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In vitro Study of Neem Bark Extract on Rumen Fermentation and Biohydrogenation of Polyunsaturated Fatty Acids

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Author,s contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

Aim: An *In vitro* gas production technique was conducted to assess effects of commercial Neem (*Azadirachta indica*) bark extract (NBE) on rumen fermentation and biohydrogenation (BH) of selected polyunsaturated fatty acids (PUFAs).
Study Design: There were three (3) treatments, each made up of a total of 32 incubation bottles i.e. 8 bottles for control (contained basal diet, rumen fluid and buffer solution), 8 bottles (basal diet, rumen fluid, buffer solution and 100 mg/l of NBE), 8 bottles (basal diet, rumen fluid, buffer solution and 200 mg/l of NBE) and 8 bottles (basal diet, rumen fluid, buffer solution and 400 mg/l of NBE).
Methodology: All three (3) treatments were incubated at a temperature of 39^{o}C for 12 hours (treatment 1), 24 hours (treatment 2) and 48 hours (treatment 3). During incubation procedure, gas production was measured by using pressure transducer after 3, 6, 9, 12, 24, and 36 hours. Consequently, pH was measured by using pH meter after 12, 24 and 48 hours of fermentation and then incubation was stopped. During this time, digested samples in gas medium were collected for analysis of PUFAs by using gas chromatography. GenStat 15th edition (version 15.1) software was

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used to analyse collected data in which statistical method of analysis of variance (ANOVA) i.e. one – way ANOVA was used to compare means between treatments.
Results: Results showed that no differences in gas production, pH, changes in concentration and BH of selected PUFAs after 48 hours of in vitro fermentation. However, concentrations (g/100g FA) of linolenic acid at levels of 200 and 400 mg/l of NBE i.e. 0.8 and 0.8 respectively were increased ($p < .05$) compared to control (0.7). Moreover, NBE at level of 400 mg/l inhibits ($p<.05$) BH of linolenic acid (92.1%) and docosahexaenoic acid (75.0%) compared to control (92.8%) and (80.3%) respectively.
Conclusion: These findings demonstrated that, NBE possesses bioactive compounds which affect BH of docosahexaenoic and linolenic acids and has little effect on improving fermentation and modifying ruminal BH of PUFAs.

Keywords: Biohydrogenation; In vitro study; neem bark extract; polyunsaturated fatty acids.

1. INTRODUCTION

Ruminant products such as meat and milk containing high amount of saturated fatty acids (SFAs) and low amount of unsaturated fatty acids (UFAs) e.g. bovine milk contains 70% SFAs and 30% UFAs [1]. While PUFAs in beef is 11% and lamb 15% which are lower than that of recommended Figure of 45% for the UK diet as a whole [2,3]. High level of SFAs in ruminant products is a result of extensive BH of PUFAs in the rumen [4]. PUFAs have been confirmed to be potential in human health as they regulate hormonal activity, blood pressure, immune response and kidney functions [1]. Also PUFAs have shown to minimize risk factor for cardiovascular disease, some cancers, asthma and diabetes [2,5-7]. Apart from that, consumption of diet with high level of SFAs increases high risk of coronary heart disease (CHD) in human [8]. By considering the merits of PUFAs against SFAs in human health, reduction of extensive BH of PUFAs in the rumen is necessary in order to increase proportional of UFAs in ruminant products. In the meantime, reduce the level of SFAs which have proven to increase the risk factor of CHD.

The application of antibiotic-growth promoters in production of farm animals have demonstrated to improve genetic potential, feed conversion efficiency, animal health, growth rate and feed efficient utilization [9]. However in January, 2006 European Union (EU) banned the application of these feed additives in production of animals in the Europe [10,11]. This was decided because of the emergence of bacterial resistance against antibiotics, cases of toxicities in animals and appearance of drug residues in meat and milk [12]. Because of this situation animal nutritionists have developed curiosity in using natural plants and their extracts to improve animal production performances as an alternative way to antibiotic-growth promoters [13,14].

