The accuracy of several in vitro methods in estimating in vivo digestibility of the tropical dairy ration

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Abstract. In vitro digestibility methods have been developed to overcome problems in the in vivo digestibility measurement, but its accuracy should be tested in a local setting. In vitro methods developed by Tilley and Terry (T2), Theodorou (T3) and Sutardi (T4) have been compared to in vivo method (T1) in a block randomized design study. Four heifers FH (337.50 ± 45.87 kg BW) were used in T1, and two fistulated FH bulls (510 ± 20 kg BW) were used as inoculant sources in the in vitro methods. Dairy cattle ration consisted of 54.0% Napier grass and 46.0% concentrate with 58.8% DM, 12.1% ash, 10.0% CP, 3.3% EE, 26.5% CF, and 61.1% TDN. The observed parameters were ration fermentability (pH, NH3, and VFA concentration) and digestibility (DMD and OMD). The data were analyzed using analysis of variance (ANOVA) followed by the Tukey test. The correlation was made before regression analysis to estimate the in vivo parameters from the in vitro. The results showed that pH values are in the normal range (6.7 – 6.8), and insignificantly different between treatments (P>0.05). The concentration of NH3 and VFA were significantly different between the treatments (P<0.05), but T2 produced similar NH3 and VFA concentrations to T1. Similar results were also found in the DMD and OMD. Correlation analysis showed that pH value of T3 correlated significantly with T1, while DMD value of T4 correlated to T1. The T1 DMD (Y) could be estimated from T4 DMD (X) using formula Y (%) = y = -0.091x² + 9.163x - 168.4. It is concluded that tropical dairy feedstuffs in vitro digestibility using Tilley and Terry's method produced similar result to in vivo digestibility method, but in vivo dry matter digestibility can be estimated accurately by in vitro dry matter digestibility using Sutardi method.

1. Introduction

Feed is an important factor in dairy cattle farming which determines the ability of the cattle to express its genetic potential [1]. Dairy cattle feed quality is not only determined by its chemical composition, but also its utilization by dairy cattle. Information of feeds utilization by dairy cattle such as feed digestibility can improve ration formulation accuracy in predicting dairy cattle performance. Feed digestibility expresses the feeds proportion that remains in the animal body and is not excreted in feces [2]. Digestibility value can be used as an early indication of nutrient available in the feed that can be...
used by the animal. High feed digestibility indicates a high proportion of feeds that can be used by the animal for maintenance and production and releases less excess nutrient to the environment [3]. According to Despal [4], chemical compositions have lower accuracy in estimating in vivo digestibility in comparison to in vitro methods such as Hohenheim gas test [5] or cellulase method [6].

The method to determine digestibility including measurement of feed intake and fecal. This method involves preliminary and collecting periods that may vary between species. The preliminary period in dairy cattle is usually about two weeks while the collecting period is for about a week [7,8,9]. In total, the digestibility of the feedstuff required at least three weeks and three cattle as replication. This method is considered expensive, time and labor-consuming, and needs a lot of samples. Therefore, many in vitro methods [10,11] have been developed to imitate the in vivo direct digestibility measurement using animals.

In vitro methods can be grouped into simple batch methods [10] or continuous culture as used by Despal [4]. These methods are different due to different rumen liquid to solid feed sample ratios, incubation time, and incubator used. Although these methods have high correlation to in vivo, however, these methods were developed in the temperate areas. In vivo digestibility process in temperate dairy cattle was different from tropical dairy cattle due to different feeds used and rumen fermentation conditions. Sutardi [12] attempted to modify Tilley and Terry [10] method by adopting tropical dairy cattle conditions, however, the result had not been compared to in vivo and other in vitro methods. Moreover, the different in vitro methods used in the study of tropical dairy ration fermentatability and digestibility might lead to different results and therefore needed to be justified. This study is aimed to compare the results of several in vitro digestibility methods to in vivo digestibility method, and to estimate in vivo digestibility value from in vitro results.

