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FERMENTATION CHARACTERISTICS AND RUMEN MICROBIAL POPULATION IN VITRO WITH ENCAPSULATED CYANIDE-DEGRADING BACTERIA AT DIFFERENT STORAGE TIMES

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ABSTRACT

This study aimed to observe the effect of encapsulated cyanide-degrading bacteria (ECDB) supplementation on in vitro fermentation with bitter cassava leaf meal-based ration. The coating materials for encapsulation consist of alginate, starch, canola oil, and lecithin. The cyanide-degrading (CDB) bacteria strains include *Sharpea azabuensis*, *Bovine rumen bacterium*, and *Lachnospiraceae bacterium*. The treatments were P0 = control ration (Napier grass: bitter cassava leaf meal:concentrate=30:30:40), P1 = P0 + ECDB (0-day storage), P2 = P0 + ECDB (7-day storage), P3 = P0 + ECDB (21-day storage), P4 = P0 + ECDB (28-day storage). Variables observed include pH value, NH_3 concentration, dry matter digestibility (DMD) and organic matter digestibility (OMD), volatile fatty acid (VFA) concentration and its proportion, methane estimation, and rumen microbial population. The data obtained were tested using analysis of variance (ANOVA), and if there was any significant difference among treatments were further tested using the Duncan Multiple Range test. The results showed that the addition of encapsulated cyanide-degrading bacteria (ECBD) with different storage times increased ($p<0.05$) rumen pH, total bacterial population, and NH_3 concentrations. On the other hand, DMD, OMD, VFA, protozoa populations, and methane estimation were treated similarly. In conclusion, the addition of encapsulated cyanide-degrading bacteria (ECBD) stored for up to 28 days at room temperature has a beneficial effect on the bacterial population and feed protein degradation.

Keywords: Microbial population, encapsulated cyanide-degrading bacteria, in vitro fermentation, bitter cassava leaf, natural resources, methane estimation

INTRODUCTION

Bitter cassava leaves have high potency as ruminant feed because they contain quite high nutrients, including 91.32% dry matter, 10.9% ash, 18.50% crude protein, 4.83% crude fat, 20.97% crude fiber, 44.80% BETN, and 60.77% TDN. However, bitter cassava leaves are weak because they contain high anti-nutrients in cyanide acid (HCN). Fresh bitter cassava leaves (flour) have an HCN content of about 501.39 ppm (Suharti et al., 2021). Cyanide toxicity is associated with inhibiting cytochrome oxidase enzyme activity in the electron transport system and causes the cells to lack oxygen (Beasley and Glass, 1998).

To optimize the use of bitter cassava leaves as ruminant feed without causing cyanide poisoning, we already isolated the bacteria that can degrade cyanide from rumen goats. Based on the molecular identification, the cyanide-degrading (CDB) bacteria had a similarity (99%) *with Sharpea azabuensis, Bovine rumen bacterium*, and *Lachnospiraceae bacterium* . Inoculating cyanide-degrading bacteria to the sheep fed 30% bitter cassava leaf meal tends to improve volatile fatty acid (VFA) production and ammonia concentration in the rumen without affecting body weight gain and feed efficiency. Furthermore, inoculating cyanide-degrading bacteria could detoxify cyanide in bitter cassava leaf meal and enhance the rumen fermentation profile (Suharti *et al*., 2021). Cyanide-degrading bacteria are capable of degrading cyanide from plant sources. Cyanide is detoxified through the hydration to formamide, sulfur incorporation to form thiocyanate, and subsequent degradation of thiocyanate into sulfite and ammonia. The conversion of cyanide into non-toxic products like formamide, thiocyanate, sulfite, and ammonia helps protect the rumen environment and the host animal (such as cattle) from the toxic effects of cyanide exposure (Gupta et al., 2010; Loh et al., 2020).

Using cyanide-degrading bacteria as a probiotic is beneficial, especially for ruminants fed with plant-rich cyanide such as bitter cassava leaf meal. However, the CDB used in the ration is unstable at room temperature and easily damaged. Therefore, to improve the viability of bacteria and protect them from unfavorable environments, the applicable technique, i.e. bacteria encapsulation, is used. Encapsulation is coating bacteria using materials that can protect and maintain bacteria viability. The encapsulation process protects the bacteria from environmental stressors like temperature and pH fluctuations by forming a protective outer layer or capsule around the bacterial cells. This capsule plays several crucial roles in bacterial defense and is usually made of proteins, lipids, or polysaccharides. The capsule acts as a physical barrier to prevent harmful substances including toxins, enzymes, and antimicrobial agents from entering the bacterial cell (Safeer Abbas et al., 2023). In addition,

Nanoencapsulation may be a viable method for masking some types of unwanted odors and scents while shielding a variety of food ingredients from particular physiological circumstances or deterioration (Kavia et al., 2024). The viability of cyanide-degrading bacteria has an essential role because to have their beneficial effects on the ruminant host, they must stay alive as far as their site of action in the rumen. The most frequently used bacterial culture encapsulation methods are freeze and spray-drying (Morgan et al., 2006). Peighambardoust et al., 92011) stated that sensitive microorganisms can be encapsulated to increase their viability and shelf life.

