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PREFACE

Indonesian Journal of Animal and Veterinary Sciences (IJAVS) has been indexed in DOAJ, CABI, EBSCO, Science and Technology Index (SINTA), Indonesian Publication Index (IPI), and Google Scholar. Since March 2018 IJAVS has fulfilled registration form to get index from Elsevier products (Scopus, Reaxys), Clarivate Analytics products (Emerging Sources Citation Index). IJAVS got S2 score from Sinta with H index 23.

In this edition, volume 23 No 2, we proudly present articles from animal and veterinary sciences including feed and nutritive technology, and chemical residue. The articles published in this edition are: “Manipulation of rumen fermentation by bioindustrial products of cashew nutshell (*Anacardium occidentale*) to reduce methane production”; “Performance of dairy calves fed diet containing Ca-palm oil fatty acid and *Sapindus rarak* fruit"; "The effect of glucomannan inclusion derived from porang tuber extract (*Amorphophallus oncophyllus*) on dietary protein utilization in broiler chicken"; "Correlation of electric conductivity values with the dairy milk quality"; “Clenbuterol residue in beef meat collected from several cities in Java island, Indonesia" and “Growth response of leucaena embryogenic callus on embryo age differences and Auxin 2,4-Dichlorophenoxyacetic acid".

We extent high appreciation to all peer reviewers who make this journal academically high value. Hopefully these articles would offer any benefit to readers and the end-users of technological innovation, and attract interests from other authors to contribute their papers to Indonesian Journal of Animal and Veterinary Sciences.

Chief Editor;

Bogor, June 2018

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LIST OF CONTENT

Manipulation of Rumen Fermentation by Bioindustrial Products of Cashew Nut Shell (*Anacardium occidentale*) to Reduce Methane Production

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ABSTRAK

Saenab A, Wiryawan KG, Retnani Y, Wina E. 2018. Manipulasi fermentasi rumen oleh produk bioindustri cangkang biji mete (*Anacardium occidentale*) untuk menekan produksi metana. JITV 23(2): 61-70. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1821

Salah satu cara menurunkan gas metana yang dihasilkan oleh ternak ruminansia adalah dengan penggunaan pakan aditif yang berasal dari tanaman. Tanaman yang cukup potensial adalah tanaman jambu mete (*Anacardium occidentale*) khususnya bagian cangkang biji. Cangkang dapat diproses menjadi 3 produk bioindustri yaitu *biofat, biochar, biosmoke*. Tujuan penelitian untuk mengevaluasi efektivitas 3 produk bioindustri terhadap produksi gas metana dan produk akhir fermentasi pakan dalam rumen secara *in vitro*. Penelitian menggunakan rancangan acak kelompok berdasarkan periode *in vitro*. Perlakuan terdiri dari Kontrol = Substrat (S), *Biofat*: S+0.25 µL/mL, S+0.5 µL/mL, S+0.75 µL/mL; *Biochar*: S+0.1 mg/mL, S+0.2 mg/mL, S+0.3 mg/mL; *Biosmoke*: S + 2.5 µL/mL, S+5.0 µL/mL, S + 7.5 µL/mL. Perlakuan diulang 2 kali dalam 4 kali *in vitro*. Variabel yang diukur adalah produksi gas total dan metana; pH; kecernaan bahan kering (BK); *organic* (BO) dan *neutral detergent fibre* (NDF); konsentrasi amonia (NH_3) dan konsentrasi VFA parsial dan total. Hasil menunjukkan bahwa *biofat, biochar* dan *biosmoke* dapat menurunkan produksi gas metana masing-masing 43,88%; 24,21%; 37,88% pada level tertinggi. Produksi NH_3 meningkat secara signifikan pada pemberian *biochar* level tertinggi. Molar proporsi asam asetat menurun dan asam propionat meningkat secara nyata ketika ditambahkan produk *biofat, biochar* dan *biosmoke* dibandingkan dengan kontrol. Nilai kecernaan BK menurun pada penambahan *biofat* dan *biosmoke* tetapi kecernaan BO tidak berbeda nyata pada penambahan *biochar* dibanding kontrol. Terdapat mekanisme yang berbeda antara biofat, biochar dan biosmoke dalam mempengaruhi fermentasi rumen. Disimpulkan bahwa *biofat, biochar* dan *biosmoke* dapat dimanfaatkan sebagai agen pereduksi gas metana dan meningkatkan asam propionat dalam rumen ternak ruminansia.

Kata Kunci: Cangkang Mete, *Biofat, Biochar, Biosmoke*, Fermentasi Rumen, Metana

ABSTRACT

Saenab A, Wiryawan KG, Retnani Y, Wina E. 2018 Manipulation of rumen fermentation by bioindustrial products of cashew nut shell (*Anacardium occidentale*) to reduce methane production. JITV 23(2): 61-70. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1821

One of the strategies to reduce methane produced by ruminants is by the application of feed additive from plant materials. One of the potential plants is cashew plant especially its shell. The cashew nut shell can be processed to become 3 bioindustrial products; ie biofat, biochar, biosmoke. The aim of this research was to evaluate the effectively of biofat, biochar and biosmoke in reducing methane and other end product of rumen fermentation. The experiment was arranged in block randomize design based on time series of in vitro to evaluate 3 levels and 3 types of bioindustrial. The treatments were Control (substrate=S), Biofat: S+0.25 µL/mL, S+0.5 µL/mL, S+0.75 µL/mL; Biochar: S+0.1 mg/mL, S+0.2 mg/mL, S+0.3 mg/mL; Biosmoke: S + 2.5 µL/mL, S+5.0 µL/mL, S + 7.5 µL/mL. Each treatment was done in duplicates and the in vitro experiment was repeated 4 times. The research measured total gas production; methane production; digestibility of dry matter, organic matter and neutral detergent fibre (NDF); ammonia concentration (NH_3); partial and total volatile fatty acids (VFA) concentration. The results showed that biofat, biochar and biosmoke reduced methane production by 43.88%, 24.21%, 37.88% at the highest level of inclusion, respectively. The NH_3 concentration slightly increased by biochar and biosmoke addition. Molar proportion of acetic acid decreased, while molar proportion of propionic acid increased by addition of the three bioindustrial products. Organic matter digestibility decreased significantly with biofat and biosmoke addition. The mechanism of biofat, biochar and biosmoke in affecting rumen fermentation was different. It can be concluded that the three bioindustrial products of cashew nut shell can be utilized as feed additive to reduce methane and increase propionic acid production in the rumen.

Key Words: Cashew Nut Shell, Biofat, Biochar, Biosmoke, Rumen Fermentation, Methane

INTRODUCTION

Global warming is a major environmental problem today. Some types of gas CO_2, N_2O and CH_4 are greenhouse gases (GHG) that cause global warming. Livestock, in particular ruminants contribute methane emissions for agricultural sector. Methane from ruminant are the result of methanogenic bacteria activity in the rumen. It was reported that dairy cows, cattle, and goat produce methane 110-145, 60-70, and 8 kg/head/year, respectively (Morgavi 2008). Therefore, methane emission from total population of ruminants in the world contributes 95% of total emissions of methane produced by livestock and humans, and approximately 18% of total GHG in the atmosphere (Kreuzer & Soliva 2008). This condition becomes a constraint in ruminant production development.

One of the strategies to reduce methane emissions is by use feed additives derived from plant extracts that contain secondary compounds, such as tannins (Beauchemin et al. 2007, Jayanegara et al. 2010), saponins (Hess et al. 2003; Wina 2012), essential oils (Patra & Yu 2012). Those three secondary compounds reported have low potency and short term effect on mitigating methane (Gerber et al. 2013). Therefore, researchers are still looking for alternatives of bioactive containing plant materials that safe both for environment and animal to mitigate methane from animal.

Cashew (*Anacardium occidentale*) are planted for its nut, whereas the nut shell which is 45-50% of the whole nut is rarely used (Muljohardjo 1991).The nut shell has 20-30% of fat content, which is ussualy extracted to produce cashew nut shell liquid (CNSL). The residue after the extraction may be thrown away. These cashew nut shells was processed to become three industrial products, namely biofat, biochar and biosmoke. The method to produce these bioindustrial products have been developed (Simpen 2008; Sudrajat & Salim 1994) and described in Saenab et al. (2016). The information on the utilization of these three products for livestock production is still very limited.

Biofat product or CNSL is hexane extracted fraction of cashew nut shell which contains several bioactive compounds (Saenab et al. 2016). The major compounds are anacardic acids, which are phenolic compounds attached to fatty acids (C_{15}). It has been reported that CNSL reduced methane and suppressed certain bacteria in the rumen (Watanabe et al. 2010; Shinkai et al. 2012; Mitsumori et al. 2014).

Biochar is a pyrolysis product from the cashew nut shell after hexane extraction. During the pyrolysis process, a mixture of liquid smoke (biosmoke) is also obtained. The BC from rice husk has been reported to have ability to suppress methane production in the rumen and increase body weight of cattle (Leng et al. 2012a). While biochar from bamboo reported increased goat's live weight (Do et al. 2006).

Liquid smoke is generally used as an anti-diarrhea for pigs (Choi et al. 2009) due to its phenolic compounds contained (Naim et al. 2012). Moreover phenolic compound can reduce methane production in the rumen (Jayanegara et al. 2009)

Therefore, the aim of study was to investigate the effectiveness of three bioindustry products (biofat, biochar and biosmoke) from cashew nut shell as feed additives to reduce methane and improve rumen fermentation end products.

MATERIALS AND METHODS

The experiment has been approved by the Animal Welfare Commission of the Indonesian Agency for Agricultural Research and Development (Balitbangtan/Balitnak/Rm/05/2016). The experiment was conducted at the Feed Laboratory of the Indonesian Research Institute for Animal Production.

Bioindustrial products of cashew nut shell (biofat, biochar and biosmoke) were used as feed additive in the experiment. The method of preparing biofat (BF) (Simpen 2008), biochar (BC) and biosmoke (BS) (Sudrajat & Salim 1994) was described in Saenab et al. (2016). Complete feed for *in vitro* study consisted of grass, *Gliricidia sepium* leaves, yellow corn, coconut cake, molasses, rice bran, urea, salt (NaCl), limestone (CaCO3), and premix. The nutrient content of complete feed used as substrate in the *in vitro* assays is presented in Table 1. The grass (*Pennisetum purpureum*) was obtained from IRIAP farm while other feed ingredients were obtained from local trader.

Table 1. The nutrient composition of complete feed used as substrate in in vitro assays

The nutrient composition (%)	Complete feed
Cp	15.63
Cf	3.83
GE (Kcal/Kg)	3785
Ash	6.85
NDF	28.41
ADF	15.02
Ca	0.69
P	0.29
TDN	69.7

In vitro rumen fermentation

In *in vitro* method, a buffer medium consisted of bicarbonate buffer solution, macro-mineral solution, micro-mineral solution, resazurin, distilled water, reducing solution and rumen fluid as described in Makkar (2003) was used. Feed samples were incubated in the *in vitro* system according to the method modified by Theodorou et al. (1994). Cashew nut shell bioindustrial products BF, BC and BS as feed additives were mixed with feed sample as substrate for *in vitro*. A total of 750 mg of substrate was weighed into the bottle. Rumen buffer solution (75 mL) was added under CO_2 atmosphere and the rubber stopper was quickly applied on the bottle. The bottle was placed in the water bath and incubated at 39°C for 48 hours. Rumen fluid was collected just before morning feeding from a rumen fistulated Friesian Holstein cow fed with commercial concentrate and elephant grass.

The total gas and methane production were measured at 3, 6, 9, 12, 24, 30, 36, and 48 hours of incubation by using syringes glass followed method developed by Tjandraatmadja (1981). At 48 hours of incubation, the supernatant was separated from the substrat by filtration using centreglass. The residue was dried in the oven with temperature 105°C for 24 hours and weighed. Ash content of feed and residue was determined according to AOAC method (2000) and NDF analysis was conducted following Goering & Van Soest (1970) by method without addition of amylase. The *in vitro* dry matter (DM) and organic matter (OM) of digested fractions were calculated from the DM and OM of initial sample minus those of residues. The DM or OM of digested fractions divided by the DM or OM of initial sample was calculated as DM or OM digestibility. Other residue samples of *in vitro* incubation were digested using NDS to obtain residual NDF fraction. The pH, ammonia (NH_3) and volatile fatty acid (VFA) were measured after 48 hours of incubation. The NH_3 content in the supernatant was determined using Conway microdiffusion technique. The supernatant for VFA analysis was kept in low pH by adding sulphuric acid. The VFA products from fermentation was analysed by using gas chromatography *(Bruker Scion 436 GC*) with capillary column BR-Wax fame containing WCOT fused silica with the length of column 30 m x 0.32 mm ID. The carrier gas was Nitrogen 25 ml/min and the burning gas was Hidrogen 30 mL/min. Injector temperature was 250ºC, while the column temperature gradient was 70 – 150ºC in 11 minutes. The detector used was FID with temperature of 275°C

Experimental design

The study used a randomized block design based on the time series of *in vitro* repetation to test treatments as follow Control (without feed additive = 0 BF, 0 BC and 0 BS), Biofat levels were 0.25 µL/mL (BF1), 0.5 µL/mL (BF2), 0.75 µL/mL (BF3); Biochar levels were 0.1 mg/mL (BC1), 0.2 mg/mL (BC2), 0.3 mg /mL (BC3), Biosmoke 2.5 µL/mL (BS1), 5.0 µL/mL (BS2), 7.5 µL/mL (BS3). Each treatment was done in duplicate and the *in vitro* was repeated 4 times.

Statistical analysis

The data collected from each addition of BF, BC and BS compared to control were analyzed separately by PROC GLM using SPSS Program Package 16. Further analysis using Duncan test was done for obtaining significant differences among treatments.

RESULTS AND DISCUSSION

Characteristics of rumen fermentation end products

Methane production profile in the rumen as a result of fermentation of feed added by different level of BF is presented in Figure 1. At 3 hours of incubation, the methane production was similar among the treatments. The production was different after 6 to 48 hours of incubation. The line regression shows the rate of methane production (10.01, 8.92, and 6.77 mL/hour) was decreasing in line with increasing level of BF addition (0.25, 0.5, and 0.75 µL/mL), respectively compared to control (12.57 mL/hour). At the different level of BC addition (Figure 2), the methane production profile of all the treatment was similar with control during the 48 hours of incubation. The methane production curves when BS added (Figure 3) were similar to those of BF addition. The higher level of BS addition (2.5, 5.0, 7.5 µL/mL) caused the lower rate of methane production (10.71, 9.59, 8.62 mL/hour), respectively, compared to control (12.57 mL/hour),

Total gas and methane production at 48 hours of incubation decline very significant ($P<0.01$) with the increasing level of BF (Table 2). Increasing level of BF from 0.25 µL/mL to 0.5 µL/mL and 0.75 µL/mL reduced significantly ($P<0.01$) total gas and methane production by 21.73%; 20.76%; 45.25% and by 20.83%; 29.79%; 43.88% respectively, compared to control. Addition of BC increased total gas production

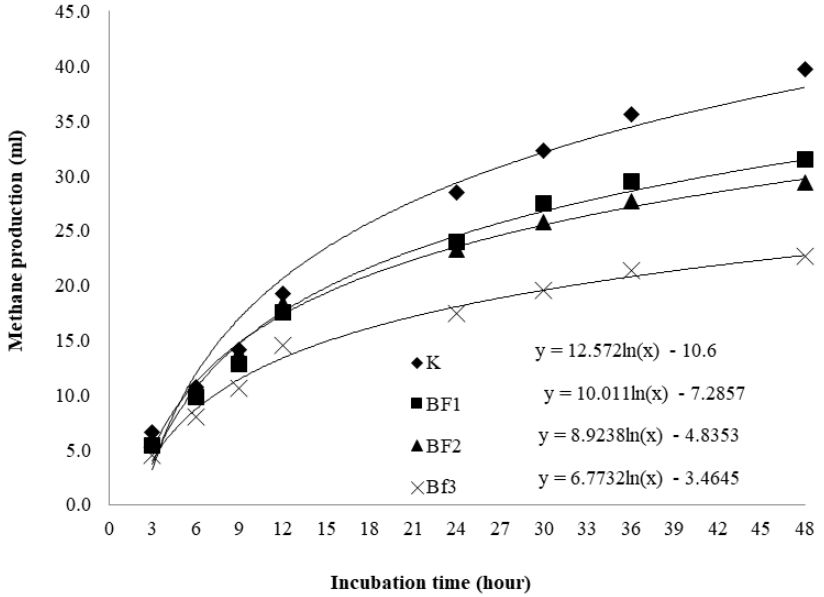

Figure 1. The profile of methane production at different level of Biofat (BF) addition during 48 hours of incubation.

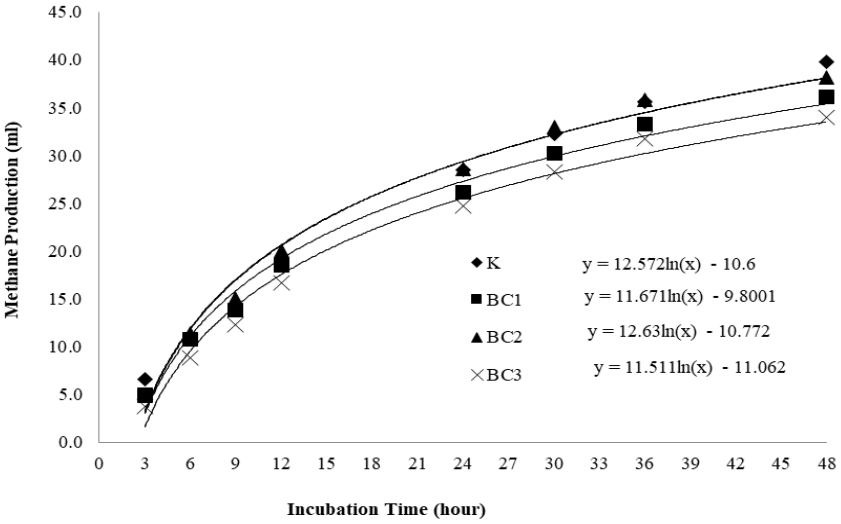

Figure 2. The profile of methane production at different level of Biochar (BC) addition during 48 hours of incubation.

by 18.76%, 14.47%, and 9.56%, but decreased methane production by 9.12%, 4.09%, and 24.21% after addition of BC 0.1 mg/mL, 0.2 mg/mL, and 0.3 mg/mL respectively, compared to control.

