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ISOLATION AND IDENTIFICATION OF BACTERIA FROM CATTLE RUMEN FLUID AND THEIR APPLICATION ON IN VITRO DIGESTIBILITY OF RICE STRAW

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ABSTRACT

This study aimed to isolate and identify bacteria from the rumen fluid of Bali cattle and to evaluate their effect as microbial inoculants on the in vitro digestibility of rice straw. The experiment was conducted using a Completely Randomized Design (CRD) consisting of four treatments and four replications. The treatments included rice straw silage with the addition of 0%, 5%, 10%, and 15% of local microorganisms (MOL) derived from cattle rumen fluid. The observed variables included the isolation and identification of bacteria from the rumen fluid, and the assessment of in vitro digestibility, including Dry Matter Digestibility (DMD), Organic Matter Digestibility (OMD), Volatile Fatty Acid (VFA) concentration, and ammonia (NH_3) production. The results showed that the addition of rumen fluid MOL significantly ($P<0.05$) improved the digestibility parameters. The highest DMD (52.44%) and OMD (47.11%) were obtained from the treatment with 15% MOL. VFA and NH_3 concentrations also increased, indicating enhanced microbial activity and fermentation efficiency. The study concludes that MOL derived from cattle rumen fluid, particularly those containing *Bacillus* sp., is effective in improving the nutritional quality and digestibility of rice straw.

Keyword: Local microorganisms, Rumen fluid, Rice straw, Digestibility, In vitro.

INTRODUCTION

The productivity of ruminant livestock is highly determined by the quality and quantity of feed, which is estimated to contribute around 70–80% to livestock production performance. Therefore, the availability of adequate feed, both in terms of quantity and nutritional value, is a key factor in ensuring the sustainability of livestock production systems. Forage is the primary source of fiber for ruminants; however, its availability is highly dependent on climatic conditions. During the rainy season, forage grows abundantly due to sufficient rainfall and humidity. Conversely, in the dry season, forage growth declines drastically due to water scarcity and low vegetation productivity, resulting in a limited supply of forage (Bira *et al*., 2024; Tahuk *et al*., 2022).

This situation highlights the need to explore alternative feed sources that are abundantly and sustainably available. Rice straw is an agricultural by-product with significant potential as an alternative feed due to its abundance after harvest. However, its use as feed is still limited because of its low nutrient content—particularly its crude protein content, which is only around 5.87%, and its high crude fiber content, approximately 28.68% (Sio *et al*., 2022). The high lignocellulose content

results in low digestibility of rice straw in the ruminant digestive system, which heavily relies on rumen microorganisms to digest fibrous feed (Karim *et al*., 2023).

The digestibility of rice straw varies depending on several factors such as plant variety, processing methods, and post-harvest treatments. Rhofita (2016) reported that the dry matter digestibility (DMD) of rice straw ranges from 34% to 52%, while organic matter digestibility (OMD) can reach 42% to 59%. Recent research by Ardigurnita *et al*. (2023) shows that fermentation of rice straw with various inoculant treatments can increase DMD from a control value of 39.44% to 57.26% under the best treatment.

One effective approach to improving the nutritional quality and digestibility of rice straw is fermentation. Fermentation is a bioconversion process carried out by microorganisms capable of breaking down complex lignocellulosic structures, producing end-products that are more easily digested by rumen microbes (Sarungu *et al*., 2020; Risdianto *et al*., 2018). The success of fermentation is influenced by several variables, including storage duration and fermentation conditions. Hopid *et al*. (2023) revealed that with proper regulation, rice straw fermentation using local microbial inoculants can improve nutritional content and feed fermentability. The addition of other ingredients, such as sugar or carbon sources, can also contribute to the efficiency of the fermentation process (Kusumaningrum *et al*., 2017). The success of fermentation largely depends on the presence of microbial inoculants involved in the ensiling process. Inoculants derived from cattle rumen fluid are a potential source of local microorganisms, as they contain various functional bacteria such as cellulolytic, amylolytic, and proteolytic bacteria. Fermenting rice straw using local microorganisms (MOL) or lactic acid bacteria can improve the nutritional content of rice straw (Aman *et al*., 2022). This process not only increases the protein content but also reduces crude fiber, thereby improving digestibility. The use of MOL from rumen fluid has been proven to improve fiber structure, reduce lignin content, and increase the availability of nutrients in rice straw (Tala & Irfan, 2018; Suryani *et al*., 2015).

