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EFFECTS OF BIONANOMINERAL SELENIUM (BIONANO-SE) AND PROBIOTICS INCLUSION TO RATION ON *IN VITRO* RUMEN FERMENTATION CHARACTERISTICS

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ABSTRACT

The absorption of the minerals produced by the probiotics in ruminants is expected to improve the absorption of the required nutrients. The study used different dietary rations with different percentage of concentrates: forages, different selenium doses, different locations and different strains of lactic acid bacteria as probiotics. The trials were performed with three repetitions using a factorial block (2x2x3x4). Samples were incubated for 48 h using *in vitro* incubation system. The parameters were pH, the kinetics of gas production, in vitro dry matter digestibility (IVDMD), in vitro organic matter digestibility (IVOMD), and NH_3-N concentration. The data were analyzed using the ANOVA, followed by the Duncan test. The results showed altered fermentation characteristics, while BioNano-Se at 25 ppm has significantly increased the rumen pH, gas production rate, IVDMD, IVOMD and decreased the total gas production significantly, but did not affect the ammonia concentration. High concentrate rations (R1) produces significantly lower pH ($P < 0.05$), but higher gas production rate and digestibility ($P < 0.05$). Higher dose of BioNano-Se significantly decreased gas production rate and ammonia production ($P < 0.05$). In conclusion, BioNano-Se and probiotics can alter rumen fermentation process.

Keywords: bionanomineral, selenium, lactic acid bacteria, in vitro rumen, fermentation characteristic.
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INTRODUCTION

The growth of the rumen microbiome is always related to the availabilit y of the balance nutrients in the rumen which including the existence of the macro-minerals and micro-minerals. The functions of these minerals are very important, particularly for metabolism, but it has received little attention. Feeding with an optimum combination of minerals would significantly impact microbial biodiversity and subsequently enhance livestock productivity (Biswas and Biswas, 2012). Selenium is among the essential minerals that can be found in the rumen especially for metabolism, because the bacteria are capable to use the Se for bacterial synthetic machinery, cellular energy production and detoxification processes during the metabolism of the digested nutrient (Serra *et al.,* 1994; Staicu and Barton, 2017).

The term biomineral refers to the mineral forms produced by microorganisms (Bhuvaneshwari *et al.,* 2011). The previous studies concerning the application of nanoparticles in animal nutrition have demonstrated that the nanoparticles can be absorbed better by the gastrointestinal tract resulting in improving the quality of the animal products (Kolkol and Wojnarowski, 2018). Some minerals important to ruminants can be found in the soil, but not all of them. However, Se is rarely found in the soil especially under anaerobic conditions (Eszenyi *et al.,* 2011). Selenium (Se) is an essential microelement for all life forms and plays a crucial role in livestock productivity (Białek and Czauderna, 2019). Several studies have examined that Se supplementation promotes the rumen microorganism populations and improves the rumen's digestive efficiency (Shi *et al*., 2011).

Organic Se or Bio-Se reported has a good application prospect because it is safer and more efficient than inorganic Se (Kišidayová *et al*., 2014), and changed the rumen microflora (Cui *et al*., 2021). BioNano-Se was synthesized by lactic acid bacteria (LAB) throughout the process of biosynthesis**.** The addition of the BioNano-Se obtained from the LAB, is expected to produce a beneficial effect on the rumen fermentation. It was

reported that the supplementation of the Nano-Se has significantly increased both the antioxidant properties and the activity of the S-transferase glutathione due to the small-size of the Se particles (Rajendran, 2013).

The optimal doses of BioNano-Se addition are needed to investigate in order to supply the ruminants with the highest availability of Se which can be absorbed effectively by the rumen microbes. Bionanomineral supplementation with the diets with the available Se aids in effectively improving the nutrient absorption in the livestock. Giving nano-Se of 10 ppm can increase sheep productivity (Kojouri *et al.,* 2012). However, it can be assumed that the need for the nano-Se in the cattle is higher than in the sheep. Rajendran (2013) proposed that the supplementation with (3 g/kg dietary DM) of the nano-Se could enhance the fermentation process resulting in the high activity of rumen microbes and consequently the feed utilization especially if the nano-Se is produced by the LAB. The LAB use as bio-cell factory in BioNano-Se production also has a function as probiotics in the rumen fermentation. The effects of the supplementation of the BioNano-Se can be analyze by measuring the fermentation variables. Therefore, this study aimed to evaluate the effects of the BioNano-Se on the rumen fermentation characteristics.

