Efficiency of Bark Extract of *Azadirachta indica* on Improving Ruminal Ammonia – Nitrogen Utilization in Ruminants: An *In vitro* study

ABSTRACT

Aims: An in *vitro gas* protocol was performed to assess the potential of Commercial bark extract of *Azadirachta indica* (BEA) on improving ruminal ammonia (NH_3) utilization in ruminants.

Study design: The experiment consisted with three (3) treatments, each made up with 16 incubation bottles i.e. four (4) replicates for control (no addition of BEA, contained mixed contents of basal diet, rumen fluid and buffer solution), four (4) replicates (mixture of basal diet, rumen fluid, buffer solution and 100 mg/l of BEA), four (4) replicates (mixture of basal diet, rumen fluid, buffer solution and 200 mg/l of BEA) and four (4) replicates (mixture of basal diet, rumen fluid, buffer solution and 400 mg/l of BEA).

Methodology: All three (3) treatments were incubated at a temperature of 39^{o}C. Treatment 1 was incubated for 12 hours, treatment 2, (24 hours) and treatment 3 (48 hours). During *in vitro* incubation, gas production was measured using pressure transducer after 3, 6, 9, 12, 24 and 36 hours and also pH by using pH meter after 12, 24 and 48 hours in order to assess fermentation process. After pH measurement, incubation was stopped and samples were collected from each replicate across each treatment and stored in the refrigerator for some days before were analysed for NH_3 determination using microtiter plate reader. GenStat 15th edition (version 15.1) software was used to analyze the data and statistical method of analysis of variance (ANOVA) i.e. one – way ANOVA was preferred to compare means between treatments.

Results: No statistical differences ($p > .05$) in ammonia production after 12, 24 and 48 hours of *in vitro* fermentation. Additionally, there was no pH variation after 12 hours of *in vitro* incubation, however pH at level of 200 mg/l was higher ($p < .05$) compared to other treatment levels. After 48 hours, pH at level of 400 mg/l was lower ($p < .05$) than the rest levels. Nonetheless, after 6, 9, 12, 24 and 36 hours of fermentation, BEA reduced gas production in all levels except at the level of 400 mg/l in which gas production were statistically similar with control after 24 and 36 hours of *in vitro* fermentation.

Conclusion: BEA at concentrations of 100, 200 and 400ml/g, cannot enhance ruminal ammonia utilization but can affects fermentation *in vitro*. This might be caused by the presence of bioactive ingredients in *Azadirachta indica* which cannot affects hyper ammonia-producing bacteria but can limits growth of other ruminal microbes which digest fiber.

Keywords: [Ammonia, Ammonia – Nitrogen, Azadirachta indica, Bark extract, PH meter, Ruminal]

1. INTRODUCTION

Nitrogen in ruminant diet is largely found in natural plants, animal protein sources and nitrogen compounds which are also known as Non Protein Nitrogen (NPN) such as urea (CH_4N_2O), urea phosphate ($CH_7N_2O_5P$), biuret uric acid and other ammonia compounds which are not protein [1]. Ruminants use the available form of dietary nitrogen (N) as a protein source [2] by using microorganisms in the rumen namely; bacteria, protozoa, fungi, archaea and viruses which are also responsible to produce enzymes necessary for fiber digestion to yield energy in the form of short chain fatty acids; notably acetate, propionate and butyrate [3]. This makes ruminant to have an ability to convert low quality feedstuffs i.e. NPN into high quality protein in beef and milk [4,47,48,49] and now days it is possible to replace portion of high quality ruminant dietary protein such as soybean meal which is expensive with NPN [3,5].

When ruminants ingest NPN sources, microorganisms in the rumen produce urease which breakdown NPN into NH_3 which combine with carbohydrate-derived keto acids to form amino acids (AA) of which 50 – 80% are absorbed in the small intestine as microbial protein [6–8]. However, excessive feeding of NPN source particularly urea in ruminants result into toxicosis due to the formation of high quantity of NH_3 in the rumen and may lead to muscle tremors, incoordination, respiratory distress, recumbency and suddenly deaths [9,10]. When ruminants feed on dietary source of protein, ruminal microbes produce proteases and peptides enzymes which breakdown peptide bonds to form AA and then, deaminate the AA by removing the amino group into NH_3 and use the NH_3 to synthesize their own microbial protein which undergo further digestion into AA and assimilated in the small intestine [11]. If there is limited energy availability in the rumen and the supply of AA exceed amount required by the body, then ruminal bacteria deaminate the AA to form NH_3 which then transformed by the liver into urea and excreted via urine [12] and other amount of urea undergo recycling via ruminal wall and salivary secretion [13].

