Rumen metabolic activities of cacao (Theobroma cacao L.) pod husk fermented with lingzhi mushroom (Ganoderma lucidum) at different concentration and incubation time

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Abstract. Cacao pod husk is a potential tropical resources that is widely utilized as animal feed. The presented study aimed to investigate rumen metabolic activities of (Theobroma cacao L.) pod husk fermented with lingzhi mushroom (Ganoderma lucidum) at different concentration and incubation time. A completely randomized factorial was applied in this study consisting of two factors; lingzhi mushroom concentration (K1 = 7.5% ; K2 = 15%) and incubation time (L1 = 15 d; L2 =30 d ; L3 = 45 d) with 3 replicates. All samples of cacao pod husk were analyzed to determine NH₃ concentration, volatile fatty acid (VFA) concentration and gas production. Gas production was periodically collected at 2, 4, 6, 8, 10, 12, 24, 48 and 72 h after incubation. The result of study indicated that there was significant interaction (P<0.01) between the concentration of G.lucidum and incubation time on NH₃ concentration of rumen liquid. The highest concentration of NH₃ was found at 7.5% G.lucidum concentration for 45 d incubation (14.70mM). G.lucidum concentration significantly affected (P<0.01) VFA concentration in which samples inoculated with the level of 7.5% G.lucidum was higher compared to that of 15% G.lucidum (125.16 mM vs 113.94 mM). This study concluded that cacao pod husk fermented with lingzhi mushroom at different concentration time influencing metabolic activities of the rumen.

1. Introduction
In the last few decades, crop residues from agricultural-industrial by-products such as rice straw, bagasse, coffee pulp, cacao pod husk produced in large quantities due to expansion of agro-industrial activities. These crop residues cannot be used optimally as animal feed because of low nutritive values. However, with microbial conversion, agriculture by-products seems to be promising alternative feed for animal production by transforming low quality feed to a high value-added of animal feed. Utilisation of crop residues from agricultural, agro-industrial and plantation by-products as animal feed has been reported [1, 2, 3]. Cacao pod husk is one of the agricultural by-products produced from cacao plantation and cacao processing factories and it contains high fibre such as lignocellulose and high anti-nutritional factors such as theobromine. In addition, cacao pod husk has detrimental effects on animal health due to high theobromine concentration.
With the amount of ca. 70-75% from the whole fruit, cacao pod husk is also beneficial as an alternative source for animal feed due to its rich mineral and antioxidant contents such as potassium and phenolic acid [4]. In addition, cacao pod husk contains fibre compounds such as cellulose, hemicellulose, lignin and pectin that can be utilized to feed animals by biotechnology application. Laconi and Jayanegara [5] reported that cacao pod husk contained high level of organic matter (87.1%) and other compounds such as crude protein (8.4%), crude fibre (55.7%), ether extract (2.5%) and nitrogen free extract (20.6%). The fraction of fibre for cacao pod husk was 41.1%, 52.2% and 10.8 for ADF, NDF and hemicellulose [6]. Its metabolizable energy content was 4.2 MJ/kg DM [6]. Due to high concentration of crude fibre in cacao pod husk, pre-treatment approaches such as physical, chemical and biological treatment are required to obtain desirable fractions for animal feed. Biotechnological innovation such as fermentation is one of treatments applied to improve nutritive values from low quality feed such as cacao pod husk. Fermentation by application of various microorganisms in the crop residues was able to decompose lignocellulosic materials produced from agro-industrial by-products to be a value-added product. The bond of indigestible lignin was broken down to be bioavailability compounds for animal feed with higher digestibility and digestibility. *Ganoderma lucidum* (lingzhi mushroom) is one of white rot fungi that can be applied in the fermentation process to improve the nutritional value of low quality feed.

*G. lucidum*, known as medical mushroom, has been used in traditional oriental medicine in China for years. *G. lucidum* contained pharmacological active constituents such as triterpenoid and polysaccharides (especially β-d-glucans) functioned as anti-hypertensive, anti-tumor, anti-angiogenic (platelet aggregation) and reduce cell from damage due to mutagens [7]. Besides its function as a medical purpose, *G. lucidum* has been also used for the fermentation process to improve the quality of animal feed due to its ability to degrade lignin and cellulose [8]. As aforementioned above, cacao pod husk contains high concentration of fibre and lignin and seems promising to be applied as inoculant for fermentation of cacao pod husk. Several studies regarding the application of biological treatment in low quality feed have been reported [9, 5]. However, the data of *G. licidum* as inoculant for animal feed fermentation was limited. Therefore, more studies in this area were needed. The purpose of this study is to investigate rumen metabolic activities of (*Theobroma cacao* L.) pod husks fermented with lingzhi mushroom (*G. lucidum*) at different concentration and incubation time.

