesis of rumen microbe protein is reported in microbe protein level (Table 2). Microbial protein after 48h fermentation *in vitro* did not show difference (P>0.05) in trash fish silage protein protected by CT of *C. calothyrsus* and *L. leucocephala* leaves.

The non-different microbial protein level of trash fish silage protected by CT of *C. calothyrsus* and *L. leucocephala* leaves indicated that not all trash fish silage protein was bound with CT. It was demonstrated from result of protein deamination of trash fish silage that provided ammonia nitrogen for microbial protein synthesis. Ammonia concentration of trash fish silage protected with *C. calothyrsus* and *L. leucocephala* leaves in this research was 15.97 – 20.85 mg/dL, sufficient for rumen microbe growth. Supplementing CT from different plant to protein enriched feed did not generally affect microbial protein synthesis in rumen (Bento et al., 2005; Tiemann et al., 2008b; Wischer et al., 2013), but slightly lowered rumen microbe population by increasing the level of CT (McSweeney et al., 2001). It was due to the lack of nutrient for rumen bacteria. Nsahlai et al. (2011) reported that bacteria inhibition was viable through the interaction of CT with membrane, cell wall or extracelluler protein.

Effects on *in vitro* ruminal nutrient digestibility

Dry matter and organic matter digestibility of trash fish silage protected with CT of *C. calothyrsus* and *L. leucocephala* leaves decreased (P<0.01) compared to that of control (Table 2). Condensed tannins of *L. leucocephala* leaves indicated different dry matter and organic matter of trash fish silage (P<0.05) from that of *C. calothyrsus*. The decrease of dry matter and organic matter of protected trash fish silage showed the reduce degradation of dry matter and organic matter of trash fish silage in rumen which was not affected by the increase of CT level. Similar result by Cortes et al. (2009) and Tiemann et al. (2010) stated that *in vitro* ruminal dry matter degradation of soybean waste in rumen decreased as the level of CT of *C. calothyrsus* and *L. leucocephala* leaves increased.

Research result demonstrated that CT of *C. calothyrsus* and *L. leucocephala* leaves could inhibited rumen microbe, not only proteolytic but also fibrolytic as observed from the decrease of ammonia nitrogen, dry matter and organic matter digestibility, and VFAs level (acetic and propionic acid) without interfering microbial protein synthesis.

Total protein digestibility (soluble in HCL-pepsin) of trash fish silage protected with CT of *C. calothyrsus* and *L. leucocephala* leaves upon 96h incubation showed difference (P<0.01) by the increase of CT level. All levels of CT supplementation showed difference (P<0.05) with control total protein digestibility, except for 4.5%/g CT of *C. calothyrsus* leaves on trash fish silage protein was not different (P>0.05) from trash fish silage without tannin.

The optimum level of CT of *L. leucocephala* leaves bound with BSA protein was 26.25 mg BSA/mg CT, equal to 4%/g trash fish silage protein, and of *C. Calothyrsus* leaves was 31.77 mg BSA/mg CT equal to 3%/g trash fosh silage protein. Under optimum level of CT of *L. leucocephala* leaves or 2%/g trash fish silage protein showed the highest total protein digestibility (P<0.05) of all CT treatment levels.

The low total protein digestibility in trash fish silage without CT (46.94 ± 3.90% DM), showed that trash fish silage protein without CT was majorly degraded in rumen. Protecting trash fish silage protein with CT either with *C. calothyrsus* or *L. leucocephala* leaves lowered rumen protein degradation, thereby increasing total protein digestibility. The increase of CT level was not followed by the increase of *in vitro* total protein digestibility, in fact it decreased. It indicated that the increase of CT level lowered protein solubility of trash fish silage in HCL-pepsin. Tannin level that showed the best total protein digestibility was on 2%/g CT of *L. Leucocephala* of trash fish silage protein and 1.5 %/g CT of *C. Calothyrsus* of trash fish silage protein.