The selection of wall material for the encapsulation of bacteria is very important since the substances used to encapsulate bacteria should be safe, biodegradable, and able to form a physical barrier between the core and its surroundings (Rajam and Subramanian, 2022). In addition, the wall material used in the encapsulation should improve bacterial cells' viability during processing and prolonged storage. The wall material should protect the bacteria cells from the factors that may cause inactivation and release them in the rumen. Microencapsulation of probiotics is often achieved with various biopolymers such as plant extrudates (gum Arabic, acacia gum), marine extracts (alginate, carrageenan), proteins (milk or whey protein, soy protein, gelatin, gluten), dietary fiber (resistant starch, maize starch), microbial and animal polysaccharide (chitosan, xanthan). Encapsulation efficiency, physio-chemical characteristics, and stability of microcapsules highly depend on the wall material compositions (Safeer Abbas et al., 2023).

There were no negative impacts or possible hazards associated with adding encapsulation bacteria to the rumen system. By micro-encapsulating film-forming and acid-resistant wall materials, it is possible to reduce bare bacteria to minute particles. On the surface of the rumen and colon mucosa, it can keep the functional flora active, strengthen the intestinal barrier, and increase its ability to colonize (Wei et al., 2022).

Our previous study reported that the encapsulation of cyanide-degrading bacteria using alginate and starch as core materials could maintain the viability of bacteria until 21 days of storage at a temperature of 27^{o}C (Mislah et al., 2018). The study about the effect of encapsulated cyanide-degrading bacteria on ruminants was not addressed in our previous study and so this is the aim of the current study. Therefore, this study aimed to evaluate encapsulated cyanide-degrading bacteria (ECBD) with different shelf lives on in vitro fermentation characteristics with bitter cassava-based rations.

MATERIALS AND METHODS

Cyanide-degrading Bacteria Culture and Encapsulation

Cyanide-degrading bacteria culture

The cyanide-degrading bacterial culture used for encapsulation was first grown in liquid brain heart infusion (BHI) media, consisting of 3.7 g BHI powder, 0.05 g glucose, 0.05 g starch, 0.1 g cysteine, 0.5 ml hemin, 0.05 ml resazurin, CMC 1 % 1 ml. All ingredients were put into a 200 ml Erlenmeyer except for cysteine, and then distilled water was added to 100 ml. The media mixture is heated until it is homogeneous, and the color changes from yellow to red and back to clear yellow. The media that has been cooled is added cysteine while flowing CO_2 gas; the media is transferred into a Hungate tube with as much as 8 ml per tube while flowing CO_2 gas and then sterilized using an autoclave at 121 ^{0}C for 15 minutes. While flowing with CO_2 gas, 1 ml of KCN and 1 ml of cyanide-degrading bacteria culture were added. In the final stage, incubation was carried out for 24 hours. The total population of cyanide-degrading bacteria used before encapsulation was 2.64 X 10^8cfu/ml based on the previous study which is the optimum level to be inoculated in the rumen system.

Cyanide-degrading bacteria capsulation

The encapsulation process was done according to Krasaekoopt et al. (2003) by modifying the encapsulation technique. Alginate and starch are used for encapsulation because of their affordability, controlled release properties, processing simplicity, gel-forming potential, and biocompatibility. They can also be utilized in several applications. The materials used included 25 ml of 0.5% alginate solution added to 25 ml of 0.5% starch solution. Then the solution was added with 50 ml of cyanide-degrading bacterial culture while flowing with CO_2 gas. After that, 50 ml of canola oil containing 0.05 ml of lecithin was added and homogenized using a magnetic stirrer for 20 minutes. 0.1 M $CaCl_2$ was added slowly through the Erlenmeyer wall as much as 50 ml and left for 30 minutes. The separation process was carried out in laminar airflow to prevent contamination with the environment. The encapsulation results are washed with 0.9% saline solution, which has added 5% glycerol, the final stage in the encapsulation process is by adding skim milk to the powder, with a comparable uniformity of the capsule. The capsules were stored at room temperature, about 37 °C, with humidity around 80%.