Cattle rumen fluid is known to contain microorganisms such as *Bacillus* sp., which can produce extracellular enzymes, including cellulase, protease, and amylase (Budiansyah *et al*., 2010). These enzymes play a role in breaking down complex compounds in rice straw into simpler, more digestible forms. Additionally, fermentation activity by rumen microorganisms produces metabolic products such as volatile fatty acids (VFA) and ammonia (NH_3), which serve as important indicators in assessing fermentation effectiveness and in vitro feed digestibility potential.

Based on the above explanation, this study was conducted with the aim of identifying the types of bacteria present in cattle rumen fluid and evaluating their effect when used as inoculants on the in vitro digestibility of rice straw. The results of this research are expected to serve as a foundation for developing strategies to utilize agricultural waste, particularly rice straw, as quality livestock feed through a local microbial biotechnology approach.

MATERIALS AND METHODS

Time and Location

The study was conducted from June to September 2020 at the Faculty of Agriculture, University of Timor. The in vitro digestibility measurements were carried out at the Feed Chemistry Laboratory, Faculty of Animal Science, Nusa Cendana University. The analysis of the characteristics of cattle rumen fluid was conducted at PT. Indolab Utama, West Jakarta

Tools and Materials

The equipment used included a 20 kg capacity plastic drum as a silo, 1000 ml plastic bottles, cutting tools, an analytical balance, writing materials, a digestion flask, Erlenmeyer flasks, an oven, a pH meter, filter paper, measuring cylinders, as well as equipment for in vitro analysis, proximate analysis, and fiber analysis. The materials used were rice straw, cattle rumen fluid, molasses, and coconut water

Research method

The research method used in this study was a Completely Randomized Design (CRD), consisting of 4 treatments and 4 replications, resulting in a total of 16 experimental units. The treatments were as follows:

R0**:** Rice Straw + 10% Rice Bran + 3% Molasses (control)

R1: Rice Straw + 10% Rice Bran + 3% Molasses + 5% MOL from cattle rumen fluid

R2: Rice Straw + 10% Rice Bran + 3% Molasses + 10% MOL from cattle rumen fluid

R3: Rice Straw + 10% Rice Bran + 3% Molasses + 15% MOL from cattle rumen fluid

The percentages of molasses and cattle rumen fluid were adjusted according to the weight of the chopped rice straw.

Research procedure

Production of Inoculum

Cattle rumen fluid was collected from the Kefamenanu slaughterhouse (RPH) and stored in plastic bottles. It was then filtered to separate it from large and coarse particles such as feed and other debris. A total of 250 ml of rumen fluid was mixed with 250 ml of coconut water and 250 ml of molasses (200 ml molasses diluted with 50 ml water), and this was prepared for 16 treatments. Coconut water functions as a microbial culture medium, while molasses serves as an energy source for the microorganisms present in the rumen fluid. The mixed rumen fluid was then placed in plastic bottles and stored in a refrigerator at 10ºC for 21 days. Before use, the inoculum was refreshed/reactivated at room temperature for 24 hours.

Rice Straw Fermentation Process

Rice straw was collected and chopped into pieces measuring 2–3 cm. The chopped straw was then mixed with rice bran and sprayed with the inoculum that had been mixed with molasses according to the treatment. The mixture was placed into a plastic drum and compacted to ensure the chopped layers were tightly packed (following the anaerobic principle). The silo (plastic drum) containing the rice straw and inoculum was then stored at room temperature for 21 days.

Table 1. In Vitro Digestibility of Rice Straw Before Fermentation

DMD (%)	OMD (%DM)	VFA (mM)	NH3 (mM)	pH Substrate
39.123	33.552	61.429	4.711	6.76

DMD=dry matter digestibility; OMD=Organic matter digestibility; VFA=Volatile Fatty Acid; NH3=Amonia.