The average total protein digestibility between trash fish silage supplemented with CT of *L. leucocephala* leaves (80.03 ± 5.95% DM) was higher than that supplemented with CT of *C. calothyrsus* (54.20 ± 4.37% DM). It demonstrated that CT of *C. Calothyrsus* leaves was too strong to bind trash fish silage protein than *L. Leucocephala*, resulting in low HCL-pepsin solubility. According to Cortes et al. (2009) and Tiemann et al. (2008a; 2010), CT of *C. calothyrsus* was more effective than that of *L. leucocephala* in protecting soybean waste. The difference across studies was likely due to different location, source and level of CT.

The decrease of dry matter and organic matter digestibility in rumen along with the increase of CT level of *L. leucocephala* leaves was not followed by the increase of total protein (post rumen), it was because trash fish protein silage bound with CT was less soluble in HCl-pepsin. However, CT under optimum level bound with BSA protein namely 2% tannin condensed of *L. leucocephala* leaves showed the highest total protein digestibility (86.32 ± 1.12% DM).

The increase of CT level of *C. calothyrsus* did not change dry matter and organic matter digestibility, volatile fatty acids (acetic, propionic and butyric) or acetic and propionic ratio. Nitrogen fraction (ammonia concentration and total protein digestibility) also remained along with the increase of CT of *C. calothyrsus.* It indicated that CT of *C. calothyrsus* leaves had higher affinity in binding trash fish silage protein and was not highly soluble in HCl-pepsin, and bound with carbohydrate so the fermentation product and digestibility was lower than that with *L. leucocephala* leaves.

In vitro test of trash fish silage protected with CT of *L. leucocephala* was better than that of *C. calothyrsus*. This result was different from previous research by Stürm et al. (2007) and Tiemann et al. (2008a, 2010) that nutrient degradation in rumen was higher in CT of *C. calothyrsus* leaves than *L. leucocephala* leaves. The difference showed binding affinity of CT of *C. calothyrsus* leaves at 1.5% trash fish silage protein, in which strong protection made it not easily degradable in rumen not soluble in HCl-pepsin. Based on binding affinity with BSA protein (Table 1), CT of *C. calothyrsus* leaves had higher affinity than that of *L. leucocephala*. Tejirian and Xu (2011) stated that CT's high

affinity with protein would also reduced cellulose activity compared to the other phenol compounds, thereby lowering cellulolytic process in rumen. The decrease of cellulose activity in rumen microbe was the corresponding factor to the non-decreasing dry matter and organic matter digestibility of trash fish silage protected with CT of *C. calothyrsus* leaves.

Conclusions

Protecting CT of *L. leucocephala* and *C. calothyrsus* leaves in trash fish silage affected rumen fermentation, lowered ammonia concentration, dry matter and organic matter digestibility in rumen, increased total protein digestibility (post rumen) without affecting microbial protein synthesis in rumen. CT of *L. leucocephala* leaves was better than that of *C. calothyrsus* leaves in protecting trash fish silage protein *in vitro*, 2% CT of *L. leucocephala* leaves and 1.5% of *C. calothyrsus* leaves in trash fish silage was more effective in protecting silage protein of trash fish compared to other cultivar.

References

AOAC (Asociation of Official Agricultural Chemists). 2005. Official Methods of Analysis. Agricultural Chemical; Contaminats; Drugs Vol. 1. Asociation of Official Agricultural Chemists, Inc. Virginia.

Bento MBL, HPS Makkar and T Acamovic. 2005. Effect of mimosa tannin and pectin on microbial protein synthesis and gas production during *in vitro* fermentation of ^{15}N-labelled maize shoots. Animal Feed Science and Technology. 123–124:365–377.

Chavan UD, F Shahidi and M Naczk. 2001. Extraction of CTs from beach pea (*Lathyrus maritimus L*.) as affected by different solvents. Food Chemistry. 75:509–512.

Cortés JE, B Moreno, ML Pabón, P Avila, M Kreuzer, HD Hess and JE Carulla. 2009. Effects of purified CTs extracted from Calliandra, Flemingia and Leucaena on ruminal and postruminal degradation of soybean meal as estimated *in vitro*. Animal Feed Science and Technology. 151:194–204.

