Effects of probiotics and encapsulated probiotics on enteric methane emission and nutrient digestibility in vitro

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Abstract. The objective of this study was to evaluate the effects of probiotics and encapsulated probiotics on enteric methane production and the in vitro nutrient digestibility in ruminants. The probiotics used were from the group of lactic acid bacteria (LAB). The experiments were conducted in three dietary treatments (control diet, probiotics addition, and encapsulated probiotics addition) and three replicates. Each replicate was performed at a different week. The experimental diets were incubated in the in vitro rumen fermentation system for 72 h. Parameters observed were pH, total gas production, methane production, total volatile fatty acids, NH3, and the in vitro dry and organic matter digestibility (IVDMD and IVOMD, respectively). Data were analyzed using variance analysis and continued with the Duncan multiple range test to compare among the different treatment means. Results showed both the probiotics and the encapsulated probiotics decreased (P<0.05) the methane production by 6.1 and 33.1% compared to the control diet, respectively. Furthermore, these probiotics and encapsulated probiotics increased (P<0.05) total gas production by 15.7 and 233% than that of control, respectively. The TFVAs, IVDMD, IVOMD, and NH3 values of the diet supplemented with probiotics were higher than the encapsulated probiotics (P<0.05). It can be concluded that both the probiotics and the encapsulated probiotics effectively mitigate the in vitro methane production while simultaneously enhancing the total gas production.

1. Introduction

Methane (CH4), as an atmospheric greenhouse gas, has climatic importance [1] because it contributes to global warming [2]. Methane is produced mainly by reducing carbon dioxide with hydrogen (CO2 + 4H2 → CH4+ 2H2O) [3] from enteric fermentation during the normal digestive process of ruminants [4]. Additionally, methane is one of the most important sources of energy loss in ruminants. It has been estimated that it is responsible for about 2–12% loss of growth energy (GE) and consequently affect animal performance [5]. Since methane is related to global warming and animal energy utilization, methane has received much attention, and many strategies have been applied regarding mitigating enteric methane emission.
Mitigating enteric methane emission in ruminants is possible through various strategies. Among these strategies, feed management approaches are considered the most developed methods in mitigating methane emissions. However, probiotics as a direct-fed microbial strategy are beneficially used in animal nutrition for improving rumen fermentation, increasing feed efficiency, balancing the rumen microflora, and inhibiting adherence and growth of pathogens [6]. Similarly, probiotics are considered as a promising approach for methane mitigation [7]. It has been hypothesized that probiotics can stimulate ruminal bacterial growth and increases the bacterial population [8] by providing them some nutrients, including metabolic intermediate and vitamins [9]. Another theory, probiotics can stimulate lactic acid-utilizing bacteria, which results in the reduction of lactic acid production and consequently stimulate the growth of cellulolytic bacteria growth and therefore fiber digestion will be improved [9]. Moreover, probiotics cause inhibition of specific rumen bacteria that produce H2 or methyl-containing compounds; thereby, CH4 will be reduced [10].

On the other hand, encapsulation technology has been used mainly in the food and nutrition field for protecting the bioactive components of the microcapsule from the undesirable environment (moisture, temperature, light, air, etc.), preventing evaporation loss and improving stability during handling and transporting process [11]. Furthermore, the technology is mainly used in animal nutrition to provide many beneficial properties for some active components, including vitamins, minerals, enzymes, and other components [12]. Until now, little is known about the application of encapsulated probiotics in animal feed and nutrition. This study aimed to evaluate the effects of probiotics and encapsulated probiotics on enteric methane production and the in vitro nutrient digestibility in ruminants.

2. Materials and methods

All the research procedures in this study were carried out at the Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong, West Java, Indonesia.

2.1. Materials

In this study, probiotics were a group of lactic acid bacteria (LAB), i.e., *Lactobacillus plantarum* TSD-10 strain. The probiotics were obtained from the Laboratory of Nutrition and Nutrigenomic, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). The strain was cultured under facultative fermentation at 30°C. Culturing was done in deMan Rogosa Sharpe (MRS) broth medium (Merck, Darmstadt, Germany) [13]. Maltodextrin, in a powder form, was purchased from Setia Guna Chemical Shop, Bogor.