MATERIALS AND METHODS

Bionanomineral Production: The production of the Bio-Se was proceeded using an isolated LAB (TSD-10 (B1), SPCE-39 (B2), IA-2 (B3), and DR-162 (B4)) strains in accordance with the method of (Eszenyi *et al*., 2011) with a bit of modification. The bacterial strains were procured from Research Center for Biotechnology-National Research and Innovation Agency (BRIN), Cibinong, West Java, Indonesia. Selenium oxide (SeO_3^{2-}) of 0.0402 g were weighed to obtain 25 ppm (D1), while 0.0804 g of Selenium oxide (SeO_3^{2-}) were weighed to obtain 50 ppm (D2). Then an isolated bacterial culture was done in accordance with Nurfitriani *et al.* (2020). Then each Se sample was added into isolates culture medium. The Erlenmeyer was sealed with cotton, gauze, and rubber bands. The insulated culture was incubated room temperature for three days using a shaking water bath. After 3 days of incubation, the isolated culture was weighed again and the results were recorded as location of BioNano-Se (L3). Then after that, the culture was centrifuged at 600 rpm for 10 minutes. The obtained supernatant, was separated from the formed sediment culture. The culture sediment was washed with autoclaved distilled water to obtain the intracellular selenium in a Nanominaral particle as location of BioNano-Se (L1). The sediment culture was then centrifuged at 2500 rpm for 15 minutes. The precipitate was discarded, while the supernatant was centrifuged at 13000 rpm for 15 minutes. The obtained sediment, an intracellular selenium compound, was washed three times with sterile distilled water. The supernatant culture was centrifuged at 10000 rpm for 15 minutes. The supernatant and the formed pellet were separated. The pellets were washed with sterile distilled water three times and centrifuged at 13000 rpm for 10 minutes. The precipitate form was the extracellular as location of BioNano-Se (L2) which was deposited with the supernatant. All these extracellular and intracellular compounds were weighted and the results were recorded. The production of the BioNano-Se was done by incubating the isolated culture in a 5 ml reaction tube for one day. After one day of incubation, the selenium was added to the 50 ml of the re-grown liquid medium and incubated for one day.

***In Vitro* Rumen Fermentation :** The rumen liquids were obtained from two fistulated local Ongole cross cattle with an average body weight of 250±30kg). These cattle were handled and maintained following the approved protocol of the animal health care and welfare of the Indonesian Animal Care and Use Committee (Indonesian Institute of Sciences No. 879/WK/HK/XI/2015). The rumen liquor was collected at 07.00 before the morning feed. The collected rumen liquor and the buffer solution were mixed with 2:1 (buffer:rumen). The samples were inserted into 50 ml of the rumen buffer solution. All the samples were incubated for 48 h using an in vitro incubation system at 39^{o}C in accordance with a modified method of (Theodorou *et al*., 1994). All the samples were incubated using 0.5 g of the substrates. Two vials were used as a blank fill in a rumen buffer. The composition of the substrate used in this study was R1; 70% concentrate and 30% grass, and R2 30% Concentrate and 70% grass (Table 1). The detail of treatment consisted of four factors with three replication, namely 1) ration; R1 (70% concentrate + 30% forage) and R2 (30% concentrate + 70% forage), 2) dose; D1 (25 ppm), D2 (50 ppm), 3) location; L1 (intracellular), L2 (extracellular), and L3 (direct of whole sample), and 4) type of bacteria factor; B1 (TSD-10), B2 (SPCE-39), B3 (IA-2), and B4 (DR-162). The variables observed in this study were as follows: 1). Gas production and gas production kinetics were measured at 2, 4, 6, 8,12, 24, and 48 h fermentation, 2) Gas production kinetics were calculated using the equation ($p = a + b\ (1\text{-}e^{-c.t})$,) of Orskov & Mcdonald (1979). Where, p is gas production at certain interval, a is the gas production (ml) at 0 h, b is the asymptotic gas production (ml), c is the gas production rate (ml.h^{-1}), and t is the incubation period), 3) The rumen pH values were recorded using a pH meter (Cyberscan pH 310 Eutech), 4) The digestibility (DMD and OMD) was calculated following Tilley and Terry (1963), and 5) Ammonia concentration was subjected to Conway and O'Malley (1942).

