Bioactive Compounds Isolated from Lignin of Empty Bunch Palm Fiber and Their Effects on *in Vitro* Rumen Fermentation

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ABSTRACT

The objective of this experiment was to study the effects of bioactive compounds isolated from purified lignin formacell (PLF) of empty bunch palm fiber as natural antimicrobes and their effects on *in vitro* rumen fermentation. The first experiment was inhibition test of 11 bioactive compounds isolated from PLF, using disc diffusion method against the growth of E*scherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus*. Four of the most potential bioactive compounds were then used in the second experiment, which was an *in vitro* test using fresh rumen liquid of Ongole grade beef cattle, to study their effects on rumen fermentation. Six treatments with 3 replications were applied in a completely randomized block (CRB) design. The treatments were R0= 0.5 g basal diet; R1= R0 + 0.3 mg Rumensin®; R2= R0 + 0.3 mg syringaldehyde; R3= R0 + 0.3 mg p-hydroxybenzoic acid; R4= R0 + 0.3 mg m-hydroxybenzoic acid; and R5= R0 + 0.3 mg oxybenzene. Isolate fraction of CC-2 (syringaldehyde), CC-3 (m-hydroxybenzoic acid), VLC-5 (oxybenzene), and VLC-9 (p-hydroxybenzoic acid) exhibited antimicrobes activity against all tested bacteria. Other isolated fractions exhibited antimicrobes activity only against 1 or 2 tested bacteria. The use of syringaldehyde, p-hydroxybenzoic acid, m-hydroxybenzoic acid, and oxybenzene improved ammonia concentration, microbial protein synthesis, and nutrients digestibility. Bioactive compounds had no effect on rumen pH but reduced total VFA concentration as well as the estimate of methane production.

Key words: bioactive compounds, natural antimicrobes, rumen fermentation

ABSTRAK

Penelitian bertujuan mempelajari pengaruh penggunaan senyawa bioaktif yang diperoleh dari lignin tandan buah kelapa sawit sebagai antimikrob dan pengaruhnya pada fermentasi rumen secara *in vitro*. Percobaan pertama adalah uji zona hambat 11 senyawa bioaktif menggunakan metode cakram pada bakteri *Escherichia coli, Salmonella typhimurium,* dan *Staphylococcus aureus*. Empat senyawa bioaktif paling baik diuji lebih lanjut dalam percobaan kedua, yaitu fermentasi *in vitro* menggunakan cairan rumen sapi peranakan Ongole, untuk mengamati pengaruhnya pada fermentasi rumen secara in vitro. Percobaan menggunakan rancangan acak kelompok lengkap dengan 6 perlakuan dan 3 ulangan. Perlakuan adalah R0= 0,5 g ransum basal; R1= R0 + 0,3 mg Rumensin®; R2= R0 + 0,3 mg siringaldehida; R3= R0 + 0,3 mg asam p-hidroksibenzoat; R4= R0 + 0,3 mg asam m-hidroksibenzoat; dan R5= R0 + 0,3 mg oksibenzena. Fraksi isolat CC-2 (siringaldehida), CC-3 (asam m-Hidroksibenzoat), VLC-5 (oksibenzena), dan VLC-9 (asam p-hidroksibenzoat) memperlihatkan aktivitas antimikrob pada ketiga bakteri patogen yang diuji. Isolat lainnya memperlihatkan aktivitas antimikrob pada 1 atau 2 saja dari bakteri patogen yang diuji. Penggunaan siringaldehida, asam p-hidroksibenzoat, asam m-hidroksibenzoat, dan oksibenzena meningkatkan konsentrasi ammonia, sintesis protein mikrob, dan kecernaan zat-zat makanan. Penggunaan senyawaan tersebut tidak mempengaruhi pH rumen, tetapi menurunkan konsentrasi VFA dan nilai estimasi produksi gas metan.

Kata kunci: senyawa bioaktif, antimikrob alami, fermentasi rumen

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INTRODUCTION

The use of antibiotics is continuously improved in animal production, especially for prevention of bacterial infection and growth promotion. However, antibiotics must be used judiciously because it could lead to the emergence and spread of resistant microorganisms and also creation of antibiotic-resistant disease in animal (Houser *et al.*, 2008; Johnson *et al.*, 2007). The increasing incidence of antibiotic-resistant pathogens has drawn the attention of scientific communities towards studies on the potential antimicrobial activity of plant-derived substances (Savoia, 2012; Fitrial *et al.*, 2008) and potential use of probiotics for improving animal performance (Hamida *et al.*, 2015). Therefore, there is a need to explore intensively the potential use of plant derived antimicrobes for controlling the gastrointestinal pathogenic microbes in animals.

