In vitro rumen fermentation and methane production as affected by rambutan peel powder

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ABSTRACT
The current study was aimed to determine the effect of rambutan peel powder (RPP) supplementation on in vitro gas production, rumen fermentation characteristics and methane production. The experimental design was a completely randomized design and the dietary treatments were different levels of RPP supplementation at 0, 4, 8, 12, 16 and 20 mg/0.5 g DM. Under this investigation, RPP supplementation did not affect gas production kinetics and in vitro digestibility (p > 0.05). The concentration of NH3-N decreased linearly with the increasing levels of RPP supplementation (p < 0.05). Propionate was increased (p < 0.05) when supplemented with RPP at 16 mg, while acetate and butyrate remained the same. On the other hand, supplementation of RPP decreased methane production (p < 0.05). This study indicated that RPP at 16 mg could be used as a rumen enhancer for manipulating rumen fermentation.

1. Introduction
Methane is one of the greenhouse gases (GHG), which is normally produced during the anaerobic enteric fermentation of feeds in many animals, especially ruminants (Patra and Saxena 2010). Methane produced in ruminants represents a substantial loss of 2–12% of gross energy intake (Johnson and Johnson 1995). Hence, decreasing methane production from ruminants is desirable for decreasing the GHG emission with improved efficiency of energy utilization. Antibiotics are used in ruminants as feed additives, to improve rumen fermentation and reduce methane production in the rumen. However, the use of antibiotics as feed additives in animals has been of increasing concern due to the potential appearance of residues in meat and milk production (Gunun et al. 2016). This trend is likely to be continued due to the growing demands for organic animal products. Recently, plant secondary metabolites have been an important area of research to substitute chemical feed additives (Patra 2012). Tannins, saponins and essential oils are a group of plant secondary compounds that have shown the possibility to modify rumen ecology, fermentation, reduce methane production and enhance animal production (Wallace et al. 2002).

Rambutan (Nephelium lappaceum L.) is one variety of the attractive tropical fruits in Southeast Asia (Thitilertdecha and Rakariyatham 2011). There are products related to rambutan such as jams, marmalade, spread, jelly and canned food (Ahmad and Alrozi 2011). After being processed, the residues consist of seeds and peels (Sun et al. 2012). Rambutan peel contains high amounts of under utilized condensed tannins and saponins (Fila et al. 2012). In a previous study, supplementation with rambutan peel had shown potential to manipulate rumen fermentation by depressing protozoa and reducing methane production in vitro (Aditya 2011). Therefore, rambutan peel has potential for use as a feed additive that can reduce the formation of methane production and improve rumen fermentation in ruminants. However, there are still a limitation of data on the use of rambutan peel as feed additives on volatile fatty acid (VFA), gas production kinetics and digestibility. Therefore, the objective of the current experiment was to investigate the effect of rambutan peel powder (RPP) supplementation on rumen fermentation and methane production by using the in vitro gas production technique.

2. Materials and methods
2.1. Experimental design and dietary treatments
The experimental replicates were arranged according to a completely randomized design (CRD) and the dietary treatments were different levels of RPP supplemements at 0, 4, 8, 12, 16 and 20 mg/0.5 g DM. Roughage to concentrate ratio at 60:40 was used as the dietary substrate, and rice straw was used as a roughage source. Rambutan peel was collected as a by-product from rambutan fruits and sundried for 3–4 days. Samples of rambutan peel, roughage and concentrates were
dried at 60°C, then ground to pass a 1-mm sieve (Cyclotech Mill, Tecator, Sweden) and used for chemical analysis and in the in vitro gas study. The samples were analysed for dry matter (DM), ash and crude protein (CP) using the procedures of AOAC (1995), while neutral detergent fibre and acid detergent fibre were determined according to Van Soest et al. (1991). The content of condensed tannins in RPP was analysed by using the modified vanillin–HCl method based on Burns (1971). Crude saponin was measured using methanol extraction following the method of Kwon et al. (2003) and modified by Poungchompu et al. (2009). The ingredients and chemical compositions of concentrate, rice straw and RPP used in this experiment are given in Table 1.