Data analysis : Data were arranged in a factorial randomized block design (2x2x3x4) with three repetitions

consisting of four factors, namely ration (portion of concentrate and forage), dose (concentration of Se), location (Intracellular, extracellular, and whole sample), and strains of bacteria (four LAB strains). Statistical model used in this research was;

$$Y_{ijklm} = \mu + \tau_{1i} + \tau_{2j} + \tau_{3k} + \tau_{4l} + \tau_{1i}\tau_{2j} + \tau_{1i}\tau_{3k} + \tau_{1i}\tau_{4l} + \tau_{2j}\tau_{3k} + \tau_{2j}\tau_{4l} + \tau_{3k}\tau_{4l} + \varepsilon_{ijklm}$$

Y_{ijklm} = the dependent variable or response variable
μ = the true value of the intercept
τ_{1i} = treatment-1 (ration) and block-i
τ_{2j} = treatment-2 (dose) and block-j
τ_{3k} = treatment-3 (location) and block-k
τ_{4l} = treatment-4 (bacteria) and block-l
$\tau_{1i}\tau_{2j}$ = treatment interactions (ration and dose)
$\tau_{1i}\tau_{3k}$ = treatment interactions (ration and location)
$\tau_{1i}\tau_{4l}$ = treatment interactions (ration and bacteria)
$\tau_{2j}\tau_{3k}$ = treatment interactions (dose and location)
$\tau_{2j}\tau_{4l}$ = treatment interactions (dose and bacteria)
$\tau_{3k}\tau_{4l}$ = treatment interactions (location and bacteria)
ε_{ijklm} = the error term.

Data were analyzed using analysis of variance (ANOVA) and then continued with the Duncan's Multiple Range test. The level of significance was accepted if *P*-value < 0.05. All the analysis procedures were done using SAS version 9.4 applications.

RESULTS AND DISCUSSION

The feed formulation and the experimental diets are presented in Table 1. The composition of concentrate and forage in the R1 and R2 treatments was based on the crude protein balance.

The results showed that the administration of rations with different formulations had a significant effect on the pH value (P < 0.05). The ration with high concentrate resulted in a lower pH (5.78) than the high forage ration (6.08). High concentrate rations will increase lactic acid production and reduce the population of fiber-degrading bacteria in the rumen (Ramos *et al*., 2021). This is in line with the study results, which suggested that the percentage of concentrate of 60% of the ration increased lactic acid so that there was a decrease in the pH value. High-concentrated feeds consist of a group of starches that are included in easily digestible carbohydrates, so they can be degraded quickly by bacteria that have glucosidase activity and produce lactic acid, high lactic acid will cause a decrease in the rumen pH (Liu *et al*., 2020; Matthews *et al*., 2019).

The normal rumen pH values range from 5.87-6.76 (Debevere *et al*., 2020). Meanwhile, the treatment of dose, location, and type of bacteria used in BioNano-Se production did not significantly affect the pH value. This response was similar to the research results from Shi *et al*. (2011) that the administration of nano-Se of 0.3 – 6 g/kg DM did not affect the pH value, which was analyzed using a linear model. There is no significant effect of inclusion of BioNano-Se, it is suspected that there are other mineral elements in the ration with higher levels in the rumen, so that the utilization of BioNano-Se in the rumen has not been seen optimally (Diyabalanage *et al*., 2021). Goff (2018) stated that absorption of the mineral Se occurs in the gastrointestinal mucosa and will enter the blood vessels. This strengthens that the administration of BioNano-Se was not seen in the pH value parameters and other parameters in the rumen. The results of the analysis between treatments showed that there was no significant interaction effect on all observed variables (Table 2 and 3).

Table 1. The feed formulation and chemical composition of the experimental diets.