Rochfort *et al.* (2008) in their review explored the potential use of medicinal properties of plants to enhance animal health in general and that of ruminants in particular. Active areas of research for plant bioactive include foam production/bloat control and methane production. The majority of plant-derived substances tested specifically for ruminants are tested in order to assess the effect on rumen ecosystem, safety aspect of rumen flora, and attempts to reduce the gram positive bacteria that may be associated with less desirable gut metabolisms. Antimicrobial effect of plant bioactives is most pronounced against gram positive bacteria, almost similar to the action of ionophores.

Salmonella has been widely reported as one of frequently found pathogenic bacteria in cattle (Hollinger *et al.*, 1998). These pathogenic bacteria could be found in the rumen and feces samples of infected cattle. *Salmonella* could enter the rumen most probably through consumption of contaminated feed. High volatile fatty acid concentrations (VFA) in the rumen may inhibit the growth of *Salmonella*, in contrary when the cattle in starvation period the growth of *Salmonella* may start to increase because of low VFA concentration. Currently, the use of probiotics and antimicrobes are intensively explored to control the pathogenic bacteria in feedlot cattle and neonatal calf. Probiotics are used to reduce the use of antibiotics in neonatal and stressed calf, enhance milk production, prevent ruminal acidosis, and to rapidly establish stable microbiota in neonatal calves (Krehbiel *et al.*, 2003).

Plant antimicrobes are phytochemicals, which are broadly grouped into phenolic compounds, terpenoids, essential oils, alkaloids, and polypeptides. Phenolic compounds are involved in plant growth to provide resistance from pathogens and predator. The structure of phenolics consists of an aromatic ring carrying one (phenol) or more hydroxyl (polyphenol) moieties. Several classes can be distinguished according to the number of phenol rings and to the structural elements that join these rings (Khadem & Marles, 2010).

Interest in the antimicrobial properties of phenolic compounds is strengthened by the finding that they affect the behavior of pathogenic bacteria and their use as natural additive in food industry (Nazzaro *et al.*, 2009).

The antimicrobial activity of phenolic compounds and flavonoids were reported by Erdemoglu *et al.* (2007) and Xia *et al.* (2011). Other study (Nohynek *et al.*, 2006) demonstrated that phenolic extracts inhibit the growth of pathogenic bacteria of human gastrointestinal tract. The mechanisms responsible for phenolic compounds to control pathogenic bacteria in gastrointestinal tract are still needs further studies. However, several mechanisms are proposed; include absorption and disruption of microbial membranes, interaction with enzymes, and metal ion deprivation (Fattouch *et al.*, 2007; Xia *et al.*, 2011). Therefore, there are lots of possibilities of exploring the use of phenolic compounds as natural antimicrobial agents in gastrointestinal tract of animals.

It has long been demonstrated that lignin is one of the major source of polyphenolic compounds available from natural biomass (Yang *et al.*, 2007). When the lignin portion of plant biomass is hydrolyzed, various phenolic acids are released (Lee *et al.*, 2012). Purified lignin formacell of empty bunch palm fiber (EBPF) contained significant amount of phenolic compounds. Since phenolic acids from different sources of lignin have specific effects on certain microbes, there is a need to study the antimicrobial effects of phenolic compounds isolated from certain sources of lignin. Objective of this experiment was to study the use of active compounds isolated from lignin of empty bunch palm fiber as natural antimicrobes and their effects on *in vitro* rumen fermentation. The bioactive compounds used were Syringaldehyde, p-Hydroxybenzoic acid, m-Hydroxybenzoic acid, and Oxybenzene.

MATERIALS AND METHODS

Lignin Fractionation

Empty bunch palm fiber was collected from local palm oil industry in Rejosari, Lampung Province, Indonesia. Lignin Formacell (LF) was isolated from ground EBPF (30-40 mesh) using pulping formacell method (Lehnen *et al.*, 2005). LF was then further purified to obtain Purified Lignin Formacell (PLF). PLF was then further analyzed using gas chromatography mass spectroscopy (GCMS) to describe its monomers.

Two methods were then applied to obtain the bioactive compounds candidate from PLF of EBPF. The two methods were used to obtain the most bioactive compounds, since certain method only effective for some type of bioactive compounds. Method 1 (Lundquist, 1973; Faustino *et al.*, 2010) was fractionation of PLF, followed by extraction, and then separation of active compounds using column chromatography. In fractionation process, 4 g PLF was diluted in 50 mL "white liquor" (NaOH 1.38 M and Na_2S 0.25 M) then heated at 170 ºC for 4 h. After reaching the room temperature, the solution was then added with 200 mL of dioxan and HCl 2 M until pH 2-3 was reached. Extraction process was conducted in 3 steps, which consisted of: (a) using 200 mL of $CHCl_3$; (b) using 100 mL of dioxan-$CHCl_3$ (1:1); and (c) using 2 x 50 mL of $CHCl_3$. Organic layer materials were obtained from this extraction process. The organic layer materials were then decanted, the dioxan-

chloroform was removed by vacuum evaporator, and then crude extract of active compounds were subjected to column chromatography (CC) using 15 g of silica and chloroform/ethyl acetate 1:10 as eluent to separate the active compounds. Crystal active compounds obtained were then analyzed using thin layer chromatography (TLC) to classify the active compounds with the same retention factor (Rf) value. All active compounds obtained were then tested against the growth of *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus*.