### Table 1. Ingredients and chemical composition of concentrate, rice straw and RPP.

| Ingredient | Concentrate | Rice straw | RPP | Chemical composition |
|------------|-------------|------------|-----|----------------------|
|            | % dry matter|            |     |                      |
| Cassava chip | 64.9        | –          | –   |                      |
| Soybean meal | 12.0        | –          | –   |                      |
| Brewery grain | 9.3         | –          | –   |                      |
| Rice bran   | 6.6         | –          | –   |                      |
| Urea        | 2.2         | –          | –   |                      |
| Molasses    | 3.0         | –          | –   |                      |
| Mineral and vitamin mixture | 1.0 | – | – |                      |
| Salt        | 0.5         | –          | –   |                      |
| Sulfur      | 0.5         | –          | –   |                      |
| Dry matter, % | 95.3        | 91.2       | 88.7|                      |
| Organic matter | 93.3       | 90.5       | 96.6|                      |
| Crude protein | 16.1        | 2.8        | 4.4 |                      |
| Neutral detergent fibre | 19.1       | 68.5       | 31.3|                      |
| Acid detergent fibre | 11.3       | 55.5       | 26.8|                      |
| Ash         | 5.7         | 9.5        | 3.4 |                      |
| Condensed tannins | –     | –          | 11.0|                      |
| Saponins    | –           | –          | 10.3|                      |

*a* Rambutan peel powder.

2.2. Animals and preparation of rumen inoculum

Two male, rumen-fistulated crossbred (Brahman × native) beef cattle with body weight (BW) of 400 ± 40.2 kg were used as rumen fluid donors. Animals were fed with concentrate (14% CP and 78% total digestible nutrient) at 0.5% of BW twice per day at 07:00 h and 16:00 h and rice straw was fed on ad libitum basis. The animals were kept in individual pens and clean fresh water and mineral blocks were offered as free choice. The animals received the diets for 14 days before the rumen fluid was collected. On day 15, 1000 mL of rumen fluid was obtained from each of steers before the morning feeding. The rumen fluid was filtered through four layers of cheesecloth into pre-warmed thermos flasks and then transported to the laboratory.

2.3. In vitro fermentation of substrates

Samples of 0.5 g of roughage to concentrate ratio (60:40) were weighed into 50 mL serum bottles and supplemented with RPP at 0, 4, 8, 12, 16 and 20 mg. For each treatment, four replications were prepared (four serum bottles per each RPP level) and there were 24 sample bottles plus 4 blanks in total 28 bottles were incubated at 10 various incubation times. Ruminal fluid from each animal was mixed with the artificial saliva solution according to Menke and Steingass (1988) in a proportion of 2:1 (mL/mL) at 39°C under continuous flushing with CO2. Forty millilitres of rumen inocula mixture were added into each bottle under CO2 flushing. Bottles were sealed with rubber stoppers and aluminium caps and incubated at 39°C (72 h) for the in vitro gas test. Thirty minutes after starting the incubation, the bottles were gently shaken and then shaken every one hour. For each sampling time, four bottles containing only the rumen inoculums were included within each run and the mean gas production values of these bottles were used as blanks. The blank values were subtracted from each measured value to give the net gas production. The 48 bottles (4 bottles per treatment × 6 treatments × 2 sampling times: 12 and 24 h incubation) were separately prepared for NH3-N, VFA and methane analyses. An in vitro true DM digestibility (IVTDMD) analysis was prepared with another set of 24 bottles (4 bottles per treatment × 6 treatments × 1 sampling times: 48 h incubation).

2.4. Sample and analysis

During the incubation, the gas production was recorded at 0, 3, 6, 9, 12, 18, 24, 36, 48 and 72 h. Cumulative gas production data were fitted to the model of Ørskov and McDonald (1979) as follows:

\[ y = a + b \left( 1 - e^{-ct} \right), \]

where \( a \) is the gas production from the immediately soluble fraction, \( b \) the gas production from the insoluble fraction, \( c \) the gas production rate constant for the insoluble fraction (b), \( t \) the incubation time, \((a + b)\) the potential extent of gas production and \( y \) the gas produced at time ‘\( t \)’.

Inoculum’s ruminal fluid was collected at 12 and 24 h post inoculations. Rumen fluid samples were then filtered through four layers of cheesecloth. Samples were centrifuged at 16,000 × g for 15 min, and the supernatant was stored at −20°C for NH3-N analysis using the micro-Kjeldahl method (AOAC 1995) and VFA analysis using high-performance liquid chromatography (Samuel et al. 1997). At 12 and 24 h post-incubation, 10 mL of the gas was withdrawn from the head space of the incubated serum bottle using a leak-proof syringe and analysed for methane production using gas chromatography (Shimadzu gas chromatograph GC-14B) (Kumar et al. 2012).