Getachew G, W Pittroff, DH Putnam, A Dandekar, S Goyal and EJ DePeters. 2008a. The influence of addition of gallic acid, tannic acid, or *quebracho* tannins to alfalfa hay on *in vitro* rumen fermentation and microbial protein synthesis. Animal Feed Science and Technology. 140: 444–461.

Getachew G, W Pittroff, EJ DePeters, DH Putnam, A Dandekar and S Goyal. 2008b. Influence of tannic acid application on alfalfa hay: *in vitro* rumen fermentation, serum metabolites and nitrogen balance in sheep. Animal. 2(3):381–390.

Hess HD, ML Mera, TT Tiemann, CE Lascano and M Kreuzer. 2008. *In vitro* assessment of the suitabilaity of replacing the low-tannin legume *Vigna unguiculata* with the tanniniferous legumes *Leucaena leucocephala, Flemingia macrophylla* or *Calliandra calothyrsus* in a tropical grass diet. Animal Feed Science and Technology. 147:105–115.

Huang XD, JB Liang, HY Tan, R Yahya, B Khamseekhiew, and YW Ho. 2010. Molecular weight and protein binding affinity of Leucaena CTs and their effects on *in vitro* fermentation parameters. Animal Feed Science and Technology. 159:81–87.

IBM SPSS Statistics. 2013. Version 22, IBM Corporation. USA.

Jayanegara A, E Wina, CR Soliva, S Marquardt, M Kreuzer and F Leiber. 2011. Dependence of forage quality and methanogenic potential of tropical plants on their fenolic fractions as determined by principal component analysis. Animal Feed Science and Technology. 163: 231–243.

Jones WT, RB Broadhurst and JW Lyttleton. 1976. The CTs of pasture legume species. Phytochemistry. 15:1407–1409.

Makkar HPS, P Siddhuraju and K Becker. 2007. Plant Secondary Metabolites. Humana Press Inc. New Jersey. Pp 67-81.

Makkar HPS. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Ruminant Research. 49:241–256.

Markom M, M Hasan, WRW Daud, H Singh and JM Jahim. 2007. Extraction of hydrolysable tannins from *Phyllanthus niruri Linn*.: Effects of solvents and extraction methods. Separation and Purification Technology. 52:487–496.

McSweeney CS, B Palmer, DM McNeill and DO Krause. 2001. Microbial interaction with tannins: nutritional consequences for ruminants. Animal Feed Science and Technology. 91:83-93.

Min BR, TN Barry, GT Attwood and WC McNabb. 2003. The effect of CTs on the nutrition and health of ruminants fed fresh temperate forages: a review. Animal Feed Science and Technology. 106:3–19.

Mohammadabadi T and M Chaji, 2012. The Influence of the plant tannins on *in vitro* ruminal degradation and improving nutritive value of sunflower meal in ruminants. Pakistan Veterinary Journal. 32(2):225-228.

Mueller-Harvey I. 2006. Unravelling the conundrum of tannins in animal nutrition and health: a review. Journal of the Science of Food and Agriculture 86: 2010–2037.

Nsahlai IV, FN Fon and NAD Basha. 2011. The effect of tannin with and without polyethylene glycol on *in vitro* gas production and microbial enzyme activity. South African Journal of Animal Science. 41(4):337-344.

Plummer DT. 1987. An Introduction to Practical Biochemistry. McGraw Hill Ltd. Bombay, New Delhi. 352 pages.

Santoso B, EW Saragih, and BT Hariadi. 2013. Effect of water extract of plants containing tannin on *in vitro* methanogenesis and fermentation characteristics of the grass Pennisetum purpureophoides. Journal of the Indonesian Tropical Animal Agriculture. 38 (1):47-54.

Sasongko WT, LM Yusiati, Z Bachruddin dan Mugiono. 2010. Optimalisasi pengikatan tanin daun nangka dengan protein *bovine serum albumin*. Buletin Peternakan. 34(3):154-158.