Isolation of Bacteria from MOL Cattle Rumen Fluid

The MOL from Bali cattle rumen fluid was sterilized with 70% ethanol for 10 minutes, followed by 2.5% sodium hypochlorite for 10 minutes, and again with 70% ethanol for another 10 minutes, then rinsed three times with sterile deionized water. One gram of the MOL endosperm from the cattle rumen fluid was mixed with 1 mL of 0.85% NaCl, and 0.1 mL of the suspension solution was taken and inoculated using the spread plate method onto sucrose agar plates (SAP), then incubated for 2–7 days. SAP contained 4% sucrose. Individual colonies were selected based on morphological differences from the mixed culture using the four-quadrant streak method. Cultures were incubated for 24 hours. This process was repeated approximately 11 times (subcultures) to obtain a pure single colony. Gram staining

was performed to confirm the purity of the colony (Suhandono *et al*., 2016).

Bacterial Identification Using the 16S rDNA Gene

A single bacterial colony was grown in liquid LB medium for 16–18 hours. The culture was then centrifuged at approximately 15,000 g (14,000 rpm) for 1 minute, and the supernatant was discarded. The pellet was resuspended in 750 µL of lysis buffer (25 mM ethylenediaminetetraacetic acid, 50 mM Tris-Cl, and 0.5% sodium dodecyl sulfate), followed by the addition of 750 µL of chloroform-isoamyl alcohol (24:1). The mixture was incubated at 80°C for 10 minutes and then centrifuged at 14,000 rpm for 3 minutes. The supernatant was collected and transferred to a new microtube, and the steps were repeated until a clear supernatant was obtained. Then, 1/10 volume of LiCl and 2.5 volumes of absolute ethanol were added, and the mixture was incubated at 20^0C for 30 minutes. The sample was centrifuged for 3 minutes at 15,000 g. The supernatant was discarded, and 200 µL of 70% ethanol was added. The sample was centrifuged again at 15,000 g for 3 minutes. The supernatant was discarded, and the sample was air-dried at room temperature. Next, 50 µL of TE buffer (pH 8.0) was added, and the sample was stored at 20^0C.

To perform molecular identification of the bacteria, the 16S ribosomal DNA (rDNA) gene marker was used. PCR was carried out using universal primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (TAC GGY TAC CTT GTT ACG ACT T) to amplify approximately 1500 bp of the 16S rRNA gene (Suhandono *et al*., 2016). The PCR products were then visualized using electrophoresis and purified using a GeneAid kit (Suhandono *et al*., 2016).

In vitro digestibility

The in vitro digestibility procedure was carried out using the two-stage method of Tilley and Terry (1963). The analysis process consists of two phases. In the first phase, 0.5 grams of sample are placed into a centrifuge tube and preheated, then a mixture of rumen fluid and buffer solution (pH 6.9) that has been saturated with CO_2 is added. The tube is sealed, incubated for 48 hours, and fermentation is stopped by adding Na_2CO_3. The sample is centrifuged, and the supernatant is filtered through nylon cloth using a vacuum pump. In the second phase, residual particles on the cloth are washed with HCl-pepsin solution and re-incubated for another 48 hours without CO_2. The sample is then centrifuged again, filtered using a thermal alumina filter, dried at 103°C, and ashed at 550°C. The resulting weights are used to calculate the organic matter digestibility coefficient.

Dry Matter Digestibility and Organic Matter Digestibility

Based on the in vitro digestibility measurement procedure described above, dry matter digestibility (DMD) and organic matter digestibility (OMD) were calculated using the formula by Nahak *et al*. (2021):

$$\text{DMD} = \frac{Initial\ DM-(Residual\ DM-Blank\ DM)}{Initial\ DM}\ x\ 100\%$$

$$\text{OMD} = \frac{Initial\ OM-(Residual\ OM-Blank\ OM)}{Initial\ OM}\ x\ 100\%$$

N-NH_3 production measurement

The N-NH_3 concentration was measured using the microdiffusion method with a Conway dish. A total of 1 mL supernatant and 1 mL NaOH were placed on opposite sides of the dish, while the center well was filled with 1 mL boric acid containing indicators. The dish was sealed tightly, gently shaken, and left at room temperature for 24 hours. The ammonia bound to the boric acid was then titrated with 0.005 N H_2SO_4 until a reddish color appeared. The N-NH_3 concentration was calculated using the formula by Nahak *et al*. (2021): N-NH3 = (ml titration x NH_2SO4 x 1000) mm.