However, ruminants have poor nitrogen use efficiency which is between 13 to 31% and also have inefficiency dietary protein utilization compared to monogastric animals [12,14,15]. This means high level amount of nitrogen in the feed is converted to NH_3 and expel in the form of urea in the urine and faeces and lead to emission of greenhouse gases and cause environmental pollution [16,17]. In recent years, plant extracts have brought prospect on improving ruminal NH_3 utilization and improve animal performances while minimizing greenhouse gases emission from ruminants and mitigates environment pollution [18].

Studies have been conducted to improve performances and productivity in ruminants as well as to optimize NH_3 utilization in the rumen by using plant extracts however, with minimal significant achievements. For the example Chanu et al [19] revealed that the blended eucalyptus oil and aqueous extract of root of *Glycyrrhiza glabra* lower rate of ruminal NH_3 production and increase nitrogen utilization efficiency and performance without affecting fiber digestibility in Murrah buffalo *(Bubalus bubalis).* Furthermore, by using *in vitro* Hohenheim Gas Test Protocol, Kapp-Bitter et al [20] proved that 7 out of 35 mature temperate-climate herbaceous meadow plant species have shown to lower ruminal NH_3 concentration without affecting fermentation after 24 hours of *in vitro* incubation. Therefore this study was focused to evaluate the potential of bark extracts of *Azadirachta indica* (Neem plant) on improving ruminal utilization of NH_3 after 48 hours of incubation by using *in vitro* gas production protocol.

2. MATERIAL AND METHODS

2.1 Experimental Design

The experiment was conducted at the laboratory in the Department of School of Sport, Equine and Animal Science in Writtle University College in the United Kingdom. There was three (3) treatments, each made up of a total of 16 incubation bottles i.e. 4 bottles for control (contained basal diet, rumen fluid, and buffer solution), 4 bottles (basal diet, rumen fluid, buffer solution and 100 mg/l of BEA), 4 bottles (basal diet, rumen fluid, buffer solution and 200 mg/l of BEA) and 4 bottles (basal diet, rumen fluid, buffer solution and 400 mg/l of BEA). Both treatments were incubated at a temperature of 39^{o}C. Treatment number 1 was incubated for 12 hours, treatment number 2, (24 hours) and treatment number 3 (48 hours).

2.2 Preparation of Diet for *In vitro* Fermentation

Hay, concentrate and linseeds each was milled by using 1 – 2 mm sieve and each was sieved to obtain uniform samples of 1 mm size and then were mixed together to obtain 1kg of ration made up of 70% hay, 25% concentrate and 3% linseeds. Thereafter 2% of fish oil was added into the mixture to form a total mixed ration which was stored in a freezer to prevent oxidation and absorption of the moisture from the atmosphere. Dry Matter (DM), Ash (Mineral), Organic Matter (OM) and Crude Protein (CP) contents of the diet were analysed according to the Association of Official Analytical Chemists [21] and its nutritional contents are presented in Table 1.

Table 1. Nutritional quality of the ration containing 70% hay, 25% concentrates, 3% linseeds and 2% fish oil before *in vitro* fermentation

Contents	Level of contents
Dry Matter (g/kg DM)	898.8
Organic Matter (g/kg DM)	837.8
Crude Protein (g/kg DM)	118.0
Ash (g/kg DM)	60.9

2.3 Preparation of Gas Production Medium

The gas production medium was prepared after mixed up together five (5) different solutions notably micro-mineral, macro-mineral, buffer solution, reducing and anaerobic indicator solutions [22–24]. The gas production medium was then stored under the room temperature (Table 2).

