Effect of Cinnamon Bark Meal (*Cinnamomun burmanni* Ness ex Bl) on *In Vitro* Methane Production and Rumen Methanogens Diversity

I Hadianto¹, L M Yusiati², Z Bachrudin², B Suhartanto², C Hanim² and A Kurniawati²

¹ Graduate Student, Faculty of Animal Science, Universitas Gadjah Mada, Jl. Fauna No. 3, Yogyakarta 55281, Indonesia
² Faculty of Animal Science, Universitas Gadjah Mada, Jl. Fauna No. 3, Yogyakarta 55281, Indonesia

liesmira@ugm.ac.id

Abstract. Plant secondary metabolites such as cinnamaldehyde have been used to manipulate methane production in the rumen. The research aimed to investigate the effect of cinnamon bark meal as cinnamaldehyde source on methane production and methanogen diversity in the rumen. Substrate of fermentation consist of *Pennisetum purpureum*, wheat pollard and soybean meal. Cinnamon bark meal was added into feed sample equal to cinnamaldehyde level as much as 200, 400, 600, 800 mg based on dry matter (DM). Parameters recorded were dry matter digestibility (DMD), organic matter digestibility (OMD), methane production, number of protozoa and communities of methanogen. Fermentation parameters were measured by incubating the sample in a rumen liquor buffer that was taken from a rumen fistulated Bali cattle. *In vitro* gas production was analyzed using Menke and Steingass gas production technique. Methanogenic communities were observed using Terminal Restriction Fragment Length Polymorphism (T-RFLP) technique. The bacterial diversity (H’ index) and evenness (E index) were calculated from the peak value. Results showed that there was no significant (P>0.05) different among treatments on IVDMD, IVOMD, methane production and number of protozoa. Based on T-RFLP, methanogenic archaea diversity was not affected by the diet. This study shows that addition of cinnamon bark meal as cinnamaldehyde source may not viable CH4 mitigation strategies to reduce methanogenic activity in the rumen.

1. Introduction

Rumen microorganisms are involved in the fermentation of substrates contained in the diet of the ruminant (carbohydrates, proteins and lipids). A symbiotic relationship is found among different groups of microorganisms due to the diverse nature of these microbial species and their adaptability and interactions also coexist. The ruminant provides the necessary environment for the establishment of microbial, while the microbial obtain energy from the host animal from end products of fermentation. However, the fermentation process is not effective because there are energy losses mainly in the form of methane gas (CH₄), which is a problem for the environment. Rumen methanogens produce methane during fermentation of feed in the rumen represent a loss of energy offered to the ruminants as feed [1]. Methane emissions reflects the loss of some energy that could not
be utilized for ruminant performance, health and productivity [2]. Methane emissions represent approximately 6 to 10% of feed gross energy which consumed by ruminant lost as CH$_4$ [3]. Microbial fermentation through the action of methanogenic archaea in rumen considerably contribute to the methane. Ruminants produce CH$_4$ approximately 80 million tonnes of methane per year [4]. Concerns about the possible environmental effects and associated economic burdens of ruminant methane emissions have lead to increased interest in manipulating methanogens and methanogenesis in the rumen [5].

Plant secondary metabolite has been reported as an effective strategy for inhibition of methanogenesis in ruminants [6]. Cinnamaldehyde is one of the secondary metabolites present in cinnamon tree, and it has been investigated because of its potential to decrease production of methane [7]. Other studies, cinnamaldehyde could be used to reduce 26-95% of CH$_4$ production from rumen fermentation by in vitro [8,9]. Although the effects of cinnamaldehyde on rumen fermentation have previously been studied, only a few were examined on rumen methanogenic archaea communities [10]. However, to understand the mechanism how cinnamaldehyde influence CH$_4$ production in the rumen, more comprehensive determination on rumen methanogenic archaea communities is essential [11]. The extent of methane production in the rumen is determined by the diversity as well as the interaction among that methanogenic archaea community.