Addition of BS to the *in vitro* fermentation caused total gas and methane production decreased significantly (P<0.05) compared to control. Methane production was reduced by 31.55%, 30.69%, and 37.88% with addition of BS 2.5, 5.0, and 7.5 µL/mL. The NH_3 concentration and pH at 48 hours of incubation (Tabel 2) were not significantly different (P>0.05) between addition of BS and control. However, the highest level of BC increased NH_3 concentration significantly (P<0.05) compared to control. The BS additional was not significantly increased NH_3 production.

Inclusion of BF to the feed (Table 3) improved proportion of propionate significantly (P<0.05),

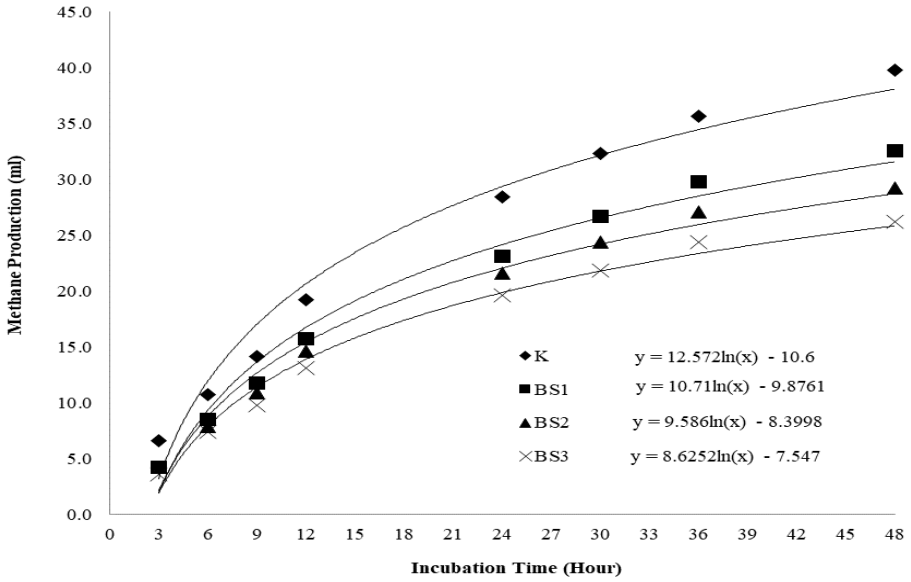

Figure 3. The profile of methane production at different level of Biosmoke (BS) addition during 48 hours of incubation.

Table 2. The effect of biofat (BF), biochar (BC) and biosmoke (BS) of cashew nut shell on total gas, methane production, NH_3 concentration and pH at 48 hours of incubation

Additive	Level	Total Gas (mL)	Methane (mL)	NH_3 (mM/g BK)	pH
BF	0	157.3^{d}	39.7^{d}	8.30	6.67
	0.25 µL/mL	143.5^{c}	31.5^{c}	8.18	6.68
	0.50 µL/mL	127.5^{b}	27.9^{b}	8.12	6.70
	0.75 µL/mL	101.4^{a}	22.3^{a}	7.53	6.71
SE		2.60	0.72	0.47	0.01
P-value		0.00	0.00	0.41	0.17
BC	0	157.3^{a}	39.7^{c}	8.30^{a}	6.67^{a}
	0.25 mg/mL	164.2^{a}	36.1^{b}	8.49^{a}	6.72^{b}
	0.50 mg/mL	164.4^{a}	38.1^{bc}	8.95^{a}	6.70^{ab}
	0.75 mg/mL	190.9^{b}	30.1^{a}	10.78^{b}	6.70^{ab}
SE		3.13	0.87	0.48	0.01
P-value		0.00	0.001	0.003	0.05
BS	0	157.3^{d}	39.7^{c}	8.30	6.67
	0.25 µL/mL	145.9^{c}	27.20^{b}	11.30	6.69
	0.50 µL/mL	121.9^{b}	27.5^{b}	10.56	6.70
	0.75 μLmL^{-1}	114.3^{a}	24.7^{a}	9.23	6.72
SE		2.98	0.76	0.89	0.01
P-value		0.00	0.00	0.14	0.11

Different letter on the same column indicates significant difference ($P<0.05$). Statistical analysis was done separately for each additive against control

Table 3. Molar proportion of acetate, propionate, butyrate, valerate, BCVFA, ratio of A/P and total VFA (mM) production in the *in vitro* rumen fermentation of feed added by biofat (BF), biochar (BC) and biosmoke (BS) of cashew nut shell after 48 hours of incubation

Additive	Level	Acetate	Propionate	Butyrate	Valerate	BCVFA	A/P	Total VFA (mM)
		----------%----------						
BF	0	60.72	23.37[a]	10.99[b]	1.82	3.10[b]	2.64	87.10
	0.25µL/mL	56.83	31.86[b]	9.10[a]	1.42	2.54[a]	1.98	112.69
	0.5 µL/mL	59.26	27.76[b]	9.18[a]	1.35	2.35[a]	2.2	96.25
	0.75µL/mL	56.24	31.37[b]	8.98[a]	1.21	2.40[a]	1.87	110.56
SE		2.543	1.568	0.442	0.172	0.106	0.191	7.546
P-Value		0.502	0.044	0.035	0.150	0.004	0.242	0.106
BC	0	60.72	23.37[a]	10.99	1.82	3.10	2.64	87.10[a]
	0.1 mg/mL	58.18	24.90[b]	11.84	1.67	4.24	2.34	120.02[ab]
	0.2 mg/mL	51.70	28.55[b]	13.51	2.00	3.08	1.85	108.38[ab]
	0.3 mg/mL	59.98	29.37[b]	11.39	1.74	3.81	2.20	125.5[b]
SE		1.143	1.419	1.080	0.169	0.444	0.092	8.393
P-Value		0.123	0.049	0.249	0.565	0.236	0.002	0.035
BS	0	60.72[c]	23.37[a]	10.99	1.82	3.10[ab]	2.64[c]	87.11
	2.5 µL/mL	49.93[a]	36.67[c]	9.91	1.49	3.29[b]	1.39[a]	107.25
	5.0 µL/mL	56.22[b]	26.32[b]	11.85	1.86	3.36[b]	2.24[c]	102.2
	7.5 µL/mL	54.20[b]	27.28[b]	12.64	2.04	3.24[a]	2.07[b]	104.37
SE		0.880	0.835	0.737	0.151	0.240	0.093	4.610
P-Value		0.000	0.000	0.110	0.130	0.026	0.000	0.043

BCVFA = Branched chain volatile fatty acid
A/P = Acetate/Propionate
Different letter on the same column shows significant difference (P<0.05)
Statistical analysis of every bioindustrial product treatment against control was conducted separately

however decreased proportion of butyrate and BCVFA significantly (P<0.05) compared with control (P<0.05). But not significantly decreased proportion of acetate and valerate and not significantly increased total VFA. The results also showed that BC addition enhanced total VFA production and proportion of propionate produced (P<0.05) (Table 3). The highest level of total VFA and proportion of propionate was obtained at the BC addition of 0.3 mg/mL. In contrast, proportion of acetate and ratio of A/P decreased significantly (P<0.05) due to BC addition compared to control.

Addition of BS increased significantly proportion of propionate and total BCVFA (P<0.01) while it reduced proportion of acetate (P<0.05) (Table 3).

Effect of bioindustrial products of cashew nut shell on rumen digestibility

The DM, OM and NDF digestibility of feed significantly (P<0.05) decreased in the presence of BF (Table 4). Increasing level of BF inclusion resulting in decreasing feed digestibility. The reduction of NDF digestibility was higher than the reduction of DM and OM digestibility. The BC addition at any level did not cause any significant effect on DM, OM and NDF digestibility. Similar to BF, the BS addition significantly (P<0.05) reduced DM, OM and NDF digestibility.

The effect of Biofat (BF) on rumen fermentation and methane mitigation

The results indicated that increasing level of BF addition followed by reducing in the production of total gas and methane. Our results on decreased methane production were due to BF was in line with previous reports (Watanabe et al. 2010). The ability of BF in reducing production of methane in the rumen could be attributed by several factors (Watanabe et al. 2010; Shinkai et al. 2012; Mitsumori et al. 2014). Watanabe et al (2010) stated that anacardic acids were the active compounds in BF that might reduce methane and caused changes in fermentation process. Saenab et al. (2016) analysed the phenolic compounds in BF indicated that there were some phenolic compounds, which are binded to the long chain fatty acids (C15).

Some of these compounds were identified as 78.16% anacardic acid, 13.18% cardol, 4.66% cardanol and 3.56% methyl cardol. The long chain lipid that binded to the phenolic structure has none, one, two or three double bond in their lipid structure (Njuku et al. 2014). Plant secondary compounds including phenolic compounds possessed antimicrobial activity that may inhibit the growth of methanogenic bacteria and some other bacteria, hence, reduced methane and total gas production (Kamra et al. 2012, Jayanegara et al. 2013). Watanabe et al. (2010) also reported that all Gram positive bacteria including rumen bacteria were highly or moderately influenced by CNSL (BF), however, several Gram negative bacteria were not influenced by CNSL (BF). The exact bactericidal mechanism of BF may be due to the surfactant properties of anacardic acids that may disrupt the bacteria cell membrane.

Reducing methane by BF addition may be due to the lower population or activity of methanogenic bacteria. As reported by Shinkai et al. (2012) that CNSL or BF reduced the activity of methanogens to produce methane. It might due to the less hydrogen availability in the rumen. The growth of hydrogen and formic acid forming bacteria may be suppressed by BF (Shinkai et al. 2012). There might also be competition in hydrogen

Table 4. The DM, OM and NDF digestibility of feed in the presence of biofat (BF), biochar (BC) and biosmoke (BS) of cashew nut shell in the *in vitro* rumen fermentation after 48 hours of incubation

Additive	Level	Digestibility		
		BK (%)	BO (%)	NDF (50
BF	0	67.96^{bc}	69.20^{c}	54.03^{c}
	0.25 µLmL^{-1}	67.37^{c}	68.80^{bc}	50.88^{bc}
	0.5 µLmL^{-1}	63.15^{ab}	64.46^{ab}	40.73^{ab}
	0.75 µLmL^{-1}	59.88^{a}	60.48^{a}	36.42^{a}
SE		1.85	1.47	2.46
P-Value		0.033	0.004	0.001
BC	0	67.96	69.55	54.03
	0.1 mg/mL	66.48	70.03	52.51
	0.2 mg/mL	65.86	67.86	50.39
	0.3 mg/mL	69.17	71.11	52.39
SE		2.37	1.89	1.66
P-Value		0.762	0.683	0.505
BS	0	67.96	69.55	54.03^{c}
	2.5 µL/mL	58.54	63.18	34.28^{a}
	5.0 µL/mL	58.51	62.10	42.82^{ab}
	7.5 µL/mL	54.89	58.55	38.73^{ab}
SE		3.99	3.47	4.44
P-Value		0.178	0.216	0.047

Different letter on the same column shows significant difference (P<0.05). Statistical analysis of every bioindustrial product treatment against control was conducted separately

utilization by methanogenic bacteria, propionic acid forming bacteria, sulfate reducing bacteria and other bacteria that consume hydrogen (Mitsumori et al. 2014). Hydrogen is released from the formation of acetic acid and butyric acid during fermentation in the rumen. The hydrogen will be taken up by bacteria because hydrogen is the main component required in producing methane and propionate. There is a competitive action of bacteria producing methane and propionate in hydrogen utilisation. The results showed that methane production decreased as propionate increased with the addition of BF, might be due to more hydrogen used by propionate forming bacteria. These results are in line with observation of Watanabe et al. (2010) who reported that the population of propionate forming bacteria (*Selemonas ruminantium*, *Megasphaera elsdenii*, *Prevotella ruminicola*) increased as increasing in the amount of hydrogen taken by these bacteria. It then reduced the amount of hydrogen taken up by other hydrogen consuming bacteria.

The lipid structure in anacardic acids may also suppress methane production as it may suppress the methanogenic process as reported by Martin et al. (2010). Lipid can reduce fiber digestibility in the rumen since unsaturated fatty acid is toxic to fiber degrading bacteria (Maia et al. 2007), hence, less fiber degrading bacteria that grew in the presence of BF. Watanabe et al. (2010) found that the addition of CNSL (BF) inhibited the growth of *Ruminococcus flavefaciens* and *Ruminococcus albus* that have the ability to digest fiber. This also resulted in decreasing the DM, OM and NDF digestibility as addition of BF increased.

The BF inclusion in the diet did not affect NH_3 production (Table 2), but it decreased BCVFA significantly (Table 3). The BCVFA is the result of degradation of branched amino acids (valine and leucine) by bacteria *Megasphaera elsdenii* and *Prevotella ruminicola* (Wolin & Miller 1997). Phenolic lipid compounds might specifically depress the growth of bacteria that disintegrate the bond of branched amino acids but it might not interfere the growth of ammonia-forming bacteria. The NH_3 is required for rumen microbial growth to synthesis rumen microbial protein. Sutardi (1994) stated that the production range of NH_3 for a good growth of rumen microbes is between 4-12 mM. This means that the concentration of NH_3 in this study (7.53 to 8.34 mM) were within the normal range for optimum growth of rumen microbes.

The effect of Biochar (BC) on rumen fermentation and methane mitigation

Result of study indicated that the administration of BC at the highest level (0.3 mg/mL) suppressed the methane production quite markedly 16.75%. However the activity of BC in suppressing methane production was not similar with those of BF activity. Lower methane production in *in vitro* fermentation was also reported due to the inclusion of BC from rice husk (Leng et al. 2012b) and BC from wood (Hansen et al. 2012). The difference on the mechanism of methane reduction by BC vs BF was due to the physical property of BC, but not due to the bioactive compounds in BC. The BC from cashew nut shell has highly porous structure (Saenab et al. 2016), which might absorb methane present in the rumen.

Addition of BC was very significant increased NH_3 production ($P<0.01$). Increasing level of BC added to the *in vitro* fermentation increased NH_3 produced (Table 2). The findings were similar with those reported by Leng et al. (2012a), who use BC from rice hull and coconut meal in the *in vitro* experiment. The BC treatment resulted a very significant increase on the proportion of propionate and total VFA compared to control (Table 3). The highest propionate and total VFA were obtained at the highest level of BC treatment (0.75 mg/mL). Reducing methane production might be followed by inreasing in propionate forming due to the hydrogen availability.

Different results with the BF or BS addition, the addition of BC had no effect on feed digestibility. These results were similar with finding of previous study reported by Leng et al. (2012a). Even though BC did not improve feed digestibility, it increased daily weight gain of cattle (Leng et al. 2012b) and goat (Do et al. 2006). Addition of BC might improve dietary energy utilization by the animals due to the shifted of methane to propionate production, which is required for animal's weight gain.

The effect of Biosmoke (BS) on rumen fermentation and methane mitigation

Profile of methane production at various levels of BS was similar to that of BF. The mechanism of methane reduction due to BS might be different from that of BF. The BS is a liquid fraction obtained from the pyrolysis process of cashew nut shell. It has low pH (3.69) and contained acidic compounds and many simple phenolic compounds that came from degradation of lignin, cellulose, hemicellulose (Naim et al. 2012; Saenab et al. 2016). While BF has neutral pH and contained phenolic compounds bonded with C_{15} fatty acids. So it is likely that ability of phenolic compounds contained in BF affects rumen fermentation and methane reduction in different form from that ability of BS. Simple phenolic compounds as reported by Jayanegara et al. (2009) could reduce methane production, as the phenolic compounds can react and destroy the cell wall of methanogenic bacteria, hence inhibit the bacterial growth.

Some rumen bacteria such as cellulolytic bacteria are very sensitive to low pH (acidic) condition. Low rumen pH as resulted by BS inclusion might suppressed the growth or activity of methanogenic and cellulolytic bacteria. This might cause reduction on the methane production, DM, OM and NDF digestibility, but increasing in propionate production (Table 2 and Table 4). The inclusion of BF and BS in the ruminant feed must be in appropriate amount as it is beneficially mitigates methane and increases propionate production, but in the same time also might depress feed digestibility in the rumen.

CONCLUSION

This research concludes that each bioindustrial product of cashew nut shell has beneficial effect on reducing methane but enhancing propionate. Biofat, biochar and biosmoke may have different mechanism on reducing methane and affecting rumen fermentation. Biofat has the highest effect on mitigating methane, followed by biosmoke and biochar.