Neem plant (*Azadirachta indica*) is the herb tree which is well recognized because; it is seeds, stems, bark, flowers, leaves and fruits have been confirmed to cure many diseases such as hypertension, ulcers and diabetes [15,16]. The plant is commonly found in tropical areas mostly in India and it has been confirmed also to have immunomodulatory, adaptogenic and anti-inflammatory properties [17]. Studies confirmed that Neem plant has potential in improving feed efficient utilization in animals e.g. Rukmini [18] confirmed increased in feed intake and body weight in rat weaners after they were fed a diet containing 20% crude protein and 10% Neem oil in contrast to groups which were fed a diet containing groundnuts oil. However, Patra et al [19] revealed that 0.5ml of Neem extracts reduced *in vitro* dry matter digestibility, acetate to propionate ratio and production of total volatile fatty acids compared to control.

The aim of this experiment was to use in vitro gas production technique in assessing effects of different levels of NBE on the rumen fermentation and biohydrogenation of UFAs. This study was important as it provide prospect of improving feed digestibility and utilization in ruminants and reducing ruminal disappearance of UFAs and increase its proportional yield against SFAs in meat and milk. By improving yield of more UFAs in ruminant products will assist in promoting consumption of more health beneficial fatty acids and therefore reducing risk factor of CHD in human.

2. MATERIALS 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 were three (3)

treatments, each treatment made up of a total of 32 incubation bottles i.e. 8 bottles for control (contained basal diet, rumen fluid, and buffer solution), 8 bottles (basal diet, rumen fluid, buffer solution and 100 mg/l of NBE), 8 bottles (basal diet, rumen fluid, buffer solution and 200 mg/l of NBE) and 8 bottles (basal diet, rumen fluid, buffer solution and 400 mg/l of NBE). Make up a total of 96 incubation bottles in all treatments and both 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). Separately, there was one treatment (freeze-dried) which was made up with four replicates of control (no addition of NBE), 100, 200 and 400 mg/l of NBE. This treatment received similar treatment like others however; it was not incubated (zero-hour) and was used in computation of rate of disappearance of individual fatty acid.

2.2 Preparation of the Basal Diet

A basal diet of 1kg was prepared by mixing up three (3) feed ingredients i.e. hay 70%, concentrate 25%, linseeds 3% and fish oil 2%. Hay, concentrate and linseeds each was milled by using 1 – 2 mm sieve, thereafter all were sieved in order to obtain uniform samples of 1 mm size and all were mixed together. The fish oil was then 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 basal diet were analysed according to AOAC [20] and its nutritional contents are presented in Table 1.

2.3 Preparation of Gas Production Medium

The gas production medium was prepared according to Theodorou et al [21] and Mauricio et al [22] by mixed up together five (5) solutions (micro-mineral, macro-mineral, buffer solution, reducing and anaerobic indicator solutions). Each solution was prepared from different chemicals by dissolving chemicals into distilled water as presented in Table 2. The medium solution was then stored under room temperature for couple of days before the start of experiment.

2.4 *In vitro* Gas Production Procedure

A total of 100 well-cleaned incubation bottles of 100 ml volume were labeled according to the treatments allocation. Then 1.0 g of basal diet was added into 96 bottles except four (4) blank bottles which were added only rumen fluid and buffer solution. Then different levels of concentration of commercial NBE were added into 72 bottles i.e. 24 bottles 100 mg/l, 24 bottles 200 mg/l, 24 bottles 400 mg/l and 24 bottles were control (no addition of NBE). Afterward 80 ml of gas production medium was added into all 100 incubation bottles including all containers of zero hour. Then all bottles containing medium were arranged in respect to their treatments allocation i.e. treatment 1 (incubated for 12 hours) made up of 8 bottles of control (no addition of NBE), 100, 200 and 400 mg/l of NBE. Similar arrangement was applied to treatment 2 (incubated for 24 hours) and treatment 3 (incubated for 48 hours) and both treatments include blanks were incubated overnight at 39^{o}C. The next morning rumen contents were collected from the abattoir from three different sheep and then rumen fluid was extracted by squeezing the rumen contents by hand using two layers of cheesecloth. The rumen fluid was collected into pre warmed containers under anaerobic condition meanwhile incubated at 39^{o}C. After that 10 ml of rumen fluid was dispersed into each container of zero hour and in all bottles and covered well immediately and shaken vigorously. Thereafter all bottles were introduced soon in the incubator to allow incubation and fermentation to occur for 48 hours at 39^{o}C. Meanwhile zero-hour samples were freeze-dried for 12 days and followed by fatty acids analysis

2.5 Data Collection

Gas production during *in vitro* incubation was measured and recorded using pressure transducer after 3, 6, 9, 12, 24, and 36 hours. Also during *in vitro* incubation, pH was measured and recorded using pH meter after 12, 24 and 48 hours. After 48 hours of incubation, four (4) replicates across each treatment were collected and freeze-dried for 12 days before fatty acids analysis using gas chromatography (GC).