In vitro degradability was determined after termination of incubation at 72 h, when the contents were filtered through pre-weighed Gooch crucibles (40 mm of porosity) and residual DM was estimated. The per cent loss in weight was determined and presented as in vitro dry matter degradability (IVDMD). The dried feed sample and residue left above were ashed at 550°C for the determination of in vitro organic matter degradability (IVOMD) ( Tilley and Terry 1963). At 48 h post inoculation, a set of sample was determined IVTDMD according to Van Soest and Robertson (1985).
2.5. Statistical analysis

All data were analysed as a CRD using the GLM procedure of SAS (1996). Data were analysed using the model:

\[ Y_{ij} = \mu + M_i + e_{ij} \]

where \( Y_{ij} \) is observation; \( \mu \) is the overall mean, \( M_i \) is the effect of the different RPP levels (\( i = 1–6 \)) and \( e_{ij} \) is the residual effect. Results are presented as mean values with the standard error of the means. Differences between treatment means were determined by Tukey’s test and differences among means with \( p < .05 \) were accepted as representing statistically significant differences. Trends of RPP level responses were performed by orthogonal polynomials.

3. Results

3.1. Gas kinetics, cumulative gas production and in vitro digestibility

Gas kinetics and cumulative gas production at 72 h were not significantly different among treatments (\( p > .05 \)) (Table 2). In addition, the IVDMD, IVOMD and IVTDMD were not affected (\( p > .05 \)) by RPP supplementation.

3.2. In vitro fermentation and methane production

Ruminal pH and NH\(_3\)-N concentrations decreased linearly (\( p < .05 \)) with the increasing level of RPP supplementation (Table 3). On the other hand, the total VFA concentration, acetate (C2), butyrate (C4) and C2:C3 were similar among treatments (\( p > .05 \)), while propionate (C3) molar proportions quadratically increased (\( p < .05 \)) in treatments supplemented with RPP at 16 mg. Interestingly, ruminal methane production was quadratically decreased (\( p < .05 \)) when supplementation with RPP at 16 mg.

4. Discussion

4.1. Gas kinetics, cumulative gas production and in vitro digestibility

Cumulative gas production (72 h incubation) was not affected by supplementation of RPP at 4–20 mg. The above results suggested that condensed tannins from rambutan peel have a weaker inhibition effect on the fermentation rate. These results were in agreement with Anantasook and Wanapat (2012), who reported that supplementation of rain tree pod meal containing condensed tannins and saponins at 4, 8 and 12 mg/0.2 g DM had no effect on cumulative gas production. In contrast, Paengkoum et al. (2015) reported that cumulative gas production was decreased by supplementation of mango-teen peel condensed tannins at 2–6% DM. Vieira and Borba (2011) suggested that supplementation of quebracho tannins at 2.5% and 5% DM suppressed cumulative gas production. The difference in the effect of condensed tannins on cumulative gas production and rumen fermentation characteristics could be attributed to the substrate used in the incubation. The extent of tannins effects on fermentation is a function of dose, type of tannins, molecular weights and substrate used (Getachew et al. 2008). The present results indicate that used whole rambutan peel containing condensed tannins, rather than pure CT as the effect on cumulative gas production.

Measurement of IVDMD and IVOMD has been widely used to assess the nutritional quality of feeds, due to its high correlation with in vivo digestibility (Getachew et al. 2004). Condensed tannins have been implicated for their inhibitory effect on feed digestion and microbial population in many experiments (Patra et al. 2006). Getachew et al. (2008) reported that the addition of quebracho tannins at 100 g/kg DM reduced IVDMD by 17%. However, supplementation of RPP had no effect on IVDM, and IVOMD was found in the present study. This suggests that the levels of RPP were insufficient to adversely affect the majority of the rumen microbial population. Several ruminal microorganisms have been identified that can tolerate relatively high concentrations of condensed tannins (Hervas et al. 2003).