2. Materials and methods

2.1. Ration preparation
Ration used in in vivo and in vitro studies were similar. It consisted of 54% Napier grass and 46% concentrate. The ration contained 58.8% DM, 12.1% ash, 10.0% CP, 3.30% EE, 26.5% CF, and 61.1% TDN. For in vivo study, 20 kg of fresh Napier grass and 4 kg of concentrate were offered and distributed into two feeding frequencies daily. Morning feeding was at 08.00 am, while afternoon feeding was at 3 pm. Water was served ad libitum. Dry matter rations offered were 2.35% of the cattle’s body weight. For in vitro study, Napier grass and concentrate were dried in an Eyela NDO 400 (Made in Japan) oven at 60 °C for 48 hours and ground using a locally made laboratory blender to pass 1 mm screen. The dried powder grass and concentrate were mixed to match a similar proportion to the in vivo study, stored in a polyethylene plastic bag, and then frozen before it was used for analysis.

2.2. In vivo digestibility study (T1)
In vivo digestibility method used in this study followed the principal of daily routine [2]. Four Frisian Holstein (FH) heifers (337.5±45.87 kg body weight (BW)) were placed in individual stalls equipped with feeding buckets and drinking bowls. Two weeks preliminary phase was conducted followed by 1 week collecting period. The heifers were offered 20 kg fresh Napier grass (22% DM, 13.59% ash, 12, 46% CP, 1.56% EE, 35.58% CF and 52.01% TDN) and 2 kg concentrate (90.85% DM, 10.35% ash, 6.91% CP, 5.25% EE, 15.89% CF and 71.7% TDN) daily which were distributed into two equal feeding frequencies. The grass was sampled daily. Feed refusal and feces were measured daily and 10% of them were dried and composited prior to grin and send to laboratory for dry matter (DM) and OM contents analysis. Feed DM and organic matter (OM) intake were calculated by subtracting the amount of DM and OM refusals from DM and OM offered, while digestibility was calculated by subtracting feces from intake. In vivo rumen conditions (pH, NH3, and VFA concentration) were measured from rumen fistulated bulls which fed with the similar ration.

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2.3. In vitro digestibility study (T2, T3 and T4)

In vitro digestibility methods compared in this study were T2 (Tilley and Terry) [10], T3 (Theodorou) [11], and T4 (Sutardi) [12]. Parameters observed including ration fermentability (pH, NH₃, and VFA concentration) and digestibility. The rumen fluid as inoculant source was collected from two fistulated dairy bulls (510 ± 20 kg BW), following standard laboratory procedures. For fermentability measurement, the samples were incubated with rumen liquor, added with buffer and aerated with CO₂ to produce an anaerobic condition. The amount of rumen liquor and buffer used were vary according to the methods. The fermentors were closed with rubber stopper and put into 39°C water shaker bath for 4 h. Supernatant were collected from the fermentability test tubes after adding 2 drops of HgCl₂ to stop the fermentation process and centrifuged at 3500 rpm for 15 minutes. The pH value were measured a Hanna HI98191 pH meter. The NH₃ concentration was measured using micro diffusion Conway, while VFA concentration was measured using steam distillation method. Fermentability measurements followed similar methods used by Riestianti et al. [13].

The digestibility (DMD and OMD) measurement consisted of two steps. Each step lasted for 24 h for T2 and 48 h for T3 and T4. The first step followed similar procedure as fermentability test. In the second stage, sample residues were incubated with 2% HCl-pepsin enzyme (50 ml for T2, 75% for T3 and 25 ml for T4). After the enzymatic digestion, the feed residues were filtered using the predetermined weight of no 41 Whatman paper. The filter paper was dried at 105°C oven to determine DM residue and then incinerated at 600°C for 4 h to determine ash residue. In vitro DMD and OMD were calculated by subtracting DM and OMD residues from samples.

2.4. Statistical analysis

This experiment used a block randomized design with 4 replications as a block. The data were analyzed using ANOVA and continued with the Tukey test. Correlation between T2, T3, and T4 to T1 was made prior to regression analysis to determine the prediction accuracy of T2, T3, and T4 in estimating T1. Statistical analysis were conducted using SPSS version 20.