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Performance of Dairy Calves Fed Diet Containing Ca-Palm Oil Fatty Acid and *Sapindus rarak* Fruit

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ABSTRAK

Wina E, Widiawati Y, Tangendjaja B. Performans sapi perah anak yang diberi pakan mengandung kalsium-asam lemak sawit dan buah *Sapindus rarak*. JITV 23(2): 71-76. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1828

Garam kalsium-asam lemak sawit (CaFA) diberikan kepada induk sapi perah terutama saat periode awal laktasi sebagai sumber energi yang padat untuk meningkatkan produksi susu. Buah lerak (*Sapindus rarak*, SrF) mengandung kadar saponin tinggi dan dilaporkan dapat meningkatkan berat badan domba, produksi susu dan menekan populasi protozoa di dalam rumen. Percobaan ini menggunakan kombinasi CaFA dan SrF untuk meningkatkan performans anak sapi perah. Tiga puluh dua ekor anak sapi FH digunakan dalam percobaan dalam rancangan blok pola faktorial (2x2). Faktor pertama adalah CaFA (tanpa dan dengan 2,5% CaFA), dan faktor kedua adalah SrF (tanpa dan dengan 0,3% SrF dalam pakan). Jenis kelamin ternak diperlakukan sebagai blok. Pakan disusun sebagai pakan komplit mengandung protein kasar 17% dan TDN min 69%, Net energi untuk pemeliharaan 1.70 Mcal/kg and Net energi untuk pertambahan bobot badan 1.03 Mcal/kg. Pakan diberikan sebanyak 3% dari berat badan ternak selama 14 minggu termasuk 2 minggu masa adaptasi. Pengumpulan feses untuk mengukur kecernaan pakan dilakukan pada akhir percobaan. Tidak ada pengaruh yang nyata pada interaksi antar kedua faktor untuk semua pengukuran. Pertambahan bobot badan harian yang menerima SrF lebih tinggi dibandingkan tanpa diberikan SrF (896,9 dibanding 853,7 g/hari) sedangkan yang diberi CaFA lebih rendah dari pada tanpa diberi CaFA (860,6 dibanding 890 g/hari) tetapi pengaruh keduanya (SrF dan CaFA) tidak signifikan (P>0,05). Konsumsi BK cenderung lebih tinggi pada yang diberi SrF (4.4 dibandingkan 4,3 kg/hari). Nilai kecernaan BK tidak berbeda diantara perlakuan. Disimpulkan bahwa pemberian tepung buah *Sapindus rarak* pada level 0,3% tidak nyata meningkatkan pertambahan bobot badan harian tetapi dapat mengurangi kasus diare pada anak sapi. Kalsium asam lemak dapat digunakan sebagai sumber energi terlindungi pada level 2,5% tanpa pengaruh negatif terhadap performans anak sapi.

Kata Kunci: Kalsium Asam Lemak Sawit, *Sapindus rarak*, Saponin, Anak Sapi

ABSTRACT

Wina E, Widiawati Y, Tangendjaja B. Performance of dairy calves fed diet containing Ca-palm oil fatty acid and *Sapindus rarak* fruit. JITV 23(2): 71-76. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1828

Calcium salts of palm oil fatty acid (Ca-FA) is a high dense energy source that is protected from degradation in the rumen. Sapindus rarak fruits (SrF) contain high level of saponin and have been reported to increase body weight sheep or cattle and reduced protozoa population in the rumen. This experiment used a combination of Ca-FA and SrF to improve the performance of weaned dairy calves. Thirty two heads of calves of Indonesian Holstein were used in factorial block design experiment (2 x 2). The first factor was Ca-FA (with 2.5% and without addition of Ca-FA) while the second factor was SrF (with 0.3% and without addition of SrF in total diet) and sex of the animal as block was applied. The feed as total mixed ration contained crude protein (CP) 17%, total digestible nutrien (TDN) minimum 69%, Net energy for maintenance 1.70 Mcal/kg and Net energy for gain 1.03Mcal/kg. It was fed 3% of body weight of the animals for 14 weeks included 2 weeks of adaptation period. Feces collection for measuring digestibility of feed was conducted at the end of experiment. Average daily gain (ADG) of calves received SrF was higher than without SrF (896.9 vs 853.7 g/day) while ADG received CaFA was lower than without CaFA (860.6 vs 890 g/day) but both effects were not significantly different (P>0.05). DM intake due to SrF treatment tended to be higher than control treatment (4.4 vs 4.3 kg/day). DM digestibility was not different among treatments. In conclusion, calves received supplementation of 0.3% SrF fruit in the diet had similar average daily gain with those given unsupplemented diet but had reduced diarrhea cases. The use of Ca palm oil fatty acid as a dense energy source at 2.5% in the diet did not show any negative effect on calves performance.

Key Words: Calcium- Fatty Acid, *Sapindus rarak*, Saponin, Dairy Calves

INTRODUCTION

Weaned calves require protein and energy to grow, however, the use of high energy in the form of fat will disturb the rumen function especially for young calves. The fat or fatty acid should be protected from degradation in the rumen as it causes negative effect to rumen function. The source of fatty acid that available abundantly in Indonesia is a byproduct from the cooking oil industry, called as palm oil fatty acid. Reaction palm oil fatty acid with calcium will produce calcium salt of palm oil fatty acid (CaFA) which will protect fatty acid from its degradation in the rumen and will not disturb the cellulolytic bacteria in the rumen, hence, does not negatively affect the rumen. The use of CaFA for dairy cow for early lactating period is very beneficial as it increased milk production (Reis et al. 2012; Rabiee et al. 2012). However, the information on the use of CaFA for calves as an energy source was only a few. It was reported an increase in weight gain for calves receiving diets containing 20% *vs* 10% (of DM) of a protected lipid source. Supplementation with 2.5% protected fat in calves feed increased feed conversion ratio, dry matter and fat digestibility of feed with low digestible protein content in the feed. Protein digestibility increased due to inclusion of protected fat in the diet (Cruywagen et al. 2003).

Sapindus rarak fruits (SrF) contain high level of saponin and several experiments showed that SrF fruit reduced protozoa population in the rumen (Wina 2005; Wina 2012). *Sapindus rarak* fruit has been proven to destroy oocytes of *Eimeria tenella in vitro* (Pasaribu et al. 2014). Saponin has been reported to show antimicrobial or antifungal activity (Hassan et al. 2010; Saha et al. 2010). Weaned calves sometimes suffer from diarrhea. Therefore, the use of SrF fruit to calves may improve the performance of calves and prevent the occurrence of diarrhea on calves.

The aim of this experiment is to evaluate the supplementation of SrF fruit and CaFA in the diet to improve the growth performance of dairy calves.

MATERIALS AND METHODS

The experiment was done at the research station of Indonesian Research Institute for Animal Production. Thirty two newly weaned calves of Indonesian Holstein (4 months old) were placed in individual cement floor stall. They were divided randomly into 4 treatments. Each treatment consisted of 4 males and 4 females of calves. The experiment was conducted in factorial block design 2 x 2 with sex of the animal as block. First factor was Calcium palm oil fatty acid (CaFA) (with the level of 2.5% CaFa of ration and without addition of CaFA and the second factor was *S.rarak* (SrF) fruit powder (with 0.3% SrF of ration and without addition of SrF) The treatments were: P1) Control feed (without SrF, without CaFA), P2) Feed with SrF, without CaFA, P3) Feed without SrF, with CaFA, P4) Feed with SrF, with CaFA. The total mixed ration contained CP 17%, Net energy for maintenance 1.70 Mcal/kg, Net energy for gain 1.03 Mcal/kg. It consisted of elephant grass, ground corn, coconut meal, palm kernel meal, rice bran, soybean meal, molasses, limestone, salt, vitamin and mineral mix (Table 1). The feeding trial was conducted for 14 weeks including 2 weeks of adaptation period. The feed was fed twice a day at the level of 3% of body weight. Body weight was recorded every two weeks. Digestibility trial was conducted at the end of feeding trial where faeces were collected. The feed, feed residue and faeces were dried in the oven at 60°C for 3 days and milled after dried. The samples were analyzed for ash, crude protein, NDF, ADF, gross energy contents. The rumen liquor was taken from each animal and measured its pH, ammonia, total bacteria and protozoa population.

RESULTS AND DISCUSSION

Addition of Calcium fatty acid (CaFA) in the diet resulted in similar intakes of DM, OM, CP, NDF, ADF, Energy among treatments (Table 2). DM intake was significantly higher as *S.rarak* powder (SrF) was added into the diet compared to control (4.45 *vs* 4.29 kg/day). Crude protein intake tended to be higher due to SrF addition. There was no interaction effect of CaFA and SrF on intakes. There was no significant effect of CaFA on any digestibility (Table 2). SrF tended to decrease NDF and energy digestibility and SrF caused a significant decrease of ADF digestibility. There was no interaction effect of CaFA and SrF on digestibility.

Rumen condition had some changes in term of pH that increased significantly due to addition of SrF (Table 3). SrF tended to reduce total protozoa while it did not affect the total bacteria. CaFA did not give any significant effect on pH, ammonia production and also did not change total bacteria or total protozoa in the rumen. There was no interaction effect of CaFA and SrF on rumen condition and microbes population.

Table 4 presents the average daily gain (ADG) and FCR of calves received SrF or CaFA for 12 weeks. ADG of calves receiving CaFA was lower than that without CaFA (860.6 *vs* 890 g/day) while ADG of calves receiving SrF was higher than that without SrF (896.9 *vs* 853.8 g/day) but both effects were not significantly different ($P>0.05$). ADG of male calves were higher than that of female (931 *vs* 819 g/day), however, the difference between ADG of male and of female calves was not significant (Figure 1).

During the experiment, it was recorded that some animals got diarrhea although it was only mild diarrhea. The diarrhea occurred to 3, 1, 5 and 7 animals for for P1) Control (without SrF and CaFA), P2) with SrF without CaFA, P3) without SrF, with CaFA, P4) with SrF and CaFA treatments, respectively.

Table 1. Diet composition and its calculated nutrient

Ingredient (%)	P1 without CaFA, without SrF	P2 without CaFA, with SrF	P3 with CaFA, without SrF	P4 With CaFA, with SrF
Elephant grass (DM)	50	50	50	50
Corn grain (ground)	12.9	12.9	9.1	9.1
Rice bran	12.7	12.7	13.8	13.9
Copra meal	12.5	12.5	12.5	12.5
Palm kernel meal	2.8	2.8	2.8	2.8
Molasses	2.5	2.5	2.5	2.4
Soybean meal	5.2	5.2	5.7	5.7
Lime stone	1	1	0.7	0.7
Salt	0.3	0.3	0.3	0.3
Ruminant premix	0.1	0.1	0.1	0.1
Ca-fatty acid (CaFA)	0	0	2.5	2.5
TOTAL	100.0	100.0	100.0	100.0
Sapindus rarak powder (SrF)	0	0.3	0	0.3
Calculated nutrient				
Crude Protein (%)	17	17	17	17
TDN min (%)	69	69	69	69
Net energy for maintenance (Mcal/kg)	1.70	1.70	1.70	1.70
Net energy for gain (Mcal/kg)	1.03	1.03	1.03	1.03
Ca min (%)	0.52	0.52	0.52	0.52
P min (%)	0.31	0.31	0.31	0.31

Weaned calves have usually problem in consuming new feed. It was also reported that the addition of Calcium fatty acid to the young calves depressed feed intake (Cruywagen et al. 2003) because calcium fatty acid might causea palatability problem. However, inclusion of 2.5% of CaFA in this experiment did not cause any negative effect on palatability. The calves did not show any rejection to any of feed containing CaFA. Surprisingly, SrF (*Sapindus rarak fruit*) increased DM intake significantly. This result was in agreement with the report that *Sapindus Saponaria* which increased OM intake of feed in sheep (Abreu et al. 2004). *Sapindus rarak* fruit contains high saponin level but saponin did not reduce intake, instead it increased intake. It was reported that addition of 3 g tea saponin/day in goat diet increased DM intake, although not significantly (Wang et al. 2012). Increased DM intake might be attributed to the increased efficiency of microbial protein synthesis (Uddin et al. 2015).

Digestibility of dry matter, organic matter and protein was not affected by the addition of CaFA or SrF. Protection process of fatty acid by calcium prevented the negative effect of fatty acid to rumen microbes. It was reported that unprotected fat would cover the feed particle and prevent ruminal bacteria to degrade feed and unsaturated fatty acid in free form was toxic to some rumen microbes.

Fiber digestibility (NDF and ADF) significantly decreased by the addition of SrF. Saponin in SrF decreased fiber digestibility (NDF) in the rumen but not total tract fiber digestibility as reported by Wina (2005). SrF saponin reduced the activity of fibrolytic enzymes significantly and the activity had more positive correlation with the number of protozoa in the rumen of sheep (Wina et al. 2005). Protozoa exerts cellulase, hemicellulase, xylanase and glycoside hydrolase as reported by Williams & Coleman (1988). In this experiment, total protozoa tended to decrease. Therefore, lower protozoa numbers might cause lower activity of fibrolytic enzymes to degrade fiber.

Table 2. Effect of Calcium fatty acid (CaFA) and *S.rarak* fruit (SrF) on daily intake of feed

	CaFA factor		SrF factor		sem	CaFA	SrF
	without	with	without	with		factor	factor
Intake, g/head/day							
DM	4374	4372	4292[a]	4454[b]	49.15	NS	P<0.03
OM	3942	3884	3844	3982	149.00	NS	NS
CP	747	699	699	748	17.80	NS	NS
NDF	1899	1854	1908	1845	80.25	NS	NS
ADF	971	959	979	951	34.25	NS	NS
GE (Mcal)	184	188	185	188	8.05	NS	NS
Digestibility (%)							
Dry matter	72.17	71.68	73.08	70.76	1.16	NS	NS
Organic matter	72.21	71.36	73.14	70.44	1.27	NS	NS
Crude protein	72.24	69.83	70.75	71.32	1.62	NS	NS
NDF	65.64	64.15	67.13	62.45	1.62	NS	NS
ADF	55.75	53.29	57.65[a]	51.39[b]	2.03	NS	P<0.04
Gross Energy	71.78	71.05	73.30	69.54	1.37	NS	NS

Table 3. Effect of Calcium fatty acid (CaFA) and *S. rarak* fruit (SrF) on rumen condition and microbial population of calves

	CaFA factor		SrF factor		sem	CaFA	SrF
	without	with	without	with		factor	factor
pH	7.06	7.08	6.97[a]	7.17[b]	0.07	NS	P<0.04
NH3, mM	10.44	10.06	11.14	9.36	1.18	NS	NS
Total bacteria (10^9)	11.81	9.99	10.83	10.97	1.52	NS	NS
Total protozoa (10^5)	8.67	10.65	12.22	7.10	1.94	NS	NS

Table 4. Effect of Calcium fatty acid (CaFA) and *S.rarak* fruit (SrF) on ADG and FCR of calves

	CaFA factor		SrF factor		sem	CaFA	SrF
	without	with	without	with		factor	factor
ADG g/day	890.0	860.6	853.8	896.9	0.05	NS	NS
FCR	4.92	5.18	5.21	4.88	0.15	NS	NS

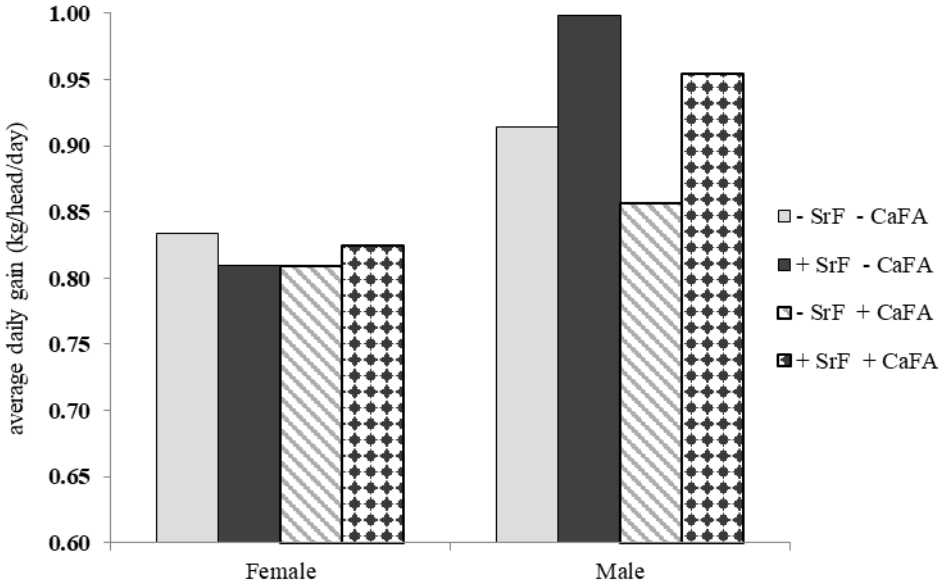

Figure 1. Average daily gain (kg/head/day) of female and male dairy calves supplemented without and with CaFA or SrF.

Several reports showed that ammonia production in the rumen was reported lower in the presence of saponin in the feed than control without saponin. Ammonia production in the rumen occurred from degradation of protein from either feed or proteolysis of bacterial protein when protozoa engulf ruminal bacteria or protozoal number. Contribution of total rumen nitrogen from protozoa was 10-40%, and therefore, when protozoa number tended to reduce, the ammonia production in the rumen might be lower. In this experiment, since the protozoa number was also slightly reduced, the ammonia production in the rumen was not affected. It was reported that the efficiency of microbial protein synthesis was greater in forages containing saponin and tannins which reduce ruminal N degradability (Uddin et al. 2015), therefore it is expected that microbial protein entered abomasum and ileum was higher in the presence of SrF, hence increase protein absorption and increase body weight gain. However, neither CaFA or SrF affected significantly the average daily gain (ADG) of weaned calves. Calcium fatty acid is a dense energy that can partly substitute corn when calcium fatty acid was included as part of the ingredients and formulated in the diet. In this experiment, 2.5% CaFA in combination with rice bran substituted 29% of corn and produced similar average daily gain with diet without CaFA. There is an opportunity to substitute partly corn during the availability of corn is limited or during off season of corn.