2.6 Fatty Acid Analysis

A total of four (4) reagents namely; saponification mixture – 5Molar potassium hydroxide (KOH), Butylatedhydroxytoluone (BHT), Internal standard, Sulphuric acid/methanol solution were prepared according to Enser et al [3] while procedures for fatty acids analysis were done in reference to Wachira et al [23].

Table 1. Nutritional quality of experimental diet containing hay 70%, concentrates 25%, linseeds 3% and fish oil 2% before *in vitro* incubation

Contents	Level of contents
Dry Matter (g/kgDM)	898.8
Organic Matter (g/kgDM)	837.8
Crude Protein (g/kgDM)	118.0
Ash (g/kgDM)	60.9
Fatty Acids (mg/g)	
14 : 0 (myristic)	2.0
16 : 0 (palmitic)	4.7
16 : 1 (palmitoleic)	1.5
18 : 0 (stearic)	1.2
18 : 1*n*-9 (oleic)	3.1
18 : 1*n*-7 (cis-vaccenic)	1.0
18 : 2*n*-6 (linoleic)	4.5
18 : 3*n*-3 (linolenic)	7.2
20 : 4 (arachidonic)	0.07
20 : 5*n*-3 (eicosapentaenoic)	2.0
22 : 6*n*-3 (docosahexaenoic)	1.4
Remaining fatty acids	**11.6**
Total fatty acids	**38.6**

Table 2. Preparation of gas production medium for in vitro incubation

Type of solutions	Chemicals used to make up a solution
a) 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 ($FeCl_3$.6H2O) 100 ml of distilled water
b) 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
c) 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
d) Reducing solution (100 ml)	0.625 g of Cysteine ($HCl.1H_2O$) 100 ml of distilled water
e) Anaerobic indicator (100 ml)	0.1 g Resazurin 100 ml of distilled water

Total fatty acids concentration in a sample was computed as

$$\text{Total fatty acids } \left(\frac{\text{mg}}{\text{g}}\right) = \frac{(\text{Total area} - \text{std area}) \times \left[\frac{\text{Amount std.added (mg)}}{\text{Std area}}\right]}{\text{Sample weight (g)}}$$

Concentration of each individual fatty acid

$$\text{Fatty acid (mg/g)} = \frac{(\text{Fatty acid area}) \times \left[\frac{\text{Amount std.added (mg)}}{\text{Std area}}\right]}{\text{Sample weight (g)}}$$

$$\text{Fatty acid in (g/100g FA)} = \left(\frac{\text{Fatty acid in (mg/g)}}{\text{Total fatty acids in (mg/g)}}\right) \times 100$$

$$\text{Disappearance (\%)of PUFA} = \left(\frac{\text{Conc. indiv FAo(g/100g FA)} - \text{Conc. indiv FA48 (g/100g FA)}}{\text{Conc. indiv FAo(g/100g FA)}}\right) \times 100$$

Conc. IndivFAo (g/100g FA) = Concentration of individual fatty acid at zero hour of incubation.

Conc. Indiv FA48 (g/100g FA) = Concentration of individual fatty acid after 48 hour of incubation.

2.7 Data Analysis

Genstat 15th edition software was used to analyse available data in which a 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 NBE on Gas Production (ml/g Om) During *In Vitro* Incubation