4.2. In vitro rumen fermentation and methane production

Excessive N supply, a release of ruminal NH\(_3\) that often exceeds its rate of incorporation into microbial protein, resulted in loss of a great part N as NH\(_3\) absorbed from the rumen (Cherdthong et al. 2014). The concentration of NH\(_3\)-N in the rumen fluid is the net result of NH\(_3\)-N production from the feed, fermentation of protein, absorption through the rumen wall and passage out of the rumen and utilization by microbes (Gunun et al. 2016). Ruminal NH\(_3\)-N concentrations were lower when supplemented with RPP at 4–12 mg when compared with the control. The reduction in NH\(_3\)-N concentration in the present experiment could be due to the result of condensed tannin in RPP. Condensed tannins have beneficial effects by forming protein–tannin complex, decreasing availability of feed protein for ruminal degradation and ammonia nitrogen release (Makkar 2003). However, ruminal NH\(_3\)-N concentrations were not affected by RPP supplementation at 16 and 20 mg in the current study. Similarly, Anantasook et al. (2016) reported that NH\(_3\)-N concentration was the lowest when supplemented with Terminalia chebula Retz. containing tannins and saponins at 12 mg/0.5 g DM, while the highest was in the 20 mg/0.5 g DM supplementation group.

Propionate molar proportion was the highest in treatment with RPP supplementation at 16 mg. Moreover, the increase in the proportion of propionate by RPP supplementation led to the reduction of methane production in this study as propionate serves as an alternative sink to methane (Moss et al. 2000). Some natural plants and fruits rich in tannins and saponins have been shown to selectively modulate the rumen microbial populations (Wallace 2004) resulting in an improvement of rumen fermentation, especially propionate and a decrease in methane production (Kamra et al. 2008). In addition, methanogens and protozoa populations were decreased when supplementation of plants rich in tannins and saponins, resulting in enhancing VFA production, especially propionate (Pilajun and Wanapat 2013; Anantasook et al. 2015), which was also reflected in methane inhibition. Anantasook et al. (2016) reported that propionate was increased, while protozoal population and methane production were decreased by supplementation of T. chebula Retz. containing tannins and saponins at
12–20 mg/0.5 g DM. However, the highest level (20 mg) of RPP supplementation did not affect propionate proportions in the current study. Low propionate proportions at high doses of RPP were a result of interaction between saponins and tannins present in it. Tannins in RPP at high doses inhibited bacteria responsible for propionate production, while tannins in low doses of RPP did not inhibit propionate-producing bacteria. But saponins in RPP inhibited protozoa with increasing doses of RPP causing the changes of propionate proportions. A meta-analyses study showed that saponins increased propionate concentrations with increasing doses, but tannins did not influence propionate concentration (Patra 2010).

There are reports indicating a decrease in methane production with the supplementation of plant containing condensed tannins and saponins (Poungchompu et al. 2009; Wanapat et al. 2014; Anantasook et al. 2016; Gunun et al. 2016). Supplementation of RPP at 16 mg reduced methane production in the present study. The results from the present study were similar to those of Denek et al. (2017), who reported that the addition of condensed tannins in dried pistachio by-product at 8% could decrease methane production by 30% in vitro. Aditya (2011) reported that protozoal population and methane production were reduced by the addition of saponins in rambutan peel at 0.2–0.6%. Patra and Saxena (2010) indicated that saponins decrease methanogenesis indirectly via inhibition of protozoa. Sterol-binding capability of saponins has been implicated in the destruction of protozoal cell membranes. Moreover, condensed tannins suppress methanogenesis by decreasing the methanogenic populations in the rumen either directly or by decreasing the protozoal populations, thereby decreasing methanogens symbiotically associated with the protozoal population (Cieslak et al. 2013). Protozoa provide hydrogen as a substrate for methanogenesis carried out by methanogens (Morgavi et al. 2010). A decrease in methane production by tannins and saponins in Samanea saman could be mediated through a direct effect on protozoa and methanogens (Anantasook et al. 2015). Poungchompu et al. (2009) reported that supplementation of mangosteen peel and soapberry fruit pellet containing tannins and saponins at 4% of DM intake resulted in a significant decrease in methane production and protozoa population, but without affecting the methanogens in dairy heifers. In the current study, the methane-inhibiting effects of condensed tannins and saponins in RPP were presumably a direct action against rumen microbes involved in methane formation including methanogens and protozoa. Further work is needed to study the effect of RPP 

