

# The Effect of Gamma-Irradiated Nitrate-Reducing Bacteria in Decreasing the *In Vitro* Production of Methane by Buffalo Rumen Liquid

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## ABSTRACT

Ruminant livestock undeniably contributes to the increase of greenhouse gases by emitting methane. One strategy used to reduce methane emission is by applying nitrate-reducing bacteria (NRB). Utilizing active NRB has a side effect of acidosis in rumen liquid in an uncontrolled condition. Alternatively, NRB can be applied in an inactive form. The aim of this study was to examine the use of gamma irradiation on NRB to be used for decreasing methane production by buffalo rumen liquid. The examination was performed *in vitro* and involved comparison to other treatments, which were active NRB, autoclaved NRB, and without NRB. The result showed that the NRB in either active or the inactive condition affected fermentation of the rumen microorganisms. There were differences in pH values, digestibility of organic matters, ammonia, total and partial volatile fatty acids, and methane production between all treatments after 24 and 48 h of incubation. All treatments showed significant differences for each parameter, except for the gas production ( $p \leq 0.05$ ). The irradiated NRB produced slightly more methane, i.e. 3.0 and 10.4 ml/200 mg after 24 and 48 h incubation, respectively, compared to the active NRB, autoclaved NRB, and no-NRB-addition treatments, i.e., 2.0 and 8.0; 7.8 and 11.7; 8.0 and 12.4 ml/200 mg, respectively. In conclusion, the irradiated NRB has a potency as a supplement feed for buffaloes to reduce methane production without the risk of acidosis in the rumen liquid.

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## INTRODUCTION

The global warming takes place today due to the increasing amount of gas emissions into the atmosphere. Methane (CH<sub>4</sub>) is one of the three primary greenhouse gasses responsible for the temperature elevation that are three times more harmful than the other gasses, i.e., carbon dioxide (CO<sub>2</sub>) and nitrous oxide (N<sub>2</sub>O) [1]. The amount of atmospheric CH<sub>4</sub> has increased 2.5-fold for over the past three centuries [2].

The ruminant livestock sector generates more greenhouse gas for it contributes 26 % of the total methane emission [3]. Most (80-95 %) of the CH<sub>4</sub> is emitted from the process of methanogenesis in the

rumen of the digestive system, and the remaining 5-20 % is from the large intestine. The gas is released into the atmosphere as the animal breathes [4]. The release of methane causes a loss of more than 15 % of the total energy ingested. Therefore, minimizing CH<sub>4</sub> production increases the efficiency of livestock production, while reducing greenhouse gasses creates a better environment [5].

Various strategies in decreasing the enteric CH<sub>4</sub> emission from ruminants have been proposed or under investigation. One approach related to the modification of ruminant feed and microorganisms is by using nitrate-reducing bacteria (NRB). Some studies showed that the NRB can compete with methanogenic bacteria for utilizing hydrogen [6-8]. The addition of active culture of NRB resulted in methane reduction without decreasing the *in vitro* true digestibility (IVTD), as compared to the

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treatments containing nitrate with autoclaved microbial cultures or without any culture. It is revealed that to reduce methane emission, the NRB can be used as probiotic to prevent the accumulation of nitrites when sodium nitrate is used [9].

The NRB in inactive condition has been used to decrease the accumulation of nitrites to the level that is not toxic to the host [10]. Inactivation by using hot steam from an autoclave makes the NRB not grow, and several enzymes inactive, but they can still reduce methane production. This method still has a weakness; therefore, it is necessary to conduct further research using alternative methods, for instance, inactivation of bacteria with gamma ( $\gamma$ ) irradiation. This method inactivates cell growth, but metabolic activities can still run [11]. Preliminary research showed that NRB can be inactivated by  $\gamma$ -irradiation with dose of 1500 Gy.

Therefore, the current study aimed to examine the use of the NRB isolated from the buffalo rumen, in both active and inactive conditions (either by irradiation and autoclaving), to reduce the methane emission.

## EXPERIMENTAL METHODS

### Inactivation of NRB

This study used isolate KS1 as nitrate-reducing bacteria. First, isolate KS1 was inoculated into a modified medium of NRB agar (0.5 g  $\text{KNO}_3$ , 0.1 ml microminerals, 5 ml macrominerals, 3 g yeast extract, and 2 g agar, with a pH of 6.8) [12] and incubated at 39 °C for 48 h in an anaerobic jar. For multiplying the cell culture, the growing cells were inoculated into 30 ml NRB broth (without agar) and incubated at 39 °C, 120 rpm, for 48 h in anaerobic condition with the addition of  $\text{CO}_2$ . Total cell concentration was determined by the dilution method. Before inactivating, the isolate was examined for Gram reaction, cell morphology, nitrate reducing ability, and purity [13]. Then, the verified NRB culture with a total concentration of  $10^6$  cells/ml was inactivated, either by gamma irradiation at a dose of 1.5 kGy with a dose rate of 20 kGy/h, or by autoclaving at 121 °C for 15 minutes.