Method 2 (Faustino *et al.*, 2010; Sticher, 2008 with slightly modification) was fractionation of PLF, followed by extraction, and then separation of active compounds using vacuum liquid chromatography. Before fractionation, PLF was macerated using methanol for 1-3 d. Methanol soluble fraction was then separated from methanol insoluble fraction by sieving. A volume of 4 N HCl (100 mL) was added under stirring liquid phase (1000 mL) giving rise to a solution of pH 6. This solution was then centrifuged at 4,500 rpm for 7 min or kept overnight, until the solid fraction and supernatant was separated. Supernatant was then decanted and extracted with ethyl acetate using ethyl acetate solution with volume ratio of 1:3 (v/v) in a single extraction step. The ethyl acetate was removed by vacuum evaporation and the crude extract was subjected to vacuum liquid chromatography (VLC) using chloroform/methanol as eluent to separate the active compounds candidate. The modification was the ratio of eluent methanol:chloroform; in this experiment non polar to polar ratio was used at 0:100 (VLC-1), 10:90 (VLC-2), 20:80 (VLC-3), 30:70 (VLC-4), 40:60 (VLC-5), 50:50 (VLC-6), 60:40 (VLC-7), 70:30 (VLC-8), 100:0 (VLC-9). Crystal active compounds were obtained which were then analyzed by using thin layer chromatography (TLC) to classify the active compounds with the same retention factor (Rf) value. All active compound candidates were then tested by using inhibition test method against the growth of *E. coli*, *S. typhimurium*, and *S. aureus*.

Antimicrobial Activity of Selected Isolates

This experiment was inhibition zone test of all bioactive compound candidates isolated from PLF of EBPF. Inhibition zone test was conducted by using disc diffusion method (Satirapathkul & Leela, 2011) and the tested pathogenic bacteria comprised of *E. coli*, *S. typhimurium*, and *S. aureus*. The bacteria were maintained by subculturing periodically on nutrient agar and were preserved at 4°C prior to usage.

Culture media. The nutrient agar was composed of 20 g agar, 5 g peptone, 1.5 g yeast extract, 1.5 g beef extract and 5 g NaCl, diluted in 1000 mL distilled water. The mixture of nutrient was then boiled and its pH was adjusted to a range of 6.4–6.8. The mixture of nutrient was then autoclaved (at temperature of 121°C and pressure of 15 psi) for 20 min. Nutrient mixture was then poured into sterilized petri plates for culture media. All of the tested pathogenic bacteria were then grown overnight (at temperature of 37°C and rotation of 120 rpm) in nutrient

broth, and part of this broth was then used for seeding the bacteria in culture media.

Antimicrobial assay. Discs of filter paper were impregnated with 0.3 mg of each diluted crystal of bioactive compound. The discs were then placed on the top part of the seeded media plate of the tested bacteria. The plates were then incubated at 37°C for 24 h. The diameters of the clearing zone in each plate was then recorded. As positive controls, the discs of the standard antibiotics; Rumensin® (Elanco) (0.3 mg per disc) were used in this experiment. Diameter of the zone of inhibition in each disc was then assumed as a measure of antibacterial activity of the tested bioactive compounds. The diameters of the inhibition zones by the bioactive compounds were then compared with that of Rumensin® discs. Each of the antimicrobial assays was conducted in duplicate.

In Vitro Rumen Fermentation

Selected potential bioactive compounds that exhibited higher inhibition activity from previous experiment were then further studied in *in vitro* rumen fermentation. This *in vitro* experiment was conducted in a completely randomized block design with 6 treatments in 3 blocks, with rumen fluid from different cattle as a block. The treatments were R_0 = 0.5 g of basal diet (contained 66.7% TDN and 14.7% crude protein); R_1= R_0 + 0.3 mg of Rumensin®; R_2= R_0 + 0.3 mg of Syringaldehyde; R_3= R_0 + 0.3 mg of p-Hydroxybenzoic acid; and R_4= R_0 + 0.3 mg of m-Hydroxybenzoic acid; and R_5= R_0 + 0.3 mg of Oxybenzene. Ingredient composition of the basal diet is presented in Table 1.