2. Materials and Methods
2.1. Experimental Materials and Treatment
Cocoa pod husks for this experiment were obtained from farmers in Seulawah district, Aceh Besar Regency. Before further process, fresh cacao pod husk was dried and ground with the size of 0.5 x 0.5 cm. *G. Lucidum* inoculum was bought from the producer of commercial mushroom located in Sumedang district, West Java and molasses for this study was purchased from the experimental farm, Animal Husbandry Department, the Faculty of Agriculture, Universitas Syiah Kuala. Rumen content required for *in vitro* digestibility in this study was obtained from a fistulated dairy cow raised at Animal Science Faculty, Bogor Agricultural University.

In the fermentation process, firstly the water content of dried cacao pod husk was adjusted to reach 60% by adding the water. Then, molasses was added in the samples with the amount of 3%. The polyethylene bags were provided to keep the samples for the fermentation process. Prior to fermentation to protect samples from other microorganism’s contamination, all samples were steamed at the temperature of 125°C for 1.5 h. Inoculation of the steamed samples with *G. lucidum* was conducted directly after the samples were cold. Each treatment was inoculated with 7% and 15% of *G. licidum* and kept for the duration of 0, 15, 30 and 45 d depending on treatments in aerobic condition. All samples was incubated at room temperature with the humidity of 75-80%.
2.2. Chemical analysis and in vitro procedure
The production of volatile fatty acid (VFA) and gas production were determined by Theodorou et al. [10] procedures. Mixtures of buffered rumen fluid were used for in vitro analysis. Rumen fluid was collected from a rumen fistulated FH cow housed at the Faculty of Animal Science, IPB. To filter rumen fluid, four layers of gauze were used prior to use. 125 ml serum bottles were provided and poured with 0.75 g samples. Then it was added with 75 ml of buffered rumen fluid. The ratio between fluid and buffer was (1:4 v/v). All serum bottles were sealed with butyl rubber stoppers and aluminium crimp prior to fermentation process. During the incubation, the water bath was maintained stable with a temperature of 39°C for 48 h. Gas production was periodically collected by using a syringe at the duration of 2, 4, 6, 8, 12, 24, 36, 48 and 72 h after incubation. After 72 h incubation, serum bottles were centrifuged to separate between supernatant and residue. Total amount of VFA and NH₃ concentration was determined from supernatant collected after 24 h incubation as described by Jayanegara et al. [11].

2.3. Statistical Analysis
The data were statistically analysed as completely randomized factorial design (CRFD) using GLM procedure of SPSS. Two factors applied in this study including concentration of G. lucidum (7.5% vs. 15%), incubation period (15, 30 and 45 d). Parameters measured in this study were gas production, NH₃ and VFA concentration. The following is the mathematical model applied in this study:

\[ Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk} \]

Where Yijk is the value of each observation, µ is the overall general mean, \( \alpha_i \) is the effect of A treatment level i, \( \beta_j \) is the effect of B treatment level j, \( \alpha\beta_{ij} \) is the interaction of A treatment level i and B treatment level j and \( \varepsilon_{ijk} \) is residual error to each observation. Results were expressed as mean ± SEM. The values were declared significantly different at P<0.05. The differences between treatments are determined by using the Duncan Multiple Range Test [12].

3. Results and discussion
3.1. Gas Production
Gas production was strongly correlated with losing rate of organic matter in vitro digestibility. High gas production during the fermentation process can be used as an indicator the optimal fermentation process in the rumen system [13], mainly quantitative cellulose rate digestibility as informed by Schofield et al. [14]. Gas production (ml g/DM) of (Theobroma cacao L.) pod husk fermented with lingzhi mushroom (Ganoderma lucidum) at different concentration and incubation time is presented in Figure 1.

The result of the study indicated that there was interaction (P<0.01) between inoculum concentration and incubation time on the gas total production after 72 h in vitro fermentation (Figure 2). Increasing inoculum concentration from 7.5% to 15% significantly improved total gas production (P<0.01) about 8.50% (72.63% vs 78.80 ml/g DM). Improvement of gas production was probably due to reducing of lignin content as reported by Syahrir et al. [15] in which reducing lignin content was as result of bioconversion process providing more spaces for fermentation process of microbes. In the study, they fermented cacao pod husk with Phanarochaete chrysosphorium and Pleurotus ostreatus for 96 h. In our study at 7.5% inoculant concentration, prolonged incubation time linearly increased gas production, it was assumed that during the bioconversion process of cacao pod husk, sufficient soluble nutrition was available for inoculum to grow and to degrade fibers.
Figure 1. Gas production (ml g/DM) of (Theobroma cacao L.) pod husk fermented with lingzhi mushroom (Ganoderma lucidum) at different concentration and incubation time (n=3 replicates). K1L1 (7.5% concentration of G. lucidum and 15 d of incubation time); K1L2 (7.5% concentration of G. lucidum and 30 d of incubation time); K1L3 (7.5% concentration of G. lucidum and 45 d of incubation time); K2L1 (15% concentration of G. lucidum and 15 d of incubation time); K2L2 (15% concentration of G. lucidum and 30 d of incubation time); K2L3 (15% concentration of G. lucidum and 45 d of incubation time).