2.2. Encapsulating of probiotics and diet preparation

In this study, the encapsulation process was done using freeze-drying technology according to [14]. Maltodextrin: probiotics (1:1) was used. Commercial concentrates’ meal was purchased from Indofeed Mini Feed mill Bogor West Java. The concentrates (GT03) contained soybean meal, rice bran, cornmeal, corn gluten feed, DDGS, and etc. The grass used was elephant grass (*Pennisetum purpureum*) as a source of forage, which grown locally in the animal farm area, was collected and dried overnight using a cupboard drier. The concentrates’ meal and the grass were ground through a 1 mm screen. Proximate analysis and fiber analysis were performed according to [15]. The results of the chemical analysis were shown in table 1.

Substrates are composed of 60% forages and 40% concentrates. Experimental diets were arranged in a randomized block design (RBD) with three treatments and three replications. The treatments were: T0 = dietary control, which contains only forages and concentrates; T1 = dietary control supplemented with inclusion 0.5 ml probiotics 10^{11} CFU/ml, and T2 = dietary control supplemented with inclusion 0.5 g encapsulated probiotics 10^{11} CFU/ml. All the treatments were triplicated. The experiments were performed in three different weeks, and each week was considered as one replicate.
### Table 1. Chemical composition of *in vitro* feed substrate of the treatments

| Item       | Steer diet | *In vitro* diet |
|------------|------------|-----------------|
| Ash %      | Forage     | Concentrate     | Forage     | Concentrate     |
|            | 2.3        | 3               | 15.3       | 17.5            |
| Moisture%  | 9.2        | 10.83           | 8.15       | 9.5             |
| CP%        | 8.25       | 16              | 15.3       | 17.5            |
| EE%        | 1.92       | 5.3             | 2.7        | 5.61            |
| CF%        | 35.8       | 15.8            | 30.13      | 15.14           |
| NDF%       | 80.5       | 44.1            | 62         | 35.44           |
| ADF%       | 52         | 33              | 40.5       | 19.82           |

Notes: CP%, crude protein; DM; dry matter digestibility, EE; ether extract, CF; crude fiber, NDF; nutrient detergent fiber, ADF= acid detergent fiber.

### 2.3. *In vitro* fermentation

Rumen buffer solutions for these experiments were prepared anaerobically [16]. The prepared buffer solution was kept in the water bath at 39ºC and continuously purged with CO₂ until the solution turned colorless. Fresh rumen fluids were collected from two rumen fistulated Ongole crossbred steers (550±30 kg). The steers were cared and handled following the animal welfare protocols approved by the Animal Care and Use Committee of the Indonesian Institute of Sciences 2015. Animals were fed a substrate that contains 60% concentrate and 40% forage two times a day, at 7:00 am 12 am. Water was freely accessible by the steers. The collection of the rumen fluids was done around 7:00 am before morning feeding. The collected rumen fluids were kept separately in pre-warm bottles after were filtered through four layers of sterile cerecloth before incubation. Each of these steers was considered as one replicate by itself. The obtained rumen contents were immediately transferred to the lab to be ready for use later for the *in vitro* incubation. After arriving in the lab, the pH of the collected rumen fluids was immediately measured using the pH meter. Three separate in vitro rumen fermentation experiments were conducted using 1,000 ml fresh rumen fluid and 2,000 ml buffer solution. The buffered medium for the in vitro fermentation was mixed anaerobically with the rumen fluid by 2:1 buffer: rumen fluid. Subsequently, 50 ml of rumen buffered medium were distributed in 100 ml serum bottles contained 500 mg ground feed substrate. The bottles were incubated according to Theodorou et al (1994) at 39ºC for 72 hours in the water bath, and another two bottles were used as a blank [17]. And bottles were shacked every 1 hour frequently. After 72 h incubation, the gas pressure in each bottle was measured according to the modified protocol of Theodorou et al (1994) using 50 ml syringe to determine the total gas production [17]. Records of the total gas production were recorded at (2, 4, 6, 8, 10, 12, 24, 48, and 72h) for gas production kinetics estimation by the Ørskov’s equation; p = a + b (1-e⁻ᵃᵇ) Ørskov and McDonald (1979). The methane gas concentration in the samples was measured using a methane analyzer (RIKEN KEIKI RX415). Results of methane concentration were recorded at 8, 10, 12, 24, 48, and 72h. After 72h incubation, all bottles were removed from the water bath of 39ºC and put in -20ºC to stop the poster microbial activities. The DMD and OMD were determined according to Tilley et al [18]. Total volatile fatty acids (VFA) were determined using the method of Chatterjee [19] and N-NH₃ using the method of [20].