After 3 hours of incubation no difference *(p>.05)* in gas production in all treatment levels of NBE i.e. control (28.2); 100 mg/l (28.1), 200 mg/l of NBE (26.6) and 400 mg/l of NBE (28.1). After 6 hours the gas productions were reduced at levels of 100 mg/l (54.8); 200 mg/l (50.3) and 400 mg/l (55.7) compared to control (56.7) but were not differ statistically (*p> .05*). Similarly after 9 hours no difference in gas production at levels of 100 mg/l (80.2) and 400 mg/l (81.6) and both were similar to control (82.7). However, least amount of gas was produced at level of 200 mg/l (73.6) which was not differ statistically (*p> .05*). Furthermore, after 12 hours of *in vitro* fermentation no difference *(p> .05)* in gas production in levels of 100 mg/l (101.1) and 400 mg/l (101.7) of NBE and both were similar to control (102.3). Conversely, the amount of gas produced in level of 200 mg/l of NBE was lowest (92.7) compared to other treatments. After 24 hours of incubation, gas production was not differ statistically in levels of 100 mg/l (147.2), 400 mg/l (149.8) compared to control (148.8) but was differ *(p<.05)* in a level of 200 mg/l (137.6) of NBE. After 36 hours there was no difference *(p>.05)* in gas production in levels of 100 mg/l (205.5), 400 mg/l (210) of NBE compared to control (207.5). However, amounts of gas produced in levels of 100 mg/l (205.5) and 200 mg/l (194.9) of NBE were similar statistically with control (207.5) and amount of gas produced in level of 200 mg/l (194.9) of NBE was lower compared to the amount produced in levels of 400 mg/l (210) of NBE as presented in Figure 1.

3.2 Effects of NBE on pH During *In Vitro* Incubation

After 12 hours of *in vitro* fermentation, there was no difference in pH between treatments i.e. control (6.7), 100 mg/l (6.8), 200 mg/l (6.8) and 400 mg/l (6.7) of NBE. Even after 24 hours of incubation, there was no pH variation between 100 mg/l (6.7) and 400 mg/l (6.7) of NBE compared to control (6.7). However, significant variation *(p< .05)* of pH was observed in 200 mg/l (6.73) of NBE. Likewise after 48 hours of incubation, pH level in 100 mg/l (6.7) and 200 mg/l (6.7) of NBE were not differ compared to control (6.7) but the pH between control and 400 mg/l (6.6) of NBE were statistically similar. However the pH in 100 mg/l (6.7) and 200 mg/l (6.7) of NBE each was differ statistically *(p< .05)* with pH in 400 mg/l (6.6) of NBE as presented in Figure 2.

3.3 Effects of NBE on Disappearance of Selected PUFAs During *In Vitro* Incubation

After 48 hours of *in vitro* fermentation, there was no difference (*p>.05*) in rate of disappearance of Eicosapentaenoic acid (EPA) (20: 5n-*3*) and Linoleic acid (18: 2*n-6*) in all treatments compared to control. However the rate of disappearance of Linolenic acid (93.1%) in treatment level of 100 mg/l of NBE was statistically highest *(p< .05)* compared to 200 mg/l (92.3%) and 400 mg/l (92.1%) of NBE but it was not differ with control (92.8%). Also there was no difference in rate of disappearance of linolenic acid in levels of 200 mg/l (92.3%) and 400 mg/l (92.1%) of NBE. Furthermore, rate of disappearance of Docosahexaenoic acid (DHA) (22: 6*n-3*) was not differ in treatment levels of 100, 200 and 400 mg/l of NBE. However, the rate of disappearance of DHA in treatment levels of 100 mg/l (78.4%) and 200 mg/l (78.6%) of NBE was statistically related with control (80.6%). Meanwhile, the lowest rate of disappearance of DHA (75.0%) was observed in level of 400 mg/l of NBE compared to control (80.6%) as presented in Table 3.

Table 3. Effects of different levels of concentrations (0, 100, 200 and 400mg/l) of NBE on disappearance (%) of polyunsaturated fatty acids after 48 hours of in vitro incubation

Fatty acids	Concentration of Neem bark extract (mg/l)					
	0	100	200	400	SED	P-Value
Linoleic acid (18:2n-6)	90.7^{a}	90.9^{a}	90.6^{a}	90.2^{a}	0.4	*NS
Linolenic acid (18:3n-3)	92.8^{bc}	93.1^{c}	92.3^{ab}	92.1^{a}	0.3	*p<.05*
Eicosapentaenoic acid (20:5n-3)	73.5^{a}	77.3^{a}	77.8^{a}	79.8^{a}	3.0	*NS
Docosahexaenoic acid (22:6n-3)	80.4^{b}	78.4^{ab}	78.6^{ab}	75.0^{a}	2.2	*p<.05*

*SED = Standard errors of differences of means; *NS = not significant; Means bearing same latter a, b and c in row not differ significantly*