In vitro Fermentation

The substrate for in vitro fermentation consists of elephant grass, bitter cassava leaf meal, and concentrate mix with a ratio of 30%:30%:40%. The research ration was prepared by mixing

the ingredients according to the treatment. The ration formulation and nutrient content of the research feedstuffs used in this study are presented in Table 1.

Table 1 Ration and nutrient composition as a substrate in the *in vitro* Fermentation

Ingredients	Proportion (%)
Pollard	2
Cassava waste	5
Molasses	1.8
Rice bran	20.5
$CaCO_3$	0.35
DCP	0.35
Bitter cassava leaf meal	30
Napier Grass	40
Total	100
Nutrient composition :	
Crude protein (%)	14.73
Crude Fiber (%)	28.01
Ether extract (%)	4.27
TDN (%)	65.70
Calcium (%)	0.87
Phosphor (%)	0.43
Cyanide acid/HCN (ppm)	155.33

The research design used in this study was a randomized block design (RBD) using five treatments and five groups. The grouping based on time of rumen fluid collection and five ration treatments and five ration treatments including:

P0 = control ration

P1 = P0 + Encapsulated cyanide-degrading bacteria with a shelf life of 0 days

P2 = P0 + Encapsulated cyanide-degrading bacteria with a shelf life of 7 days

P3 = P0 + Encapsulated cyanide-degrading bacteria with a shelf life of 21 days

P4 = P0 + Encapsulated cyanide-degrading bacteria with a shelf life of 28 days

The rumen fluid used in this study came from the fistula of Ongol Peranakan cattle from LIPI (Indonesian Institute of Sciences). Rumen fluid is taken in the morning before feeding. Before taking rumen fluid, a flask filled with water with a temperature of 39-40^{o}C was prepared. A gauze is used to squeeze rumen fluid, which is then put into a thermos after the water is removed.

In vitro fermentability testing was performed according to the method of Tilley and Terry (1963). Total of 500 mg of substrate for each treatment was put into the fermenter tube, and then added 40 mL of McDougall's solution and 10 mL of rumen fluid. The tube is shaken with CO_2 gas for 30 seconds. The fermenter tube is inserted into the available rack in the water bath with a temperature setting of 39-40^{o}C. The fermenter tube is closed with a vented rubber cap. Incubation was carried out for 4 hours to measure protozoa, total bacteria, pH, and NH_3 and 48 hours incubation to measure the digestibility of dry matter and organic matter digestibility.

Sampling and Analysis

Using a pH meter, the pH value was measured following the feed fermentation process. A pH 4 and pH 7 solution were first used to calibrate the pH meter. The pH of each rumen sample was measured following a 4-hour fermentation procedure.

Measurement of the dry matter digestibility (DMD) and organic matter digestibility (OMD) using the method of Tilley and Terry (1963). Ammonia concentration was measured using the Conway Microdiffusion technique (Suharti et al., 2015) . Gas Chromatography (GC) was used to measure the concentration of VFA and its proportions. Shimadzu Crop., Kyoto, Japan, uses a type GC 8A gas chromatography (GC) system with an SP-1200 column and 1% H3PO4 on 80/100 Cromosorb WAW.

Total bacteria were counted using plate count with the roller-tube method, and the population of protozoa was enumerated using a microscope set to x100 magnification. Brain Heart Infusion (BHI) media and a modified roller tube technique were utilized to determine the total bacterial population. A ten-fold serial dilution was performed. Subsequently, 0.5 mL of samples from dilutions 6 through 10 were transferred into a Hungate tube containing BHI media and evenly rotated using a roller tube. The samples were then incubated at a temperature of 37–40°C for 48 hours. The bacterial population was estimated using the following formula: $BP = C \times 10^{n} \times 2$, where C represents the number of colony-forming units, n denotes the dilution level, and BP signifies the bacterial population (Suharti et al., 2017). The estimation of methane production was calculated from the proportion of VFA with the following equation (mM)= 0.45 C2-0.275 C3+ 0.40 C4 (Moss et al., 2000).

Data Analysis

Analysis of variance (ANOVA) was used to examine the collected data using SPSS version 16. The Duncan multiple-range test was used to further investigate the significant differences between treatments.