Table 2. Preparation of Gas Production Medium before *in vitro* fermentation

	Name of the solution	Chemical used to make up a solution
1)	Micro-mineral solution (100 ml)	13.2 g Calcium chloride ($CaCl_2.2H_2O$) 10 g of Manganese chloride ($MNCl_2.4H_2O$) 1.0 g of Cobalt chloride ($CoCl_2.6H_2O$ 8.0 g of Iron chloride ($FeCl_3.6H_2O$) 100 ml of distilled water
2)	Macro-mineral solution (1000 ml)	9.45 g of Di-sodium hydrogen ortho-phosphate ($NaHPO_4.12H_2O$) 6.20 g of Potassium di-hydrogen ortho-phosphate (KH_2PO_4) 0.60 g of Magnessium sulphate 7-hydrate ($MgSO_4.7H_2O$) 1000 ml of distilled water
3)	Buffer solution (100 ml)	4.0 g of Ammonium hydrogen carbonate (NH_4HCO_3) 35 g of Sodium hydrogen carbonate ($NaHCO_3$) 100 ml of distilled water
4)	Reducing solution (100 ml)	0.625 g of Cysteine ($HCl.1H_2O$) 100 ml of distilled water
5)	Anaerobic indicator (100 ml)	0.1 g Resazurin 100 ml of distilled water

2.4 *In vitro* Gas Production Technique

A total of 48 well-cleaned incubation bottles of 100 ml volume were labeled according to the treatments. Then 1.0 g of basal diet was added into all 48 bottles followed by different levels of concentration of commercial BEA in 36 bottles i.e. 12 bottles 100 mg/l, 12 bottles 200 mg/l, 12 bottles 400 mg/l and 12 bottles were control (no addition of BEA). Afterward 80 ml of gas production medium was added into all 48 incubation bottles and then were arranged in respect to their treatments allocation i.e. treatment 1 (12 hours incubation) made up of 4 replicates of incubation bottles of control (no addition of BEA), 100, 200 and 400 mg/l of BEA. Similar arrangement was applied to treatment 2 (24 hours incubation) and treatment 3 (48 hours incubation). The next morning rumen contents were collected from the abattoir from three (3) different sheep and their fluid content was extracted by squeezing the rumen contents by hand using two layers of cheesecloth direct into the pre warmed containers under anaerobic condition in the meantime incubated at 39^{o}C. After that 10 ml of rumen fluid was dispersed into bottles and covered well immediately and shaken vigorously. This procedures were adopted from Theodorou et al [24], Mauricio et al [22] and Mbisha [23]. Thereafter all bottles were soon placed in the incubator to allow incubation and fermentation to occur for 48 hours at 39^{o}C. During incubation, gas production was measured using pressure transducer after 3, 6, 9, 12, 24 and 36 hours and also pH by using pH meter after 12, 24 and 48 hours in order to assess fermentation process. Additionally, after 12, 24 and 48 of *in vitro* incubation and pH measurement, samples were collected from each replicate across each treatment and stored in the refrigerator for some days before were analysed for ammonia determination using microtiter plate reader.

2.5 Ammonia-Nitrogen Determination

The method of ammonia-nitrogen analysis was adopted from Rhine et al [25] whereby four (4) reagents were prepared namely; 2-phenylphenol-nitroprusside, citrate, hypochlorite and standard solution. Each reagent was prepared by using different chemicals (Table 3).

Table 3. Preparation of Reagents for Ammonia-Nitrogen Determination

Name of the Reagent	Chemicals used to make up a reagent
1) 2-Phenylphenol-nitroprusside (100 ml)	3.22 g of 2-phenylphenol sodium salt ($C_{12}H_9NaO \cdot 4H_2O$). 0.015 g of sodium nitroprusside (Sodium nitroferricyanide (III) dihydrate) ($C_5H_4FeN_6Na_2O_3$) 100 ml of distilled water.
2) Citrate ($C_6H_8O_7$) (100 ml)	5.0 g of Trisodium citrate ($Na_3C_6H_5O_7$). 80 ml of distilled water. Drops of 0.1 M Hydrochloric acid (HCl) to adjust the pH until pH of 7 was attained. Distilled water was added to make up 100 ml of a solution.
3) Hypochlorite (ClO^-) (100 ml)	1.0 g of Sodium sulphate (Na_2SO4) 80 ml of distilled water. 10 ml of 0.7 M of household bleach (NaOCl) Drops of 2 M of Sodium hydroxide (NaOH) was added to the mixture to adjust the pH until it become 13 Distilled water was added to make up 100 ml of a solution
4) Standard solution (1000 ml)	0.9346 g of dried $(NH4)_2SO4$ Distilled water to make a volume of 1000 ml of standard solution.

2.6 Analysis of Ammonia-Nitrogen by using Microtiter Plate Reader

A total of seven (7) solutions of 50 ml each with different concentrations were prepared by using different concentrations of standard solution (200 mg/l) by diluting the quantity of standard solution with distilled water (Table 4).