### Table 2. Effect of RPP supplementation on gas kinetics, cumulative gas production (72 h) and in vitro digestibility.

| RPP (mg) | Gas kinetics<sup>a</sup> | Degradability<sup>b</sup> (%) | IVTDMD<sup>c</sup> (%) |
|----------|--------------------------|-----------------------------|----------------------|
|          | a | b | c | a + b | Gas (72 h)(ml/0.5 g DM substrate) | IVDMD | IVOMD |
| 0        | 8.3 | 94.7 | 0.05 | 103 | 105 | 62.1 | 69.6 | 70.5 |
| 4        | 8.9 | 95.6 | 0.05 | 105 | 106 | 64.7 | 71.8 | 71.2 |
| 8        | 8.0 | 99.4 | 0.05 | 107 | 108 | 67.1 | 73.6 | 70.4 |
| 12       | 7.7 | 99.5 | 0.05 | 103 | 105 | 65.3 | 72.1 | 67.8 |
| 16       | 8.4 | 99.9 | 0.05 | 108 | 109 | 65.3 | 72.0 | 68.2 |
| 20       | 7.5 | 101.6 | 0.05 | 109 | 111 | 64.7 | 71.2 | 69.8 |
| SEM      | 0.5 | 1.94 | 0.002 | 1.92 | - | 0.98 | 1.46 | 1.02 |

Orthogonal polynomials
- Linear
- Quadratic
- Cubic

Notes: ns: non-significant.
<sup>a</sup>a = the gas production from the immediately soluble fraction, b = the gas production from the insoluble fraction, c = the gas production rate constant for the insoluble fraction (b), (a + b) = the potential extent of gas production.
<sup>b</sup>IVDMD: in vitro dry matter degradability, IVOMD: in vitro organic matter degradability.
<sup>c</sup>IVTDMD: in vitro true dry matter digestibility.

### Table 3. Effect of RPP supplementation on rumen fermentation and methane production (CH₄).

| RPP (mg) | pH | NH₃-N (mg/dl) | Total VFA (mmol/l) | C₂ (%) | C₃ (%) | C₄ (%) | C₂:C₃ | CH₄ (mL/0.5 mg DM) | CH₄ (mL/0.5 g IVDOM<sup>b</sup>) |
|----------|----|--------------|--------------------|--------|--------|--------|-------|-------------------|-------------------------------|
| 0        | 6.6<sup>a</sup> | 29.0<sup>a</sup> | 16.5 | 68.5 | 23.5<sup>a</sup> | 8.1 | 2.9 | 4.1<sup>a</sup> | 5.8<sup>a</sup> |
| 4        | 6.6<sup>a</sup> | 20.8<sup>b</sup> | 14.5 | 65.5 | 25.1<sup>a</sup> | 9.5 | 2.6 | 4.0<sup>a</sup> | 5.2<sup>ab</sup> |
| 8        | 6.6<sup>a</sup> | 19.8<sup>b</sup> | 15.5 | 66.6 | 24.3<sup>a</sup> | 10.1 | 2.7 | 3.6<sup>a</sup> | 4.6<sup>ab</sup> |
| 12       | 6.5<sup>a</sup> | 20.3<sup>b</sup> | 19.7 | 67.9 | 23.5<sup>a</sup> | 8.7 | 2.9 | 3.3<sup>ab</sup> | 4.5<sup>ab</sup> |
| 16       | 6.4<sup>a</sup> | 22.9<sup>ab</sup> | 15.5 | 64.8 | 27.0<sup>a</sup> | 8.3 | 2.4 | 2.8<sup>b</sup> | 3.7<sup>ab</sup> |
| 20       | 6.5<sup>a</sup> | 27.1<sup>b</sup> | 14.3 | 67.8 | 23.9<sup>a</sup> | 8.3 | 2.8 | 3.6<sup>a</sup> | 4.6<sup>ab</sup> |
| SEM      | 0.01 | 2.63 | 2.18 | 1.68 | 0.42 | 1.10 | 0.32 | 0.15 | 0.18 |

Orthogonal polynomials
- Linear
- Quadratic
- Cubic

Notes: ns: non-significant.
<sup>*p < .05.</sup>
<sup>**p < .01.</sup>
<sup>aC₂ = acetate, C₃ = propionate, C₄ = butyrate.</sup>
<sup>bIVDOM = in vitro degrade organic matter.</sup>
<sup>a,bValue on the same column with different superscripts differed (p < .05).</sup>
on protozoa and methanogens to clarify the mechanism with which the methane production is inhibited by RPP.

5. Conclusions

The current study indicated that supplementation of RPP at 16 mg could improve the in vitro rumen fermentation and decrease methane production without an adverse effect on gas production kinetics and digestibility. However, further work is recommended on the use of RPP in in vivo studies.

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