At 15% of inoculum concentration, the increase of total gas production can be used as indication of high fermentation activities, mainly the capacity of digested fiber fraction, despite no effect of incubation time on total gas production. It was assumed that soluble nutrition, which was available during the fermentation process, was imbalanced with inoculum concentration. Therefore, at 15 d incubation time, the microbe activities to degrade fibers was not optimal. Agustin et al. [16] also reported that the loss of dry and organic matter in rice straw fermented with G. lucidum relates to the growth of mycelium. In vitro gas production had correlated with feed chemical compositions, especially the crude fiber content and structural polysaccharides [17]. In addition, macro-fungi produced some metabolite bioactive which is integral parts from fungi cell walls and had antimicrobial functions [18].
3.2. NH$_3$ Production

NH$_3$ concentration in the rumen liquid reflected fermentation activities of feed protein and protein synthesis by rumen microbes [19]. NH$_3$ was the result of protein degradation from feed utilized by microbes as a source of protein [20]. NH$_3$ concentration in the rumen liquid incubated with lingzhi mushroom for 24 h is presented in Figure 3.

The results of the study indicated that there was interaction (P<0.01) between inoculum concentration and incubation time on NH$_3$ concentration after 24 h incubation (Figure 4). Incubation time significantly affected (P<0.05) on NH$_3$ concentration. In our study, at 7.5% inoculum concentration linearly increased NH$_3$ concentration along with incubation time. However, at 15% inoculum concentration significantly decreased NH$_3$ concentration at 30 d incubation time, then increased again at 45 d incubation time. The concentration of NH$_3$ in this study was above 8.8 mM which was required by ruminant animals to digest fibres optimally [15].
Figure 3. NH$_3$ concentration (mmol/L) of (Theobroma cacao L.) pod husk fermented with lingzhi mushroom (Ganoderma lucidum) at different concentration and incubation time (n=3 replicates). K1L1 (7.5% concentration of G.lucidum and 15 d of incubation time); K1L2 (7.5% concentration of G.lucidum and 30 d of incubation time); K1L3 (7.5% concentration of G.lucidum and 45 d of incubation time); K2L1 (15% concentration of G.lucidum and 15 d of incubation time); K2L2 (15% concentration of G.lucidum and 30 d of incubation time); K2L3 (15% concentration of G.lucidum and 45 d of incubation time).

Improvement of NH$_3$ concentration at the 7.5% inoculum concentration was supposed from mycelium protein continuously increasing with incubation time treatment. At the treatment of 15 % incubation time, the NH$_3$ concentration reduced at 30 d incubation time, it was assumed due to nutrition limitation, the growth of mycelium was disturbed, and laccase enzyme has not optimally worked yet. After 30 d incubation, enzyme activities to degrade cell wall improved and released monosaccharaides to be used by microorganisms as substrates during metabolism activities [20]. Syahrir et al. [15] reported NH$_3$ concentration in cacao pod husk fermented with Phanarochaete chrysosphorium and Pleurotus ostreatus was higher compared to cacao pod husk without fermentation treatment. Rice straw fermented in vitro with G. lucidum improved NH$_3$ concentration and the peak of NH$_3$ concentration was reached at 10 d fermentation without urea administration (17.88 mg/100 ml) and 20 d with urea administration (19.08 mg/100 ml) [20]. In the rumen system, the production of NH$_3$ depended on the content and soluble protein in feed, retention in the rumen and rumen pH [21].
3.3. VFA production
Fermentation in the rumen system produced VFA as main product to supply energy and carbon for growth and maintenance of microbe communities. The number of VFA production was strongly influenced digestibility and quality of fermented feed [22]. VFA concentration (mM/l) of (Theobroma cacao L.) pod husk fermented with lingzhi mushroom (Ganoderma lucidum) at different concentration and incubation time was presented in the Figure 5.