Table 4. Effects of supplementing a forage and concentrate (70:30) diet with graded levels of Neem bark extract (mg/l) on the concentrations of selected fatty acids (g/100g FA) after 48 hours of incubation in rumen fluid *in vitro*

Fatty acids	Concentration of Neem bark extract (mg/l)					
	0	100	200	400	SED	P-Value
Stearic acids (18:0)	25.0^{ab}	24.7^{a}	25.2^{b}	25.9^{ab}	0.6	*p<.05*
Vaccenic acid (18:1 *trans* 11)	12.2^{a}	11.9^{a}	12.4^{a}	12.1^{a}	0.2	*NS
Linoleic acid (18:2n-6)	0.7^{a}	0.7^{a}	0.7^{ab}	0.7^{b}	0.1	*p<.05*
Linolenic acid (18:3n-3)	0.7^{a}	0.7^{ab}	0.8^{bc}	0.8^{c}	0.0	*p<.05*
Conjugated linoleic acid	0.1^{a}	0.1^{a}	0.1^{a}	0.1^{a}	0.0	*NS
Arachidonic acid (20:4)	0.1^{a}	0.1^{a}	0.1^{a}	0.1^{a}	0.0	*NS
Eicosapentaenoic acid (20:5n-3)	0.8^{a}	0.7^{a}	0.7^{a}	0.6^{a}	0.1	*NS
Docosahexaenoic acid (22:6n-3)	0.4^{a}	0.4^{a}	0.4^{a}	0.5^{a}	0.0	*NS

*SED = Standard errors of differences of means; *NS = not significant; Means bearing same latter a, b and c in row not differ significantly.*

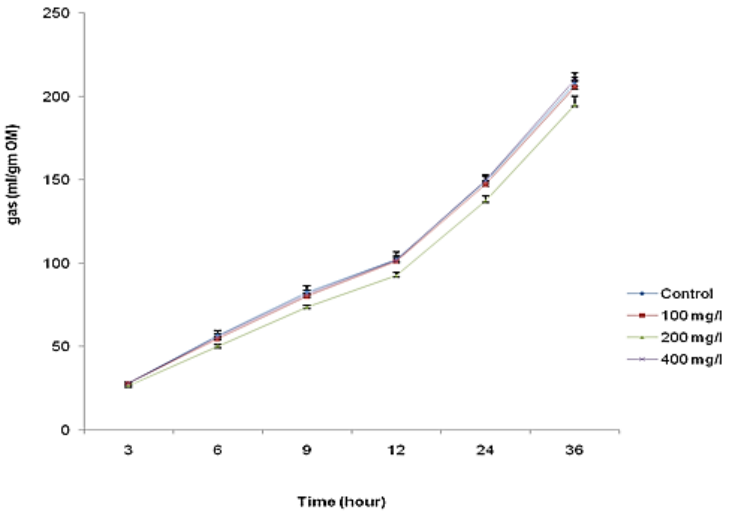

Fig. 1. The *In vitro* effects of different levels of concentration (0, 100, 200 and 400mg/l) of Neem bark extract on gas production

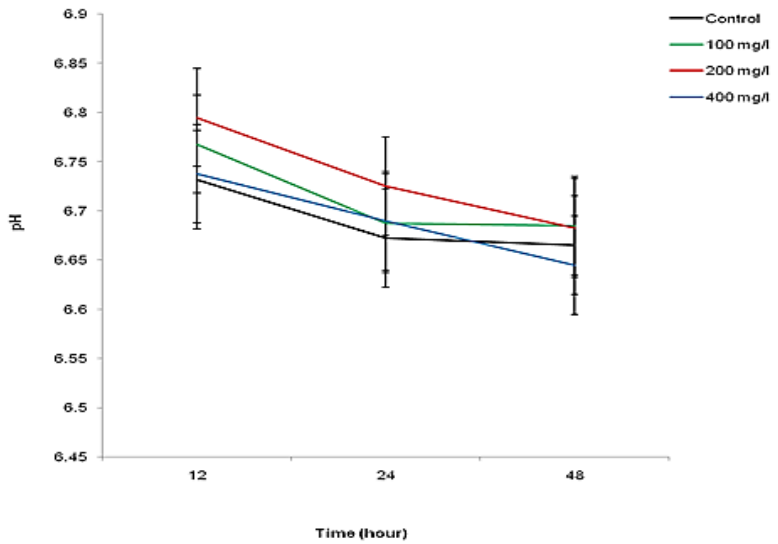

Fig. 2. The *in vitro* effects of different levels of concentration (0, 100, 200 and 400mg/l) of Neem bark extract on pH