Total VFA production measurement

A 5 mL supernatant obtained from centrifugation was mixed with 1 mL of

H_2SO_4 and stored in a refrigerator. Then, 2 mL of the preserved supernatant was distilled to obtain 100 mL of vapor. After adding 3 drops of phenolphthalein indicator, the solution was titrated with 0.01 N NaOH until a pink color appeared. The titration volume was recorded, and the VFA concentration was calculated using the formula from Nahak *et al*. (2021).

$$\text{VFA} = \frac{(Titration\ Volume\ x\ N\ NaOH\ \ x100)}{2\ ml\ sample}\ x\ Dilution$$

Data Analysis

The isolation and identification of rumen fluid bacteria were presented using descriptive qualitative analysis, while the in vitro digestibility data were analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test. The data were processed using SPSS version 26.

RESULTS AND DISCUSSION

Isolation and Identification of Endophytic Bacteria from Cattle Rumen Fluid

Laboratory analysis results showed that the endophytic bacteria identified were of the *Bacillus* sp. type. *Bacillus* sp. is a probiotic capable of inhibiting the growth of pathogenic bacteria (Suhandono *et al*., 2016), thereby increasing the population of beneficial bacteria in the digestive tract without competition from harmful bacterial growth (Salaki, 2011). It does not produce toxins, is easy to cultivate, can withstand high temperatures, and does not produce metabolic by-products (Linggarjati *et al*., 2013). Furthermore, Jlidi *et al*. (2022) stated that this bacterium has the ability to secrete protease, lipase, and amylase enzymes. In addition, the presence of *Bacillus* sp. enhances the aroma of fermented straw, giving it a more pleasant smell (similar to fermented cassava or "tape"), which is expected to improve the palatability of rice straw.

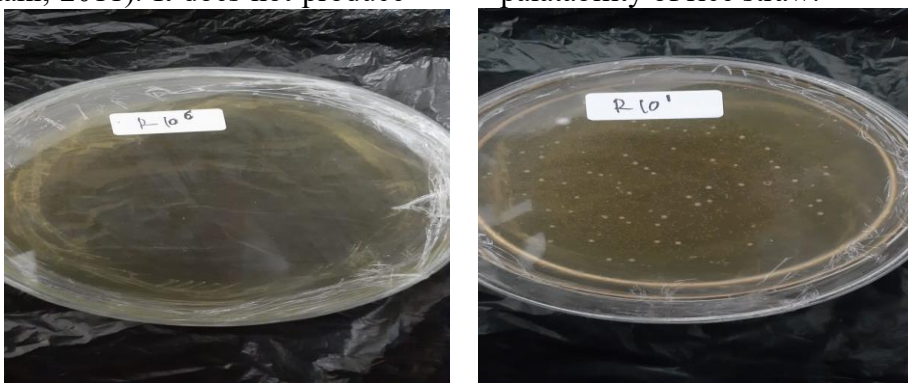
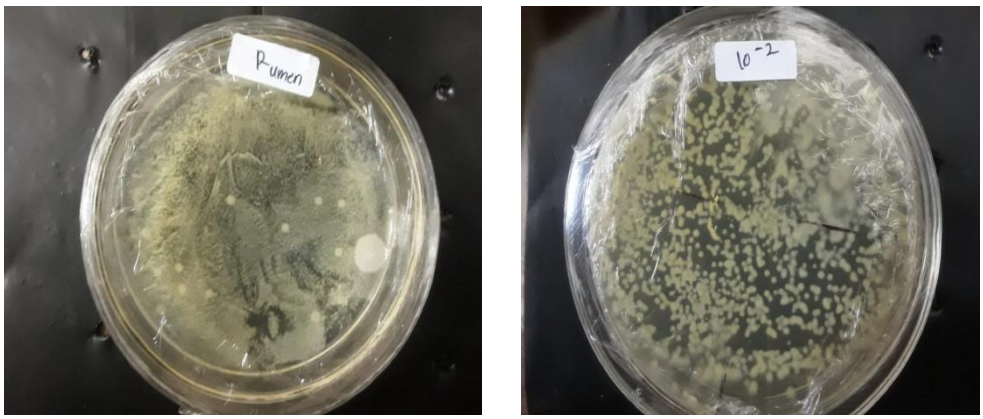