The difference ADG between SrF and non-SrF fed calves was only 43g/day (5%) was too small compared to the high variation of ADG obtained by individual calf (26%). The use of SrF at the level of 0.3% might be too low to give any significant effect on ADG. In sheep experiment, SrF was used at the level of 0.7% and significantly increased ADG of male sheep (Thalib et al. 2010; Wina 2005). In this experiment, although it was not significantly different, ADG of male calves were higher (112 g) than that of female. It was in agreement as reported in sheep when fed 40 mg/kg of Quillaja saponin, the male goat had higher daily gain than female goat. This experiment showed that the male calves had faster growth rate than female calves and they were very potential as meat type animal.

In this experiment, SrF reduced diarrhea problem in young animal. It is the first report on the effectiveness of SrF reduced diarrhea case *in vivo*. It was reported previously that Yucca saponin reduced the incidence of coccidiosis on calves when they were infected with Eimeria (Rambozzi et al. 2011).

Saponin in SrF effectively reduced protozoa population in the rumen and also reduced the growth of *E.coli* in newly weaned calves. Feeding of SrF to weaned calves should be taken carefully since their rumen has not developed well and saponin in SrF may depress the growth and activity of ruminal microbes.

CONCLUSION

The addition of Calcium palm fatty acid (CaFA) and *Sapindus rarak* fruit (SrF) at the level of 2.5% and 0.3%, respectively gave similar average daily gain with dairy calves fed without CaFA or SrF. Addition of *Sapindus rarak* fruit reduced diarrhea cases on calves.

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The Effect of Glucomannan Inclusion Derived from Porang Tuber Extract (*Amorphophallus oncophyllus*) on Dietary Protein Utilization in Broiler Chicken

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ABSTRAK

Khanifah, Suthama N, Wahyuni HI. 2018. Pemanfaatan protein ransum pada ayam broiler akibat penambahan glukonaman dari ekstrak umbi porang (*Amporphopallus onchophyllus*). JITV 23(2): 77-81. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1834

Penelitian bertujuan untuk mengkaji pengaruh penambahan glukomanan dari ekstrak umbi porang (GPTE) terhadap kemampuan produktivitas ayam broiler. Ternak yang digunakan adalah ayam broiler strain New Lohmann umur 1 hari sebanyak 160 ekor dengan bobot badan rata-rata 42,08±0,86g. Penelitian disusun dalam rancangan acak lengkap (RAL) dengan 5 perlakuan dan 4 ulangan, masing-masing ulangan dengan 8 ekor ayam. Perlakuan yang diberikan yaitu T0 = ransum basal, T1 = T0 + 0,05% GPTE,T2 = T0 + 0,10% GPTE, T3 = T0 + 0,15% GPTE dan T4 = T0 + 0,20% GPTE. Parameter yang diamati meliputi koefisien cerna protein, retensi nitrogen, massa kalsium daging, dan massa protein daging. Hasil penelitian menunjukkan bahwa penambahan GPTE nyata (P<0,05) meningkatkan koefisien cerna protein, massa kalsium daging dan massa protein daging, tetapi tidak mempengaruhi retensi nitrogen. Koefisien cerna protein dan massa kalsium daging tertinggi pada penambahan 0,2% GPTE (T4) tetapi tidak berbeda dengan T3, sedangkan massa protein daging tertinggi pada T2 tetapi tidak berbeda dengan T3 dan T4. Kesimpulan penelitian yaitu penambahan GPTE 0,15% (T3) pada ransum ayam broiler dapat meningkatkan koefisien cerna protein, massa kalsium dan protein daging, serta pertambahan bobot badan harian, meskipun retensi nitrogen sama.

Kata Kunci: Glukomanan, Koefisien Cerna Protein, Retensi Nitrogen, Massa Kalsium, Protein Daging, Broiler

ABSTRACT

Khanifah, Suthama N, Wahyuni HI. 2018. The effect of glucomannan inclusion derived from porang tuber extract (*Amorphophallus oncophyllus*) on dietary protein utilization in broiler chicken. JITV 23(2): 77-81. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1834

The aim of this study was to evaluate the effect of glucomannan inclusion derived from porang (*Amorphophallus oncophyllus*) tuber extract (GPTE) on production performance of broiler chicken. A total of 160 one day old of New Lohmann broiler chickens with an average initial body weight of 42.08±0.86g were used in this study. The study was arranged in a completely randomized design with 5 treatments and 4 replications (8 birds each). The treatments applied were T0 = basal ration, T1 = T0 + 0.05% GPTE, T2 = T0 + 0.10% GPTE, T3 = T0 + 0.15% GPTE and T4 = T0 + 0.20% GPTE. The parameters observed were protein digestibility, nitrogen retention, meat calcium and protein mass. The results showed that dietary addition of GPTE significantly (P<0.05) increased the coefficient of protein digestibility, meat calcium and protein mass, but had no effect on nitrogen retention. The highest protein digestibility and meat calcium mass were shown in T4, but they were not significantly different from those in T3, and T2 for protein digestibility. While the highest meat protein mass was in T2 but it was not different with than in T3 and T4. The conclusion of the study is that dietary supplementation of glucomannan derived from porang tuber extract in broiler up to 0.15% (T3) increases protein digestibility, meat calcium and protein mass, and daily weight gain with similar nitrogen retention.

Key Words: Glucomannan, Protein Digestibility, Nitrogen Retention, Meat Calcium, Protein Mass, Broiler

INTRODUCTION

Diet is a crucial determinant factor in poultry productivity maintenance, including broiler chickens. Since diet is the largest cost of broiler chicken production, an effective effort to improve the diet utilization and production efficiency is needed. Feeding non-antibiotic feed additive is one effort that can be performed to avoid the remaining residue in the product. Haryati et al. (2010) reported that the impact of the continuous and inappropriate use of antibiotics led to bacterial resistance to the animal and consumers. Prebiotic is one possible health-friendly nonantibiotic

feed additives that can be used in poultry feed and feeding.

Prebiotics are additives substances that could not be digested by the gastrointestinal tract, but were selectively fermented by lactic acid bacteria thereby increasing the activity of beneficial bacteria in the gut (Daud et al. 2007). Prebiotic compounds often used in poultry was a non-digestible oligosaccharide, including fructooligosaccharide, manan-oligosaccharide, and xylooligosaccharide. The prebiotics used in this study were glucomannan derived from porang tuber extract.

Glucomannan from porang tuber is an oligosaccharide group. An oligosaccharide is a type of prebiotic that could be applied to poultry (Haryati et al. 2010). Prebiotics oligosaccharide were difficult to be digested but it could be fermented by beneficial bacteria present in the gastrointestinal tract (Haryati et al. 2010). Glucomannan from the porang tuber is potentially used as a source of prebiotics. Glucomannan could be categorized as a source of prebiotic oligosaccharides (Zhang et al. 2014). The porangtuber as a source of oligosaccharide prebiotics contained glucomannan more than 60% (Rahayu 2013).

Addition glucomannan as prebiotic would stimulate the development of lactid acid bacteria. Additive oligosaccharide, including inulin, can be fermented by intestinal bacteria, especially lactic acid bacteria, to produce short-chain fatty acid such as acetate, butyric and propionate that inducing low intestinal pH (Fanani et al. 2016). Low pH conditions suppressed the growth of pathogenic bacteria but increasing the beneficial bacteria which leads to the better microflora balanced and improved the digestive tract health. The improvement of digestive tract health indirectly improves the digestion and absorption of nutrients, especially protein. Digestion and protein absorption increased meat calcium and protein mass (Wijayanti 2016).

The aim of this research was to evaluate the effect of glucomannan inclusion fromporangtuberon productive ability of broiler in terms of protein digestibility coefficient, nitrogen retention, meatcalcium mass, and meat protein mass. This researchcan be used as a basis of scientific informationfor the farmer community concerning the beneficial effect of glucomannan from porang tuber extract as prebiotic to increase broiler productivity.

MATERIALS AND METHODS

One day old New Lohman broiler chickens with initial body weight of 42.08±0.86 g were used in this study. This study was arranged in a completely randomized design with 5 treatments and 4 replications, 8 chicks per replication. The treatments were T0 = basal diet, T1 = T0 + 0.05% glucomannan from porang tuber extract (GPTE), T2 = T0 + 0.10% GPTE, T3 = T0 + 0.15% GPTE and T4 = T0 + 0.20% GPTE. The GPTE addition was in percentage of total diet basis. Composition and nutritional content of experimental feed are presented in Table 1.

Table 1. Composition and nutrient content of the experimental diet

Ingredients	Composition
Yellow corn, (%)	54.00
Rice bran, (%)	14.20
Soybean meal, (%)	18.00
Meat bone meal, (%)	5.75
Poultry meat meal, (%)	6.75
Dicalcium phospate, (%)	0.50
L-Lysine, (%)	0.10
DL-Methionine, (%)	0.20
Calcium carbonate, (%)	0.25
Premix, (%)	0.25
Total	100.00
Nutrient content[1)]	
Metabolisable energy (Kcal/kg)[2)]	2965.69
Crude protein, (%)	21.33
Ether extract (%)	4.68
Crude fiber (%)	4.45
Methionine [3)] (%)	0.55
Lysine[3)] (%)	1.16
Ca (%)	1.03
P (%)	0.71

[1]Analyzed at the Laboratory of Animal Nutrition and Feed Science, Faculty of Animal and Agricultural Sciences, Diponegoro University
[2]Calculated based on Balton formula (Siswohardjono 1982) cyted by Wahyuni et al. (2008)
[3]Calculated based on NRC (1994)

This research was started with the preparation of glucomannan extract, diet and cage and its equipment. Glucomannan extract was made from porang tuber according to the modified method of Tatirat & Charoenrein (2011). The chickens were offered dietary treatments since one day old until 35 days old (slaughtered age). The birds from 1 to 14 days old were kept in litter floored-cage, and they were moved and kept in the individual cage (battery) there after. Vaccinations were provided onday 4 for Newcastle

Disease (ND), on day14 for gumboro, and on day 20 for ND2.

The GPTE was based on the treatment levelswas mixed with a small portion of feed and offered in the morning to ensure the amount was completely consumed. The feed without GPTE for daily requirement and drinking water were provided *ad libitum*.

Protein digestibility coefficient was measured using total collection method combined with indicator according to Indreswari et al. (2009). Excreta was collected from 1 chicken per replication on day 31, 32, 33 and 34. Crude protein (CP) consumption was obtained from the amount of feed consumed during total excreta collection was performed. The protein digestibility coefficient was calculated using the formula of McDonald et al. (2002), namely:

$$PDC = \frac{CCP - CPE}{CCP} \times 100\%$$

PDC = Protein digestibility coefficient
CCP = Consumption of Crude Protein
CPE = Crude Protein of Excreta

Nitrogen retention was measured based on the difference of the amout of nitrogen consumed and fecal nitrogen.

Calcium and protein mass was measured from meat samples. Meat samples of approximately 100 g were obtained from each part of carcassof one birdin each replication. The samples were mixed and finely ground and then taken as much as 20 g for the analysis of calcium, protein and moisture contents. The protein and calcium mass of meat was calculated based on Suthama (2003) as follows:

$$\text{Meat Calcium Mass (g)} = \text{Meat Calcium Content (\%)} \times \text{Meat Weight (g)}$$

$$\text{Meat Protein Mass (g)} = \text{Meat Protein Content (\%)} \times \text{Meat Weight (g).}$$

Data were subjected to analysis of variance with F test to determine the effect of treatment. When the treatment indicated significant effect, it was continued to Duncan multiple range test at 5% probability level.

RESULTS AND DISCUSSION

The addition of GPTE as a prebiotic source in broiler diet on protein consumption, protein excreta, protein digestibility coefficient and nitrogen retention are presented in Table 2. Dietary addition of GPTE indicated significant effect only on protein digestibility coefficient. Protein digestibility coefficient of T0 treatment was significantly ($P<0.05$) lower that that of other treatments, and that of T1 was also lower as compared to that of T4 but no diferences among other treatments. The addition of GPTE at 0.20% (T4) increased protein digestibility to the highest value even though no difference was found compared to T2 and T3. Feeding GPTE in broiler as compared to control diet improved protein digestibility coefficient. Protein digestibility coefficient was closely related to digestive tract health due to the addition of prebiotic glucomannan (GPTE). The increase in protein digestion coefficient was assumed that GPTE played a role as a source of "food or nutrient" that could be exploited by the beneficial endogenous bacteria in the digestive tract.

Better digestive tract health brought about the increased protein digestibility coefficient Muhammad et al. (2015) stated that the addition of prebiotics could balance the microflora in the gastrointestinal tract by increasing beneficial bacteria counts, and decreasing pathogenic bacteria population. This gastrointestial condition provided an impact on the increase in nutrient digestibility such as protein digestibility coefficient. Guilloteau et al. (2010) reported that the addition of prebiotics in chickens can increase short-chain fatty acids leading to the decrease in pH of the gastrointestinal tract and then increasing the activity of beneficial bacteria. Similarly, feeding probiotic inulin in crossbred local chicken increased lactic acid bacteria and decreased the population of *Escherichia coli* (Krismiyanto et al. 2015), thereby positively affecting the health of the gastrointestinal tract leading to the increase in protein digestibility (Fanani et al. 2016). Research result of Muhammad et al. (2015) also showed that the addition of prebiotic increased protein digestibility.

The different pattern was found with nitrogen retention (Table 2) that there was no significant effect caused by the addition of GPTE even though protein digestibility coefficient increased. Nitrogen retention was not significantly different due to the same dietary protein content and nitrogen consumption. The present result was in accordance with the report of Primacitra et al. (2014). Ma'rifah et al. (2013) also mentioned that nitrogen retention was influenced by nitrogen consumption, because nitrogen retention was calculated based on the value of nitrogen consumption. Wijayanti (2016) also supported the present result that the dietary addition of prebiotic soybean oligosaccharide (SOS) in broiler significantly increased protein digestibility coefficient, but nitrogen retention was the same. Nitrogen retention was not statistically different, but the addition of GPTE tended to increase its value numerically. This phenomenon suggested that dietary inclusion effect of GPTE would be significant when added at higher level. However, this condition indicated that the addition of GPTE improved the efficiency of

protein utilization since the higher meat protein mass can be resulted (Table 3) although with the same nitrogen retention (Table 2).

Dietary additional effect of GPTE as a prebiotic source on meat protein and calcium mass is presented in Table 3. Both parameters were significantly affected by dietary inclusion of GPTE.

Meat protein mass in T0 and T1 treatments indicated the same values, and both were significantly ($P<0.05$) lower than that in T2, T3 and T4, but there was no different among that in T2, T3 and T4. This was in accordance with the increase in protein digestibility coefficient. The increased protein digestibility coefficient was assumed to be due to a higher increase in protein intake even though nitrogen retention was the same. Therefore, the increased meat protein mass was an evidence of the improvement rate of muscle protein synthesis due to feeding effect of GPTE up to 0.2%. Wijayanti (2016) reported that supplementation of prebiotic soybean oligosaccharide (SOS) was able to improve protein digestibility coefficient that brought about higher protein supply as a substrate for the increase in meat protein mass. The higher meat protein mass indicates the better protein deposition due to utilization efficiency of dietary protein for tissue synthesis.

Similar value of meat calcium mass was found among T0, T1, and T2 treatments and they were significantly ($P<0.05$) lower than that in T3 and T4, but there was no difference between T3 and T4 treatments (Table 3). Meat calcium mass showed the same pattern with meat protein mass. The calcium mass of meat in T3 and T4 increased along with the increase in protein digestibility coefficient. The increase in calcium mass of meat due to the addition of GPTE was in accordance with protein digestibility coefficient and meat protein mass. The increase in protein digestibility coefficient in this study was assumed to play an important role in binding calcium called calcium binding protein (CaBP), thereby increasing the absorption of calcium which causes the increase in meat calcium mass. This was in accordance with the result reported by Syafitri et al. (2015) that the addition of beluntas leaf extract as feed additive can increased meat calcium mass supported by the improved protein digestibility coefficient and higher calcium retention bringing about the high calcium deposition in the meat. Fanani et al. (2016) stated that feeding prebiotic inulin improved protein digestibility coefficient that closely related to the absorption of calcium. As it has been previously discussed that calcium was absorbed together with a protein called CaBP, thus, it can be assumed that increasing protein digestibility coefficient followed by increased calcium absorption resulting in the increase in meat calcium mass.

The increased meat protein mass was closely related to the improved body weight gain. This relationship was indicated by the pattern between meat protein mass and body weight gain was almost the same. Body weight gain of T2, T3 and T4 treatments were higher than that of T0 and T1 (Table 3). However, when GPTE was added at the level of 0.15% (T3) resulted high growth and better meat product similar to those of T4 (0.20% GPTE), but T3 more efficient because lower amount of porang tuber extract was used. Output

Table 2. Protein digestibility coefficient and nitrogen retention in broiler chicken fed diet with addition of glucomannan from porang tuber extract (GPTE)

Parameter	Level GPTE (%)				
	T0 (0)	T1 (0.05)	T2 (0.10)	T3 (0.15)	T4 (0.20)
Protein consumption (g/bird/day)	28.04	27.61	28.77	27.90	27.53
Protein in excreta (g)	6.55	5.49	5.24	4.91	4.64
Coefficient of protein digestibility (%)	76.70^{c}	80.14^{b}	81.78^{ab}	82.28^{ab}	83.09^{a}
Nitrogen retention (g/bird/day)	3.44	3.54	3.76	3.68	3.66

$^{a-c}$ Different superscript at the same raw shows significantly difference ($P<0.05$)

Table 3. Meat protein and calcium mass of broiler fed diet with addition of glucomannan from porang tuber extract (GPTE)

Parameter	Level GPTE (%)				
	T0 (0)	T1 (0.05)	T2 (0.10)	T3 (0.15)	T4 (0.20)
Meat protein mass (g/bird))	150.39^{b}	161.98^{b}	187.45^{a}	181.62^{a}	182.10^{a}
Meat calcium mass (mg/bird)	59.51^{b}	64.20^{b}	68.18^{b}	84.32^{a}	85.28^{a}
Body weight gain (g/bird/day)	39.59^{b}	40.50^{ab}	42.30^{a}	41.60^{a}	42.13^{a}

$^{a-b}$ Different superscript at the same raw shows significantly difference ($P<0.05$)

specification produced in T3 treatment can be categorized as better meat quality due to its rich in calcium and protein deposition with high body weight. The determinant of meat quality was chemical compositions including protein and calcium (Dewi 2013). Thus, T3 resulted better meat quality with high body weight and more efficient as compared to T4.