4. DISCUSSION

4.1 Effects of different levels of NBE on gas production i*n Vitro*

The results from figure 1 showed that, after 3, 6, 9, 12, 24 and 36 hours of *in vitro* incubation, the gas production was not affected by NBE at different levels of concentrations i.e. 100, 200 and 400 mg/l compared to control. Gas productions were slightly reduced after 6, 9 and 12 hours of incubation in all treatments. However after 24 and 36 hours of incubation, the gas productions in level of 400 mg/l of NBE were slightly increased but statistically were similar to control. These results mean that at higher level of supplementation, NBE inhibits rumen fermentation. This was also supported by Patra et al [19] when they used 0.25 and 0.5 ml of methanol (M), ethanol (E) and water (W) extracts from seed kernel of *Azadirachta indica* and gas productions (ml/g DM) were 188 (M), 184 (E) and 164 (W) for 0.25 ml and 162 (M), 161 (E) and 174 (W) for 0.5 ml compared to control 174 (M), 164 (E) and 149 (W). Nonetheless, the results were also suggested by Yang et al [24] when they found 20 and 40g/kg dietary supplementation with Neem seed oil in beef cattle reduces fibre digestibility by 0.77 for 20g/kg and 0.71 for 40g/kg compared to control 0.79. This effect is probably due to bioactive components which are found in *Azadirachta indica. In vitro* gas productions reflect digestibility of organic matter and nutrients utilization in ruminants [25,26] and the amount of gas produced is equivalent to the amount of fermented feed [27].

4.2 Effects of NBE on pH During *In Vitro* Incubation

There is a strong relationship between volatile fatty acids (VFA) production and pH in the rumen, when VFA produced and accumulate in the rumen it causes drop in pH [28]. During *in vitro* gas production technique, microbes in culture medium ferment substrates to form volatile fatty acids and ammonia [29]. The *in vitro* gas production reveals the digestion of dietary carbohydrates to form acetate, propionate and butyrate which are principal source of energy in ruminants [30]. However, the pH values which were obtained from this experiment, all were within the range. This was also proved by Beauchemin [31] when suggested, rumen microbes produce VFA's under anaerobic condition at 36 – 41^{o}C and pH between 5.7 – 7.3 and their normal growth depends on types of nutrients and their availability in the diet. However, rumen pH is not static but it keeps on changing depends on type of the diet, feeding frequency and ruminant species i.e. on roughage based ration the pH is 6.0 – 7.0 while on concentrate based ration the rumen pH is 5.5 – 6.5 and also 7.5 – 8 in anorexic animals due to persistence saliva secretion containing bicarbonate and phosphate which increase rumen alkalinity [32-34]. Fiber-digesting microbes flourish better at the pH of 6.0 – 7.0 [35] while starch-digesting microbes are more active at pH below 5.5 [36] and the normal pH equilibrium in digestion of well balanced mixed diet of fiber and starch is suggested to be 6.5 – 6.7 [37].

4.3 *In vitro* biohydrogenation of PUFAs

After 48 hours of *in vitro* fermentation (Table 2) highest rate (93.1%) of disappearance of PUFAs was observed to linoleic acid in a level of 100 mg/l of NBE. Similar effect was also happened in linoleic acid (90.9%) at the same level of 100 mg/l of NBE although statistically both were similar across treatments. Generally, the rate of disappearance of eicosapentaenoic and docosahexaenoic acids was lower than linoleic and linolenic acids across treatments. The least significant rate of disappearance of docosahexaenoic acid (75.0%) of was found in treatment level of 400 mg/l of NBE however statistically was not differ compared to the rate of disappearance of docosahexaenoic acid in other treatments except control. The effect of disappearance of PUFAs during *in vitro* fermentation was largely associated with microbes present in rumen fluid which catalyze the breakdown of dietary lipid by using microbial lipase to form free fatty acids [38]. The free unsaturated fatty acids were then hydrogenated by rumen microbes i.e. rumen bacteria and fungi to form more saturated fatty acids [39-41]. Furthermore, rumen bacteria are more responsible for hydrogenation of unsaturated free fatty acids than the rumen fungi, whereby the end product of fungal biohydrogenation of unsaturated free fatty acids is vaccenic acid [42]. Biohydrogenation (BH) of free unsaturated fatty acids by rumen bacteria to form saturated fatty acids was interpreted as a defense mechanism of rumen bacteria against toxic effects from PUFAs [43]. Based on the effect of NBE on disappearance of PUFAs, these findings were also related with findings of Castañeda-Gutiérrez et al [44] after supplemented calcium salts of fish oil and unprotected fish oil in Holstein cows and