Table 4. Preparation of seven (7) solutions by using standard solution (200 mg/l)

Name of solution	Concentration (mg/l)	Volume of standard solution (ml)	Volume of distilled water (ml)
1	0.0	0.0	50.0
2	0.5	0.1	49.9
3	1.0	0.2	49.8
4	2.0	0.5	49.5
5	5.0	1.2	48.8
6	10.0	2.5	47.5
7	20.0	5.0	45.0

After that, samples were removed from the fridge and defrosted and centrifuged for 10 minutes at 3000 rpm and each was diluted with distilled water at a ratio of 5 µl of sample per 45 µl of distilled water to make up 50 µl of standard samples. Consequently 50 µl of each

standard sample was placed separately into their respective microtiter wells followed by addition of 50 µl of each prepared solution into their specific wells from solution 1 to 7. Subsequently 50 µl of citrate reagent was added into all wells containing standard samples and other solution and after one minute, 50 µl of 2-Phenylphenol-nitroprusside reagent, 25 µl of hypochlorite reagent and 100 µl of distilled water were added into all wells containing solution. Then the microtiter well was agitated for 30 seconds and was allowed to incubate for 45 minutes at a room temperature and then was analysed at 660nm by using microtiter plate reader.

2.7 Data Analysis

The available data of ammonia in each treatment were then analysed by using GenStat 15th edition software (version 15.1) and statistical method of analysis of variance (ANOVA) i.e. one – way ANOVA was used to compare means between treatments.

3. RESULTS

3.1 Effects of different levels of concentration (mg/l) of BEA on ammonia production (µg/l) during *in vitro* incubation

After 12 hours of *in vitro* incubation, ammonia gas production (µg/l) in both treatments level were similar statistically ($p > .05$) i.e. Control (11.8), 100 mg/l (12.7), 200 mg/l (10.9) and 400 mg/l (12.7). Moreover, even after 24 hours of fermentation, there was no differences in ammonia gas production (µg/l) ($p > .05$) i.e. Control (13.2), 100 mg/l (13.6), 200 mg/l (13.8) and 400 mg/l (13.6). Furthermore, there was no statistical differences ($p > .05$) in amount of ammonia gas production (µg/l) in all treatments even after 48 hours of *in vitro* incubation i.e. Control (14.8), 100 mg/l (14.3), 200 mg/l (14.9) and 400 mg/l (13.9). During all over the period of *in vitro* fermentation, BEA at concentration of 100 mg/l produced cumulative highest amount of ammonia (40.6µg/l) followed by 400 mg/l (40.1µg/l), then control (39.8µg/l) and last 200 mg/l (39.6µg/l). However both were statistically similar to each other as presented in Figure 1.

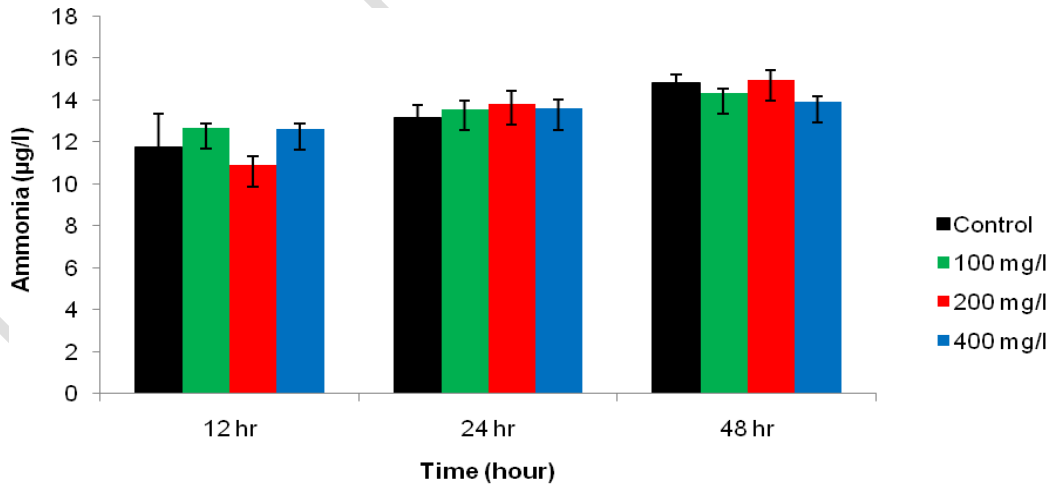

Figure 1: The *in vitro* effects of different levels of concentration of Neem bark extract (NBE) on ammonia production