The result of the study indicated that there was interaction (P>0.05) between inoculum concentration and incubation time on the VFA total after 24 h incubation (Figure 6). Increase of inoculum concentration from 7.5% to 15% significantly reduced (P<0.01) VFA total about 8.96% (125.16 vs 113.94 mmol/L). Even though, statistically no interaction, concentration of VFA total tended to increase along with incubation time at the 7.5% inoculum concentration. However, at the 15% inoculum concentration VFA total reduced at 30 d incubation time and increased again at 45 d incubation. Interestingly, the pattern of VFA total was similar to the pattern of NH₃ concentration. NH₃, produced in the rumen, was utilized by rumen microbes mainly cellulolytic bacteria as N source for microbial protein synthesis.
Figure 5. VFA concentration (mM/l) of *(Theobroma cacao L.)* pod husk fermented with lingzhi mushroom *(Ganoderma lucidum)* at different concentration and incubation time (n=3 replicates). K1L1 (7.5% concentration of *G.lucidum* and 15 d of incubation time); K1L2 (7.5% concentration of *G.lucidum* and 30 d of incubation time); K1L3 (7.5% concentration of *G.lucidum* and 45 d of incubation time); K2L1 (15% concentration of *G.lucidum* and 15 d of incubation time); K2L2 (15% concentration of *G.lucidum* and 30 d of incubation time); K2L3 (15% concentration of *G.lucidum* and 45 d of incubation time).

Increase of microbe population resulted in increasing of enzymes produced to digest feed [20]. Activities of rumen microbes effected on rumen metabolic profiles, mainly VFA produced from carbohydrate degradation [23]. *In vitro* study, cacao pod husk fermented with *Phanarochaete chrysosphorium* and *Pleurotus ostreatus* significantly increased VFA total compared to treatments without fermentation [15]. This is because the existence of lignin in the cacao pod husk strengthened cell wall structure and not easy to dissolved and withstand from microbe attacks [24]. High feed digestibility was not always followed by high of fermentation products. It indicated that digested organic feed was probably more used to synthesize microbe biomass rather than fermentation process [15].

Table 1. The VFA concentration (mM/L) of cacao *(Theobroma cacao L.)* pod husk fermented with lingzhi mushroom *(Ganoderma lucidum)* at different concentration and incubation time (n=3 replicates).

| VFA Concentration (mmol/L) | K1L1 | K1L2 | K1L3 | K2L1 | K2L2 | K2L3 |
|---------------------------|------|------|------|------|------|------|
| Acetate                   | 29.30| 26.12| 25.32| 29.44| 30.05| 31.04|
| Propionate                | 8.14 | 7.35 | 5.96 | 7.13 | 8.25 | 8.11 |
| n-Butyrate                | 5.03 | 4.37 | 3.69 | 4.52 | 5.21 | 5.16 |
| Iso-Butyrate              | 1.59 | 0.98 | 0.50 | 0.56 | 1.02 | 1.75 |
| n-Valerate                | 0.61 | 0.63 | 0.43 | 0.53 | 0.64 | 1.04 |
| Iso-Valerate              | 1.13 | 0.95 | 0.73 | 0.93 | 1.09 | 1.25 |

Note; K1L1 (7.5% concentration of *G.lucidum* and 15 d of incubation time); K1L2 (7.5% concentration of *G.lucidum* and 30 d of incubation time); K1L3 (7.5% concentration of *G.lucidum* and 45 d of incubation time); K2L1 (15% concentration of *G.lucidum* and 15 d of incubation time); K2L2 (15% concentration of *G.lucidum* and 30 d of incubation time); K2L3 (15% concentration of *G.lucidum* and 45 d of incubation time).
In this study, VFA partial was presented in description. The concentration of VFA partial in 15% inoculant concentration produced higher lactic acid compared to 7.5% inoculant concentration meaning that it had more digested crude fiber. Generally, the high amount of acetate with the low number of propionate produced high concentration of methane due to high hydrogen production. Kuivand and Kafilzadeh [25] found out that there was positive correlation between crude fiber fractions from different kinds of grass with the concentration of methane production. The high amount of methane production indicated the energy losing and as the result, the production of VFA reduced. It can be explained why the gas production total was higher at the 15% inoculant concentration, followed by the high concentration of NH$_3$ and VFA total.

4. Conclusions
In conclusion, cacao pod husk fermented with lingzhi mushroom at different concentration time influencing metabolic activities of the rumen. There was significantly interaction (P<0.01) between the concentration of G.lucidum and incubation time on NH$_3$ concentration of rumen liquid. The highest concentration of NH$_3$ was found at 7.5% G.lucidum concentration for 45 d incubation (14.70 mM). G.lucidum concentration significantly affected (P<0.01) VFA concentration in which samples inoculated with the level of 7.5% G.lucidum was higher compared to that of 15% G.lucidum (125.16 mM vs 113.94 mM).

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