causes greater than 85% ruminal BH of eicosapentaenoic acid for calcium salts and more than 75% ruminal BH of docosahexaenoic acid for unprotected fish oil. Furthermore, in their feeding trial Jack et al [45] revealed BH of oleic, palmitic, stearic, and linoleic acids were not significantly affected after West African dwarf rams were dietary supplemented with 0, 2.5, 5.0, 7.5 and 10% of water-washed Neem fruits for 90 days, Conversely, Privé et al [46] have confirmed reduction in BH of linoleic acid after sunflower oil was heated at a temperature of 110 or 150^{o}C for 1, 3 and 6 hours before *in vitro* incubated with ruminal content. However, it has led to negative implications by reducing production of CLA and trans-C18:1 and increase synthesis of trans-10 isomer.

4.4 Effects of NBE on concentrations of selected Fatty Acids

Concentrations of CLA, 18:1 trans 11, arachidonic acid, linoleic, eicosapentaenoic, docosahexaenoic and stearic acids all were not affected by NBE in all treatments after 48 hours of *in vitro* incubation (Table 4). However concentration (g/100g FA) of linolenic acid was increased to 0.8 and 0.8 at levels of 200 mg/l and 400 mg/l of NBE respectively compared to control (0.7). Inhibition of BH of linolenic and docosahexaenoic acids induced by effects of NBE during *in vitro* incubation increases their concentrations i.e. 0.8 and 0.5 respectively. However, statistically concentration of docosahexaenoic acid (0.5) was similar to control (0.4). The results was supported by Kesava Rao et al [47] when they found increased amount of unsaturated fatty acids (UFA) in Longissimus dorsi muscle in goats after supplemented water washed Neem seed kernel cake (WWNSKC) for 180 days. Addition of 15% WWNSKC was found to increase total UFAs to 45.99 ± 0.47% meanwhile 25% of WWNSKC increased total UFAs to 47.52 ± 0.68% compared with control (41.69 ± 0.44%). Nevertheless 15% of WWNSKC was found to reduce total amount of SFAs to 54.07 ± 0.5% while 25% of WWNSKC reduced SFAs to 52.46 ± 0.79% compared to control 58.31 ± 0.82%. Moreover, Verma et al [48] revealed decrease in fat content in Longissimus dorsi muscle and overall increased in lean to fat ratio in goat meat. This happened after goats were supplemented with 15 and 25% of WWNSKC in their diet and comparison was made against the control group which was fed conventional oil seed cake. Inhibition of BH of PUFAs in the rumen is necessary in order to increase the yield of PUFAs and meanwhile reduces the level of SFAs in meat and milk [49]. This is due to the fact that high consumption of SFAs can cause CHD in man [2] while UFAs are health-promoting fatty acids in human which reduce risks of CHD, asthma and cancer [50].

5. CONCLUSION

This *in vitro* experiment revealed that concentration of NBE at different levels of 100, 200 and 400mg/l statistically did not affect gas production and pH. Also, NBE at level of 400 mg/l inhibits biohydrogenation of linolenic and docosahexaenoic acids. Meanwhile at levels of 200 and 400 mg/l NBE improves yield (g/100g FA) of linolenic acid. Based on the above findings, NBE demonstrated little effects on improving forage digestibility in ruminants and modifying ruminal biohydrogenation of PUFAs**.** Therefore more research in this area are needed to optimize utilization of plant extracts in improving feed utilization in ruminants and inhibition of disappearance of UFAs in the rumen so as to yield more UFAs in ruminant products which has proven to reduce risk factor for coronary heart disease in human.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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