RESULTS

Rumen Microbe Population

The results showed that inoculation of encapsulated cyanide-degrading bacteria (ECBD) stored at 7, 21 and 28 days in the rumen that used bitter cassava leaf-based rations significantly ($P<0.05$) increased the total bacterial population but had no significant effect on the rumen protozoa population compared to the control (Table 2).

Table 2 Total bacteria dan protozoa population in vitro in the addition of encapsulated cyanide-degrading bacteria (ECBD) with different storage times

Variables	Treatments				
	Control (C)	C + ECDB shelf lives 0 d	C + ECDB shelf lives 7 d	C + ECDB shelf lives 21 d	C + ECDB shelf lives 28 d
Total bacteria (Log cfu ml^{-1})	5.16 ± 0.58^{d}	11.22 ± 0.58^{a}	10.55 ± 0.44^{b}	9.63 ± 0.48^{c}	9.52 ± 0.51^{c}
Total Protozoa (Log cell ml^{-1})	4.22±0.11	4.32±0.09	4.31±0.11	4.32±0.06	4.37±0.07

Note: ECDB=Encapsulated cyanide-degrading bacteria; Different superscripts in the same row show significant differences ($P\leq0.05$)

Rumen fermentation characteristics *in vitro*

Inoculation of encapsulated cyanide-degrading bacteria (ECBD) significantly ($P\leq0.05$) decreased the pH value but significantly increased ($P\leq0.05$) ammonia concentration. The dry matter digestibility (DMD), organic matter digestibility (OMD), total VFA production and its proportional (acetate, propionate, butyrate, valerate), C2/C3 ratio, and methane estimation were similar among treatments (Table 3).

Table 3. *In vitro* Digestibility, ammonia, VFA production and methane estimation in the presence of encapsulated cyanide-degrading bacteria (ECBD) with different storage times

Variables	Treatments				
	Control (C)	C + ECDB shelf lives 0 d	C + ECDB shelf lives 7 d	C + ECDB shelf lives 21 d	C + ECDB shelf lives 21 d
DMD (%)	71.01±0.73	71.06±0.28	71.25±0.81	69.96±2.47	70.64±0.48
OMD (%)	69.76±0.43	69.55±0.27	69.79±0.47	69.76±0.23	69.85±0.58
pH	7.06±0.09^{a}	6.94±0.11^{b}	6.90±0.10^{b}	6.88±0.08^{b}	6.88±0.08^{b}
NH_3 (mM)	12.13±2.91^{b}	16.53±6.78^{a}	17.54±2.39^{a}	16.52±0.79^{a}	15.84±1.95^{a}
VFA Total (mM)	86.04±7.50	83.13±7.77	81.33±13.96	88.26±7.98	80.99±9.64
Proportional VFA (%)					
Acetate (C2)	59.45±3.15	54.18±5.35	56.32±3.68	58.25±2.62	56.30±1.92
Propionate (C3)	22.74±3.00	26.58±4.27	25.22±3.96	24.62±0.54	23.60±1.80
Butirate (C4)	13.46±0.71	14.73±1.98	13.95±2.05	13.15±3.06	15.24±1.50
Valerate (C5)	4.35±0.53	4.51±0.31	4.51±1.05	3.98±0.76	4.86±0.66
C2 : C3	2.65±0.45	2.09±0.53	2.28±0.48	2.37±0.06	2.40±0.26
Methane (mM)*	22.36±3.49	19.05±2.73	19.52±4.09	21.80±1.99	20.26±3.32

Note: ECDB=encapsulated cyanide-degrading bacteria; Different superscripts in the same row show significant differences (P≤0.05).

DISCUSSION

Rumen Microbe Population

The increasing total bacteria population indicates that encapsulation of cyanide-degrading bacteria (ECBD) could maintain the viability of bacteria until 28 days of storage at room temperature. The encapsulation using alginate and starch as coating materials could inhibit the oxidation of membrane lipids and protein denaturation and prevent the denaturation of macromolecules in bacterial cells. Gunzburg et al., (2020) reported that cellulose sulphate (CS) encapsulation could protect bacteria from stomach acid and bile and survive at low pH in vitro for at least four h without appreciable loss in viability as compared to their respective non-encapsulated counterparts. Oberoi et al., (2021) stated that combination of alginate-

xanthan microcapsules exhibited the highest encapsulation efficiency of up to 95% and showed a remarkable improvement in the survival rate of microencapsulated probiotics under simulated gastric conditions. A combination of alginate-xanthan gum microcapsules has a significant potential to deliver large numbers of probiotic cells to the intestines, where cells can be released and colonized for the consumer's benefit. Long-term benefits of higher rumen bacterial populations include better nutrient absorption, fiber digestion, and overall microbial balance, all of which can enhance animal performance.