### 2.4. Statistical analysis

All the treatments were arranged in 3 × 3 block randomized design with three treatments and three replicates. Data were analyzed by using analysis of variance (ANOVA) and performed using SPSS software version 22 (SPSS, Inc., IBM, Chicago). All significant differences between the treatments were determined by Duncan's multiple range test method. Significant differences were accepted if P<0.05. The figure was represented using MS Excel.
3. Results and discussion

Total gas production and the gas kinetics were summarized in Table 2. Methane production was presented in Table 3 and Figure 1. It was proposed the total gas involves CO$_2$, CH$_4$, and small amounts of H$_2$, N$_2$, and O$_2$ and the total gas is produced mainly through the microbes’ during the fermentation process [21]. Probiotics (T1) significantly ($p<0.05$) increased total gas production (mg/g DM), gas fraction, and methane (mg/g DM), particularly in the initial time. The presence of the material substrates and gas production were eliminated by the absence of the nutrient substrates in the rumen [22]. The same result was reported by Astuti et al (2018) [23]. Indeed, the gas fraction increase corresponds with total gas production—the higher gas rate production links with the maximum gas produced and the higher total gas produced. Encapsulation (T2) in this study increased the total gas significantly ($P<0.05$) as compared with non-encapsulated probiotics because the Maltodextrin was considered as a readily fermentable carbohydrate [24]. Our finding in this experiment did not agree with the previous work of [25]. Even though treatments showed a remarkable enhancement in gas production, the readily fermentable materials could lower the rumen pH because of the rapid fermentation rates. Over time, methane production (ml/g DM) increased significantly ($P<0.05$) by adding probiotics and the encapsulated probiotics. On the contrary, supplementation of probiotics (T1) significantly ($P<0.05$) reduced the accumulative percentage of methane per total gas production as compared with the control diet (T0). It was hypothesized that the inclusion of probiotics causes reduction in H$_2$ production, during the fermentation process, which is responsible for methane reduction therefore Jeyanathan et al (2014) and Takahashi et al (1997) stated that H$_2$ is used by methanogens to reduce CO$_2$ to CH$_4$ [26,27]. The same result was reported by Akin [28]. Encapsulation, on the other hand, an effective method for methane reduction due to the slow release, which will contribute in maximization of the probiotics availability especially in late time where methane at maximum production. Furthermore, Maximum utilization of nutrient by animals can be obtained due to encapsulation process because methane represents sources of energy losses [29].

Table 2. The effects of treatments on the in vitro gas production and gas kinetics.

| Treatment/ hours | Gas production (mg/g DM) | Gas kinetics |
|-----------------|--------------------------|--------------|
|                 | 8 | 10 | 12 | 24 | 48 | 72 | TGP | L | B | C |
| T0              | 51.34a | 68.60ab | 81.34a | 124.57ab | 170.43ab | 196.43abc | 120.67a | 0.30a | 94.26a | 0.41a |
| T1              | 65.36b | 82.95ab | 96.65b | 142.10ab | 189.34ab | 260.40abc | 148.33b | 0.90b | 101.34b | 0.46b |
| T2              | 241.63c | 294.36c | 324.71c | 406.34c | 502.05c | 545.39abc | 302.78c | 1.63c | 238.53c | 0.93c |
| SEM             | 30.58 | 36.54 | 39.38 | 45.74 | 53.91 | 71.83 | 6.39 | 0.20 | 23.60 | 0.009 |
| P-value         | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.108 | 0.006 | 0.017 | <0.001 | 0.002 |

Table 3. The effects of treatments on methane production.

| Treatment/ hours | Methane production (mg/g DM) | CH$_4$/TGP % |
|-----------------|-----------------------------|--------------|
|                 | 8 | 10 | 12 | 24 | 48 | 72 |
| T0              | 2.53bc | 5.78bc | 8.67bc | 19.49bc | 31.03bc | 37.89bc | 14.18a |
| T1              | 3.97bc | 7.93bc | 11.18bc | 23.79bc | 35.69bc | 44.34bc | 12.94b |
| T2              | 9.75a | 18.06a | 23.84a | 40.81a | 59.60a | 70.79a | 8.65a |
| SEM             | 1.70 | 2.00 | 2.49 | 3.81 | 5.18 | 6.09 | 0.67 |
| P-value         | 0.002 | 0.002 | 0.004 | 0.020 | 0.018 | 0.022 | 0.025 |