Rumen contents were collected just about 3 h after morning feeding from 400 kg ruminally fistulated Ongole grade beef cattle in LIPI Cibinong Indonesia, which were maintained on basal diet composed of 40% forage and 60% concentrate. The rumen fluid was filtered through a double layer cheese cloth which was then used as inoculum. The substrate for the fermenta-

Table 1. Ingredient composition and calculated nutritional content of basal diet

Ingredients	Dry matter (%)	Nutrient contents (% DM)			
		TDN	Crude protein	Ca	P
Forage					
Pennisetum purpureum	25.0	15.43	2.88	0.17	0.06
Gliricidia sepium	15.0	11.25	4.01	0.02	0.02
Concentrate					
Cassava waste	21.1	13.34	0.91	0.04	0.01
Rice bran	11.1	7.27	1.42	0.01	0.19
Corn meal	9.0	6.84	0.79	0.01	0.02
Tofu waste	9.3	6.88	2.48	0.02	0.00
Coconut oil meal	7.5	5.65	2.22	0.01	0.01
Bone meal	1.0	0.00	0.00	0.32	0.11
Vitamin and mineral mix	1.0	0.00	0.00	0.05	0.02
Total	100.0	66.65	14.71	0.63	0.45

tion was a basal diet composed of 40% forage and 60% concentrate, containing 66.5 % TDN and 14.7% crude protein (CP).

In vitro rumen fermentation was conducted according to the method of Tilley & Terry (1963). An amount of 500 mg of substrate and 40 mL of McDougal buffer solution were added into individual fermentation tube, and then 10 mL of strained rumen fluid was inoculated to the medium. McDougal buffer solution contained $NaHCO_3$ 58.8g, $Na_2HPO_4.7H_2O$ 42 g, KCl 3.42 g, NaCl 2.82 g, $MgSO_4.7H_2O$ 0.72 g, $CaCl_2$ 0.24 g, and H_2O in 6 L solution. The fermentation medium was stirred and flushed with carbon dioxide to establish anaerobic condition and the tubes were then sealed with a rubber cork fitted with the gas release valve. Fermentation tubes were then incubated in a shaker water-bath at 39 ^oC for 24 h.

Individual VFA of the rumen fluid aliquots were analyzed by using gas chromatography (Filípek & Dvořák, 2009). About 1 mL of rumen fluid sample was added with 200 μL of metaphosphoric acid (25%) and formic acid (3:1) mixture. After 10 min of centrifugation (12000 rpm), the clear supernatant was x10 diluted in water, then 1 μL of it was injected in a 6820 GC System gas chromatograph, Agilent Technologies. A FFAP capillary column was used, 30 m x 250 μm x 0.25 μm (Quadrex Corporation). Nitrogen was used as a gas carrier, flow 1.0 mL/min; temperature programme: detector – FID (300ºC); injector (250ºC). Ammonia concentration of the rumen fluid aliquots was analyzed using the micro diffusion method (Conway, 1962). Rumen microbial protein synthesis was determined as acid prepicitable nitrogen, described by Makkar *et al.* (1982).

Data Analysis

All data collected were subjected to analysis of variance (ANOVA) followed by least significant difference (LSD) test. Computation was performed by using SPSS 13.0 for windows evaluation system and Microsoft Excel 2010.

RESULTS AND DISCUSSION

Results of GCMS analysis revealed that purified lignin formacell of empty bunch of palm fiber contained 19 bioactive phenolic compounds. Percentage of these detected bioactive compounds ranged from the lowest 2,3-Dimethylphenol (0.16%) to the highest Syringol (23.75%). List of phenolic compounds detected in PLF of EBPF are presented in Table 2. Among the 19 detected bioactive compounds, syringol, p-hydroxybenzoic acid, guaiacol, acetosyringone, and 2-methoxy-4-methylphenol were 5 among the phenolic compounds detected that had higher percentage in the PLF (> 9% of PLF).

Results of two methods of fractionation, extraction, and separation processes revealed that purified lignin formacell (PLF) of empty bunch of palm fiber (EBPF) contained 11 isolate fractions. Four isolate fractions were obtained from Method 1 (column chromatography, CC) and 7 isolate fractions were obtained from Method 2 (vacuum liquid chromatography, VLC). The isolates fractions were differentiated based on their Rf value from thin layer chromatography (TLC) test. The isolate fractions isolated from PLF of EBPF and it retention factor (Rf) value are presented in Table 3.

