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Evaluation of noni (*Morinda citrifolia*) leaves and fruits on methane emission and rumen fermentation parameters *in vitro*

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Abstract. Various attempts were made to reduce emissions of methane, among others was through use bioactive compound of the herbs. One of the herbs that can use is Noni (*Morinda citrifolia*), which has been reported contain tannin, saponin and possess antimicrobial activities. The objective of this study was to evaluate the use of the *Morinda citrifolia* leaves (MCL) and *Morinda citrifolia* fruits (MCF) on methane emission and rumen fermentation *in vitro*. These MCL and MCF were added to the incubation bottles containing *Penisetum purpureum* grass (PPG) with the following treatments (performed in four replicates): R0: control substrate (100% PPG), R1: 90% PPG + 10% MCL, R2: 80% PPG + 20% MCL, R3: 70% PPG + 30% MCL, R4: 60% PPG + 40% MCL, R5: 90% PPG + 10% MCF, R6: 80% PPG + 20% MCF, R7: 70% PPG + 30% MCF, and R8: 60% PPG + 40% MCF. Parameters measured were total gas production, methane emission, microbial population, and ammonia. The results showed that the highest total gas production and a decrease in methane gas were obtained at R3. In conclusion, addition of *Morinda citrifolia* leaves or fruits have a potential to reduce methane emission and improves feeding value of *Penisetum purpureum*.

1. Introduction

Carbon dioxide (CO₂) and methane (CH₄) are the largest contributors to greenhouse effects. However, methane has the ability to retain heat 25 times greater than CO₂ [1]. Beauchemin [2] said that one of the contributors to the accumulation of anthropogenic methane gas, which around 28% of total methane, is from livestock, especially ruminants. Methane emissions affect global warming, furthermore it is represent of significant energy loss in ruminant livestock, which is about 8–14% of total energy intake digestible [3].

Various attempts were made to reduce emissions of methane, among others was through feeding strategies; such as by using antibiotics as a feed additive, however it had a negative effect on livestock [4]. This result triggered exploration of a variety of natural compounds that could replaced antibiotics in reducing methane emissions. Plant secondary metabolites, including the tannins and saponins are among natural compounds that could potentially be used as feed additives in mitigating methane emissions from ruminant.



Morinda citrifolia is one of plants that contain tannin and saponin in its leaf and fruit. This study aimed to evaluate the use of *Morinda citrifolia* leaves (MCL) and *Morinda citrifolia* fruits (MCF) on methane emission and rumen fermentation *in vitro*.

2. Materials and method

2.1. Sample preparation and analysis

Feed substrate used in this study was a mixture of *Penisetum purpureum* grass (PPG) and *Morinda citrifolia* leaves (MCL) or fruits (MCF). These materials were oven-dried at 50°C for 24 h and ground to pass a 1 mm sieve by using a hammer mill. Further, they were subjected to chemical composition analysis that included crude protein, ether extract, neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin contents. Substrates were homogeneously mixed according to the following dietary treatments: R0: 100% PPG, R1: 90% PPG + 10% MCL, R2: 80% PPG + 20% MCL, R3: 70% PPG + 30% MCL, R4: 60% PPG + 40% MCL, R5: 90% PPG + 10% MCF, R6: 80% PPG + 20% MCF, R7: 70% PPG + 30% MCF, and R8: 60% PPG + 40% MCF. These dietary treatments were subjected to *in vitro* incubation with buffered-rumen fluid mixture.

2.2. In vitro rumen fermentation procedure

In vitro incubation was conducted by a modified method of Theodorou (1994) as described in Jayanegara (2017) [5,6]. Briefly, 1 g of each dietary treatment was added into each incubation bottle. Rumen fluid was taken from a fistulated Friesian-Holstein cow before morning feeding, and then brought to the laboratory to be filtered and mixed with buffer solution (rumen fluid : buffer = 1 : 4 v/v). The buffer solution was comprised of 385.6 ml bicarbonate, 193.6 ml macro minerals, 0.256 ml micro minerals, 0.975 ml resazurine, 36.8 ml reducing solution and 579 ml distilled water. Rumen-buffer solution was saturated with CO₂ during the mixing process. Similarly, the incubation bottles were saturated with CO₂ before closing in order to ensure the anaerobic condition. An amount of 100 ml rumen-buffer solution was added into each incubation unit and then immediately closed with rubber cap.