Formulation (%)	R1	R2
Pollard	25	5
Cassava Press Waste	13	5
Molasses	1.8	1.8
Coconut Meal	15	3
Soybean Meal	5	10
Rice bran	10	5
$CaCO_3$	0.2	0.2
Forages (*Pennietum purpureum*)	30	70
Chemical composition (%)		
Dry Matter (DM)	91.22	96.31
Ash (%DM)	4.18	3.07
Crude Protein (CP %DM)	14.92	14.30
Ether Extract (EE %DM)	1.84	2.05
Crude Fiber (CF %DM)	25.88	32.34
TDN* (%DM)	66.29	62.02

*TDN= total digestible nutrients. Calculated according Hartadi *et al.,* (1980), NFE; Nitrogen-free Extract, TDN = 92.464 – (3.338 x CF) – (6.945 x EE) – (0.762 x NFE) + (1.115 x CP) + (0.031 x CF^2) – (0.133 x EE^2) + (0.036 x CF x NFE) + (0.207 x EE x NFE) + (0.1 x EE x CP) – (0.022 x EE^2 x CP).

Gas production is an indicator of carbohydrate degradation by microbes in the rumen (Almaraz-Buendía *et al*., 2018). Table 2 shows that there is no significant effect from rations, dose, location, and strain of bacteria used in the production of BioNano-Se on the total gas values and a+b. The kinetics of gas production (Figure 1) also confirmed that gas production was not affected by any treatments in this research.

The kinetics of gas production shows the results of carbohydrate degradation by rumen microbes. The effect of the addition of selenium for ruminants is still varied, generally based on the binding of selenium to feed components in the rumen (Goff, 2018). In addition, ruminants have lower absorption of Se minerals than non-

ruminants. This difference occurs due to the reduction process of selenite and selenate compounds in selenide, which is less available in ruminants than in non-ruminants (Meschy, 2010). This absorption process occurs by simple diffusion according to the amino acid absorption mechanism (Mehdi *et al*., 2013). Other factors that affect the fermentation yield of selenium in the rumen are the presence of other microminerals and the technique used (Almaraz-Buendía *et al*., 2018). The use of different rations and doses of BioNano-Se significantly affected the rate of gas formation (c value). The addition of a dose of 25 ppm showed a faster gas formation rate than 50 ppm. This occurs because of the effect of BioNano-Se in the first hour of the feed degradation process, but then the benefits are not visible so that the total gas production and the a+b value show no significant results.

The use of different ration treatments affected the digestibility of dry matter and organic matter ($P < 0.05$). High concentrate rations provide higher digestibility compared to high forage rations. This is in accordance with Kumar *et al*. (2013), the higher the percentage of concentrate in the ration, the higher the dry matter digestibility of the ration in the rumen. High concentrate rations have non-structural carbohydrate compounds that are easily degraded, and they are easily digested faster than forages that have more structural carbohydrates. Higher organic matter digestibility occurred in rations containing more grain feed ingredients (Lima *et al*., 2016).

Table 2. The effects of the treatments on the rumen pH value, gas production, and gas production kinetics.

Treatment	Variables observed			
	pH	Gas Total (ml)	a+b (ml)	c (ml. h^{-1})
Ration				
R1	5.78[b]	87.11	88.23[b]	0.078[a]
R2	6.08[a]	86.12	96.47[a]	0.048[b]
P-Value	<0.01	0.54	<0.01	<0.01
Dose				
D1	5.94	85.81	91.27	0.065[a]
D2	5.93	87.42	93.43	0.062[b]
P-Value	0.77	0.32	0.15	0.01
Location				
L1	5.94	85.62	91.33	0.065
L2	5.93	86.85	93.53	0.062
L3	5.93	87.37	92.19	0.064
P-Value	0.91	0.66	0.48	0.21
Strain of LAB				
B1	5.93	86.26	91.51	0.064
B2	5.93	87.54	93.30	0.064
B3	5.95	85.64	92.17	0.061
B4	5.91	87.03	92.41	0.063
p-Value	0.87	0.85	0.86	0.51
Interaction		p-Value		
Ration*Dose	0.50	0.58	0.73	0.01
Ration*Location	0.96	0.57	0.85	0.01
Ration*Strain of Bacteria	0.87	0.84	0.91	0.67
Dose*Location	0.24	0.83	0.80	0.81
Dose*Strain of LAB	0.96	1.00	0.85	0.68
Location* Strain of LAB	0.99	0.94	0.89	0.93

Mean in the same column with different superscripts differ significantly ($P < 0.05$). R1 (70 % concentrate + 30 % forage) and R2 (30 % concentrate + 70 % forage), D1 (25 ppm), D2 (50 ppm), L1 (intracellular), L2 (extracellular), and L3 (direct), B1 (TSD 10), B2 (SPCE 39), B3 (IA2), and B4 (DR162), LAB; lactic acid bacteria, (a+b); potential gas production, c; gas production rate.