Classic cultivation techniques are commonly used to obtain information about community structure of rumen methanogenic archaea. The detection of rumen microbial population by simple culture methods may give unreliable results, which probably only account for 10 to 20 percent of the rumen microbial population [12]. In a recent study the microbial diversity archaea (methanogens) in the rumen was assessed by more precise molecular techniques and shorter analysis duration. Methyl coenzyme-M reductase (mcrA) gene sequencing is one of the basic genetic markers for the characterization of methanogenic archaea community [13]. It encodes the enzyme (methyl coenzyme-M reductase) involved in the terminal step of methanogenesis and has been shown to be a good marker for the presence of methanogens [14]. The biomolecular techniques were such as terminal restriction fragment length polymorphism (T-RFLP) have been successfully used for the analysis of methanogenic archaea community in rumen [15,16]. T-RFLP (Terminal Restriction Fragment Length Polymorphism) method is used to determine the dynamics of microbial community. Therefore, this study was conducted to investigate the effects of cinnamon bark meal as cinnamaldehyde source on methanogenic archaea diversity, which contribute to methane production for mitigating livestock methane emissions.

2. Materials and methods

2.1. Sample preparation

Feed sample for in vitro for fermentation consisted of Pennisetum purpureum, which cut before flowering stage, wheat pollard and soy bean meal, with ratio 60:30:10 based on dry matter (DM) basis. Cinnamon bark meal accounting cinnamaldehyde for 2.17% (purity >80%) and feed samples were prepared by drying in dryer incubator at 55°C and grounded to pass through a 1-mm screen. Inoculum for the in vitro gas production was obtained from a rumen fistulated Bali cattle fed a diet consisting of Pennisetum purpureum and wheat pollard 60:40 DM basis TDN 62.01% and CP 13.16% which offered in equal proportions twice a day, at 07:00 and 14:00 h. Rumen fluid was collected in the morning prior feeding, and squeezed through polyester cloth into prewarmed vacuum flask.

2.2. In vitro gas production

In vitro gas production were used in this study according to method explained by [17]. Substrate of 300 mg was put into the syringe and incubated for 48 h under anaerobic condition at 39°C. Rumen fluid and buffer mixture (1:2) was pumped in 30 ml doses into syringes. The dietary treatments were: P0 (60% elephant grass + 30% wheat bran + 10% soybean meal), P1 (P0+1.16% cinnamon bark meal or equal to cinnamaldehyde with 200 mg/kg DM basis) P2 (P0+2.3% cinnamon bark meal
or equal to cinnamaldehyde with 400 mg/kg DM basis), P3 (P0+3.5% cinnamon bark meal or equal to cinnamaldehyde with 600 mg/kg DM basis), P4 (P0+4.5% cinnamon bark meal or equal to cinnamaldehyde with 800 mg/kg DM basis). Each treatments was examined in triplicate. At the end of incubations gas samples were collected using calibrated syringe and 10 ml of gas were transferred into 10 ml plain vacuum tube for methane determination using gas chromatography. In vitro Dry matter (IVDMD) and in vitro organic matter digestibility (IVOMD) were determined by filtered the bottle content, and residual feed were collected for dry matter and organic matter analysis. Proximate analysis was used for chemical composition of residual feed [18]. Sample for protozoa calculation were prepared by pipetting 1 ml of bottle content and be added to 0.8ml of formaldehyde saline (1ml of 37%formaldehyde + 9 ml 0.9% NaCl). One microliter sample then transferred to haemocytometer for direct calculation under microscope according to method explained by [19].

2.3. T-RFLP analysis

Methanogen communities were determined using terminal restriction length polymorphism (T-RFLP) following method by [20]. DNA was isolated from liquid media after 48 h incubation by in vitro gas production method. A total of 15 ml of liquid phase was centrifuged at 7000 rpm speed for 15 minute and pellet was used for DNA extraction. Genomic DNA was extracted by using ZymoBioticTM DNA Mini Kit (Zymo Research Cat No D4300). Specimen DNA was pooled from each treatment (five DNA samples in total).