Incubation was carried out in a water bath at 39°C for 48 h. Total gas production was recorded at 4, 6, 9, 12, 24, 30, 36, and 48 h after incubation by using a gas syringe (Sigma-Aldrich Z314382-1EA, Poulten and Graf GmbH, Wertheim, Germany) equipped with an injection needle (BD REF Precision Glide Needle TM 302 008, Singapore). Measurement of methane production was conducted based on Jayanegara [6]. In brief, gas production was flown into 5 N NaOH. Carbon dioxide, the main gas produced during *in vitro* rumen fermentation, was bound by NaOH and methane volume was read in another syringe connected to the system. Determination of ruminal ammonia concentration followed the description of Jayanegara (2017) whereas total bacteria and protozoa were enumerated according to Antonius (2015) description [6,7].

2.3. Data analysis

The experiment was based on a completely randomized design, and each treatment was performed in four replicates. Data obtained from this experiment were analyzed by analysis of variance (ANOVA). When a parameter showed significantly different at $P < 0.05$, Duncan's multiple range test was conducted for comparing among treatment means. All the statistical analyses were performed by using SPSS statistical.

3. Results and discussion

3.1. Nutrient composition

Nutrient composition of PPG, MCL and MCF used as substrates in the present experiment is presented in table 1. Among these materials, MCL contained the highest crude protein content whereas MCF was the lowest. Contents of ether extract of PPG, MCL and MCF were low. Both MCL and MCF had

substantially lower NDF and ADF contents as compared to PPG but their lignin were higher. *Penisetum purpureum* is a grass species that commonly used by farmers as a main forage in Indonesia. Primarily *Pennisetum purpureum* is a source of fiber and in this experiment it contained 14.4% crude protein which was relatively higher in comparison to other reports [8,9]. The NDF content of the grass species was quite high, could be expected to affect NDF content of the rations and their digestibility values. Main part of NDF is comprised of hemicellulose, cellulose and lignin, and the linkage between lignin and cellulose is difficult to digest by rumen microbes [10]. The NDF content, besides affecting the digestibility, also affects the emission of methane gas; methane emission increases with increasing NDF content.

Table 1. Nutrient composition of MCL, MCF and PPG (% dry matter)

Nutrient	MCL	MCF	PPG
Crude protein	16.7	6.9	14.4
Ether extract	3.2	1.6	3.0
NDF	50.1	40.8	86.9
ADF	45.8	32.5	52.3
Lignin	15.7	10.7	9.7

Feed Science and Technology Laboratory, IPB University (2016)

Morinda citrifolia leaves had high crude protein content, i.e. above 15% DM, and therefore it could be used as a protein source for ruminants. Despite its high protein content, this forage also contained high NDF and ADF so there was a possibility of negatively affecting rumen fermentation and digestibility. In this study, the mixtures of *Pennisetum purpureum* and *Morinda citrifolia* leaves or fruits were used. The use of these forages for livestock were expected that the two would complement each other as a source of fiber and protein, and in addition to the expected higher digestibility of these mixtures and to simultaneously lowering methane emissions during fermentation process in the rumen. Both *Morinda citrifolia* leaves or fruits have been reported to contain various phenolic compounds [11,12] that may influence rumen fermentation and methanogenesis.

3.2 In vitro rumen fermentation and methanogenesis

Total gas production, methane production and concentration of dietary treatments was as a result of microbial fermentation is presented in table 2.

Table 2. Total gas production, methane production and concentration of dietary treatments

Treatment	Total gas (ml)	Methane (ml)	Methane (%gas)
R0	70.3 ^a	23.3 ^a	29.3 ^{ab}
R1	68.3 ^a	20.9 ^a	30.6 ^b
R2	125.0 ^d	38.1 ^c	30.5 ^{ab}
R3	87.0 ^b	22.9 ^a	26.3 ^a
R4	111.8 ^c	33.9 ^b	30.3 ^{ab}
R5	111.3 ^c	33.1 ^b	29.7 ^{ab}
R6	111.5 ^c	33.5 ^b	30.0 ^{ab}
R7	109.8 ^c	33.1 ^b	30.2 ^{ab}
R8	126.3 ^d	36.5 ^{bc}	28.9 ^{ab}

R0: 100% PPG, R1: 90% PPG + 10% MCL, R2: 80% PPG + 20% MCL, R3: 70% PPG + 30% MCL, R4: 60% PPG + 40% MCL, R5: 90% PPG + 10% MCF, R6: 80% PPG + 20% MCF, R7: 70% PPG + 30% MCF, and R8: 60% PPG + 40% MCF.