CONCLUSION

Inclusion of glucomannan derived from porang tuber extract (GPTE) in broiler chicken diet at 0.15% (T3) can improve protein digestibility, and produce better and more efficient meat quality based on calcium and protein mass and daily body weight gain.

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Correlation of Electric Conductivity Values with the Dairy Milk Quality

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ABSTRAK

Yanthi ND, Said S, Anggraeni A, Damayanti R, Muladno. 2018. Korelasi nilai electric conductivity dengan kualitas susu sapi perah. JITV 23(2): 82-88. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1694

Susu sebagai sumber makanan utama bagi kehidupan mammalia memiliki kandungan elektrolit untuk menggantikan cairan tubuh yang hilang oleh karena aktivitas ataupun proses metabolisme. Konsentrasi total elektrolit dapat diukur berdasarkan nilai konduktivitas yang dihasilkan susu berdasarkan pada komposisi nutrisi yang dikandungnya. Dengan demikian hubungan parameter kualitas susu dengan nilai konduktifitas dapat menjadi tolok ukur untuk kualitas dan nilai jual susu sehingga lebih mudah diaplikasikan di lapangan. Penelitian ini bertujuan menganalisis hubungan antara electric conductivity (EC) dengan nilai kandungan susu sapi. Susu diambil dari 10-30 ekor sapi dari peternakan di Kabupaten Bandung Barat (Lembang), Kabupaten Bandung (Pangalengan), Tasikmalaya, Sumedang, Subang, Sukabumi dan Bogor Provinsi Jawa Barat. Susu ditampung dalam falcon steril 50 ml. Probe EC count-meter CT-3031 digunakan untuk mengukur EC sedangkan Probe MilkoScan^{TM} FT 120 (Foss) digunakan untuk mengukur kualitas susu. Kualitas susu dicerminkan oleh kandungan Protein, Fat, Total Solid (TS), Solid Non Fat (SNF), Lactose, Density, Acidity, dan Freeze Point Deviation (FPD). Hasil penelitian menunjukkan bahwa nilai EC pada susu memberikan pengaruh positif yang sangat nyata ($p = <0.01$) terhadap Total Solid (TS), Solid Non Fat (SNF), Lactose dan Freeze Point Deviation (FPD). Selanjutnya nilai EC berpengaruh nyata ($p = < 0.05$) terhadap nilai density pada susu. Oleh karena itu nilai EC dapat digunakan untuk memprediksi nilai kualitas susu.

Kata Kunci: Korelasi, Electric Conductivity, Kualitas Susu

ABSTRACT

Yanthi ND, Said S, Anggraeni A, Damayanti R, Muladno. 2018. Correlation of electric conductivity values with the dairy milk quality. JITV 23(2): 82-88. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1694

Milk, as the prime source of food for mammals, has an electrolyte to replace the loss of body fluid caused by activity or metabolism process. The total electrolyte concentration can be measured based on conductivity value from the nutritional content. Therefore, the parameter of the quality of milk with conductivity value can be a benchmark for quality and selling value of milk, making it simpler to be implemented in the field. The aim of this research is to analyze the relation between electric conductivity (EC) with the content value of cow milk. The milk was taken from 10-30 cows from a farm in Lembang (district of West Bandung), Pengalengan (district of Bandung), Tasikmalaya, Sumedang, Subang, Sukabumi and Bogor of West Java Province. The milk was put in 50 ml of sterile falcon. The Probe EC count-meter CT-3031 was used to measure EC while the quality of milk was measured by Probe MilkoScan^{TM}FT 120 (Foss). The milk quality is reflected by protein content, Fat, Total Solid (TS), Solid Non-fat (SNF), Lactose, Density, Acidity and Freeze Point Deviation (FPD). The results of this study show that the EC value in the milk gives a very real positive effect ($p=<0.01$) to Total Solid (TS), Solid Non-fat (SNF), Lactose, and Freeze Point Deviation (FPD). The value of EC also significantly affect ($p=<0.05$) the value of density in milk. Therefore, the value of EC can be used to predict the quality value of milk.

Key Words: Correlation, Electric Conductivity, Milk Quality

INTRODUCTION

Electric conductivity value is determined by the concentration of anion and cation condition. The electrical conductivity or well known as conductivity is how far a solution may conduct electricity (Irwan & Afdal 2016), so that the conductivity value of a solution is a measure of total concentration of electrolyte in a solution. Electrolyte solution is a solution consisting of negative and positive ions. In the electrolyte solution, its molecules are dissociated into positive and negative electricity charged particles. These ions then may conduct the electricity.

Milk is a solution consisting of essential nutritional components to humans needed to improve physiological and biological functions that affect the metabolism and health. These essential components consist of the ideal diet composition that is all chemical components in the form of six main nutrients: water, fat, protein, carbohydrate (including lactose), mineral and vitamin (Chandan 2006; Rezaei et al. 2016). Vegarud et al. (2000) stated that protein, fat, carbohydrate, minerals (Na, K, Ca, I etc.) and elements (Fe, Zn, Cu, ets.) contained in milk. Cow's milk is in a chemical equilibrium with free ion content present in greater quantities than any other form of dairy products. The chemical composition of milk can be influenced by many factors such as breed, environment condition, milking period, and nutrient status of animal (Vegarud et al. 2000; Schanbacher et al. 1998; Korhonen & Pihlanto-Leppälä 2004; Gobbetti et al. 2007; Kalač et al. 2010; Pereira 2014). Nutrients contained in milk consist of complex compounds that have 100,000 chemical molecules separated from each other (Chandan 2006). These chemical molecules contain free cation and anion elements that can bind to other free ions. The use of EC as a milk analysis has been widely reported. Mammadova & Keskin (2013) stated that the value of EC was used to detect to detect changes in milk composition associated with an udder condition infected with mastitis is another measure of evidence used in increasing the frequency of milk production. Based on a research result resulted by Norberg et al. (2004b), milk electric conductivity might be used as an indicator of phenotypic and genetic of cow mastitis. Research result of Diaz et al. (2012) also showed that the use of EC to detect the subclinical mastitis as well as its initial condition effectively.

Juozaitienė et al. (2017) said that the electric conductivity might be used to collect information about electric conductivity of milk in the genetic evaluation to improve milk quality. In its application, the measurement using EC is a reading done by capturing the positive and negative ions of a solution. The body requires Na^+ and Cl^- ions in a small, balanced concentration. But it is necessary for the metabolism process in the body. The electrolyte value of a solution may change due to the condition of the body and the environment. It becomes more practical and easy to know by using EC.

MATERIALS AND METHODS

The dairy cow milk samples analyzed were from 10-30 cows in the farms around West Java, consisting of West Bandung District (Lembang), Bandung District (Pangalengan), Tasikmalaya District, Sumedang District, Subang District, Sukabumi District and Bogor District.

Milk is stored in a 50 ml sterile falcon tube then stored in an ice box while on the go in order to maintain milk quality. Milk quality observation was conducted at Animal Health Laboratory, Biotechnology Research Center-Indonesian Institute of Science, Cibinong.

Measurement of electro conductivity of milk was done by using EC count-meter CT-3031. This tool is a fast, reliable and inexpensive method to measure the concentration of solution ions, as well as saving battery because it uses electric w/ATC so also saving battery. The Unit S1 conductivity was Siemen per meter (s/m). This type of pen conductivity meter is not only lightweight and compact but also waterproof and will provide a +/- 2% accuracy reading with a range of 0-19.99 mS. The end of "Conductivity/TDS Probe" is immersed in the solution, until submerged. Shake the probe several times to remove air bubbles from internal examination. When the screen reading reached stable, it was the measured conductivity value.

Milk quality measurement was done by using MilkoScan™ FT 120 (Foss) mechine. This MilkoScan tool can analyze milk quickly and accurately on raw milk, intermediates and the final products. In addition, this tool can also analyze various parameters of milk Fatty Acids, Urea, Fats, Proteins and Lactose in various dairy products. With the completeness of analysis of milk and its dairy products, this tool has a large shape that can't be placed anywhere and expensive, so it can only be placed only in the laboratory. The MilkoScan™ FT 120 (Foss) probe was dipped in 35-40 ml of milk then did the reading of milk quality value. The data obtained are: protein, fat, total solid, solid nonfat, lactose, density, FPD, and acidity. The obtained data were processed by using SPSS 17 to see correlation between milk quality and EC value.

RESULTS AND DISCUSSION

The quality of milk

Essential nutrients in milk in the form of proteins, minerals, and other essential elements are needed as a source of food for growth and body maintenance (Singh et al. 2015; Enb et al. 2009). Milk quality data presented in Table 1 were protein, total solid (TS), Solid Non Fat (SNF), lactose, density, Freeze Point Deviation (FPD), acidity, Electric Conductivity (EC), with comparable milk values based on Indonesian National Standard (SNI). This standard establishes good quality requirements of fresh milk including protein content at a minimum value of 2.8%, a minimum fat content of 3.0%, a minimum SNF of 7.8%, TS of 10.8%, density of 1.027 g/ml, FPD of -0.520 - -0.560, acidity of 6.0 - 7.5°SH and other requirements in the form of microbiological contamination. Thus if the fresh milk content tested has a value below the

minimum value of SNI then the fresh milk value is bad. Chemically, the composition of milk consists of fatty emulsions in water containing sugar, mineral salts, and proteins in the form of colloidal suspense, which means that the main components of milk are water, fat, protein (in the form of casein and albumin), lactose (sugar milk) and ash. Besides the water in the milk component, there were TS and SNF components. Each component contained in milk has a concentration that can be affected by several factors, including disease (mastitis), stages in the lactation period, season/climate and food conditions (Bernabucci et al. 2015; Milani 2016). So these factors can change the concentration and condition of milk in quality and quantity.

The result of milk quality analysis obtained from milk samples showed the mean fulfill the quality of SNI. But the minimum and maximum value obtained in the measurement of mastitis milk was still very wide. The minimum protein value of the samples obtained was still very far from the standard of 0.1. Likewise, the maximum value was well above the standard value. For other parameters, the fat concentration, TS, SNF and acidity had a very wide range of values as well. The minimum and maximum value distance of the milk quality parameter content is influenced by many factors. This is reinforced by the statement of Elmoslemany et al. (2010) that basically the quality of milk was influenced by the quality of feed offered. It was also influenced by management. The comparison between concentrate and forage feed can also affect the value of the concentration of milk quality. In addition to feed, livestock and climate conditions can also determine the concentration of milk quality (Milani et al. 2015; Gellrich et al. 2015). Healthy cows will have better milk quality and qualify for SNI.

The correlation between EC and milk quality

Table 2 shows the relationship between EC and the composition of milk content which shows the relationship of each parameter with EC. In addition, each parameter also has a close relationship with each other. Proteins had a very real sequence of TS, SNF, lactose, FPD, and Acidity. Then fat had a very real relationship to the TS and density and had a real relationship to the EFF. TS had a very real relationship to the other three variables in addition to protein and Fat were SNF, density, and FPD. Besides, TS also had a very real sequence with EC. SNF parameters had a similar relationship to TS plus a very strong relationship to lactose. Lactose had a very real relationship to the four parameters of protein, SNF, density, and EC. Density had a very real relationship with five parameters of fat, TS, SNF, lactose, and FPD, also to EC. The FPD was a parameter that had a very significant correlation between the five parameters of protein, fat, TS, SNF and density and the apparent relationship of acidity besides FPD also had a very real relationship to EC. The acidity parameter had a very real relationship to the protein and the data relationship to the EFF. Of the eight milk quality parameters of protein, fat, TS, SNF, lactose, density, FPD and acidity, the most obvious correlation to EC were TS, SNF, lactose, and FPD and a significant relationship with density.

Table 1. Milk quality of dairy milk in West Java

Parameter	Minimal	Maximal	Mean	SNI
Protein	0.1	6.95	2.81±1.060	2.8
Fat	0.31	11.19	3.05±1.944	3.0
TS	7.8	19.17	11.21±2.202	10.8
SNF	1	10.32	7.53±2.061	7.8
Lactose	0.34	9.34	5.03±1.857	-
Density	1015	1037	1029.45±4.692	1.027
FPD	0.311	0.648	0.50±0.59	-0.520-0.560
Acidity	3.43	14.43	6.70±1.663	6.0-7.5
EC	1.33	9.47	4.59±1.278	-

Table 2. Pearson correlation of milk quality and electric conductivity value

		Protein	Fat	TS	SNF	Lactose	Density	FPD	Acidity	EC
Protein	Pearson Correlation	1	-.035	.421**	.503**	-.512**	-.143	.274**	.274**	-.142
	Sig. (2-tailed)		.689	.000	.000	.000	.105	.002	.002	.106
Fat	Pearson Correlation	-.035	1	.834**	-.054	.003	-.524**	.179*	-.017	-.125
	Sig. (2-tailed)	.689		.000	.545	.976	.000	.042	.846	.156
TS	Pearson Correlation	.421**	.834**	1	.328**	-.144	-.273**	.538**	.061	-.312**
	Sig. (2-tailed)	.000	.000		.000	.101	.002	.000	.489	.000
SNF	Pearson Correlation	.503**	-.054	.328**	1	-.706**	.340**	.517**	.103	-.525**
	Sig. (2-tailed)	.000	.545	.000		.000	.000	.000	.245	.000
Lactose	Pearson Correlation	-.512**	.003	-.144	-.706**	1	.308**	.148	-.077	.261**
	Sig. (2-tailed)	.000	.976	.101	.000		.000	.092	.386	.003
Density	Pearson Correlation	-.143	-.524**	-.273**	.340**	.308**	1	.575**	.053	-.219*
	Sig. (2-tailed)	.105	.000	.002	.000	.000		.000	.549	.012
FPD	Pearson Correlation	.274**	.179*	.538**	.517**	.148	.575**	1	.201*	-.376**
	Sig. (2-tailed)	.002	.042	.000	.000	.092	.000		.022	.000
Acidity	Pearson Correlation	.274**	-.017	.061	.103	-.077	.053	.201*	1	-.058
	Sig. (2-tailed)	.002	.846	.489	.245	.386	.549	.022		.510
EC	Pearson Correlation	-.142	-.125	-.312**	-.525**	.261**	-.219*	-.376**	-.058	1
	Sig. (2-tailed)	.106	.156	.000	.000	.003	.012	.000	.510	

* P<0.05 ** P<0.01

The increasing of EC value of milk may also indicate substantial variation in the absence of mastitis this may be due to many factors such as lactation stages, cow age, milking intervals and cow-milk conditions (Biggadike et al. 2000). In addition, according to Kaşikçi et al. (2012), EC value increase is a change that occurs to the mineral content in milk. Factors such as milk temperature, pH and fat concentration in milk also have an effect on EC measurement (Qayyum et al. 2016)

This research result is the EC value had a very significant positive effect ($P \leq 0.01$) on the four parameters of milk composition i.e., TS, SNF, Lactose and FPD. Total Solid was a dissolved solid and/or total suspended solids. This parameter had a very large influence on the milk content. TS is a component consisting of proteins, fats, carbohydrates, lactose, minerals, and vitamins. Under normal conditions the milk produced in a healthy udder will contain a balanced and healthy chemical composition of milk, making it feasible for consumption. The balance of the chemical composition of milk is obtained from a balanced diet between forage and concentrate (Kholif et al. 2014; Villeneuve et al. 2013).

Protein in milk consists of 80% casein which is resistant to low pH and 20% whey (Malacarne et al. 2002; Pegoretti et al 2015). Chemically, a protein is a polymer made of 20 amino acid proteinogenic. Amino acids contained in casein consist of histidine, methionine, phenylalanine, and other non-essential amino acids, arginine, glutamine, proline, serine and tyrosine. With the acid-resistant properties of casein, the amino acids constituent are amino acids that are soluble in an acid-containing solvent. Amino acid content in whey consists of leucine, isoleucine, and valine (Boisen et al. 2000). Mohan et al. (2014) stated that whey protein in cow's milk has a negative charge at pH 6.6 (normal milk pH), so whey is a dissolved protein fraction. This is reinforced by the statement of Pereira (2014) that the fraction of proteins can be divided into two i.e., soluble and insoluble proteins. The soluble protein is represented by whey. Thus the concentration of milk protein will greatly affect the value of electrical conductivity. Some of these amino acids are composed of amino acids that have different polarities with each other. This level of polarity will determine the value of the ions to be produced, so this will also affect the value of EC. According to Wu (2013), amino acids have a very extraordinary role in life ranging from metabolic processes, growing physiology and immunity.

Milk fat is composed of triacylglycerol and some other fats. Fat is one tool in assessing the quality of milk. Fat has a low solubility level of normal diluted, but it can dissolve in acid solvents. Vitamins contained in milk are vitamins A, D, B, K, C, and E. These vitamins are highly dependent on feed intake and sun exposure is obtained (Gorewit 2016). Mineral milk content is composed of monovalent ions namely Na, K, and chloride (Cl). These ions are the most common mineral type of ion suspension present in a free state in milk. This salt ion collectively contributes for more than 25% of the total osmolarity of cow's milk. There are also divalent ions: calcium (Ca), magnesium (Mg), citrate, phosphate (P) and sulfate and some other elements of copper, iron, silicon, zinc, and iodine. These mineral ions have a great influence during lactation in physiological conditions to open and close the intersections between epithelial cells in the milk glands (Li 2016). In addition, minerals also play an important role in the stability of milk proteins (Mohan et al. 2014). This difference in composition, fat, protein, vitamins and minerals will affect the value of EC. Because there are components of these nutrients can conduct electricity.