The methyl-coenzyme M reductase – subunit (mcrA) gene was amplified using primers specific for mcrA primer MLf (5′-GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC-3′) and MLr (5′-TTC ATT GCR TAG TTW GGR TAG TT-3′) [21]. The forward primer for the PCR reaction was labeled on the 5′ end with 6-fluorescein amidite (FAM) marker to allow the terminal fragment to be tracked. Polymerase chain reaction (PCR) amplification was carried out in 30 μl reaction mixtures, with three replicates prepared for each sample. The reaction mixture contained of 4 μL dissolved DNA (<250 ng), 12.5 μl GoTaq® Green Master (Promega Corporation), 9.5 μl DNase free water, and 2 μl (10 pmol/μl) of each primer. The PCR was performed using a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA) and a thermal profile of an initial 5 min denaturing step at 95°C, followed by 35 cycles of a 95°C denaturing step for 1 min, 55°C annealing step for 45 s, and 72°C extension step for 1 min, with a final 10 min of extension at 72°C. All PCR products were confirmed on a 1% (w/v) agarose gel at 90 V for 45 min and quantified with a ladder of known. DNA concentration. Triplicate tubes of each sample were then combined and digested enzymatically with restriction enzymes. Restriction digestion with TaqI (FastDigest ThermoFisher Scientific) was carried out in a reaction volume of 20 μl containing 16 μl of PCR product, 2 μl 10X FastDigest® buffer and 2 μl of TaqI, with digestion at 37°C for 5 min then immediately placed on ice. DNA fragments digestion product was analyzed using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA) and interpreted using the GeneMapper® v 5.0 analysis software. T-RFLPs and diversity analysis for each sample, the TRFs (terminal restriction fragments) peak size between 50 bp and 500 bp and peak high more than 1% from total peak high were further analyzed to determine the phylogenetic relationship. The TRFs from the same sample with differences size less than 0.5 bp digested with one restriction enzymes were grouped as one TRF [22]. Prediction of T-RFs was performed by using the MiCAIII microbial diversity database [23]. Diversity of microbial populations was determined based on the Shannon index method, as previously described by [24].

2.4. Data analysis

Data of fermentation were analyzed using one-way analysis of variance with SPSS 16 for Microsoft Windows and statistically significant differences between means were determined by Duncan’s Multiple Range Test (DMRT) when the effects of treatment (P<0.05) were detected [25], microbial diversity data were analyzed descriptively.
3. Results and discussion

3.1. In vitro digestibility
Data in vitro dry matter digestibility and organic matter digestibility were presented in Table 1. There was no effect of cinnamon bark meal addition up to 4.5% on IVDMD and IVOMD.

Table 1. Effects of cinnamon bark meal (Cinnamomum burmanni Ness ex Bl) addition as cinnamaldehyde source on in vitro digestibility.

| Variable (%) | Treatments |
|--------------|------------|
|              | P0         | P1         | P2         | P3         | P4         |
| IVDMD<sup>ns</sup> (%) | 53.36±2.07 | 52.56±2.46 | 52.07±3.24 | 50.83±3.38 | 50.44±3.46 |
| IVOMD<sup>ns</sup> (%) | 51.34±3.69 | 50.67±3.86 | 50.42±3.91 | 49.35±3.91 | 48.87±4.09 |

<sup>ns</sup> Non significant.

IVDMD = in vitro dry matter digestibility; IVOMD = in vitro organic matter digestibility

Effect of plant secondary metabolites on IVDMD and IVOMD digestibility were varies. The in vitro dry matter and organic matter digestibility were not affected by addition of Kaempferia galangal L up to 100 mg/L [26]. Other study showed addition of java cardamom as essential oil source reduced dry matter digestibility at levels 100 mg/l [27]. This different finding might be caused by different their chemical composition of plant secondary metabolite. Plant secondary metabolites activities were influenced by their chemical nature and concentration. Purity also influenced activity of plant secondary metabolite. Activities of essential oils also depend on their component and dose. In the same study, IVDMD and IVOMD pattern parallel to gas production pattern (unpublished observation), gas production commonly used to predict IVDMD and IVOMD. Total gas production reflect the substrates degradability on in vitro nutrient digestibility [28].