### Determination of *in vitro* gas production

The syringes containing the substrate were prepared with four treatments, designated as A, B, C, and D, as follows: A is the culture with active or live NRB; B with irradiated NRB; C with autoclaved

NRB; and D without NRB. A total of 3 ml culture, according to the treatments, was added to each of the prepared syringes and incubated at 39 °C for 24 and 48 h. The parameters measured were: pH; ammonia production; total volatile fatty acids (VFA); acetic, propionic, and butyric acid as partial VFA; microbial protein synthesis by radioisotope of  $^{32}\text{P}$  [14]; digestibility of organic matters (DOM); gas production and methane production. The pH was measured by a pH meter; ammonia production was determined by Conway microdiffusion technique; total and partial VFA concentrations were examined by gas chromatography method; digestibility of organic matters was measured by gravimetric method; and concentration of gases was examined by an MRU Gas Analyzer®. Each treatment was performed in triplicates.

### Statistical analysis

The data was analyzed by using one-way analysis of variance which was performed by SPSS Statistics version 20.0 (IBM Inc.). The means comparison by Duncan's Test were applied with the significance level of 0.05.

## RESULTS AND DISCUSSION

The results obtained showed that the NRB, both in active and inactive conditions, affected fermentation by rumen microorganisms. There were differences in pH values, digestibility of organic matters (DOM), ammonia production, total VFA, partial VFA, and  $\text{CH}_4$  production under all treatments after 24 and 48 h of incubation (Table 1). All the treatments showed significant differences for each parameter, except for gas production ( $p \leq 0.05$ ).

Another study reported, differently, that the addition of NRB leads to the increase of pH in rumen liquid [9]. The pH of the cultures in our study tended to decline until 24 h and slightly increased until 48 h of incubation. NRB need more time to adapt in the rumen liquid before they carry out normal activities. Another study showed that the addition of NRB leads to the neutral pH, *i.e.*, 7 [6]. pH is one of the factors that support the success of the fermentation process because it influences the growth and enzymatic activity of microorganisms that play a role in the fermentation process [15]. Therefore, the pH in the four treatments did not cause any significant problems since they were all in the optimal range of pH for rumen microbial activities, *i.e.*, 6.5-7.

**Table 1.** Measurement results of the parameters under the four treatments

Parameter	Time (h)	Treatments			
		A	B	C	D
pH	0	7.2 <sup>a</sup>	7.2 <sup>a</sup>	7.2 <sup>a</sup>	7.2 <sup>a</sup>
	24	6.9 <sup>a</sup>	6.9 <sup>a</sup>	7.0 <sup>a</sup>	6.9 <sup>a</sup>
	48	6.6 <sup>a</sup>	7.0 <sup>c</sup>	6.9 <sup>b</sup>	6.9 <sup>bc</sup>
Amonia (mM)	0	2.4 <sup>a</sup>	2.4 <sup>a</sup>	2.4 <sup>a</sup>	2.4 <sup>a</sup>
	24	2.7 <sup>b</sup>	2.6 <sup>b</sup>	2.4 <sup>a</sup>	2.3 <sup>a</sup>
	48	2.8 <sup>c</sup>	2.6 <sup>a</sup>	2.5 <sup>b</sup>	2.5 <sup>bc</sup>
VFA total (mmol/100 ml)	0	108.1 <sup>a</sup>	107.0 <sup>a</sup>	106.0 <sup>a</sup>	105.7 <sup>a</sup>
	24	88.9 <sup>a</sup>	92.0 <sup>a</sup>	92.3 <sup>a</sup>	115.0 <sup>b</sup>
	48	90.2 <sup>a</sup>	91.7 <sup>a</sup>	88.1 <sup>a</sup>	118.3 <sup>b</sup>
Acetic (mmol/100 ml)	0	69.5	68.9	69.1	70.0
	24	45.9	48.5	51.0	72.0
	48	46.8	49.3	48.4	74.6
Propionic (mmol/100 ml)	0	35.0	34.5	33.5	37.0
	24	39.2	40.0	38.2	38.5
	48	39.6	39.3	36.5	39.1
Butiric (mmol/100 ml)	0	3.6	3.6	3.5	3.7
	24	3.8	3.4	3.0	4.6
	48	3.9	3.1	3.0	4.6
Ratio Acetic : Propionic	0	2.0	2.0	2.1	1.9
	24	1.2	1.2	1.3	1.9
	48	1.2	1.3	1.3	1.9
DOM (%)	24	30.0 <sup>b</sup>	27.5 <sup>c</sup>	32.8 <sup>a</sup>	26.9 <sup>c</sup>
	48	57.8 <sup>a</sup>	57.2 <sup>a</sup>	56.1 <sup>a</sup>	53.4 <sup>b</sup>
Ratio Protozoa : Bacteria	0	2.0 <sup>a</sup>	2.0 <sup>a</sup>	2.0 <sup>a</sup>	2.0 <sup>a</sup>
	24	0.7 <sup>c</sup>	1.3 <sup>b</sup>	1.3 <sup>b</sup>	1.8 <sup>a</sup>
	48	0.8 <sup>a</sup>	0.9 <sup>a</sup>	0.6 <sup>a</sup>	0.8 <sup>a</sup>

