Evaluation of nutritive values and digestibility’s 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 has been widely utilized as animal feed. The purpose of this study is to evaluate nutritive values and digestibility of fermented cacao pod husk with different concentration and incubation time. A completely randomized factorial design consisting two factors; lingzhi mushroom concentration (K₁ = 7.5% ; K₂ = 15%) and incubation time (L₁ = 15 d; L₂ =30 d ; L₃ = 45 d) was employed in this study (n=3 replicates). Proximate analysis was performed to determine nutritive values of fermented cacao pod husk. Fermented samples were then subjected to an in vitro digestibility with rumen fluid and McDougall’s buffer mixture. The results of study reveal that cacao pod husk fermented with the concentration of 15% at 15d and 30 d significantly increased (P<0.05) crude protein content but not for other parameters. Dry and organic matter digestibility of cacao pod husk fermented with 7.5% of lingzhi mushroom at 45 d significantly improved (P<0.05) in vitro dry and organic matter of treatment. In conclusion, cacao pod husk fermented with different concentration and incubation time was able to improve the nutritive values and in vitro digestibility of cacao pod husk.

1. Introduction
The main constrain to satisfy nutritional requirement of ruminant animals for production and reproduction is the shortage of animal feeds such grass and legumes. Crop residues from agricultural, agro-industrial and plantation by-products play an important role in providing an alternative feedstuff for ruminant animals [1–3]. Cacao pod husk from cacao plantation by-product (up to 76%) is one of alternative feedstuffs for ruminant animals due to its abundant availability and not expensive. Utilisation of cacao pod husk as animal feed has been widely reported by many scientists [4,5].

Cacao pod husk as locally available feed resources contains not only bioactive compounds such as pectin, antioxidant compounds and theobromine [6] but also other nutritional contents such as crude protein, fibre, ash and NFE. Laconi and Jayanegara [4] stated that cacao pod husk contained 87.1%, 8.4%, 55.7%, 2.5% and 20.6% for organic matter, crude protein, crude fibre, ether extract and nitrogen free extract respectively. In addition, cacao contain theobromine with the amount of 6.79 mg /100 g dry weight [7]. Cacao pod husk as by-product either from cacao plantation or from cacao industries has low nutritional quality indicated by high fibre and low protein content. Kim et al.[8] stated that feedstuffs with low nutritional quality should be treated physically, chemically or biologically before
being fed to animals. Fermentation is biological processes involving microorganisms to improve nutritional values and functional properties of low quality feed. Many studies have been reported that fermentation was able to improve bioavailability of nutrients [9] and decreased the concentration of anti-nutritional factors in the origin products [10].

*Ganoderma lucidum* (lingzhi mushroom) is recognised as traditionally medical mushroom with heath-stimulating properties in China. Lingzhi mushroom contains abundant antioxidants for pharmaceutical purpose. Parumul and Kalaichelvan [11] reported that *G. lucidum* was able to degrade lignin (delignification) and cellulose using the extracellular enzyme such as lignin peroxidase, laccase and cellulose. Cacao pod husk contained high concentration of fibre and lignin, which has low digestibility for animal. Inoculation of *G. lucidum* in agricultural by-products as animal feed assumed improve the nutritional quality of feed and digestibility. Misra et al. [12] reported that the straw fermented with *G. lucidum* improving protein content and *in vitro* digestibility and reduced lignin content. Information relating to *G. lucidum* as source of fermentation material for animal feed is very limited. Therefore, it is required to investigate the effect of *G. lucidum* as fermentation material by using cacao pod husk as animal feed. The purpose of this study is to evaluate nutritive values and digestibility of cacao pod husk fermented with the lingzhi mushroom at different concentration and incubation time.