Figure 1. Condition of the inoculum solution before mixing with coconut water and sugar water



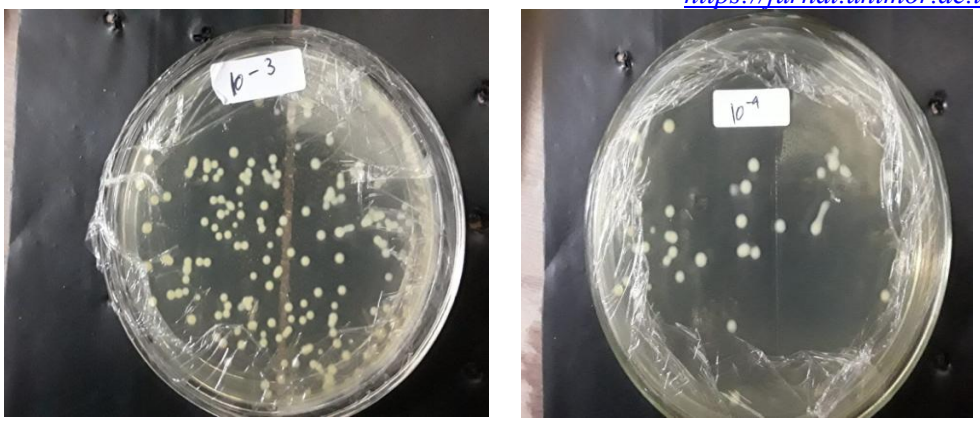


Figure 2. Microorganisms that grew after mixing rumen fluid, coconut water, and sugar water

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus cereus

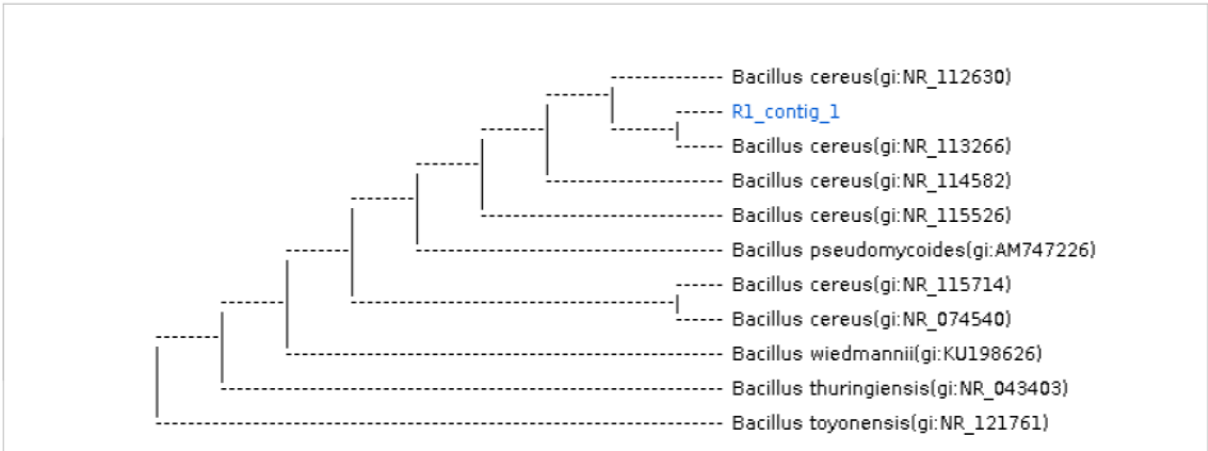


Figure 3. Construction of phylogenetic trees for isolates

Based on the results of this study (Figure 3), the bacteria that grew were identified as *Bacillus* sp. This bacterium can produce extracellular enzymes, not only cellulase but also protease, which hydrolyzes peptide bonds in proteins into amino acids (Yogyaswari *et al*., 2016). Suardana *et al*. (2007) further stated that this bacterium belongs to the lactic acid bacteria group, with broad antimicrobial activity against both Gram-positive and Gram-negative bacteria, and it grows optimally between 48 and 168 hours after inoculation (Gordon *et al*., 1973). These characteristics support the fermentation process of rice straw silage, as indicated by improved nutritional value, reduced fiber content, and increased in vitro digestibility.