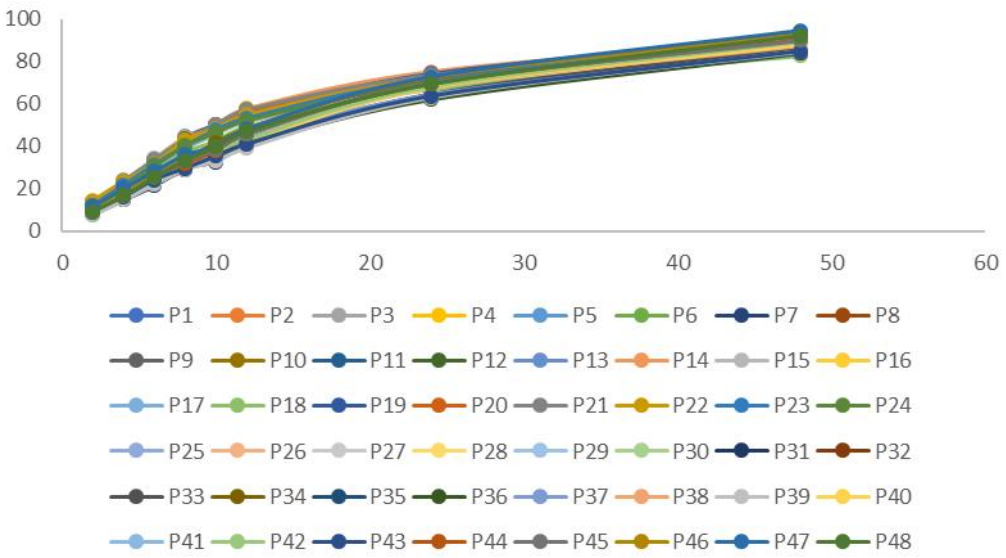

Figure 1. Kinetics Gas Production All Treatments

Table 3. The effects of the different treatments on the nutrient digestibility and the ammonia concentration.

Treatments	Variables observed		
	DMD (%)	OMD (%)	NH_3-N (mM)
Ration			
R1	66.58[a]	66.69[a]	21.27
R2	56.00[b]	58.89[b]	21.24
p-Value	<0.01	<0.01	0.98
Dose			
D1	62.08	63.59	22.93[a]
D2	60.50	61.99	19.59[b]
p-Value	0.36	0.26	<0.01
Location			
L1	60.99	62.07	20.97
L2	61.63	63.33	21.16
L3	61.25	62.97	21.65
p-Value	0.95	0.75	0.85
Strain of LAB			
B1	60.19	62.19	20.84
B2	61.89	63.30	21.94
B3	60.96	61.99	20.95
B4	62.12	63.67	21.30
p-Value	0.85	0.81	0.87
Interaction		**p-Value**	
Ration*Dose	0.25	0.15	0.98
Ration*Location	0.02	0.02	0.64
Ration*Strain of LAB	0.68	0.61	0.83
Dose*Location	0.29	0.61	0.54
Dose*Strain of LAB	0.97	0.69	0.95
Location* Strain of LAB	0.91	0.83	0.42

Mean in the same column with different superscripts differ significantly ($P < 0.05$). R1 (70 % concentrate + 30 % forage) and R2 (30 % concentrate + 70 % forage), D1 (25 ppm), D2 (50 ppm), L1 (intracellular), L2 (extracellular), and L3 (direct), B1 (TSD 10), B2 (SPCE 39), B3 (IA2) and B4 (DR162), DMD; dry matter digestibility, OMD; organic matter digestibility, LAB; lactic acid bacteria

The propionate can inhibit methane production due to the utilization of hydrogen, but excessive propionate conditions can cause ketosis and acidosis in the rumen (Lima *et al*., 2016). Different dose, location, and LAB strain of BioNano-Se did not affect the digestibility of dry matter and organic matter. The BioNano-Se minerals are utilized by microbes for their growth so that they have not been utilized for livestock production. The bacteria and archaea groups utilize Se minerals as electron acceptors during the anaerobic respiration process to form nanospheres which are insoluble forms of Se (Almaraz-Buendía *et al*., 2018). This causes BioNano-Se cannot bind to other compounds so that it is not optimally absorbed into the post-rumen.