2. Materials and methods

2.1. Experimental materials and treatment

Fresh cocoa pod husk in this study was collected from farmers in Seulawah district, Aceh Besar Regency. After collection, all sample materials were dried and ground in the crumble form. *G. Lucidum* inoculum was purchased from commercial mushroom producer in Sumedang district, West Java and molasses was obtained from experimental farm, animal husbandry department, the Faculty of Agriculture, Universitas Syiah Kuala. Rumen content for *in vitro* digestibility was collected from a fistulated dairy cow raised at Animal Science Faculty, Bogor Agricultural University.

Prior to fermentation, dried cacao pod husk was mixed thoroughly with water to adjust the moisture content to 60% and added molasses 3% of total samples. Samples were kept in the polyethylene bags for steaming process at the temperature of 125°C for 1.5 h. After cooling, the steamed samples were inoculated with *G. lucidum*. The concentration of *G. lucidum* for each treatment was 7% and 15% then stored in aerobic condition for 15, 30 and 45 d at the room temperature with the humidity around 75-80%.

2.2. Chemical analysis and *in vitro* procedure

Before chemical analysis, the treated cacao pod husk was dried at the temperature of 60°C for 24 h and ground to pass 1 mm sieve with hammer mill. Proximate analysis was performed to determine the chemical composition, i.e. dry matter, crude protein, crude fibre, ether extract and ash by the procedure of AOAC [13]. Nitrogen free extract (NFE) was calculated according to the following equation: NFE=100% - (the content of ash+crude protein+ether extract and crude fibre).

*In vitro* analysis followed the procedure of Tilley and Terry [14] in which each treatment of cacao pod husk was incubated by using McDaugalls’ buffer mixture with rumen fluid. Rumen fluid in this study was collected in the morning before feeding from a rumen fistulated FH cow at at Animal Science Faculty, Bogor Agricultural University. Sample (0.75 g) was weight and poured into 125 ml serum bottles. Subsequently, 75 mL rumen fluid and McDaugalls’ buffer were added in the tubes. Before incubation, the tubes were closed with a rubber cup, then placed in the automatic shaker water bath for anaerobically incubation. Temperature of incubation was maintained stable at 39 °C for 24h. After anaerobically incubation, the caps were opened and added HgCl₂ with the amount of 0.2 mL, followed by centrifugation of the samples for 10 min with the 10,000 rpm. The supernatant was removed and the residue was added 0.2% pepsin (20 mL) with acidic condition and conducted for
another a-24h incubation. The residue from the two-stage in vitro incubation procedure was filtered by a Whatman paper no. 41 to determine in vitro dry matter digestibility (IVDMD) and in vitro organic matter digestibility (IVOMD). The incubation was conducted for three replicates with the representation of two tubes for each treatment.

2.3. Statistical analysis
The statistical model in this study included the effects of concentration of G. lucidum (7.5% vs.15%), incubation period (15, 30 and 45d), and their interactions. All data were statistically analyzed and designed to completely randomized factorial design (CRFD) as follow:

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

(1)

Where, \( Y_{ijk} \) is the value of each observation, \( \mu \) is the overall general mean, \( \alpha_i \) is the effect of A treatment level i, \( \beta_j \) is the effect of B treatment at level j, \( \alpha\beta_{ij} \) is the interaction of A treatment at level i and B treatment level j and \( \epsilon_{ijk} \) is residual error to each observation. The statistical analysis was performed by using SPSS software. Results were expressed as least squares means and standard error of the means (SEM). The statistical significance was declared (P<0.05). The means among treatments were applied by using Duncan Multiple Range Test [15].