The results showed that treatment significantly ($P < 0.05$) to total gas production. Greater total gas production was observed in the inclusion of *Morinda citrifolia* leaves or fruits in comparison to control

($P < 0.05$) except for R1. This may suggest that *Morinda citrifolia* leaves or fruits are good sources of feeds and easily digested and fermented by rumen microbes. Gas produced by rumen microbes during incubation, in part a product of the metabolism of the microbes digest and ferment fed or substrated, while the other part was from the process of buffering solution of artificial saliva (buffer solution) of the volatile fatty acids generated [13].

Methane production is according to the total gas produced. Methane concentrations of diets with *M. citrifolia* inclusion were similar to control except for R3 that tended to be lower. The tendency of methane concentration decrease observed in R3 may occur because of the contents of tannins and saponins in *M. citrifolia*. Tannins may reduce methane emission through direct elimination of methanogen population [14]. The mechanism of inhibition of methane production in ruminants has been initiated by Tavendale (2005) namely (1) indirectly by inhibiting the digestion of fiber that reduces the production of H_2 , and (2) directly inhibits the growth and activity of methanogens [15]. In addition, tannins also inhibit the growth of protozoa which became one of the main host of methanogens [16]. While the mechanism of saponin in reducing methane emissions is through the elimination of rumen protozoa population. This can be seen in table 3.

Table 3. Population of total bacteria and protozoa and NH_3 concentration.

Treatment	Bacteria (cell/mL)	Log Protozoa (cell/mL)	NH_3 (mM)
R0	7.75 ^c	5.02 ^d	10.95 ^{cd}
R1	9.50 ^d	3.72 ^c	9.89 ^{cd}
R2	5.00 ^b	5.55 ^f	6.31 ^a
R3	11.00 ^e	3.33 ^b	10.66 ^{de}
R4	5.75 ^b	6.13 ^g	11.74 ^{fg}
R5	10.00 ^d	2.79 ^a	12.01 ^g
R6	3.75 ^a	6.36 ^h	8.52 ^b
R7	10.25 ^{de}	5.38 ^e	10.46 ^{cde}
R8	10.00 ^d	3.41 ^b	9.63 ^c

R0: 100% PPG, R1: 90% PPG + 10% MCL, R2: 80% PPG + 20% MCL, R3: 70% PPG + 30% MCL, R4: 60% PPG + 40% MCL, R5: 90% PPG + 10% MCF, R6: 80% PPG + 20% MCF, R7: 70% PPG + 30% MCF, and R8: 60% PPG + 40% MCF.

Beauchemin (2008) stated that the activity of saponin in reducing the number of rumen protozoa associated with saponin capacity for binding to the sterol present in the cell membrane of protozoa, causing cell lysis of the protozoa [2]. Rumen protozoa elimination increased the number of amylolytic bacteria, but big protozoa was a predator for amylolytic bacteria. With the reduction of the population of protozoa in the rumen, amylolytic activity of bacteria increased, and generated more propionic acid and less methane. A number of methanogenic bacteria lived in the rumen and stuck on protozoa cell surfaces [17]. With the reduction of the population of methanogenic protozoa due to loss of habitat, resulted in the production of methane decreased. From statistical analysis, it was found that 70% *Penisetum purpureum* and 30% *Morinda citrifolia* leaves (R3) and 60% *Penisetum purpureum* and 40% *Morinda citrifolia* fruits (R8) gave a tendency of reducing emissions of methane gas in the rumen. It can be caused by dosage or concentration of tannins and saponins additives in *Morinda citrifolia* leaves and fruits in the treatments were in an optimal state.

With the declining of protozoa population, methanogens bacteria was declined, due to loss of habitat, resulting in the decreasing of methane production. Wina (2005) reported in her study, saponin extract from *Sapindus rarak* fruit flesh given to fistula sheep significantly reduced the population of protozoa in the long-term experiments [18]. The population of rumen protozoa had direct effect to the production of methane, which means that the production of methane was reduced when the protozoa in the rumen decreased [20]. Similar result occurred in population of methanogenic bacteria in the treatments were able to reduce emissions of methane gas. When both combined, the population of methanogens as metanogenesis agent and protozoa population as a supplier of hydrogen was eliminated simultaneously

which then lower the methane emissions from rumen. Effect of the declining protozoa population was attributed to a mechanism protozoa disorders as a result of the bond between the saponin with membrane sterols found in protozoa. This mechanism did not occur in bacteria because bacteria did not have in their membrane sterols [19].

4. Conclusion

70% *Pennisetum purpureum* and 30% *Morinda citrifolia* leaves (R3) numerically can reduce methane emissions, so that these feed can reduce emissions of methane production in ruminant livestock. This could be due to the optimal combination of content of tannins and saponins of *Morinda citrifolia*.

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