3.2. Number of protozoa and methane production
Data on number of protozoa and methane production were presented in Table 2. Addition cinnamon bark meal as cinnamaldehyde source had no effect on total numbers of protozoa. Cinnamaldehyde effects on protozoal numbers are varies. Some studies report different effect of plant secondary metabolite and cinnamaldehyde on protozoal numbers. There were no changes in ruminal protozoa counts with 600 mg/kg DM or 1g/day of cinnamaldehyde, respectively [29,30]. Addition essential oil of red ginger as plant secondary metabolite reduced protozoa number [31]. Population of protozoa appears to be not that sensitive to the presence of cinnamaldehyde in the rumen. Patra and Saxena [1] suggested that plant secondary metabolite present in all types of plant is not equally effective on protozoa.

Table 2. Effects of cinnamon bark meal (Cinnamomum burmanni Ness ex Bl) addition as cinnamaldehyde source on number of protozoa and methane production.

| Variable (%) | Treatments |
|--------------|------------|
|              | P0         | P1         | P2         | P3         | P4         |
| Protozoa (sel x10<sup>5</sup>) | 1.16±0.11 | 1.13±0.12 | 1.10±0.05 | 1.09±0.01 | 1.08±0.01 |
| CH<sub>4</sub> (ml) | 5.76±0.34 | 5.47±0.32 | 5.34±0.35 | 5.27±0.78 | 5.23±0.53 |
| CH<sub>4</sub>/DM digested (ml/g) | 38.20±2.55 | 37.23±3.62 | 36.70±4.15 | 37.17±6.18 | 37.15±4.51 |

<sup>ns</sup> Non significant.

Cinnamon bark meal as cinnamaldehyde source at level 0, 1.16, 2.3, 3.5 and 4.5% did not affect significantly on total methane production and methane productions per digested dry matter. In agreement with our results, addition of 132 mg/L cinnamaldehyde had no effect on CH<sub>4</sub> production [9]. This result support that the effect of cinnamaldehyde on methane production is a secondary effect...
as on number of protozoa. It has been known that ruminal protozoa are the host of some methanogens and may contribute to methane emission. Thus protozoal population has a correlation with some population of methanogen [2]

3.3. T-RFLP profile of methanogenic community

The DNA band as result of PCR amplification were produced a single band of DNA that measure approximately 500 bp size at 1% agarose (Figure 1).

![Figure 1. The product of PCR amplification of mcrA genes with 500 bp at 1% agarose; M = DNA marker](image)

In this study, the amplification of mcrA genes with primer MLf and MLr produced a single band of DNA approximately 500 bp sized at 1% Agarosa (Figure 1). A single major PCR product of expected size (464 to 491 bp) was generated using the primers MLf and MLr [21]. The amplified products were used for T-RFLP analysis with TaqI restriction enzyme and produced 8 TRFs sizes from 57 to 441 bp. TaqI restriction enzyme could be used to produce an accurate representation of the methanogen archae community [32]. The size of TRF on electropherogram was identified using the Microbial Community program analysis III (MiCA). TRF size details on each of the eight filotipe are TRF peak size 52 bp, filotipe identified and classified from the uncultured Methanomicrobiales archaeon clone: Chem-m-D02; TRF peak size 163 bp, identified belongs to uncultured euryarchaeote clone MCR-U3SP-18; TRF peak size 207 bp, identified methanogenic belongs to Methanocorpusculum parvum; TRF peak size 224 bp, identified archae belongs to uncultured archaeon clone B09; TRF peak size 234 bp, identified methanogenic belongs to uncultured Methanobacteriales archaeon; TRF peak size 279 bp, identified archae belongs to uncultured archaeon clone CLI36; TRF peak size 306 bp, identified methanogenic belongs to uncultured methanogenic archaeon clone LLM-ADT43-40, whereas the TRF peak size 441 bp, identified archae belongs to uncultured archaeon clone ATB-EN10721 (Table 3).