3. Results and discussion

3.1. Nutritive values of fermented cacao pod husk
Nutritive values of fermented cacao pod husk by using G. lucidum with different concentration and incubation time is presented in the Table 1. Based on the statistical analysis, there was strong interaction (P<0.01) between isolate concentration and incubation time on crude protein content of substrate. Inoculation of 7.5% G. lucidum in the cacao pod husk increased protein content linearly and the highest of crude protein content was at the 45 d incubation time (8%). Application of 15% G. lucidum in the cacao pod husk only increased up to 30 d incubation time and then decreased after 45 d incubation time. Improvement of protein cacao content from 6.87% to 10.6% also reported by Syahrir et al. [16] using substrate combination of Phanarochaete chrysosporium and Pleurotus ostreatus (8g/kg and 15g/kg). In addition, Han et al. [17] stated that G. lucidum was be able to improve protein content of corn from 11% to 16.5%. Yulistiani et al. [18] reported that solid state fermentation by inoculation of microorganisms in the lingocellulosic material was be able to improve protein content. In our study, decrease of protein content at the 15% G. lucidum concentration at 45 d incubation time was probably due to imbalance of nutritive availability for substrate resulting in the growth of mushroom was hampered [19]. Increase of protein content in solid state fermentation is because yeast utilized carbohydrate from starch hydrolysed to be glucose during degradation process to synthesis mycelium biomass [18]. In addition, Agustin et al. [20] stated that increase of mycelium quantity resulted in improving protein content and when the number of mycelium declined, the protein content reduced as well. Addition of nitrogen in the fermented media with G. lucidum was be able to improve mycelium production as reported by [17].

The ether extract and NFE content significantly reduced (P<0.05) by incubation time. This is because yeast utilized simple carbohydrate as sources of energy and lipid by using lipase enzyme [21]. In accordance with [18] during fermentation process, mushroom used firstly soluble cell fraction for their activities. It was approved by Han et al. [17] in which decrease of corn starch content fermented with G. lucidum from 64.5% to 25.3% and the lipid content reduced from 10.3% to 8.5%. Similar results in rice brand and coffee skin and pulp fermented with Rhizopus oligosporus resulted in reducing NFE content and increasing substrate crude fibre [22].
Table 1. The nutritive values of cacao (*Theobroma cacao* L.) pod husk fermented with lingzhi mushroom (*Ganoderma lucidum*) at different concentration and incubation time (n=3 replicates).

| Nutritive Value (%) | Treatment | P value |
|---------------------|-----------|---------|
|                     | K1L1      | K1L2    | K1L3    | K2L1    | K2L2    | K2L3    |
| DM                  | 55.11±0.95| 54.18±0.41| 54.74±1.49| 54.73±0.49| 53.25±0.99| 52.87±0.89| 0.731   |
| CP                  | 5.73±0.05 | 7.42±0.17 | 8.00±0.13 | 6.35±0.13 | 7.91±0.11 | 6.34±0.24 | 0.000   |
| CF                  | 35.70±2.11| 37.83±1.85| 37.28±1.02| 36.38±0.67| 38.57±0.82| 40.21±0.66| 0.635   |
| EE                  | 0.77±0.13 | 0.50±0.08 | 0.41±0.15 | 1.09±0.26 | 0.43±0.13 | 0.50±0.20 | 0.515   |
| NFE                 | 47.87±2.07| 44.05±1.85| 44.21±1.05| 46.04±0.52| 43.01±0.85| 42.14±1.09| 0.924   |
| Ash                 | 9.93±0.05 | 10.19±0.23| 10.09±0.16| 10.14±0.16| 10.08±0.25| 10.81±0.32| 0.161   |

Means followed by different letters within column are significantly different (P<0.05).

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).

DM=dry matter; CP=crude protein; CF=crude fiber; EE=ether extract; NFE=nitrogen free extract.