A: active NRB; B:  $\gamma$ -irradiated NRB; C: autoclaved NRB; D: without NRB; DOM: Digestibility of Organic Matters. Different superscript letter (<sup>abcd</sup>) through a column shows a significant difference ( $p < 0.05$ ).

The changes in pH value were influenced by the content of ammonia and VFA produced by the rumen microorganisms. Ammonia resulted from the reduction of nitrate by NRB and was derived from protein degradation [16]. The content of ammonia caused the pH to be more alkaline. The content of ammonia in the treatment with either active or inactive NRB was higher than that without the addition of NRB. These results confirmed that the inactivated NRB have metabolic ability. On the contrary, the VFA values, both from the active or inactive NRB, were lower than those without the addition of NRB. It was assumed that the NRB stimulated the increasing of rumen microbial growth. The VFA values decreased since they were assimilated into monomers in synthesizing carbohydrate, lipid, and protein for the growth of rumen microorganisms [17].

The composition of the partial VFA affected methane production by rumen microorganisms, especially the group of methanogens. Acetic acid was the highest content of partial VFA resulting from all of the treatments. There was an intermediate compound produced by types of acetotrophic

bacteria before the formation of methane [18]. The presence of propionic and butyric acids indicated the inhibition of acetate production, which resulted in lower production of methane. Reduction of methane can be detected from the decrease in acetate and propionate ratio [18]. In this study, the acetate and propionate ratio decreased in all treatments. The ratio after 48 h of incubation in the order from the lowest to the highest was the treatment of active (1.18), irradiated (1.25), autoclaved (1.33), and without NRB (1.91). The NRB obtained their final electron acceptor as hydrogen from acetic acid, then transferred them to the denitrification process that requires hydrogen to make some ammonia [20].

The results from the measurement of DOM supported the detection of ammonia and VFA. The DOM value showed polysaccharides, lipids, and proteins contained in forage sorghum substrate degraded by microorganisms in the rumen liquid. The addition of both active and inactive NRB lead the DOM value higher than those without the addition of NRB. The DOM value after 48 h of incubation in the order from the highest to the lowest were the treatment of active (57.8 %), irradiated (57.2 %), autoclaved (56.1 %), and without NRB (53.4 %).

The bacteria and protozoa in the rumen liquid are indigenous biota that might be affected by the addition of the active or inactive NRB. The protozoa:bacteria ratios from the treatment of either active or inactive NRB were lower than those without the addition of NRB. This shows that the NRB suppressed the growth of protozoa and may result in a decrease of methane production. The rumen protozoa and bacteria are well-known, and they have a mutualistic relationship in which the protozoa provide habitat for methanogenic bacteria. Methanogenic bacteria are associated with protozoa because of their role in the process of methanogenesis in the hydrogen transfer.  $H_2$  and  $CO_2$  from the feed fermentation in the rumen are converted into methane by methanogenic bacteria through hydrogen transfer. Consequently, the decline in protozoa community may cause a decrease of methane production [21].

From the 8<sup>th</sup> to the 24<sup>th</sup> hour of fermentation, the total gas increased in a high rate, then turned into steady stationary until the end of incubation (Fig. 1(a)). The pattern of gas production under all treatments were not significantly different. However, methane production was different among all treatments (Fig. 1(b)). After 24 and 48 h of incubation, the order of the methane production from the lowest to the highest was the treatment of active (2.0 and 8.0 ml/200 mg), irradiated (3.0 and

10.4 ml/200 mg), autoclaved (7.8 and 11.7 ml/200 mg), and without NRB (8.0 and 12.4 ml/200 mg).