The results of this study also indicate that the Bali cattle slaughtered at the Kefamenanu slaughterhouse (source of the rumen fluid sample) primarily consumed forage with a relatively high fiber content. Essentially, the type of feed consumed by livestock influences the types of microbes that develop in the rumen. The presence of *Bacillus* sp. suggests that the animals consumed more fibrous feed such as rice straw and dried grasses (which aligns with the current climatic conditions). This is supported by Suryani *et al*. (2014), who noted that livestock fed with forages high in cellulose content will have higher populations of cellulolytic microbes in the rumen.

Dry Matter Digestibility (DMD) and Organic Matter Digestibility (OMD)

Table 1 shows that the DMD and OMD values of rice straw without the addition of liquid rumen MOL (R0) were only 39.123% and 33.552%, respectively. After adding rice bran as an additive and

liquid rumen MOL, the average DMD and OMD values increased to 50.63% and 44.57% (DM basis). This indicates that the addition of liquid rumen MOL as a bacterial starter can enhance both DMD and OMD of the silage. This finding is supported by Dewi et al. (2012), who reported that fermenting rice straw and corn stover using buffalo rumen contents improved in vitro digestibility. The lower DMD and OMD values in the R0 treatment are due to the absence of rumen MOL, resulting in higher fiber content (lignin, cellulose, hemicellulose) (Aman *et al*., 2022).

According to Table 2, the percentage DMD values were as follows: R0 = 41.06±0.97, R1 = 48.61±3.99, R2 = 50.84±0.93, and R3 = 52.44±0.24. Statistical analysis showed that the treatment had a significant effect (P<0.05) on the DMD value of rice straw silage. The higher the level of rumen MOL inoculum, the higher the DMD value. This is due to the use of liquid rumen MOL, which contributes to the breakdown of fiber bonds, making the structure softer, easier to digest, and more usable by livestock. Suryani *et al*. (2015) stated that feed digestibility is influenced by its chemical composition, with the fibrous fraction having a major effect. A higher fiber fraction results in lower DMD values. This study also showed a decrease in crude fiber content (Sio *et al*., 2022), contributing to increased DMD.

The OMD values (% of DM) obtained in this study were: R0 = 37.19±0.65, R1 = 42.10±3.23, R2 = 44.50±2.03, and R3 = 47.11±1.36. Statistical testing showed that the treatment had a significant effect (P<0.05). The OMD trend followed the same pattern as DMD, since most organic matter is a component of dry matter, excluding ash. The increase in OMD is attributed to the higher crude protein (CP) content in the silage, which supports the growth of microorganisms that digest feed material (Santi *et al*., 2012). The addition of rice bran also contributed to the increased DMD and OMD, as it provides energy for rumen microbes, thereby enhancing digestibility. This is consistent with Surono *et al*. (2003), who stated that rice bran is a source of soluble carbohydrates for lactic acid bacteria during the ensiling process.

Feed digestibility depends on the activity of rumen microorganisms, which play a central role in fermentation (Pendong *et al*., 2022). Rumen microbial activity, in turn, is influenced by the nutritional content of the feed (Wahyuni et al., 2014). Therefore, the higher the DMD and OMD values, the more active the rumen microbes become with increasing levels of rumen MOL. A reduction in the crude fiber content of rice straw also improves organic matter digestibility (Ilham *et al*., 2018). Additionally, fungi present in the rumen fluid contribute significantly to fiber degradation by forming colonies on cellulose tissue in the feed, which opens up the feed structure and facilitates degradation by rumen bacterial enzymes (Suryani *et al*., 2015).