Satter LD and LL Slyter. 1974. Effect of ammonia concentration of rumen microbial protein production *in vitro*. British Journal of Nutrition. 32:199–208.

Shahidi F and M Naczk. 2004. Phenolics in Food and Nutraceuticals. CRC Press. Washington. 566 pages.

Sivakumaran S, AL Molan, LP Meagher, B Kolb, L. Foo, GA Lane, GA Attwood and K Fraser. 2004. Michael Tavendale a Variation in antimicrobial action of proanthocyanidins from Dorycnium rectum against rumen bacteria. Phytochemistry. 65:2485–2497.

Smith AH, E Zoetendal and RI Mackie. 2005. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. Microbial Ecolology. 50:197–205.

Stürm CD, TT Tiemann, CE Lascano, M Kreuzer and HD Hess. 2007. Nutrient composition and *in vitro* ruminal fermentation of tropical legume mixtures with contrasting tannin contents. Animal Feed Science and Technology. 138: 29–46.

Susanti S dan E Marhaeniyanto. 2014. Kadar saponin daun tanaman yang berpotensi menekan gas metan secara *in vitro*. Buana Sains. 14(1):29-38.

Tejirian A and F Xu. 2011. Inhibition of enzymatic cellulolysis by fenolic compounds. Enzyme and Microbial Technology. 48:239-247.

Tiemann TT, CE Lascano, HR Wettstein, AC Mayer, M Kreuzer and HD Hess. 2008a. Effect of the tropical tannin-rich shrub legumes *Calliandra calothyrsus* and *Flemingia macrophylla* on methane emission and nitrogen and energy balance in growing lambs. Animal. 2(5):790–799.

Tiemann TT, LH Franco, G Ramírez, M Kreuzer, CE Lascano and HD Hess. 2010. Influence of cultivation site and fertilisation on the properties of CTs and *in vitro* ruminal nutrient degradation of *Calliandra calothyrsus, Flemingia macrophylla and Leucaena leucocephala*. Animal Feed Science and Technology. 157:30–40.

Tiemann TT, P Avila, G Ramírez, CE Lascano, M Kreuzer and HD Hess. 2008b. *In vitro* ruminal fermentation of tanniniferous tropical plants: plant-specific tannin effects and counteracting efficiency of PEG. Animal Feed Science and Technology. 146:222–241.

Tilley JMA and RA Terry. 1963. A two-stage technique for the *in vitro* digestion of forage crops. Journal of the British Grassland Society. 18(2):104.

Utomo, R. 1997. Kemungkinan penggunaan daun kaliandra sebagai pengganti daun lamtoro untuk pakan kambing. Bulletin Peternakan. 21(1):56-62.

Utomo R. 2010. Modifikasi metode penetapan kecernaan *in vitro* bahan kering atau bahan organik. Bulletin Sintesis. ISSN: 0853-9812. 14(1):1 - 11.

Wheatherburn MW. 1967. Phenol-hypoclorite reaction for determination of ammonia. Analytical Chemmistry. 39: 971-974.

Wischer G, J Boguhn, H Steingass, M Schollenberger, and M Rodehutscord. 2013. Effects of different tannin-rich extracts and rapeseed tannin monomers on methane formation and microbial protein synthesis *in vitro*. Animal. 7(11): 1796-805.

Yusiati LM, Z Bachrudin, C Hanim dan WK Ratriningtyas, 2007. Pengaruh Daun Ketepeng Cina Terhadap Produksi Gas Methan, Asam Lemak Volatile Dan Mikrobia Pada Fermentasi Substrat Dengan Rasio Hijauan Dan Konsentrat Yang Berbeda Menggunakan Mikrobia Rumen Secara *In Vitro*. Proseding Seminar Nasional Dies Natalis ke-38 Fakultas Peternakan UGM. Yogjakarta. ISBN: 978-979-1215-10-7. Hal. 102-107.