Antimicrobial Activity of Selected Isolates

Table 4 indicated that 4 isolate fractions, which was fraction CC-2, fraction CC-3, fraction VLC-5, and

Table 2. List of phenolic compounds detected in purified lignin formacell (PLF) of empty bunch palm fiber (EBPF)

Phenolic compounds*)	%	Formula	Molecular weight
Syringol	23.75	$C_8H_{10}O_3$	154
p-Hydroxybenzoic acid	14.19	$C_7H_6O_3$	138
Guaiacol	11.21	$C_7H_8O_2$	124
Acetosyringone	9.79	$C_{10}H_{12}O_4$	196
2-Methoxy-4-methylphenol	9.53	$C_8H_{10}O_2$	138
3-Methoxy-pyrocatechol	8.33	$C_7H_8O_3$	140
3-Methoxyacetophenone	6.46	$C_9H_{10}O_2$	150
Monophenol	5.15	C_6H_6O	94
Syringaldehyde	2.63	$C_9H_{10}O_4$	182
Acetovanillone	2.09	$C_9H_{10}O_3$	166
Vanillin	1.90	$C_8H_8O_3$	152
Eugenol	1.65	$C_{10}H_{12}O_2$	164
o-Cresol	0.95	C_7H_8O	108
3-Methoxy-2,5,6-trimethylphenol	0.57	$C_{10}H_{14}O_2$	166
2-Methoxy-4-methylphenol	0.54	$C_8H_{10}O_2$	138
Coniferyl alcohol	0.45	$C_{10}H_{12}O_3$	180
p-Ethylphenol	0.35	$C_8H_{10}O$	122
Homovanillic acid	0.31	$C_9H_{10}O_4$	182
2,3-Dimethylphenol	0.16	$C_8H_{10}O$	122

Note: *)Analyzed using Shimadzu GCMS-QP 2010.

Table 3. Phenolic compounds of purified lignin formacell (PLF)

Fractionation results	***TLC Test		
	Rf value	Eluent	Ratio eluent
Method 1*			
Fraction of CC-1	0.72	EtOAc : $CHCl_3$	10:01
Fraction of CC-2	0.66	EtOAc : $CHCl_3$	10:01
Fraction of CC-3	0.83	EtOAc : $CHCl_3$	10:01
Fraction of CC-4	0.55	Dioxan : air	1:01
Method 2**			
Fraction of VLC-2	0.38	MeOH : $CHCl_3$	80:20:00
Fraction of VLC-3	0.69	EtOAc : $CHCl_3$	10:01
Fraction of VLC-4	0.79	EtOAc : $CHCl_3$	10:01
Fraction of VLC-5	0.38	MeOH : $CHCl_3$	0.479
Fraction of VLC-6	0.45	MeOH : $CHCl_3$	0.479
Fraction of VLC-8	0.50	MeOH : $CHCl_3$	0.479
Fraction of VLC-9	0.71	MeOH : $CHCl_3$	0.479

Note: *CC= Column Chromatography; **VLC= Vacuum Liquid Chromatography: ***TLC= Thin Layer Chromatography; Rf= retention factor.

fraction VLC-9 exhibited antimicrobial activity against all tested bacteria (*E. coli, S. typhimurium,* and *S. aureus*). Other isolated fractions exhibited antimicrobial activity against only 1 or 2 tested bacteria. Level of antimicrobial activity of all isolate fractions obtained was still lower compare with that of Rumensin® (one of commercial antibiotic product).

Pan *et al*. (2009) classified the level of antimicrobial activity based on diameter of inhibition zone as follow: inhibition zone < 3 mm was considered low, 3-6 mm was medium, and > 6 mm was high. Based on this classification, fraction CC-1 only had high activity against *S. aureus*; fraction CC-2 had high activity against all tested pathogenic bacteria; fraction CC-3 had medium activity against all tested pathogenic bacteria; fraction CC-4 and VLC-6 had medium activity against *S. aureus*; fraction VLC-2, and VLC-8 had high activity against *S. aureus*; fraction VLC-5 had high activity against *S. aureus*, medium activity against *S. typhimurium*, and low activity against *E. coli*; and fraction VLC-9 has high activity against *S. typhimurium* and *S. aureus*. No inhibition zone was observed for fraction VLC-3 and VLC-4.

Table 4 also showed that more inhibition zone was demonstrated by the isolate fractions against the *S. aureus*, compared with that of *E. coli* and *S. typhimurium*. This result revealed that *S. aureus* was more susceptible to all isolated fractions compared to *E. coli* and *S. typhimurium*. *S. aureus* is a gram positive bacteria, which is more susceptible to antimicrobial agent compared with gram negative bacteria (Gonzales *et al*., 2006). The outer part of cell membrane of gram positive bacteria contains small amounts of lipopolysaccharides that is not sufficient to protect the cell from penetration by antimicrobes.

Four of most potential isolate fractions among the 11 isolate fractions obtained were then further analyzed by using gas chromatography mass spectroscopy (GCMS) to describe its monomers. Result of the GCMS analysis of the most potential bioactive compounds is presented in Table 5. Bioactive compounds obtained from the isolate fractions (crystal) were Oxybenzene (VLC-5 fraction), p-Hydroxybenzoic acid (VLC-9 fraction), Syringaldehyde (CC-2 fraction), and m-Hydroxybenzoic acid (CC-3 fraction). It is known that p-Hydroxybenzoic acid and m-Hydroxybenzoic acid is polyphenol compounds of non flavonoid sub-group of benzoic acids.