3.2 Effects of different levels of concentration (mg/l) of BEA on pH during *in vitro* incubation

After 12 hours of *in vitro* fermentation, there was no statistical differences (*p >.05*) in pH between treatments. Even after 24 hours of incubation, there was no pH variation between 100 and 400 mg/l of BEA compared to control. However, pH variation (*p < .05*) was observed in 200 mg/l level of BEA. Moreover, after 48 hours of in *vitro* fermentation, pH in concentration levels of 100 and 200 mg/l of BEA both were similar to control. However, each was differ statistically (*p< .05*) with pH level in 400 mg/l of BEA as presented in Table 5.

Table 5. Effects of different levels of concentration (mg/l) of BEA on pH after 12, 24 and 48 hours of *in vitro* fermentation

Duration of *in vitro* incubation (Hours)	Concentration of Neem bark extract (mg/l)					
	0	100	200	400	SED	*P* – Value
12	6.7^{a}	6.8^{a}	6.8^{a}	6.7^{a}	0.04	0.5
24	6.7^{a}	6.7^{a}	6.7^{b}	6.7^{a}	0.02	0.04
48	6.7^{ab}	6.7^{b}	6.7^{b}	6.6^{a}	0.01	0.03

SED = Standard error of the difference; Means bearing same latter "a" not differ statistically

3.3 Effects of different levels of concentration (mg/l) of BEA on gas production (ml/g OM) during *in vitro* incubation

After 3 hours there was no difference *(p>.05)* in gas production in all treatment levels of BEA. After 6 hours the gas productions were reduced at levels of 100, 200 and 400 mg/l of BEA compared to control but were not differ (*p> .05*). Likewise after 9 hours, there was also no difference in gas production at levels of 100 and 400 mg/l and both were similar to control. However, least amount of gas production was observed at level of 200 mg/l which was not differ statistically (*p> .05*). Also after 12 hours there was no difference *(p> .05)* in gas production in levels of 100 and 400 mg/l of BEA and both were similar to control meanwhile, the lowest amount of gas production was still observed in level of 200 mg/l of BEA compared to other treatments. After 24 hours gas production was statistically similar in levels of 100 and 400 mg/l compared to control but was differ *(p<.05)* in a level of 200 mg/l of BEA. After 36 hours there was no difference *(p>.05)* in gas production in levels of 100 and 400 mg/l of BEA compared to control. However, the amount of gas production in levels of 100 and 200 mg/l of BEA were similar with control while the amount of gas produced in level of 200 mg/l of BEA was lower compared to the amount produced in level of 400 mg/l of BEA as presented in Table 6.

Table 6. Effects of different levels of concentration (mg/l) of BEA on gas production (ml/g OM) after 3, 6, 9, 12, 24 and 36 hours of *in vitro* fermentation

Duration of *in vitro* incubation (Hours)	Concentration of Neem bark extract (mg/l)					
	0	100	200	400	SED	*P* – Value
3	28.2^{a}	28.1^{a}	26.6^{a}	28.1^{a}	0.8	0.2
6	56.7^{b}	54.8^{ab}	50.3^{a}	55.7^{ab}	2.7	0.1
9	82.7^{b}	80.2^{ab}	73.6^{a}	81.6^{b}	3.5	0.1
12	102.3^{b}	101.2^{b}	92.7^{a}	101.7^{b}	4.1	0.1
24	148.8^{b}	147.2^{b}	137.6^{a}	149.8^{b}	4.2	0.03
36	207.5^{ab}	205.5^{ab}	194.9^{a}	210.0^{b}	6.2	0.1

SED = Standard error of the difference; Means bearing same latter "a" not differ statistically