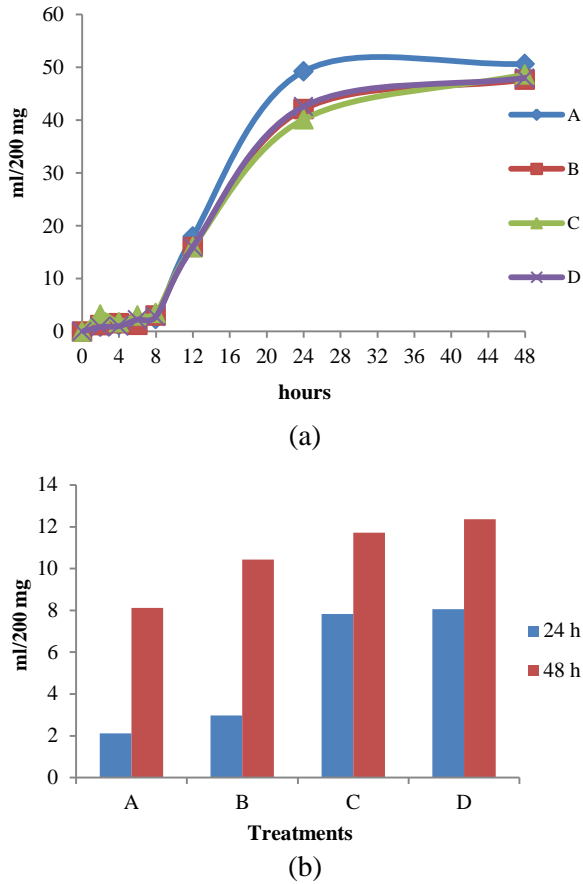


Fig. 1. Total gas (a) and methane production (b).

The percentages of differences in methane production between active and irradiated NRB were about 10.7 % and 18.6 % after 24 and 48 h incubation, respectively. The values were not significant for application as ruminant supplement. This proves that  $\gamma$ -irradiated NRB has the potential to be used as a reducing agent despite lower activity than active NRB because it can prevent the effects of acidosis in rumen liquid.

Increasing the population density of nitrate-reducing bacteria in the presence of nitrates was an effective means of inhibiting methanogenesis [6]. The addition of NRB such as *Escherichia coli* to the diet of sheep reduced the accumulation of nitrite in the rumen, though a further reduction in methane production was not detected [22]. Those responses differ from ours in that we observed a further decline in methanogenesis as a result of the addition of NRB. Besides, the inoculated isolate prevented the accumulation of nitrite; it is suggested that they possibly also protect against nitrite toxicity when nitrates are used. This process gives the benefit of lowering methane production in the rumen.

A model is proposed to illustrate the metabolisms of the active and inactive NRB (Fig. 2). When the active NRB are inoculated into rumen liquid, they will have the potency to grow and release the enzyme. Meanwhile, when the inactive NRB that had been either irradiated or autoclaved are inoculated into the rumen, they lose the ability to grow but still have the ability to produce the enzyme. The  $\gamma$ -irradiated NRB has a higher capacity to produce the enzyme than the autoclaved NRB. However, the damage by radiation is not as severe as that by autoclaving. The hot steam and pressure from the autoclaving method bring about cell death, and the enzymes become denatured. Consequently, the enzyme production and activity of the  $\gamma$ -irradiated NRB are higher; thus, they survive better in the rumen liquid than the autoclaved NRB. Therefore, when the  $\gamma$ -irradiated NRB are applied as a part of a feed supplement, they can suppress methane production in ruminants.

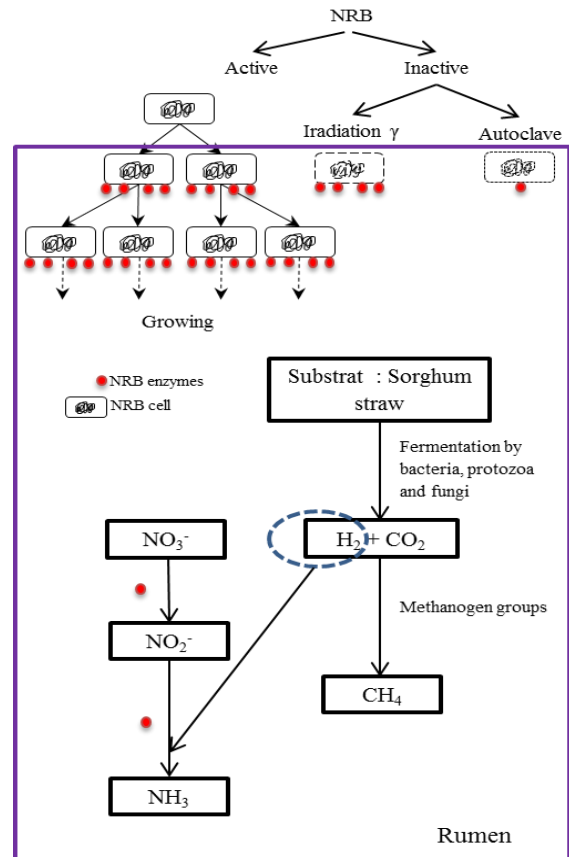


Fig. 2. The proposed model for active and inactive NRB metabolism in the rumen.

## CONCLUSION

The  $\gamma$ -irradiated NRB has a potency for use as a supplement feed for buffaloes to decrease methane production without the risk of acidosis in the rumen liquid.

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