In Vitro Rumen Fermentation

Effects of treatment on ruminal metabolism variables of *in vitro* fermentation are presented in Table 6. The used of Rumensin®, syringaldehyde, p-hydroxybenzoic acid, m-hydroxybenzoic, and oxybenzene did not change the rumen pH, which were stable in the range of 6.60-6.70, which were considered normal pH for optimal rumen fermentation. This result indicates that the use of these bioactive compounds in this experiment did not have adverse effects on rumen metabolism. Rochfort *et al*. (2008) stated that the plant-derived substances should not have deleterious effect on ruminal flora, since ruminants drive much of their nutrition through bacterial gut fermentation. The same result is reported by Ozturk *et al*. (2010) that the rumen pH remains unchanged (6.8) when propolis ethanolic extract is given as antimicrobial product in rumen microbial fermentation using rumen simulation technique (Rusitec).
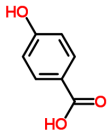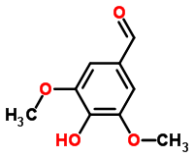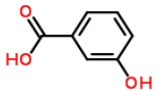
Table 6 also shows that ammonia concentration of fermentation medium significantly increased (P<0.05) by addition of Rumensin®, syringaldehyde, p-hydroxybenzoic acid, m-hydroxybenzoic, and oxybenzene. However, an ammonia concentrations of Rumensin® treatment was still higher than that of syringaldehyde, p-hydroxybenzoic acid, m-hydroxybenzoic, and oxybenzene treatment. These results indicate that bioactive compounds have positive effect on ammonia supply

Table 4. Diameter of inhibition zone against *E. coli, S. typhimurium,* and *S. aureus* obtained from inhibition test of isolate fractions

Compounds	E coli		S. typhimurium		S. aureus	
	Treatment	Rumensin®	Treatment	Rumensin®	Treatment	Rumensin®
	--------------------------------(in mm²)--------------------------------					
Method 1*						
Fraction of CC-1	-	14.00±0.09	3.63±0.03	14.05±0.13	8.56±0.39	14.50±0.35
Fraction of CC-2	8.28±0.34	13.93±0.20	9.17±0.06	14.18±0.12	10.23±0.80	16.18±0.00
Fraction of CC-3	5.52±0.20	13.08±0.03	4.42±0.13	11.05±0.28	5.48±0.36	13.60±0.35
Fraction of CC-4	-	12.88±0.10	-	11.22±0.03	4.92±0.11	12.45±0.97
Method 2**						
Fraction of VLC-2	-	14.25±0.26	-	15.58±0.23	9.10±0.20	15.88±0.81
Fraction of VLC-3	-	13.17±0.06	-	12.38±0.20	-	12.58±0.36
Fraction of VLC-4	-	12.60±0.09	-	11.73±0.24	-	10.70±0.31
Fraction of VLC-5	2.38±0.23	13.63±0.08	3.13±0.03	13.02±0.06	13.33±0.65	13.37±0.08
Fraction of VLC-6	-	11.82±0.03	-	13.27±0.14	4.46±0.16	12.80±0.23
Fraction of VLC-8	-	13.52±0.38	-	12.63±0.29	9.67±0.19	14.31±0.33
Fraction of VLC-9	5.47±0.13	14.10±0.05	7.42±0.14	13.18±0.15	10.75±0.00	15.10±0.00

Note: *CC= Column Chromatography; **VLC= Vacuum Liquid Chromatography.

Table 5. Description of the most potential isolate fractions obtained from purified lignin formacell (PLF) of empty bunch palm fiber (EBPF)

Crystal	Bioactive compounds*)	Formula	Molecular weight (Da)	Structure
VLC-5 fraction	Oxybenzene	$C_6 H_6 O$	94.111	
VLC-9 fraction	p-Hydroxybenzoic acid	$C_7 H_6 O_3$	138.121	
CC-2 fraction	Syringaldehyde	$C_9 H_{10} O_4$	182.173	
CC-3 fraction	m-Hydroxybenzoic acid	$C_7 H_6 O_3$	138.121	

Note: *)Analyzed using Shimadzu GCMS-QP 2010.