SNF is a milk component consisting of nonfat dry material. The SNF content consisting of protein, lactose, and mineral (Chandan 2006). Concentrations of proteins, lactose, vitamins, and minerals will greatly affect the value of EC. According to Norberg et al. (2004a), the infected udder of bacteria will increase Na^+ and Cl^- concentration in milk which produce high conductivity. High values of Na^+ and Cl^- cause the value of milk content will decrease.

The FPD is a measure of the milk condition to the level of milk freezing. The freezing point in milk is affected by the dissolved components in milk, especially the components of amino acids (proteins), lactose, vitamins and minerals. This component is very influential on dairy products both in yogurt, cheese and others. According to McCarthy & Singh (2009), the rate of decrease in freezing point will be proportional to the molality of a solution. The content of milk consisting of lactose, chloride salt and other water-soluble ingredients (such as calcium, potassium, magnesium, lactate, phosphate, citrate, etc.) each contribute to a decrease in freezing points of about 55, 25, and 20%. The amount of ions in the solution is also affected by the dissolved solids in it. The greater the amount of dissolved solids in solution, the more likely the number of ions in the solution will increase, so that the electrical conductivity value will also increase (Mohan et al. 2014).

In addition, EC also has a very real effect on lactose. Lactose is the major carbohydrate in cow's milk is a disaccharide consisting of one glucose residue and one galactose residue (Mohan et al. 2014). The concentration of lactose in milk is about 4.8-5.2% of the total weight of cow's milk volume (O'Mahony & Fox 2014). In solution, lactose undergoes mutarotation between α and β forms, involving the interchanging of OH and H groups in the reducing group. Mutarotation of lactose depends on temperature and pH (Mohan et al.

2014). The low concentration of lactose values in milk can increase the value of EC. This is in accordance with the statement of Fahmid et al. (2016). Mastitis conditions affect lactose levels where the condition of the erythrocyte. Inflammation is caused by bacterial mastitis and can increase the concentration of Na^+ and Cl^- ions and decrease the levels of K^+ and lactose ions so that the EC value will increase. Even though electrolyte dissolved sugar does not provide an increase in the value of positive and negative ions, but with the destruction of cell tissues in the infected udder as a milk making "factory" in the mastitis, udders can increase Na^+ and Cl^-. Varman & Sutherland (2001) has explained that lactose makes a major contribution to the milk's colligative properties, such as the osmotic pressure, the depression freezing point, and the boiling point.

Another effect that gives significant results on cow milk content so that it can also affect the value of EC is the value of density. The density value had a significant correlation to EC ($P \leq 0.05$). The density value describes the mass weight of a substance or solution. Density of milk consists of milk-forming mass such as protein, fat, lactose and minerals, so the density value will affect the EC value because milk mass formers consist of milk forming components. Variations in electrical conductivity caused by changes in Na, K and Cl milk is highly likely due to several factors such as nutrition, age, type, parity, milking intervals and milk composition other than mastitis (Norberg 2005).

CONCLUSION

The overall milk analyzed showed mean value that still remained on the value of milk nutrient quality based on the SNI. Analysis value using EC had correlation to milk quality. The components of milk that had significant effect ($P<0.01$) on EC value were Total Solid, Solid Non Fat, Lactose, FPD. The component that had significant effect ($P<0.05$) to EC value was Density. EC had a close correlation with milk quality, and EC can be used to predict the value of milk quality.

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Clenbuterol Residue in Beef Meat Collected from Several Cities in Java Island, Indonesia

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ABSTRAK

Widiastuti R, Anastasia Y. 2018. Residu clenbuterol pada daging sapi yang dikoleksi dari beberapa kota di Pulau Jawa, Indonesia. JITV 23(2): 89-94. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1621

Clenbuterol (CLB) merupakan obat hewan ilegal golongan β-agonis yang digunakan sebagai pemacu pertumbuhan pada berbagai hewan ternak. Keberadaan residu CLB pada produk ternak dapat menimbulkan keracunan pada manusia seperti tremor, takikardia, mual dan pusing. Tujuan dari penelitian ini adalah memvalidasi metode deteksi residu CLB pada daging sapi secara kromatografi cair kinerja tinggi (KCKT) serta mengetahui keberadaan residu CLB pada 74 sampel daging beku dan segar dari beberapa kota di pulau Jawa. Sampel diekstraksi dengan asetonitril dan isopropanol, kemudian dianalisis secara KCKT menggunakan kolom RP ODS C_{18} dan fasa gerak campuran 50 mM NaH_2PO_4 dan asetonitril (80:20, v/v) serta dideteksi menggunakan detektor photodiode array pada panjang gelombang 210 nm. Hasil validasi metoda untuk parameter uji perolehan kembali pada penambahan CLB adalah 103,45; 89,27 dan 89,53% untuk masing-masing penambahan konsentrasi sebesar 2, 5 dan 10 ng/g CLB. Nilai limit deteksi dan limit kuantitasi 0,10 ng/g dan 0,31 ng/g. Hasil analisis terhadap 74 sampel, menunjukkan bahwa residu CLB terdeteksi pada 8 sampel dengan kisaran konsentrasi 2,40 hingga 15,06 ng/g dan telah melebihi batas maksimum residu CLB sebesar 0,2 ng/g. Untuk menghindari bahaya dari keberadaan residu CLB, diperlukan adanya monitoring terhadap bahan pangan asal ternak untuk menjamin keamanan pangan bagi konsumen.

Kata Kunci: Clenbuterol, Residu, Daging Sapi, KCKT

ABSTRACT

Widiastuti R, Anastasia Y. 2018. Clenbuterol residue in beef meat collected from several cities In Java Island, Indonesia. JITV 23(2): 89-94. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1621

Clenbuterol (CLB) is an illegally animal drug of the β-agonist group that used as a promoter of growth in various farm animals. The presence of CLB residues in livestock products can cause poisoning in humans, such as tremor, tachycardia, nausea and dizziness. The purpose of this research is to validate CLB residue detection method on beef meat detected using a high performance liquid chromatography (HPLC) and to determine the presence of CLB residue on 74 samples of frozen and fresh beef meat from several cities in Java. Samples were extracted with acetonitrile and isopropanol, then analyzed chromatographically using RP ODS C_{18} column and mixed mobile phases of 50 mM NaH_2PO_4 and acetonitrile (80:20, v/v) and detected by photodiode array detector at a wavelength of 210 nm. The recoveries were 103.45, 89.27 and 89.53% for each additional spiked at concentrations of 2, 5 and 10 ng/g of CLB. The detection limit and the quantitation limit were 0.10 ng/g and 0.31 ng/g, respectively. Analysis of 74 samples showed that CLB residue was detected in 8 samples in a concentration range of 2.40 to 15.06 ng/g and had exceeded the CLB residue maximum limit of 0.2 ng/g. To avoid the risk of the presence of CLB residues, it is necessary to regularly monitor the presence of residue of animal products to ensu re of food safety for consumers.

Key Words : Clenbuterol, Residue, Beef Meat, HPLC

INTRODUCTION

Clenbuterol (CLB) is one of β-agonist group of human medicine that licensed as a bronchodilator (treatment for asthma) in many countries. Its chemical structure (see Figure 1) is related to catecholamine's able to interact with adrenergic receivers, generally of the type β_2 (Valladares-Carranza et al. 2014). Clenbuterol with a common trade name Ventipulmin is not licensed as a growth promoting drug in European Union (EU) (EC 2010), and also China and United States, but some countries still have approved it for food producing animals, and sometimes used at 5-10 times higher than common therapeutic doses. Therefore, this drug is also used illegally to promote animal livestock (including beef meat) growth, causing a considerable increase in muscular mass and, at the same time, a decrease in fat accumulation and improved feed efficiency (Trejo et al. 2013). The free fatty acids produced by CLB stimulation belonging to the β group

of adrenergic agonists to the fatty tissue will be used as an alternative energy source of muscle protein synthesis. The use of CLB has a positive impact on the environment due to reduce excretion of livestock.

The use of CLB for growth-promoting purposes has been prohibited in European countries, Mexico and some other countries (EC 2010; Valladares-Carranza et al. 2014). The reason of banning CLB use as a growth promoter in Europe is because its classification of class A animal drug with anabolic effects. Livestock products (meat and liver) containing CLB residues have the potential to increase heart rate and blood pressure, tachycardia, excessive anxiety, chills and muscle tremor (Brambilla et al. 2000). The maximum residue limit (MRL) for CLB permitted by WHO and Codex Alimentarius in meat is 0.2 ng/g and in liver is 0.6 ng/g (CAC 2012). The Codex Alimentarius Commission recommends MRLs for cattle are of 0.2 ng/g in muscle and fat, 0.6 ng/g in liver and kidney, and 0.05 ng/mL for cattle milk, expressed as parent drug (CAC 2012).

In some cases, the presence of non-negligible amounts of drug residues in meat is a real public health problem and causing food poisoned from eating liver and meat contained CLB residues. Fifteen people in Italy poisoned from eating beef contained CLB with concentrations of 1140-1480 ng/g (Brambilla et al. 2000), as well as in 50 people in Portugal from ingesting CLB residue in meat lamb contained 0.3 mg/kg and liver lamb contained 1.4 mg/kg (Barbosa et al. 2005). In China, a person was died in Guangdong province in March 2006, also 300 people were poisoned in Shanghai in September 15, 2006 (Lai et al. 2008). The use of CLB in Indonesia also prohibited refer to the Act No. 18 of 2009 on Animal Husbandry and Animal Health (Articles 50-51) which had been amended by Act No. 41 of 2014 (Articles 19-23) and reinforced with Circular Letter of the Director General of Animal Husbandry and Animal Health Number 30059/HK.340/F/11/2011 and the Regulation of the Minister of Agriculture Number 14/PERMENTAN/ PK.350/5/2017.

Efficient methods are required for monitoring residue levels to ensure safety of food supply for human (Schneider et al. 2007) that might be done using a rapid screening method such as ELISA and lateral-flow assay (He et al. 2009; Lai et al. 2008) or confirmative method such as high performance liquid chromatography (Chang et al. 2005; Trejo et al. 2013). So far, no published reports on CLB residues exist on frozen and fresh beef meat circulated in Indonesian markets except official data published by BPMSPH (2015) after the case was revealed in local newspaper. This study reports the presence of CLB residue in beef meat samples originated from imported beef, fattening beef and local beef collected from several cities in Java Island using an adopted method developed by Trejo et al. (2013).

MATERIALS AND METHODS

Chemical materials

The standard of clenbuterol hydrochloride (≥95%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Chemicals such as methanol (p.a grade) acetonitrile (hplc grade), isopropanol (p.a grade), NaCl, anhydrous MgSO4, anhydrous Na_2SO_4, NaH_2PO_4 were obtained from Merck (Germany) and PTFE 0.45 µm (Waters, USA) microfilter. All aqueous solvents use water type 1 (ultra pure water/UPW) from Purelab Flex (Elga LabWater, UK).

Sampling locations and collection

Altogether 74 frozen or fresh meat samples (approximately 250 gr) collected and assisted by each District Livestock officers were collected during March to August 2013 from Tanjung Priok Port Quarantine for imported beef meat and a slaughterhouse in Jakarta for fattening beef meat, supermarkets for imported and traditional markets for fattening/local beef meat in Bandung, Semarang and Yogyakarta. The samples were grounded finely, stored in plastic bags and refrigerated at -20°C until analysis. Samples were thawed at room temperature before analysis, then extracted and analyzed for CLB residue as described in the following method.

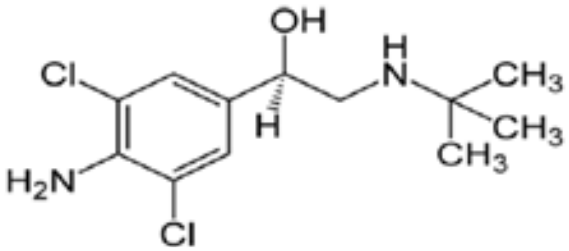

Figure 1. Chemical structure of clenbuterol (Valladares-Carranza et al. 2014).

Extraction and determination of CLB residue by HPLC

The extraction process of the samples was adopted from a method developed by Trejo et al. (2013). A total of 5 g finely grounded beef meat was put into a 50 mL centrifuge tube and added by 4 mL of acetonitrile and 2 mL of isopropanol, then shaken for 30 seconds. The mixture was then added with NaCl of 1.2 g and vortexed for 2 min, and then centrifuged for 10 min at 3500 rpm. Then the mixture was added with 4 g of anhydrous Na_2SO_4 and 0.5 g of $MgSO_4$ and vortexed for 2 minutes and centrifuged for 15 minutes at 3500 rpm. The resulting supernatant was then transferred into a 5 mL vial and dried using nitrogen at 40°C until dry. The dry residue was then reconstituted using 1 mL of ultra pure water (UPW) and filtered using a 0.45 µm filter and ready to be analyzed with an HPLC.

The HPLC instrumentation used was a Shimadzu Prominence equipped with LC-20AD pump, SPD M20A photo diode array detector (Shimadzu, Japan), set at a wavelength of 210 nm. Analytical separation was conducted using Shimp pack VP ODS column (4,6 x 150 mm, i.d 5 µm, Shimadzu, Japan). The mobile phase was a mixture of 50 mM NaH_2PO_4 (pH 3.0)/acetonitrile (80 : 20, v/v), filtered with a 0.45µm PTFE filter membrane (Waters, MA, USA) and was sonicated for 10 minutes and run at flow rate of 0.8 mL/min.

RESULTS AND DISCUSSION

Method development for CLB detection

The extraction method adopted from Trejo et al. (2013) for CLB residue detection in meat sample in this study is relatively fast, efficient, effective, environmentally friendly and does not require a purification process. The sample was extracted using acetonitrile followed by isopropanol to remove the fat from the sample matrix. Then the salt, sodium sulfate and magnesium sulfate were added to withdraw water from the sample matrix.

The CLB residue was then eluted isocratically using a mobile phase of a mixture of 50 mM NaH_2PO_4 (pH 3.0) and acetonitrile (80 : 20, v/v) and detected by HPLC equipped with a photo diode array (PDA) detector at a wavelength of 210 nm with a typical chromatogram is shown in Figure 2. The CLB residue elutes at 6.0 min. The use of PDA detector can confirm the positive samples by scanning the wavelength.

Several validation test parameters (recovery test, limit of detection and limit of quantitation) was carried out to verify this method meets the requirements to be applied in real field samples. The extraction efficiency (recovery test) of clenbuterol (CLB) was determined in triplicates at 2, 5, and 10 ng/g and subjected to extraction using the aforementioned method. The recovery of this study was assessed by analyzing a sample of known concentration and comparing the measured value to the true value and gave the results of 103.45%, 89.27%, and 89.53% at the spiking concentration level of 2, 5, and 10 ng/g, respectively (see Table 1). The values in line with those obtained by Trejo et al. (2013) in the range of 82 to 111.7% for spiking of CLB at concentration range of 5.24 to 41.96 ng/g. The values in line with those obtained by Trejo et al. (2013) in the range of 82 to 111.7% for spiking of CLB at concentration range of 5.24 to 41.96 ng/g.

The calculations of detection limit and quantitation limit were 0.10 ng/g and 0.32 ng/g, respectively. These values were close to the study conducted by Chang et al. (2005) namely 0.1 ng/g, and still below the MRL established by the United Nations Food and Agricultural Organization for CLB of 0.2 ng/g (CAC 2012).

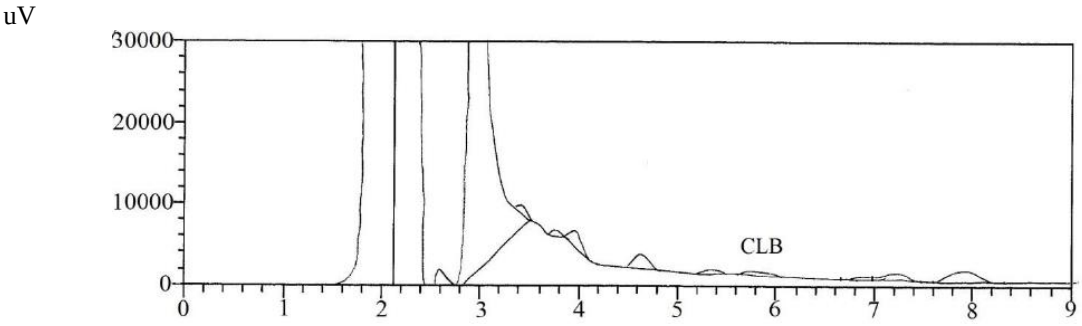

Figure 2. Chromatograms obtained from a positive sample in the presence of CLB residue.

Determination of CLB residues on field samples

The use of CLB for cattle defattening is not allowed internationally. The major biotransformation pathways of CLB were 4-*N*-oxidation, 4-*N*-sulfation, and oxidative *N*-dealkylation of the parent compound. Unchanged CLB accounted for about 20 and 50% of the radioactivity detected in urine and feces, respectively, and for a very large part of the radioactivity detected in organs (≥90%), and therefore, the toxicity of CLB residues remaining in tissues of cattle treated with this β-agonist toward the consumer is expected to be solely related to unchanged CLB (Zalko et al. 1998).