3. Results and discussion

3.1. Feeds fermentability and digestibility

Comparison feed fermentability and digestibility between methods are shown in Table 1. The table shows that pH in all treatments was not significantly different (P>0.05), while NH₃ and VFA concentrations were significantly different (P<0.05). The NH₃ and VFA values in T1 and T2 were significantly higher than T3 and T4. Fermentability of dairy ration using in vitro method developed by Tilley and Terry (T2) [10] produced insignificantly different results with in vivo (T1). Digestibility (DMD and OMD) measured using different methods also significantly different between treatments. The T1 and T2 methods produced higher DMD and OMD in comparison to T3 and T4. Digestibility of dairy ration DM and OM measured using in vitro method developed by Tilley and Terry (T2) [10] resulted insignificantly different to in vivo (T1).

From this data, it can be seen that Tilley and Terry’s method (T2) is more relevant to be used as in a vitro method in accessing fermentability and digestibility of tropical dairy ration. The similar results between T1 and T2 methods may be caused by the dairy cattle used in the experiment were FH breeds. As we might have known, the breed was originated from Europe where the T2 method was developed [10]. The use of FH breed in this experiment because so far FH cattle is the only authorized dairy cattle breed in Indonesia [1]. Although, the T2 is the most frequently in vitro digestibility method used in Indonesia, however, utilization of this method to imitate feedstuffs fermentability and digestibility for local beef cattle might need an adjustment such as attempted by Sutardi [12].

Different Fermentability and digestibility results between T1 and T3 methods might be caused by the principal measurement in the T3 method [11]. Although this method was developed to determine the nutritive value of tropical feedstuffs for ruminants, however, this method is based on substrate
fermentability and makes use of France et al. [13] mathematical equations to quantify gas production profiles. This method provides detailed information on the fermentation kinetics of ruminant feeds. The T3 method provides a better description of the gas production data and is capable of describing sigmoidal trends. The T3 is a suitable method for determining the fermentation kinetics of ruminant feeds and related them to their in vitro fermentability [11]. As other gas methods, this method is indirect method. The digestibility relies upon an inverse relationship between gas accumulation and degradation of the feedstuff. Menke et al [5] estimated OMD using simple formula OMD (%) = 7.65 gas production + 353 or by multiple parameters that included CP and EE data. Digestibility in the T3 was calculated directly using the by-difference method as used by Jayanegara et al [14].

Modification of T2 by Sutardi (T4) was aimed at adjusting the method to local conditions. However, in this case, it fails to show the similarity result to in vivo (T1). It might be caused by the dairy cattle used in this experiment were not local dairy cattle breed. So far, there is no local dairy cattle breeds have been registered. The utilization of T4 method in assessing tropical digestibility using local cattle such as used by Ifani et al [15] produced more accurate result.

| Parameters | T1 | T2 | T3 | T4 |
|------------|----|----|----|----|
| pH         | 6.44±0.42 | 6.8±0.05 | 6.70±0.06 | 6.72±0.06 |
| NH₃ (mmol) | 10.05±1.17a | 10.11±0.71a | 7.60±0.45b | 5.30±0.19c |
| VFA (mmol) | 134.65±13.81a | 134.93±15.08a | 101.57±12.73ab | 96.10±14.34b |
| Digestibility | | | | |
| DMD (%)   | 58.77±4.07a | 57.26±3.80a | 48.57±2.86b | 45.03±3.84b |
| OMD (%)    | 60.34±3.73a | 56.67±3.53a | 47.77±3.60b | 43.16±2.14b |

T1 = in vivo, T2 = Tilley and Terry, T3 = Theodorou, T4 = Sutardi, NH₃ = ammonia, VFA = volatile fatty acids, DMD = dry matter digestibility, OMD = organic matter digestibility, different superscripts on the same row show significant differences (P<0.05).