Table 6. Ruminal metabolism variables at different ration treatments

Variables	Treatmeants					
	R0	R1	R2	R3	R4	R5
pH	6.63± 0.12	6.60± 0.00	6.67± 0.06	6.70± 0.00	6.67± 0.06	6.63± 0.06
NH$_3$, mM	5.35± 0.56^c	10.01± 4.67^a	7.46± 3.30^b	7.40± 2.60^b	7.64± 4.32^b	7.83± 2.79^b
Total VFA, mM	90.30± 5.45^a	84.23± 2.91^{ab}	84.93± 6.39^{ab}	75.84±12.89^c	74.86± 5.87^c	83.78± 3.83^b
- Acetic acid (C2), mM	62.32± 4.68	56.68± 3.56	58.49± 4.63	52.70±10.20	49.95± 5.18	56.25± 2.48
- Propionic acid (C3), mM	19.28± 1.54	18.57± 0.48	17.86± 1.11	15.88± 2.02	16.10± 2.21	18.96± 1.52
- Iso-butyric acid, mM	0.55± 0.10	0.43± 0.01	0.43± 0.04	0.85± 0.27	1.34± 0.65	0.68± 0.02
- n-Butyric acid (C4), mM	6.70± 0.65	7.01± 0.30	6.85± 0.71	5.41± 0.78	5.98± 0.53	6.61± 0.22
- iso-Valeric, mM	0.50± 0.10	0.55± 0.04	0.52± 0.07	0.41± 0.03	0.65± 0.08	0.50± 0.05
- n-Valerate (C5), mM	0.95± 0.25	0.98± 0.10	0.79± 0.18	0.60± 0.09	0.84± 0.20	0.78± 0.12
- Iso acid	2.00± 0.44^b	1.96± 0.09^b	1.73± 0.29^b	1.86± 0.37^b	2.69± 0.27^a	1.96± 0.15^b
- C2/C3 ratio	3.24± 0.33^{ab}	3.06± 0.27^{ab}	3.28± 0.18^{ab}	3.31± 0.40^a	3.13± 0.47^{ab}	2.98± 0.20^b
- Non-Glucogenic Ratio (NGR)$^{a)}$	3.80± 0.35^{ab}	3.67± 0.25^{ab}	3.92± 0.18^a	3.90± 0.40^a	3.74± 0.45^{ab}	3.57± 0.26^b
- Estimate of methane production$^{b)}$	25.43± 2.17^a	23.20± 1.65^b	24.15± 2.10^{ab}	21.51± 4.46^c	20.44± 2.29^c	22.74± 1.01^b
Microbial protein synthesis, mg/mL	26.52± 7.88^b	33.52± 7.64^a	29.22± 6.68^{ab}	31.41± 2.16^{ab}	32.81± 2.09^a	32.31± 1.65^{ab}
Total gas, mL/g BO	168.17±27.97	161.10±18.88	165.47±16.12	166.94±36.48	164.21±24.38	164.86±23.47

Note: Means with different superscript at the same rows differ significantly (P<0.05), tested by LSD Test. R0= Basal diet; R1= R0 + Rumensin® 0.3 mg; R2= R0 + Syringaldehyde 0.3 mg; R3= R0 + p-Hydroxybenzoic acid 0.3 mg; R4= R0 + m-Hydroxybenzoic acid 0.3 mg; R5= R0 + Oxybenzene 0.3 mg.
$^{a)}$NGR calculated as (C2 + 2C4 + C5)/(C3+C5) (Abrahamse et al., 2008).
$^{b)}$CH$_4$ calculated as 0.45 C2 – 0.275 C3+ 0.40 C4.

for rumen bacterial growth, because ammonia is well known as the preferred source of nitrogen for microbial protein synthesis in the rumen. Rumen microbial protein synthesis data confirmed this theory and revealed that addition of Syringaldehyde, p-Hydroxybenzoic acid, m-Hydroxybenzoic, and Oxybenzene slightly increased the rumen microbial protein synthesis.

Increasing rumen microbial protein synthesis is mostly followed by reducing ammonia concentration (Mwenya et al., 2005). However, since nitrogen for bacterial protein synthesis is also supplied by amino acids and peptides, in certain condition improving in rumen bacterial protein synthesis is not always followed by reducing in ammonia concentration. Rumen bacterial yield usually increases when amino acids are included in the growth medium. Result of this experiment indicates that higher rumen microbial protein synthesis in Syringaldehyde, p-Hydroxybenzoic acid, m-Hydroxybenzoic, and Oxybenzene treatment were possibly also supported by nitrogen from amino acids and peptides in medium as main products of protein digestion. Crude protein digestion data (Table 7) confirmed this situation.

Table 7. Nutrient digestibility of ration treatments (%)

Nutrients digestibilities	Treatments					
	R0	R1	R2	R3	R4	R5
Dry matter digestibility	69.67±0.94[c]	71.44±2.53[bc]	71.65±0.49[abc]	73.75±0.89[a]	71.46±3.45[bc]	73.41±1.78[ab]
Organic matter digestibility	64.25±0.82[c]	66.67±3.47[ab]	66.90±0.89[ab]	69.31±0.60[a]	66.45±4.33[bc]	68.87±2.46[ab]
Crude protein digestibility	75.44±1.47[c]	80.17±2.52[a]	77.33±0.16[bc]	78.25±0.89[ab]	77.63±2.26[b]	77.63±2.71[b]
Crude fiber digestibility	39.92±5.48[c]	43.15±6.12[bc]	47.38±11.02[b]	60.00±1.82[a]	57.06±3.87[a]	54.60±6.27[a]

Note: Means with different superscript at the same rows differ significantly (P<0.05), tested by LSD Test. R0= Basal diet; R1= R0 + Rumensin® 0.3 mg; R2= R0 + Syringaldehyde 0.3 mg; R3= R0 + p-Hydroxybenzoic acid 0.3 mg; R4= R0 + m-Hydroxybenzoic acid 0.3 mg; R5= R0 + Oxybenzene 0.3 mg.