Analysis of 36 local/fattening beef meat samples collected from several cities in Java (Jakarta, Bandung, Semarang and Yogyakarta) collected from March to August 2013 is summarized in Table 2 and shown 7 (19.44%) out of 36 samples were positive for CLB residue and exceeded the MRL set by Codex (0.2 ng/g) of about 12 to 75 times higher to Codex's recommendation. Those results above indicated there was a misuse that local/fattening cattle were possibility of being treated by CLB and slaughtered without paying attention to its the withdrawal time of 4 weeks for proper therapeutically treated livestock (1.6 µg/kg/day for 7 consecutive days) (Elliot et al. 1995). A study on CLB contamination from treated veal calves (2.5 mL/100 kg, twice a day for 10 days) to untreated pen mates showed its probably due to licking the mouth of the treated animal or saliva from the treated animal spoiling the floor (Groot et al. 2013).

Whereas Table 3 which summarized results for frozen imported beef meat samples showed only one (2.63%) out of 38 samples was positive for CLB residue which meant that the supervision of the importing country was almost really well implemented.

Table 1. Average recoveries, detection limit and quantitation limit of CLB spiked in beef meat sample

Average recoveries (%) at 3 different spiking levels (n = 3)			Detection limit (ng/g)	Quantitation limit (ng/g)
2 ng/g	5 ng/g	10 ng/g		
103.45	89.27	89.53	0.10	0.32

Table 2. Residue analysis of CLB in local/fattening beef meat samples from Jakarta, Bandung, Yogyakarta, and Semarang collected from March to August 2013

Origin of samples	Number of samples	Number of positive samples	Concentration (ng/g)
Jakarta	10	5	7.09 to 15.06
Bandung	9	nd	-
Semarang	6	nd	-
Yogyakarta	11	2	2.40 and 2.42
Total	36	7	2.40 to 15.06

nd = not detected at concentration ≤ 0.09 ppb

Table 3. Residue analysis of CLB in imported beef meat samples from Jakarta, Bandung, Yogyakarta, and Semarang collected from March to August 2013

Origin of samples	Number of samples	Number of positive samples	Concentration (ng/g)
Jakarta	22	1	5.13
Bandung	4	Nd	-
Semarang	5	Nd	-
Yogyakarta	7	Nd	-
Total	38	8	5.13

nd = not detected at concentration ≤ 0.09 ppb

A surveillance study in 2015 in Indonesia conducted by Balai Pengujian Mutu dan Sertifikasi Produk Hewan, in Bogor (BPMPSH) revealed that 10 (14.49%) out of 69 beef samples and 26 (22.22%) out of 117 liver beef samples were positive for on detection of beta agonist (clenbuterol/salbutamol) using ELISA (BPMSPH 2015) that also meant there is no difference on the β-agonist (CLB/salbutamol) residue status in beef meat in 2013 and 2015 in Indonesia. The main drawback of ELISA detection method was there no specification of types of β-agonist applied due to its inability to differentiate β-agonist drugs (CLB or salbutamol) from the same class.

This study better than those of Betancurt et al. (2008) in Mexico that received 16.6% of 90 meats in the range of 0.1-2.3 ng/g and Trejo et al. (2013) in 78 cow liver samples showing 62% of which contained more than 0.6 ng/g (above the MRL in America and FDA). Similarly, the results of research Hajrulai-Musliu et al. (2013) which detected 21 positive samples from 55 beef samples in Macedonia in the range of 1.19 ng/g to 0.5 ng/g.

Administration of clenbuterol as a growth promoter in pig production could lead to residues in meat for human consumption up to 7 days after treatment discontinuation (Pleadin et al. 2010). Prolonged administration of a growth-promoting dose of CLB to cattle could lead to residue accumulation in the muscle tissue (meat) and liver as an edible tissue, which may induce a pharmacotoxicologic reaction in consumers. Several human poisoning cases had been reported in the presence CLB residues. Martínez-Navarro (1990) reported 135 people hospitalized in Spain in 1990 suffered the characteristic symptoms CLB poisoned (increased heart rate, muscle tremors, headache, nausea, fever, and chills) in fresh liver that contained 160 ng/g to 291 ng/g of CLB residue.

On the other hand, a strict monitoring of the CLB usage in Mexico showed a decline in CLB residue from 20% in 2006 to 7.1% as observed in 1221 samples consisting of various types of samples such as meat, liver, urine, cow serum in 2005-2010 (Almazan et al. 2012). However, data from inspection on 200 slaughterhouses in 2015 in Mexico still released that CLB residue was found on 58 out of them as the government gave suspension to those slaughterhouses (AP 2016).

Previous published report in Indonesia of another β-agonist drug of ractopamine which still permitted to be used (Widiastuti & Anastasia 2015) on 14 imported beef meat also showed none of those samples positive for ractopamine residue, also meant that the importing country puts strong supervision on the use of these drugs. In contrast, health risks study on 14 β-agonist residue (including ractopamine and clenbuterol) were assessed in cattle, chicken and swine livers at the wet market and the environmental impacts of wastewater from livestock farms in Selangor State, Malaysia showed 5 beta-agonists were detected in the wastewater samples (Sakai et al. 2016).

Urine and hair are the most frequently analyzed material for the detection of CLB (Sniegocki et al. 2003) which can be taken in both farm- and slaughter-houses. However, urine has a limitation that the concentration of β-agonists are below the limit of detection of most assays after a withdrawal time of 3 to 4 days, which makes urine suitable only for detection during treatment and less suitable for the detection of illegal use. Whereas, hair promised a better binding capacity of CLB due to presence of melanin in hair and made the residue could be accumulated up to 70 days after withdrawal (Li et al. 2013) and promised as an easy way of collecting samples before slaughtering the animal. Since the presence of CLB residues in food consumed can cause health problems for humans, the most important thing to improve public health is continous monitoring and prevention of CLB misuse is needed and to ensure the absence of contamination to the farm/livestock environment (Lee et al. 2007).

CONCLUSIONS

The detection of CLB in meat can be conducted by HPLC with the diode array detector after the sample extracted with acetonitrile and isopropanol. Analysis of CLB residues in 74 field samples consisting of imported beef, fattening and local beef meat from various cities in Java showed 8 (10.81%) of those samples were detected CLB at concentrations of 2.40 and 15.06 ng/g that has exceeded the CLB's MRL recomendation by Codex and WHO (0.2 ng/g). Continuously monitoring and prevention of CLB misuse should always be done to avoid further risk to human health.

ACKNOWLEDGEMENT

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Growth Response of Leucaena Embryogenic Callus on Embryo Age Differences and Auxin 2,4-Dichlorophenoxyacetic Acid

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ABSTRAK

Manpaki SJ, Prihantoro I, Karti PDMH. 2018. Respon pertumbuhan kalus embriogenik tanaman lamtoro terhadap perbedaan umur embrio dan hormon Auksin 2,4-Dichlorophenoxyacetic acid. JITV 23(2): 95-102. DOI: http://dx.doi.org/10.14334/jitv.v23i2.1621

Lamtoro (*Leucaena leucocephala* cv. Tarramba) merupakan hijauan sumber protein dari famili leguminosa. Varietas Tarramba mampu beradaptasi dengan baik di daerah tropis dan kering yaitu Nusa Tenggara Barat dan Nusa Tenggara Timur. Penelitian ini bertujuan untuk mengidentifikasi respon pertumbuhan dan morfologi kalus embriogenik tanaman lamtoro (*L. leucaena* cv. Tarramba) terhadap umur embrio yang berbeda dan level pemberian zat pengatur tumbuh auksin 2.4-D. eksplan yang digunakan berasal dari tanaman lamtoro (*L. leucaena* cv. Tarramba) sebanyak 400 eksplan. Penelitian ini terdiri dari 16 perlakuan dengan dua faktor, faktor pertama adalah pemberian konsentrasi ZPT 2,4-D 0.5 mg/L, 1 mg/L, 1.5 mg/L, dan 2 mg/L. Faktor kedua adalah jenis embrio mature embryo, cotyledon, heart, dan pre-globular. Masing-masing perlakuan diulang sebanyak 25 ulangan. Penelitian ini menggunakan rancangan lingkungan acak lengkap (RAL) dua faktor dengan analisis ragam (ANOVA). Penelitian ini diuji lanjut menggunakan Duncan. Analisis ragam menunjukkan pemberian ZPT 2,4-D pada konsentrasi 1.5 mg/L dan jenis mature embryo terhadap parameter pertambahan tinggi kalus, pertambahan diameter kalus, dan skor warna kalus berbeda nyata ($P<0.05$). parameter tekstur kalus menunjukkan hasil yang seragam pada seluruh perlakuan yaitu kompak. Kalus embriogenik tanaman lamtoro (*Leucaena leucocephala* cv. Tarramba) menunjukkan respon yang optimal pada konsentrasi ZPT 2,4-D 1.5 mg/L dan jenis mature embryo.

Kata Kunci: Auksin 2,4-D, Embrio, Embriogenik, Kalus, Lamtoro cv. Tarramba

ABSTRACT

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Leucaena (*Leucaena leucocephala* cv. Tarramba) is a source of protein from the legume family. Tarramba varieties able to adapt well in tropical area such as West Nusa Tenggara and East Nusa Tenggara. This study aimed to identify the growth response and embryogenic callus morphology of lamtoro (*L. leucaena* cv. Tarramba) in embryos different ages and auxin 2,4-D levels. This research was used explants derived from lamtoro (*L. leucocephala* cv. Tarramba) as much 400 explants. this study conducted of 16 treatments with two factors, the first factor is the provision of PGR 2,4-D concentration of 0.5 mg/L, 1 mg/L, 1.5 mg/L and 2 mg/L. The second factor was the type of embryo such as mature embryo, cotyledon, heart, and pre-globular. Each treatment was repeated 25 replications. This study uses a randomized complete design (CRD) with two factors. Data were analyzed using analysis of variance and if there was significant difference, data were further analyzed using Duncan's multiple range test. Analysis of variance showed that PGR 2,4-D at a concentration of 1.5 mg/L and the type of optimum embryo was mature embryo callus on parameters as height, diameter increment callus, and callus color scores were significantly different ($P<0.05$). callus texture parameter indicates results that are uniform throughout the treatment that was compact callus. Lamtoro plant embryogenic callus (*L. leucocephala* cv. Tarramba) indicate an optimal response at the concentration of PGR 2,4-D 1.5 mg/L and the type of embryo was mature embryo.

Key Words: Auxin 2,4-D, Callus, Embryo, Embryogenic, Lamtoro cv. Tarramba

INTRODUCTION

Forage is the main source of ruminants feed. Forage play an important role in basic needs of cattles living, growth, power source, as well as an important component in supporting the production and reproduction of livestock. The forage requirement for ruminants is still relatively high, but the quality of productivity in the tropics is still relatively low. In general, forages are divided into two large families, Graminae and Leguminouse. Legume family is a source of protein. One type of legume that has a relatively high

crude protein and is known well by the breeder is Leucaena (*Leucaena leucocephala*). Lamtoro plant has crude protein content of 23.7% - 34% and has a high palatability (Yumiarty & Suradi 2010).

Lamtoro plants can adapt well in the tropics. Additionally, lamtoro able to adapt to the soil with moderate acidity between pH 5.5 - 6.5 and temperate climate with an annual rainfall of over 760 mm in the region of East Nusa Tenggara and West Nusa Tenggara. One lamtoro varieties that are well developed in Indonesia is Tarramba varieties. Based on research of Yumiarty & Suradi (2010) lamtoro varieties Tarramba (*Leucaena leucocephala* cv. Tarramba) has the advantage of a leafhopper resistant to pests and resistant to drought. Manpaki et al. (2016) states lamtoro Tarramba varieties are grown through somatic phase lasting at pH 5.5. Utilization of tissue culture technology enables to perform fast and accurate assessment. Furthermore, tissue culture techniques allow for the rapid multiplication of seeds produced, uniform quality, and facilitate the standardization of plant seeds.

Provision of plant parts such candidates protoplasm, the cell, group of cells, tissues and organs can be performed with tissue culture methods that will produce meristematic or embryogenic sections. Culture of lamtoro is efficient and allows to get a diversity of genetic variation is a callus. Callus is an organized collection of cells comprising paranchyma cells. Selected candidates callus is embryogenic explants. Selection of embryogenic explants to produce derivative irreversible means superior properties of genetic diversity decrypted result is not returned to normal.

Mechanism of callus growing derived from the embryo plant varieties Tarramba lamtoro done carefully because the span of embryo growth can not be ensured in practice. Growing young embryos performed at optimal environmental *in vitro* condition. Techniques using embryonic explants derived from plants have been used to obtain seeds from the crosses between species of plants. The major advantage in using callus explant embriogenic is able to accelerate the acquisition of callus as used are very young embryos, so the development of the callus is not rapidly decreasing and potentially extend the life of callus in a long time. The use of increasingly younger explants will improve in vitro culture. Age significantly affected the development of the embryo to corn embryo explants *in vitro* (Binott et al. 2008).

The development of biotechnology through forage breeding through tissue culture can be carried out with the help of plant growth regulator (PGR). Plant growth regulators is one important factor in the successful growth of callus cultured. In general, plant growth regulators can be divided into two plant growth regulators or phytohormones called endogenous and exogenous growth regulating substances or synthetic. Plant growth regulators on the plant consists of five groups: auxin, cytokinin, gibberellin, ethylene and abscisic acid. In the development of callus, auxin hormones from the class provides the greatest impact. Auxin is a compound effect on cell growth, raising the osmotic pressure, increase protein synthesis, increase the cell permeability to water and flex or soften the cell walls that followed reduced pressure cell wall so that water can get into the cell, along with the increase in cell volume. Therefore, in plant growth regulator substances have a significant influence in explants growth. Auxin used in this study was 2.4-Dichlorophenoxyacetic acid (2,4-D) as a synthetic hormone that is more stable and not easily decomposed by changes in temperature and enzymatic.

Forage breeding using tissue culture techniques is expected to give good results for growth, as well as the properties of uniform and superior in plant embryogenic callus lamtoro Tarramba varieties selected at the level of plant growth regulator auxin 2.4-D. This study aims to identify of growth response and embryogenic callus morphology of plants lamtoro (*L. leucaena* cv. Tarramba) of the embryos different ages and levels of growth regulator auxin giving 2.4-D.

MATERIALS AND METHODS

This research was conducted at the laboratory of Plant Biotechnology, Feed Science and Technology Department, Division of Forages and Pasture, Faculty of Animal Science, Bogor Agricultural University. This research was conducted for 3 months starting in September 2016 until December 2016. The material used in this study are a lamtoro peas (*L. leucocephala* cv. Tarramba) different from the common garden collection, Field Laboratory of Forages and Pasture, Faculty of Animal Science, Bogor Agricultural University, materials sterilization form 70% alcohol, 96% alcohol, soap, clorox 10% to 20%, distilled water, plant growth regulator auxin (2.4 D), MS medium (*Murashige and Skoog*) basal, $AlCl_3$, explant culture equipment, laminar airflow, as well as observation equipment parameters.

This study consisted of two phases of research, there are preparation of explants embryos and embryonic callus induction on media treatment. Explants embryos to be used is derived from plant pods lamtoro (*L. leucocephala* cv. Tarramba) of different ages. Lamtoro pods thoroughly washed with soap and then sterilized using 20% Clorox for 7 minutes, then Clorox 15% for 7 minutes, and soaked in Clorox 10% for 7 minutes. After soaking, rinse the pod in distilled water for 5 minutes. Sterile pods in place in a petri dish to do the stripping. The pods are taken consisted of four age groups,

namely pre-globular, heart, cotyledon and mature embryos.

The main media used was MS medium plus growth regulator (PGR) 2.4 dichlrophenoxyacetic acid for callus induction at a concentration of 0.5 mg/L, 1 mg/L, 1.5 mg/L, and 2 mg/L. The explants used were pre-globular, heart, cotyledon and mature embryos were transferred into the medium with a subculture technique in a laminar airflow. Each bottle contains 20 ml of media were planted 1 embryos in accordance with the treatment given. Callus induction was observed for five weeks weeks. Explants grown into a green compact callus indicates the use of a good growing medium. The design environment used in the study of this phase is completely randomized design (CRD) Factorial with A factor is a given plant growth regulator and factor B is a type of plant embryos were used as explants. PGR used at one stage was 2.4 dichlorophenoxyacetic acid by 4 levels of treatment there are 0.5, 1.0, 1.5, and 2.0 mg/L. Each treatment was repeated 20 times with the unit observation in the form of a vial containing MS medium and 1 explant. Data will be analyzed using analysis of variance (ANOVA) using SPSS instrument 16, then if there is difference will be test with Duncan test.

RESULTS AND DISCUSSION

Plant height and callus diameter (*L. leucocephala* cv. Tarramba)

Height and diameter of the callus is a major variable in the morphological characteristics of the plant. Height callus of lamtoro (*L. leucocephala* cv. Tarramba) are presented in Table 1.