Therefore, managing the composition and diversity of the microbial community is crucial for ensuring that the long-term effects on rumen health and animal performance are beneficial.

The total protozoa were similar among treatments indicating that encapsulated cyanide-degrading bacteria did not alter the growth of protozoa. The amount used in this experiment may be insufficient to compete with the ciliate protozoa and change their population in the rumen. Protozoa and cyanide-degrading bacteria do not seem to directly compete with one another in the rumen due to the differences in their ecological roles and substrates. While bacteria that break down cyanide are specialized for detoxifying from cyanide, protozoa are mostly involved in bacterial grazing and fiber digestion. The absence of direct rivalry or interaction implies that the rumen's bacteria and protozoa can coexist while playing different ecological roles. Similar findings were also made by (Paudel et al., 2023), who discovered that supplementing nursing cross-bred cattle with various strains of bacterial probiotics did not significantly alter the population or individual rumen ciliate protozoa.

Rumen fermentation characteristics *in vitro*

Although the pH values were slightly reduced in the presence of encapsulated cyanide-degrading bacteria (ECBD), all pH values of each treatment were still in the normal range. The normal rumen pH range was between 5.7-7.3 (Suharti et al., 2019). The pH values obtained in this study ranged from 6.88-7.06 due to the presence of phosphate and bicarbonate that maintained rumen pH values (Counotte et al., 1979). The decrease in the pH value in the treatment indicates that the fermentation process in the feed is going well because the pH value is an indicator that the feed degradation process is going well, where the digestibility value in this study is high in line with the decreased pH value and the NH_3 value in this study which is increases where the increased NH_3 value will increase the total bacterial population so that the fermentation process in the feed goes well so that the pH value in this study decreases. Dijkstra et al. (2012) suggested that the optimum pH value indicates that the feed is well degraded because, at this pH, the microbes that produce crude fiber digestive enzymes can live well in

the rumen. Nitrogen metabolism is closely related to the increase in ammonia concentration in the rumen, which is a source of nitrogen for microbial development as well as a sign of possible abnormalities in the animal's overall nitrogen status, microbial activity, and protein digestion.

The encapsulation of cyanide-degrading bacteria did not affect the DMD and OMD, as the composition of the feed ingredients used in each treatment was relatively the same. The average value of dry matter digestibility in this study ranged from 69-71%. The increase in ammonia concentration in the rumen was due to an increase in the total bacterial population in the presence of cyanide-degrading bacteria. This result also indicates that cyanide-degrading bacteria could degrade cyanide into ammonia and formate. Under anaerobic conditions, cyanide can be degraded by producing fermentation products like formic acid and ammonia. *Pseudomonas fluorescens* NCIMB 11764 can hydrolyze cyanide to produce formic acid, and *Pseudomonas pseudoalcaligenes* CECT5244 can degrade cyanide by producing ammonium which is then incorporated with amino (Luque-Almagro et al., 2005).

The use of encapsulated cyanide-degrading bacteria did not affect total VFA, and its proportion, indicating that encapsulated cyanide-degrading bacteria did not alter feed degradation and fermentation by rumen microbe. There was a slight increase in propionate proportion can indicate an increase in the activity of microbes that ferment starch or sugars. Although the main function of cyanide-degrading bacteria is to detoxify cyanide, their inclusion may indirectly impact microbial fermentation pathways that result in increased propionate synthesis. Cyanide-degrading bacteria may either directly or indirectly promote the growth of propionate-producing microorganisms by supplying substrates (like formate) or by altering the general microbial environment.

Methane estimation was also similar among treatments because encapsulated cyanide-degrading bacteria did not inhibit rumen methanogenesis. The study found no significant difference in methane production since the encapsulated cyanide-degrading bacteria are not involved in the particular processes of methane generation (such as methanogenesis or acetogenesis).

CONCLUSION

Inoculation of encapsulated cyanide-degrading bacteria (ECBD) with different shelf lives increased rumen pH, total bacterial population, and NH_3 concentrations. In contrast, dry matter digestibility (DMD), organic matter digestibility (OMD), volatile fatty acids (VFA), and protozoa populations were similar among treatments. In conclusion, the addition of

encapsulated cyanide-degrading acid bacteria stored up to 28 days at room temperature has a beneficial effect on the bacteria population and feed protein degradation.

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CONFLICT OF INTEREST

No conflict of interest exists with any party related to the materials discussed in the paper.

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