Data on estimate of methane production revealed that the use of Syringaldehyde, p-Hydroxybenzoic acid, m-Hydroxybenzoic acid, and Oxybenzene significantly reduced the estimate of methane production. The exact mechanism of how these bioactive compounds affected methanogens need further study. Moreover, various attempts have been made for methane abatement through rumen microbial manipulation (Kobayashi, 2010). Some of the examples are the use of chemicals, antibiotics, and natural products such as oils, fatty acids and plant extracts. However, the use of alternative natural materials is becoming more attractive due to health concerns regarding antibiotics.

Methane is produced as a by-product of rumen metabolism and represents a loss of energy. Some related studies reported the reduction of methane by plant-derived extracts. Broudiscou *et al.* (2000) observed that *L. officinalis* (lavender) and *S. virgaurea* promote the extent of fermentation and that *E. arvense* and *S. officinalis* (sage) had a possible inhibitory effect on methane production in continuous culture experiment using rumen microbes. Patra *et al.* (2006) conducted an *in vitro* experiment using rumen liquor of buffalo to evaluate the the effect of five plant extracts on methanogenesis. The result showed that plant extracts of *Terminlaia chebula* could be used to reduce methane production.

Table 6 also demonstrates that the addition of Syringaldehyde, p-Hydroxybenzoic acid, m-Hydroxybenzoic acid, and Oxybenzene significantly reduced (P<0.05) the total VFA concentration. Reducing VFA concentration also occurred in Rumensin® treatment. This result was in contrary with the work of Felix *et al.* (2012) who reported that increasing dietary monensin did not affect ruminal VFA concentrations *in vivo*. Further experiment should be designed to investigate this contradiction, especially in a more dynamic fermentation system such as in continuous culture fermentation or *in vivo* experiment.

Data on nutrients digestibility of the *in vitro* fermentation are presented in Table 7. Table 7 shows that dry matter, organic matter, crude protein, and crude fiber digestibility were improved by Rumensin®, Syringaldehyde, p-Hydroxybenzoic acid, m-Hydroxybenzoic acid, and Oxybenzene. This result indicated that addition of antimicrobial materials to the rumen fermentation system could improve the performance of fermentation process. These findings were in accordance to the review of Wallace *et al.* (2002), who stated that plant secondary compounds had great potential as 'natural' manipulators of rumen fermentation, to the potential benefit of the animal production.

In general, these experiments revealed that Syringaldehyde, p-Hydroxybenzoic acid, m-Hydroxybenzoic acid, and Oxybenzene demonstrated antimicrobial effect against the pathogenic bacteria and could slightly modify the rumen fermentation to potential benefit of host animal. Antimicrobial effect on pathogenic bacteria could be further explored to be used for controlling gastrointestinal pathogenic bacteria in ruminant, especially in newborn ruminant. Effect of these bioactive compounds on rumen fermentation still needs more intensive exploration, since these materials could acts not only as antimicrobial but also as 'rumen modifier' or 'natural' manipulator of rumen fermentation. Rochfort *et al.* (2008) suggested that studies on the use of plant-derived bioactive compounds should also focus on the dynamic of ruminal microflora. Moreover, regarding the use of plant-derived bioactive compounds, rumen microbiology studies are important since some species of rumen bacteria are more sensitive to certain structures than others, for example, *P. anaerobius* is extremely sensitive to both high and low molecular weight polymers (Sivakumaran *et al.*, 2004). Additionally, many studies have relied on *in vitro* experiment. Therefore, further experiments should also explore more controlled *in vivo* research to verify the benefit of plant-derived bioactive compounds.

CONCLUSION

Purified lignin formacell from empty bunch palm fiber contained 19 phenolic compounds. Syringaldehyde, p-Hydroxybenzoic acid, m-Hydroxybenzoic acid and Oxybenzene were 4 amongst the 11 phenolic compounds isolated exhibiting the antimicrobial activity; although antimicrobial activity of all bioactive compounds was lower than that of Rumensin®. The use of Syringaldehyde, p-Hydroxybenzoic acid, m-Hydroxybenzoic acid, and Oxybenzene improved the ammonia concentration, microbial protein synthesis as well as *in vitro* dry matter, crude protein, and crude fiber digestibilities. However, the bioactive compounds had no effect on rumen pH and reduced the total volatile fatty acid concentration as well as estimate of methane production.

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