Addition of cinnamon bark meal as cinnamaldehyde source had no chance on the distribution and composition of methanogen compare control. The most abundant phyla across all five treatments were 279 bp (range of relatives abundance 24.29 to 27.29%), 306 bp (19.29 to 21.53), 225 bp (16.38 to 18.87), 57 bp (9.69 to 14.07), 163 bp (9.53 to 11.37). Other less abundant phyla were 207, 234 and 441 (3.92 to 6.16, respectively) (Figure 2).

| Table 3. Terminal restriction fragment of methanogenic archaea community uses restriction enzyme TaqI. |
|--------------------------------------------------|
| Restriction enzyme | No | Methanogen community | Size of DNA fragment |
|---------------------|----|----------------------|---------------------|
|                     |    |                      |                     |
In the present study, addition of cinnamon bark meal as cinnamaldehyde source cointained eight filotype of methanogen archae, of which the most dominant was uncultured archaeon clone CLI36 at all treatments. This is consistent with previous studies, which also suggest that many of the organisms in the rumen are currently uncultured [33]. Limitations of T-RFLP analysis provides estimates of filotype based only on existing taxonomies [34].
Diversity index of methanogen archaea are shown in Table 4. Addition of cinnamon bark meal as cinnamaldehyde source had no effect on rumen methanogenic archae diversity.

**Table 4.** Diversity of methanogen archae community on cinnamon bark meal (*Cinnamomum burmanni* Ness ex Bl) addition as cinnamaldehyde source.

| Parameter (%) | Treatments |
|---------------|------------|
|               | P0         | P1         | P2         | P3         | P4         |
| Richness (S)  | 8          | 8          | 8          | 8          | 8          |
| Shannon (H')  | 1.89       | 1.90       | 1.90       | 1.88       | 1.88       |
| Evenness (E)  | 0.91       | 0.91       | 0.91       | 0.90       | 0.90       |

In this study, increasing level of cinnamon bark meal given similar diversity index value (richness, shannon and evenness) for each treatment. A similar diversity index in the rumen indicated that the composition of the methanogenic filotope detected based on the MiCA III for all treatments is relatively stable. It showed that synergistic effect methanogens archae diversity on methane production. The absence of effect of cinnamon bark meal on methanogen archae diversity in our study is consistent with the other result in which there was no change on CH$_4$ production. It shows that the CH$_4$ production associated or correlated with methanogen population diversity. Microbial patterns were reflected in the fermentation parameters, a change in growth rates results in changes in the proportion of rumen methanogens populations, resulting in changes in the fermentation profile [35]. This is similar to results reported by [36] that there was no change in ruminal fermentation or number of bacterial cells because of the sequence in which the soybean oil was included in the diet.

4. Conclusion

Addition of cinnamon bark meal as cinnamaldehyde source to the diet up to 4.6% of DM feed or equivalent to cinnamaldehyde with 800 mg per kg DM feed did not effect on DMD, OMD, number of protozoa and methane production. The methanogenic communities consist of eight filotope namely: uncultured *Methanomicrobiales* archaeon clone: Chem-m-D02, uncultured euryarchaeote clone MCR-U3SP-18, *Methanocorpusculum parvum*, uncultured archaeon clone B09, uncultured *Methanobacteriales* archaeon, uncultured archaeon clone CLI36, uncultured methanogenic archaeon clone LLm-ADT43-40 and uncultured archaeon clone ATB-EN10721. The methanogens dominated of all treatments was uncultured archaean clone CLI36.

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