The content of dry matter, crude fiber and ash were not significantly difference (P>0.05) amongst treatments. Dry matter content was slightly constant amongst treatments with the percentage of 52-55%. It was assumed that no evaporation process during fermentation in which substrate was inserted in closed polyethylene bag and opened after all substrate were covered by mycelium. [15] also reported that cacao pod husk fermented by combination between *P. chrysosporium* and *P. ostreatus* was still high of dry matter content. High dry matter content was probably due to limited available nutrition and short incubation time. Therefore, degradation enzyme such as ligninolytic enzyme did not optimally work to degrade other fiber compound such as lignin. It is supported by Agustin et al. [20] in which during fermentation process, all easily fermentable carbohydrate sources were totally used, while lignin compound which was difficult to be degraded still in the substrate. The high lignin content in the substrate in this study was since ligninolytic enzyme did not optimally degrade lignin compound. The activity of ligninolytic enzyme was determined the growth of mushroom depending on the availability of nutrition and types of used media. In our study, we did not provide the treatment with slow-release carbohydrate and we supplied energy substrate from molasses to stimulate fermentation process at the beginning of incubation [23]. The fiber content was directly proportional with the ash content since ash was part of cell wall. The high of crude fibre content positively influenced on ash substrate content [24].

3.2. In vitro digestibility of fermented cacao pod husk

*In vitro* dry mater digestibility (IVDMD) and *In vitro* organic matter digestibility (IVOMD) of (*T. cacao* L.) pod husk fermented with lingzhi mushroom (*G. lucidum*) at different concentration and incubation time is presented in the Figure 1. In our in vitro study indicated that there was significantly interaction (P<0.01 ; Figure 2) between concentration of *G. lucidum* and incubation time on IVDMD and IVOMD. The value of IVDMD and IVOMD had similar pattern since the difference between them was only ash content from samples [24]. Inoculation of sample with 7.5% *G. lucidum* linearly increased IVDMD and IVOMD up to 45 d incubation time. On the other hand, inoculation of the samples with 15% *G. lucidum* decreased IVDMD and IVOMD after 30 d incubation time.
Figure 1. In vitro dry matter digestibility (IVDMD) and in vitro organic matter digestibility (IVOMD) 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).

Figure 2. Interaction treatment of In vitro dry matter digestibility (IVDMD) and In vitro organic matter digestibility (IVOMD) 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).
Generally, the value of IVDMD and IVOMD was higher in the 7.5% isolate concentration compared to administration of 15% isolate concentration in the substrate. The high value of in vitro digestibility by administration of 7.5 isolate concentration at 45 d incubation time indicated that wide accessibility of rumen microbes to degrade substrate. It was assumed that at the 7.5% isolate concentration was more availability of protein and energy, hence the ability of microbe to degrade lignin in fermentation process was optimal. Decrease of lignin in cacao pod husk after bioconversion process created the ideal condition for microbial fermentation in the rumen system[25], lignin gave a negative effect on the cell wall digestibility by protecting from hydrolyses enzymes [26]. Improvement of IVDMD and IVOMD values has been reported Syahrir et al. [16] by application of Phanarochaete chrysosporium and Pleurotus ostreatus with the amount of (8g/kg and 15g/kg)

The correct value of pH in the rumen is very important for optimal feed degradation by rumen microbes. For example, with the pH < 6.2 fibre digested slowly and < 5.4 endangered for the live of bacteria rumen and improved lactic acid bacteria resulting in acidosis to animals. Even though the value of IVDMD and IVOMD at the 7.5 % isolate concentration improved, pH value in the rumen liquid was still in normal range for crude fibre fraction digestibility (>6.5, Figure 3). In our study, pH value amongst treatments was in the range of 6.80 – 6.94. Isolate concentration and incubation time were not significantly different (P>0.05) on pH value. Structural carbohydrate was slowly hydrolysed and organic acids produced were lower compared to non-structural carbohydrate, therefore the change of pH value was relatively low [27].

Figure 3. The pH values 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).
4. Conclusions
The results of our study concluded that cacao pod husk fermented with different concentration and incubation time was able to improve the nutritive values and in vitro digestibility of cacao pod husk. Inclusion of G. lucidum 7.5% in substrate at 45 d incubation time assumed to be a good treatment as indicated by improvement of nutritive values, dry and organic matter digestibility. The pH value in our study ranged in normal value for all treatments and they were suitable for bacteria microbes to live and to degrade feed from fibrous materials.

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