Table 2. In Vitro Digestibility of Rice Straw Silage

Variable	Treatment				
	R0	R1	R2	R3	P-Value
DMD (%)	41.06±0.97^{c}	48.61±3.99^{b}	50.84±0.93^{ab}	52.44±0.24^{a}	<0.001
OMD (%BK)	37.19±0.65^{c}	42.10±3.23^{b}	44.50±2.03^{ab}	47.11±1.36^{a}	<0.001
VFA (mM)	64.49±0.93^{d}	67.45±1.57^{c}	70.11±0.95^{b}	73.00±2.36^{a}	0.208
NH_3 (mM)	4.80±0.02^{d}	6.13±0.73^{c}	6.97±0.62^{b}	7.79±0.38^{a}	0.153

Data are presented as mean ± SD; R0: Rice Straw + 10% Rice Bran + 3% Sugar Water (Without Rumen Fluid MOL / Control); R1: Rice Straw + 10% Rice Bran + 3% Sugar Water + 5% Rumen Fluid MOL; R2: Rice Straw + 10% Rice Bran + 3% Sugar Water + 10% Rumen Fluid MOL; R3: Rice Straw + 10% Rice Bran + 3% Sugar Water + 15% Rumen Fluid MOL; Different superscripts in the same row indicate significant differences (P<0.05);

DMD = Dry Matter Digestibility; OMD = Organic Matter Digestibility; VFA = Volatile Fatty Acids; NH_3 = Ammonia.

VFA dan NH_3

VFA production is one of the indicators of rumen microbial activity in fermenting feed (Bain *et al*., 2015). Table 2 shows that the in vitro VFA production of rice straw silage was as follows: R0 = 64.49±0.93, R1 = 67.45±1.57, R2 = 70.11±0.95, and R3 = 73.00±2.36. The results indicate that the treatment had a significant effect ($P<0.05$). The higher the level of rumen fluid MOL, the greater the VFA production. This occurs because rumen microbes become more active in fermenting rice straw, making it more easily utilized to support their own productivity. VFA is the primary energy source for ruminant animals (Zhen *et al*., 2023.

The VFA concentrations in treatments R2 and R3 are within the normal and optimal range for rumen microbial growth, as stated by Tillman *et al*. (1998), which is between 70–130 mM. In contrast, the VFA levels in R0 and R1 were below optimal, likely due to lower microbial populations and limited capacity to degrade fiber for microbial activity. This is supported by Mardalena. (2015), who stated that low total VFA concentration can be influenced by the availability of non-structural carbohydrates in the feed. Thus, the concentration of VFA can reflect how easily carbohydrates are utilized by rumen microbes—higher VFA levels indicate that more carbohydrates and proteins are being fermented in the rumen, and vice versa. Bata (2008) explained that an increased number of microbes leads to greater fermentation of organic matter into VFA by rumen microbes. Weimer (2022) added that rumen microbes obtain energy, carbon, and nitrogen for their development through fermentation processes carried out by the microbes themselves.

The NH_3 concentration (mM) observed in this study was: R0 = 4.80±0.02, R1 = 6.13±0.73, R2 = 6.97±0.62, and R3 = 7.79±0.38. Statistical analysis showed that the treatment had a significant effect ($P<0.05$). It is evident that higher levels of rumen fluid MOL increase NH_3 concentration. The NH_3 concentrations for all treatments remained within the optimal range to support rumen microbial activity, as suggested by Jin *et al*. (2023), who stated that a minimum of 3.57 mM is required. Microbes utilize NH_3 and also produce lactic acid, which can react with NH_3 (Bata, 2008), leading to increased NH_3 concentration. NH_3 and VFA concentrations in the rumen are influenced by several factors, including protein and energy content in the feed, time after feeding, rumen absorption rate, protein and energy degradation, and microbial utilization (Tarigan *et al*., 2018). This study suggests that the protein and energy content in rice straw are readily degradable by microorganisms.

CONCLUSION

It is concluded that the endophytic bacteria successfully isolated and identified from the rumen fluid of Bali cattle slaughtered at the Kefamenanu slaughterhouse is *Bacillus* sp. The use of rumen fluid-based MOL as an inoculum in the rice straw fermentation process can increase the in vitro digestibility of rice straw.

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