3.2. Estimation of in vivo fermentation and digestibility from in vitro methods

Utilization of in vitro fermentation and digestibility studies needs an adjustment to the in vivo results especially for T3 and T4 methods that shows significantly different value with in vivo (T1). The correlation between in vivo and in vitro results are shown in Table 2. The table shows that some in vitro parameters have high correlation (R > 0.5) to T1 [16] but only pH of T3 and DMD of T4 had significant correlation to T1 (P <0.05) due to small sample size used in the correlation (4 replications). To achieve P value < 0.05 at sample less than 6, it needs coefficient correlation more than 0.9 [17]. The T2 produced a high correlation to T1 for pH, DMD, and OMD values, while The NH₃ and VFA have a low coefficient correlation. The T3 mostly has a negative correlation to T1, except for the pH value. The T4 has a high correlation of DMD and OMD as well as NH₃ to T1, but a low correlation for pH and VFA. Although T2 produced insignificant different results from T1, but its correlation to T1 was lower than T4. It means adjustment of the T4 using regression produced more accurate T1 estimation value than the T2. Negative and low correlations of T3 to T1 in this experiment due to the direct method used in calculating DMD and OMD [14]. The T3 method is a gas method that should use the indirect method. The gas method relies upon an inverse relationship between gas accumulation and feed degradation [11]. Digestibility using gas method should be estimated from gas produced during fermentation and other chemical data such as CP and EE content in feeds [5].

Due to the different digestibility values found between in the vivo to in vitro results, therefore an adjustment should be conducted to achieve similar results. Adjustment can be done using highly correlated parameters such as DMD and OMD results from T4 methods. Regression analysis to estimate T1 DMD and OMD using T4 results are shown in Figure 1 and Figure 2.
Table 2. Correlation (R) between in vivo (T1) with in vitro results.

| Parameters | T2   | T3   | T4   |
|------------|------|------|------|
| Fermentability |      |      |      |
| pH         | 0.53 |      | **0.95** |
| NH₃        | 0.13 | -0.32| 0.78 |
| VFA        | -0.01| -0.50| 0.29 |
| Digestibility |      |      |      |
| DMD        | 0.50 | -0.26| **0.97** |
| OMD        | 0.67 | -0.78| 0.82 |

T1 = in vivo, T2 = Tilley and Terry, T3 = Theodorou, T4 = Sutardi, NH₃ = ammonia, VFA = volatile fatty acids, DMD = dry matter digestibility, OMD = organic matter digestibility, Bold numbers show a statistically significant correlation (P < 0.05)

Figure 1. Estimation of in vivo DMD from Sutardi DMD in vitro result.

![Graph of DMD estimation](image)

The graph shows formula to estimate DMD and OMD in vivo value from T4 followed quadratic equation. The formula found in this study have higher coefficient determination than what was reported by Despal [4] using Hohenheim gas test and Cellulase in vitro methods. This results were also higher than Indah et al [18] finding that estimate dry matter digestibility of tropical forage using chemical composition information. High coefficient determination found in this study due to small sample used in making the model. Sample used in this model was as many as the replication. According to Jenkins and Quintana-Ascencio [19], false positive and negative might occure at N < 8. To be able to identified a clear data shape, N > 8 was suggested. Furthermore, Jenkins and Quintana-Ascencio [19] reported that at high variance data, accurate inference was stable at N > 25. Although the data used in developing this model have very low variance because it was generated from experimental data, but in this study, we used 4 replications (N = 4) which may resulted in false positive or negative.

4. Conclusion
From this study, it can be concluded that in vitro Tilley and Terry method produced similar result to in vivo. The Tilley and Terry method is more accurate in determining in vivo digestibility of tropical dairy feedstuffs. Utilization of Sutardi method need an adjustment to match the value of in vivo
digestibility results. The adjustment is following the formula \( Y_1(\%) = -0.091X_1^2 + 9.1632X_1 -168.41 \) and in vivo \( Y_2(\%) = 2.1021X_2^2 - 176.56X_2 + 3760.3 \), where \( Y_1 \) = in vivo DMD, \( Y_2 \) = in vivo OMD, \( X_1 \) = Sutardi in vitro DMD, \( X_2 \) = Sutardi in vitro OMD. It is suggested to increase the number of replications to better estimate in vivo digestibility parameters from Sutardi in vitro methods.

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