4. DISCUSSION

4.1 Effects of different levels of BEA on ammonia production *in vitro*

The results in Figure (1) indicated that BEA at concentration levels of 100, 200 and 400 mg/l cannot improve NH_3 utilization after 12, 24 and 48 hours of *in vitro* fermentation and also has little effects on ruminal pH (Table 5) as well as fermentation (Table 6). These findings were related to Yang et al [26] when they found significant increase in concentration of NH_3-N (1.14 mmol-1) compared to control (0.83 mmol-1) in feedlot cattle after they were fed a diet supplemented with Neem oil seeds at 20 g/kg of DM, even at 40 g/kg of DM no significant effect was observed. However, Adelusi et al [27] proved that, least significant amount of rumen ammonia nitrogen was produced from West African dwarf goats after were supplemented 40 g/day leaves of Neem tree for 70 days. Nevertheless, Verma et al [28] found that prolong supplementing goats for 180 days dietary concentrates with water washed Neem seed kernel cake which containing 34 – 40% CP at levels of 12 and 25% cause significant decrease in NH_3-N in rumen liquor. NH_3 in the rumen is formed after catabolism of dietary protein and NPN sources mainly carbon and sulphur by hyper ammonia-producing bacteria after deamination of AA in the rumen [29,30]. The potential of improving NH_3 utilization in rumen brings direct effects on nitrogen use efficiency, dietary protein utilization and minimize urea excretion via urine and faeces and therefore helps to mitigate emission of greenhouse gases which cause environmental pollution [31].

4.2 Effects of BEA on pH During *In Vitro* fermentation

Fermentation in the rumen leads to production of volatile fatty acids (VFA) and when accumulates it cause drop in pH [32]. *In vitro* gas protocol, rumen microorganisms in gas medium digests substrate to yield VFA and NH_3 [33]. The *in vitro* gas production protocol demonstrates fiber digestion to yield acetate, propionate and butyrate which are major source of energy in ruminants [34]. The pH values observed in this study (Table 5) both were within the range as suggested by Beauchamin [35] ruminal microbes produce VFA's in anaerobic condition at a temperature between 36 – 41^{o}C and pH between 5.7 to 7.3 and their population varies depend on the types of nutrients and it is availability in the diet. Moreover, ruminal pH is dynamic, usually vary depends on the species of the ruminant, type of the diet and feeding frequency i.e. on the roughage based diet the pH is 6.0 to 7.0, on concentrate based diet the pH is 5.5 to 6.5 and in anorexic animals the pH is 7.5 to 8 due to rumen alkalinity caused by constant saliva secretion which containing bicarbonate and phosphate [36–39]. Rumen microbes which digest fiber grow well at the pH between 6.0 and 7.0 [40] while microbes which digest starch flourish better at the pH less than 5.5 [41] and proposed standard pH in digestion of a proper mixed diet of fiber and starch is 6.5 to 6.7 [42].

4.3 Effects of BEA on gas production i*n Vitro*

Results (Table 6) showed that, after 3, 6, 9, 12, 24 and 36 hours of *in vitro* fermentation, gas production was not much affected by BEA across treatments compared to control. However, were slight decreased after 6, 9 and 12 hours of incubation in all treatments but after 24 and 36 hours, the gas productions in level of 400 mg/l of BEA were increased but statistically were similar to control. This reveals that at high level of supplementation, BEA prevents rumen fermentation. The later findings were related to Patra et al [43] when they discovered 0.25 ml of methanol (M), ethanol (E) and water (W) extracts from seed kernel of *Azadirachta indica* increased gas productions (ml/g DM) i.e. 188 (M), 184 (E) and 164 (W)) meanwhile at level of 0.5 ml reduced gas production i.e. (162 (M), 161 (E) and 174 (W)) compared to control 174 (M), 164 (E) and 149 (W). Nevertheless, Yang et al [26] proposed that 20 and 40g/kg dietary supplementation of Neem seed oil in beef cattle reduces fiber digestion by 0.77 and 0.71 respectively compared to control 0.79. This effect is perhaps due to bioactive compounds which are found in *Azadirachta indica* which may have antimicrobial properties

against rumen microbes. In ruminants gas production reflect digestion of organic matter and nutrients utilization [44,45] and the amount of gas produced is equivalent to the amount of fermented feed [46].

5. CONCLUSION

This *in vitro* study demonstrated that three (3) different levels of 100, 200 and 400mg/l of BEA cannot affect the growth of hyper ammonia producing bacteria which digests dietary protein into NH_3 in the rumen and therefore cannot influence ruminal NH_3-N utilization in ruminants. However, BEA revealed to interfere growth of other rumen microorganisms which breakdown dietary fiber into volatile fatty acids and thus affects ruminal pH. This might be caused by antimicrobial properties of *Azadirachta indica.* Therefore based on the above research findings, more studies are required in this field in order to optimize ruminal NH_3-N utilization and dietary protein utilization in ruminants. This will advance further animal production performances and help to mitigates environmental pollution caused by emission of the greenhouse gases from ruminants.

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