Table 1. Height growth of embryogenic callus (*L. leucocephala* cv. Tarramba)

PGR doses 2,4-D (mg/L)	Embryo types				
	Mature embryo	Cotyledon	Heart	Pre-globular	
	----------cm^{-1}----------				
1 WAP					
0.5	0.285±0.152	0.255±0.005	0.088±0.004	0.000±0.000	0.157^{c}
1	0.115±0.011	0.255±0.114	0.200±0.008	0.000±0.000	0.142^{c}
1.5	1.085±0.230	0.935±0.179	0.595±0.022	0.000±0.000	0.653^{a}
2	0.921±0.198	0.815±0.093	0.535±0.018	0.000±0.000	0.567^{b}
	0.602^{a}	0.565^{a}	0.354^{b}	0.000^{c}	
2 WAP					
0.5	0.700±0.277	0.365±0.067	0.275±0.008	0.000±0.000	0.335^{b}
1	0.520±0.058	0.470±0.202	0.235±0.098	0.000±0.000	0.306^{b}
1.5	1.645±0.377	1.215±0.195	0.580±0.041	0.000±0.000	0.860^{a}
2	1.785±0.262	0.985±0.093	0.675±0.078	0.000±0.000	0.861^{a}
	1.162^{a}	0.758^{b}	0.441^{c}	0.000^{d}	
3 WAP					
0.5	0.875±0.201	0.400±0.097	0.280±0.008	0.000±0.000	0.388^{b}
1	0.755±0.066	0.435±0.042	0.550±0.166	0.000±0.000	0.435^{b}
1.5	1.705±0.640	0.925±0.205	0.635±0.087	0.110±0.047	0.843^{a}
2	1.790±0.120	1.030±0.121	0.675±0.078	0.000±0.000	0.873^{a}
	1.281^{a}	0.697^{b}	0.535^{c}	0.027^{d}	
4 WAP					
0.5	0.345±0.191	0.275±0.133	0.185±0.010	0.130±0.047	0.233^{d}
1	0.745±0.184	0.305±0.068	0.430±0.028	0.150±0.050	0.407^{c}
1.5	1.265±0.491	1.255±0.211	0.490±0.148	0.055±0.001	0.766^{a}
2	1.245±0.088	0.880±0.136	0.145±0.019	0.135±0.074	0.601^{b}
	0.900^{a}	0.678^{b}	0.312^{c}	0.118^{d}	

PGR = Plant Growth Regulation, WAP = Weeks After Planting
Different superscript within the same row and column shows significant ($P<0.05$) difference

The lowest of callus height growth in first week after planting occurred in all PGR concentrations of 2,4-D. Type of pre-globular embryo that is equal to 0 cm and the highest occurred in PGR concentration of 2,4-D 1.5 mg/L in type of mature embryo that is equal to 1,085 cm. Results of analysis of variance showed PGR giving 2,4-D at all levels of concentration and type of embryos significantly different with high accretion callus ($P<0.05$). Interaction between PGR giving 2,4-D and high-gain types of embryo against callus. The pattern of high accretion callus from the first week to the fourth week after planting that is contained in the delivery of PGR maximum peak of 2,4-D 1.5 mg/L while the minimum peak occurs at 0.5 mg/L. Type of embryos that showed the best performance on high accretion callus every week observation is mature embryos. A similar incident occurred in the fourth week after planting there is high accretion lowest callus persists on the type of pre-globular embryo of 0.055 cm while the highest occurred in PGR concentrations of 2,4-D 1.5 mg/L in type of mature embryo of 1.265 cm.

Growth in mature embryos showed better results compared with other types of embryos. The influence of this difference is due to the superiority of the embryo is already ripe. Sources of nutrients from embryos that are ripe will bring good nutrition to the embryo than other types of embryos is not yet complete so that the process will be slower compared to embryos that had matured. Arsyad (2013) states that the mature zygotic embryos have a regenerative capability better than the old embryo or embryos are too young. These results are consistent with research Mirici et al. (2009) showed that mature zygotic embryo has the activity of cell division, growth and the ability regeneratf organelles were high compared to the older embryos. The content of endogenous or exogenous hormones affect plant regeneration was lamtoro. These results are consistent with studies showing that high performance best callus occur in the plant growth regulator auxin 2,4-D 1.5 mg/L. Uranbey (2011) states that the appropriate use of embryos as explants will increase the amount of callus produced plantlets along with the use of endogenous or exogenous hormone auxin (IAA). Therefore, the use of mature embryo at a concentration of plant growth regulator auxin 2,4-D 1.5 mg/L is an appropriate alternative.

Effect of 2,4-D and type embryo embryogenic callus diameter ratio is the result of a reaction to exogenous auxin uptake activity were given. These results are consistent with Sari et al. (2014) research which states that the absorption of exogenous and endogenous auxin will cause cell division continuously increasing the number of cell tissue. Diameter growth lamtoro callus (*L. leucocephala* cv. Tarramba) are presented in Table 2.

The lowest of diameter growth in the first week after planting occurred in all PGR concentrations of 2,4-D. The type of pre-globular embryo that is equal to 0 cm and the highest occurred in PGR concentration of 2,4-D 1.5 mg/L in type of mature embryo that is equal to 1.460 cm. Results of analysis of variance showed PGR giving 2,4-D at all levels of concentration and types of different embryonic callus significantly on the increase in diameter ($P<0.05$). Interaction between PGR giving 2,4-D and type embryos to increase the diameter of the callus. The pattern of the increase in diameter of callus from the first week to the fourth week after planting that is contained in the delivery of PGR maximum peak of 2,4-D 1.5 mg/L while the minimum peak occurs at 0.5 mg/L. type of embryos that showed the best performance on high accretion callus every week observation is mature embryos. A similar incident occurred in the fourth week after planting there is high accretion lowest callus persists on the type of pre-globular embryos is 0.222 cm while the highest occurred in PGR concentration of 2,4-D 1.5 mg/L in type of mature embryo is 1.330 cm.

Callus diameter growth is the influence of PGR provision of 2,4-D that has no interaction on the type of embryo. Callus diameter accretion process described in the study of Sari et al. (2014) that the use of 2,4-D PGR influence on callus diameter. Auxin absorption will cause cell division continuously increasing the number of cells. In general, the four types of embryo provide developmental responses in diameter, but the speed and regeneration of the cells are different.

Rahmawati (2016) is stated that dosing PGR 2,4-D 1 mg/L shows the effect of the growth and development of plant callus lamtoro Tarramba best cultivars. The study is in line with the results of this research are giving PGR best 2.4 occurred at a dose of 1.5 mg/L, so that there is an increase in dose to grow new ones. Callus diameter growth is presented in Figure 1.

Figure 1 shows the linear relationship and interaction between the type of embryo and plant growth regulator concentration of 2,4-D to increase callus diameter (cm^{-1}). Callus diameter growth every week on PGR concentration of 2,4-D 1.5 mg/L relatively high compared with 2,4-D PGR 2 mg/L, 1 mg/L, and 0.5 mg/L. Type embryos showed the best performance based on the increase in diameter respectively callus is mature embryo, cotyledon, heart, and pre-globular. Figure 1 shows the same pattern every week callus growth slowdown that occurred in the third week after planting up to four weeks after planting, this means that in the third week after planting an optimum peak PGR callus growth on medium with 2,4-D.

Table 2. Diameter growth of embryogenic callus (*L. leucocephala* cv. Tarramba)

PGR dose 2,4-D (mg/L)	Embryo types				
	Mature embryo	Cotyledon	Heart	Pre-globular	
	----------cm-1----------				
1 WAP					
0.5	0.485±0.111	0.220±0.014	0.090±0.005	0.000±0.000	0.198^{c}
1	0.395±0.010	0.145±0.082	0.235±0.095	0.000±0.000	0.193^{c}
1.5	1.460±0.195	0.580±0.032	0.605±0.163	0.000±0.000	0.661^{a}
2	0.945±0.060	0.430±0.013	0.500±0.015	0.000±0.000	0.468^{b}
	0.821^{a}	0.343^{b}	0.357^{b}	0.000^{c}	
2 WAP					
0.5	0.685±0.013	0.615±0.023	0.345±0.051	0.000±0.000	0.411^{d}
1	0.520±0.105	0.415±0.130	0.375±0.011	0.000±0.000	0.327^{c}
1.5	3.285±0.225	1.220±0.514	0.670±0.020	0.000±0.000	1.293^{a}
2	2.435±0.108	0.760±0.150	0.670±0.022	0.000±0.000	0.966^{b}
	1.731^{a}	0.752^{b}	0.515^{c}	0.000^{d}	
3 WAP					
0.5	1.060±0.476	0.955±0.029	0.515±0.071	0.000±0.000	0.633^{d}
1	1.160±0.296	1.090±0.107	0.600±0.091	0.000±0.000	0.712^{c}
1.5	3.685±0.225	2.220±0.048	0.720±0.015	0.000±0.000	1.656^{a}
2	3.085±0.153	1.515±0.074	0.685±0.036	0.000±0.000	1.321^{b}
	2.247^{a}	1.445^{b}	0.630^{c}	0.000^{d}	
4 WAP					
0.5	0.450±0.211	1.085±0.140	0.605±0.051	0.225±0.055	0.591^{b}
1	0.925±0.190	1.030±0.097	0.720±0.040	0.230±0.047	0.726^{a}
1.5	1.330±0.065	0.640±0.019	0.730±0.023	0.240±0.066	0.735^{a}
2	0.935±0.142	0.480±0.069	0.800±0.040	0.222±0.041	0.609^{b}
	0.910^{a}	0.808^{b}	0.713^{c}	0.229^{d}	

PGR = Plant Growth Regulation
WAP = Weeks After Planting
Different superscript within the same row and column shows significant (P<0.05) difference

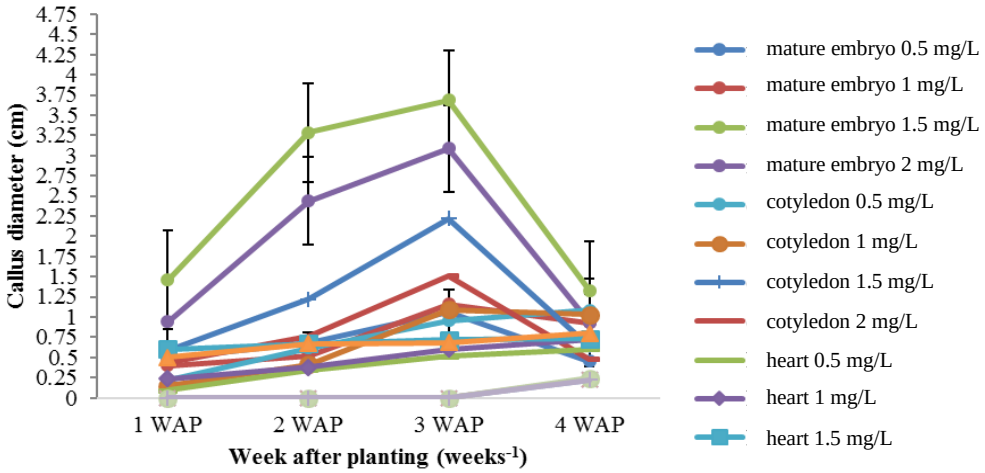

Figure 1. Callus diameter growth of lamtoro (*L. leucocephala* cv. Tarramba).

Color and callus texture morphology of Leucaena (*L. leucocephala* cv. Tarramba)

Callus color is one of the variables of the morphological characteristics of the most visible callus, while the texture of the callus callus conveniently indicates the ability to regenerate. More and crumb texture of the callus regeneration ability, the better. Callus color of lamtoro (*L. leucocephala* cv. Tarramba) are presented in Table 3.

Leaf color change in the concentration of 2,4-D PGR against this type of embryos having the same mean score on mature embryo, cotyledon, and a heart that is with a score of 4 (green), while on the type of pre-globular embryos has mean score of 1.80 (tawny). Analysis of variance results showed that administration of 2,4-D PGR concentrations on callus morphology were not significantly different colors while the color types of the morphology of embryos callus was significantly different ($P<0.05$).

There are very significant differences between mature embryo, cotyledon, heart, and pre-globular embryos. Colors on mature embryo, cotyledon, and heart has a score of 4 on 1 WAP to 4 WAP there is green, while the pre-globular embryo has a score of 1.7 to 1.9 that is yellow. Arsyad et al. (2013) states that the callus color difference caused by the accumulation of phenolic compounds. Embryos subjected to browning occurs in the regeneration inhibition compared with embryos that do not undergo browning. Callus conveniently indicates normal generally yellow-green to green (Figure 2). Rahmawati (2016) adds that brown-colored callus indicates the influence of PGR toxicity of 2,4-D that causes brown colored callus. In addition, the explants were too young to be quickly issued a phenolic compound compared with mature explants. Based on research Moallem et al. (2013) in the early phase of callus induction, the explants browning around the polyphenol oxidation reaction that occurs in the area of injury explants react with the media. The compound will inhibit the growth of plant tissue and cause the plant to die.

Table 3. Morphology of embryogenic lamtoro callus colour (*L. leucocephala* cv. Tarramba)

Embryo types	PGR (mg L)				
	0.5	1	1.5	2	
Mature embryo	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00[a]
Cotyledon	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00[a]
Heart	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00[a]
Pre-globular	1.95±0.82	1.70±0.47	1.75±0.43	1.80±0.41	1.80[b]
	3.48	3.42	3.43	3.45	

PGR = Plant growth regulation
Leaf colour chart = 6 (yellow); 5 (dark green); 4 (green); 3 (light green); 2 (greenish white); 1 (white)
Different superscript within the same row and column shows significant ($P<0.05$) difference

Figure 2. Morphology of embryogenic lamtoro callus (*L. leucocephala* var. Tarramba) with PGR 2,4-D 1.5 mg/L doses using 100x zooms. (A1) *Mature embryo*, (B1) *Cotyledon*, (C1) *Heart.*

Table 4. Morphology of embryogenic lamtoro callus texture (*L. leucocephala* cv. Tarramba)

Embryo types	PGR (mg/L)			
	0.5	1	1.5	2
Mature embryo	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
Cotyledon	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
Heart	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
Pre-globular	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00

PGR = Plant Growth Regulation
Callus texture score = very compact (5); compact (4); crumb (3); very crumb (2)
Different superscript within the same row and column shows significant (P<0.05) difference

Callus texture consists of a compact type of texture and crumb texture. Compact texture has a cell structure that is solid, tied to each other, and gather, while the texture of the crumb has a rare cell structure, hollow, dispersed, and has a crumb spot. Changes of callus texture on the entire PGR concentrations of 2,4-D and type embryos have the same texture is compact. Good callus crumb texture to make it easier to regenerate into plants. Crumb callus morphological characteristics indicate that the cells have properties for producing many embryos embryogenic somatic and easy to divide in plant regeneration. The morphology of the callus texture (*L. leucocephala* cv. Tarramba) are presented in Table 4Callus crusts are composed of cells with sufficient space and cavity width. Rahmawati (2016) suggests that the interaction between the cotyledons explants types of plants and plant growth regulator 2,4-D 1 mg/L shows the morphological characteristics of most well after gamma-ray irradiation. Based on Hopkins (2010) research, the use of high auxin dose levels would cause infected cells, enlarged, and undergo rapid cell division is not normal. The statement is in accordance with the findings that there is callus which has a superior texture morphology at doses of 2,4-D PGR 2 mg/L.

CONCLUSION

Embryogenic callus growth response and lamtoro morphology (*L. leucocephala* cv. Tarramba) is optimal in 1.5 mg/L of 2,4-D PGR concentration. based on the embryo used, mature embryo shows the most optimal manner of morphophysiology. Embryogenic callus such as mature embryo, cotyledon, and heart showed a faster growth rate than pre-globular embryos. There is no interaction between the administration of PGR concentration of 2,4-D and type embryos.

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   Example of reference writing

   Primary paper:
   Bhanja SK, Anjali DC, Panda AK, Sunder GS. 2009. Effect of post hatch feed deprivation on yolk-sac utilization and young broiler chickens. Asian-Aust J Anim Sci. 22:1174-1179.

   Book:
   a. Lawrence TLJ, Fowler VR. 2002. Growth of farm animals. 2nd ed. New York (USA): CABI Publishing.
   b. Bamualim A, Tiesnamurti B. 2009. Konsepsi sistem integrasi antara tanaman padi, sawit, dan kakao dengan ternak sapi di Indonesia. In: Fagi AM, Subandriyo, Rusastra IW, penyunting. Sistem integrasi ternak tanaman padi, sawit, kakao. Jakarta (Indones): LIPI Press. p. 1-14.
   c. Paloheimo M, Piironen J, Vehmaanpera J. 2010. Xylanases and cellulases as feed additives. In: Bedford MR, Partridge GG, editors. Enzymes in farm animal nutrition. 2nd ed. New York (USA): CABI Publishing. p. 12-53.

Proceeding:
Umiyasih U, Antari R. 2011. Penggunaan bungkil inti sawit dan kopra dalam pakan penguat sapi betina berbasis limbah singkong untuk pencapaian bobot badan estrus pertama >225 kg pada umur 15 bulan. Prasetyo LH, Damayanti R, Iskandar S, Herawati T, Priyanto D, Puastuti W, Anggraeni A, Tarigan S, Wardhana AH, Dharmayanti NLPI, editors. Proceeding of National Seminar on Livestock Production and Veterinary Technology. Bogor (Indones): Indonesian Center for Animal Research and Development. p. 192-199.

Thesis:
Krisnan R. 2008. Kombinasi penggunaan probiotik mikroba rumen dengan suplemen katalitik pada pakan domba (Thesis). [Bogor (Indones)]: Institut Pertanian Bogor.

Electronic magazines:
Wina E, Tangendjaja B, Dumaria. 2008. Effect of *Calliandra calothyrsus* on *in vitro* digestibility of soybean meal and tofu wastes. Livest Res Rural Develop. Vol. 20 Issue 6. http://www.lrrd.org/lrrd20/6/wina 20098.htm.

Institution:
a. [NRC] National Research Council. 1985. Nutrient requirements of sheep. 6th revised. Washington DC (USA): National Academic Press.
b. [CDC] Centers for Disease Control. 2006. Standard operating procedure for the direct Rapid Immunohistochemistry Test (dRIT) for the detection of rabies virus antigen. [accessed December 20th 2011]. http://www.rabiesblue print.com/IMG/pdf/DRIT_SOP.pdf.

Patent:
Blanco EE, Meade JC, Richards WD. 1990. Ophthalmic ventures, assignee. Surgical stapling system. United States patent US 4,969,591. 1990 Nov 13.

10. **Citation in text:**
Citation consists author's last name and publication year.

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