Ammonia is representative of the results of protein degradation by rumen microbes. The different rations did not show a significant change ($P > 0.05$) on the levels of NH_3-N. Meanwhile, the administration of BioNano-Se with different doses gave a significant effect ($P < 0.05$) but did not show a significant effect on the administration of different locations and bacterial strains. A similar response was found in Eun *et al*. (2013) study, which gives 50 mg kg^{-1} Se does not produce a significant effect on ammonia production. The increase in ammonia production in a dose of 50 ppm was thought to be due to protein selenium binding. Bond *et al*. (2019) reported that the presence of selenium-protein bonds resulted from the isolation of the rumen cell wall in the plasma membrane. This indicates that some BioNano-Se binds to single proteins and is absorbed through the rumen wall, but some are converted to insoluble Se by bacterial and archaeal activity (Almaraz-Buendía *et al*., 2018). This assumption needs to be further analyzed regarding the absorption of BioNano-Se, specifically by identifying the rumen cell wall and isolating bacteria and archaea in the rumen.

Gas production and gas production rate are usually in line with the rumen fermentation process can be used as an indicator in adding probiotics on rumen conditions. The constant a + b is the assumption of the maximum amount of gas production of infinite t (time), while the value of c is the gas production rate constant for the insoluble fraction. Although there was no significant difference in the gas production among the ration, the highest (a+b) was scored by R2, while the highest gas production rate was produced by R2. This means that the substrates can vary in the gas, the total production and the kinetics of the gas production. This could be because of the difference in the chemical composition of the substrates.

The extent rate (c value) was linearly increased with the increasing level of concentrate (Anantasook and Wanapat, 2012). This is because the composition of digestible carbohydrates was high in the ration so that it is faster to be degraded by microbes and results in faster gas production than rations high in forage. A dose of 25 ppm (D1) BioNano-Se shows the highest c value. Selenium supplementation suggests a greater microbial fermentation rate in the rumen (Wei *et al.,* 2019). The addition of Nano-Selenium could significantly improve rumen fermentation (Xun *et al.,* 2012). The gas production rate was decreased with 50 ppm addition of BioNano-Se, which is because of inhibitor activity of rumen microbes. Ruminal microbes reduced much of the dietary Se to insoluble forms (Shi *et al.,* 2011).

The reduction of the gas production rate is because of the reduction of the insoluble fraction in line with the addition of the BioNano-Se. The effect of direct addition BioNano-Se which was given without intracellular and extracellular separation was assumed to be influenced by bacteria that were still growing in the sample, which that capable of increasing the feed degradation in the rumen.

The highest DMD and OMD showed with the treatment high concentrate (R1), 25 ppm dose (D1), and direct BioNano-Se production (L3). Shi *et al*., (2011) reported that the inclusion of nano-Se at 3 g/kg DM base has improved that nutrient digestibility. BioNano-Se was proposed to stimulate the activity of the rumen microbes, digestive microorganisms and enzyme activity. The decreasing of dry matter and organic matter digestibility in 50 ppm was assumed to occur due to chemical bond reactions between bionanomineral Se and other compounds contained in rumen liquor, so the inhibition of rumen microbe in degrading feed occurred. One of the chemical compounds that can inhibited Se was Calcium (Ca). Harrison and Russell (1984) reported that the existence of the Ca in the ration can inhibit the absorption of Se, therefore it reduces dry matter digestibility. According to Bell and Vallee (2009) Se mineral was able to convert the form of protein bonds as with a Cd where previously Cd-tuning in the form of protein with low molecular weight converted to high molecular weight.

Conclusion: The BioNano-Se with doses 25 ppm have a function as stimulator in the rumen fermentation. High concentrate rations (R1) produces significantly higher gas production rate and digestibility. Higher dose of BioNano-Se (50 ppm) significantly decreased gas production rate and ammonia production.

Conflict of interest: The authors have declared that there is no conflict of interest.

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