T0, control diet, T1, control diet supplemented with probiotics, T2, control supplemented with capsulated probiotics, DM, dry matter, TGP, total gas production, L, lag time, B, maximum gas production, C, GP rate, SEM, standard error of mean, superscript follow the same order with the latters (a-c) at significance level $p<0.05$ among the treatments. Means were taken from three replicates.
The pH value was suggested as a good indicator for appropriate rumen conditions and the proper fermentative actions and nutrient digestibility [30,31]. Table 4 showed that supplementation with probiotics (T1) resulted in a slight reduction in the rumen pH. The treatment increased the dry matter and organic matter digestibility slightly compared with the control diet (T0). Moreover, a significant (P<0.05) increase in the total VFAs and NH₃ concentration was observed. It was stated that probiotics help stabilize the rumen pH in a normal range [11]. That subsequently improves the nutrient digestibility and increases the total volatile fatty acid production. Furthermore, NH₃ concentration will also improve. The increase of nutrient digestion was proposed due to the positive stimulatory effects of probiotics on the fermentation process [32]. The same result was found previously by Ridwan et al [33]. According to Sheikh et al (2017) volatile fatty acids are chiefly produced due to the rapid fermentation of concentrates [34]. However, it was reported that the concentration of the VFAs is correlated positively with OM digestion [35,36]. Similarly Rahman et al (2013) showed that there is a strong correlation between the total VFAs produced and the total gas production if the fermentation of the feed substrates leads to a high proportion of acetate [37]. NH₃ concentrations were influenced significantly (P<0.05) by the addition of probiotics. Probiotics were suggested can increase the deamination. The experiment of Astuti et al (2018) was similar to our study [23].

Table 4. The effects of probiotics and capsulated probiotics on the nutrient digestibility and rumen fermentation

| Treatment | pH  | IVDMD (%) | IVOMD (%) | TVFAs(mmol/g) | NH₃(mg/ml) |
|-----------|-----|-----------|-----------|---------------|------------|
| T0        | 6.74ab | 63.54ab | 72.05ab | 58bc | 30.82bc |
| T1        | 6.73ab | 65.72ab | 73.85ab | 124a | 56.04a |
| T2        | 5.90c | 57.42c | 67.12c | 72bc | 25.36bc |
| SEM       | 0.14 | 2.43 | 1.90 | 11.01 | 4.95 |
| P-value   | <0.001 | 0.066 | 0.084 | 0.020 | 0.008 |

Note: T0, control diet; T1, control diet + probiotic supplementation; T2, control diet + encapsulated probiotic supplementation; IVDMD, in vitro dry matter digestibility; IVOMD, in vitro organic matter digestibility; SEM, standard error of mean; TVFs, total volatile fatty acids; NH₃, ammonia concentration (mg/ml). Means of PH, IVDMD, and IVOMD were taken from four replications whereas the average mean of TVFAs (mmol/g) and NH₃ were taken from three replications. Means followed by different later from (a-c) in a row differ significantly at P-value (p<0.05) among the treatments.
In contrast, the addition of encapsulation probiotics increased the total volatile fatty acid profile due to the microbial activity on the Maltodextrin that is easily fermented. However, the reduction of the pH value was due to the accumulation of VFAs. The same result was highlighted by of Antonius [7]. Furthermore, a significant reduction (P<0.05) in organic and dry matter digestibility and NH₃ concentration could be due to decreased pH value, which directly affects the microbial activity. Although encapsulation is an effective method to slow our bioactive components, the coating materials should be chosen carefully on orient at which part of ruminal gut those components are needed to be active to achieve the target successfully.

4. Conclusion
Probiotics and encapsulated probiotics are effective strategies for methane mitigating in ruminants and can be considered as promising approaches in the future. Nevertheless effectiveness of the technique for the in vitro nutrient digestibility is still limited, therefore the in vivo experiments are needed to be conducted to determine the effects of encapsulation on the nutrient digestibility in the ruminants.

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