Diversity and physical properties of rumen microbial ecosystem of West African dwarf rams fed ammonium sulphate-fortified diets

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Abstract *Corresponding author: topes1725@yahoo.com; +2348034425735

*Ammonium sulphate is a feed additive used in ruminant feeds mainly for stabilization of the intestinal flora, enhance the development of the adult rumen microflora, improve digestion and nitrogen flow towards lower digestive tract and improve meat and milk production. In the study the diversity of rumen microbial ecosystem of WAD rams fed ammonium sulphate-fortified diets at 0, 2.5, 5.0 and 7.5g/kg to the concentrate diet as T1, T2, T3 and T4 respectively was assessed. Sixteen WAD rams with average body weight of 12.20±0.12 kg were used and fed for 109 days, each treatment had four replicates. In a completely randomized design and differences among treatment means were examined using least significant difference, each ram was fed at 5% of their body weight at 60:40 levels of experimental diet and wilted guinea grass as basal diet. Variables measured were: *S. typhimurium*, *E. coli*, bacteria, fungi and protozoa. The results indicated that establishment of the microbial population of diets consisting of 7.5g/kg (NH₄)₂SO₄ were significantly higher in bacteria and fungi than the control diet (P<0.05). It was concluded that fortification at 7.5g/kg (NH₄)₂SO₄ give a better understanding of the role of rumen microbes and the establishment of the microbial population, which help to maintain the host's health and improve animal performance.*

Keywords: Ecosystem, microbes, health status, WAD rams, ammonium sulphate

Introduction

Ruminants are defined by their mode of plant digestion, and have evolved a fore-stomach, the rumen, that allows partial microbial digestion of feed before it enters the true stomach. Ruminants do not produce the enzymes needed to degrade most complex plant polysaccharides, and the rumen provides an environment for a rich and dense consortium of anaerobic microbes that fulfil this metabolic role. These rumen microbes ferment feed to form volatile fatty acids that are major nutrient sources for the host animal and contribute significantly to ruminant productivity. The host also uses microbial biomass and some unfermented feed components once these exit the rumen to the remainder of the digestive tract. These were also important factors in their domestication, allowing conversion of human-indigestible plant material into readily-accessible animal

goods, especially dairy products, meat, and useful fibres. Ruminants have thus played a vital role in sustaining and developing many human cultures, as well as being used as draft animals and having religious and status values.

The rumen is a complex anaerobic microbial ecosystem, that harbors a wide variety of microorganisms, including bacteria, protozoa, archaea, fungi and viruses (Shaopu *et al.*, 2017) interact in ways that have a significant influence on the health of the ruminant (Kamra, 2005). A principal function of the micro-biome is the conversion of plant materials into digestible compounds that can be used by the animal host (Mackie, 2002). This function is of tremendous importance as it allows the conversion of solar energy stored in plant fibers into food products, such as milk and meat. In addition, as the micro-biome in the rumen undergoes long-term selection and

evolution, the microbes and host form an inter-inhibitive and interdependent homeostatic relationship that has an important role in maintaining host health, improving performance, reducing environmental pollution, and ensuring food and animal product safety (Rawls *et al.*, 2004). In the rumen, *Bacteria* are the predominant microorganisms that convert plant biomass into fermentation end products such as short-chain fatty acids (SCFA) and amino acids; these are then utilized by the ruminant host (Zhou *et al.*, 2009). Rumen microbes can be assigned to different functional groups, such as cellulolytics, amylolytics, proteolytics, etc., which degrade the wide variety of feed components or further metabolize some of the products formed by other microbes (Hungate 1966). For example, methanogens, the methane-forming archaea, are among those that metabolize hydrogen formed by some fermentative microbes to form methane. The methane generated during this fermentation contributes to global anthropogenic greenhouse gas emissions (Ripple 2014) and represents a 2–12% loss of feed energy for the animal (Johnson and Ward, 1996). Differences in rumen microbial communities underlie variations in methane formation (Kittelman, 2014) and the conversion of feed to animal products (Jami *et al.*, 2014). Therefore, understanding these communities is key to understanding ruminal transformations of plant material to both undesirable and useful ruminant products. Therefore, an improved understanding of rumen microbial ecology can contribute to solutions for nutritional and environmental concerns in the livestock industry. Advances in rumen microbiology may also contribute insights into other complex interacting anaerobic microbial ecosystems. Many previous studies have

shown that the bacterial and archaeal communities related to the production of fermentation end products and methane emissions are affected by factors, such as ruminant species, diet, feed additives, season and geographic region (Kim *et al.*, 2011). The nitrogen: sulphur ratio in the microbial proteins is 14.5:1, and a sulphur deficiency in the diet causes changes in the microbial fermentation. In this context, it is interesting to note that the utilization of non-protein nitrogen (NPN) is decreased by low levels of sulphur in the rumen fluid, implying that the microbial growth is attenuated (Cassio *et al.*, 2014). Such a deficiency also reduces the utilization of lactate by the rumen bacteria, resulting in its accumulation. Consequently, there is an appreciable reduction in the digestion of cellulose, possibly due to the reduction of the microbial growth. For this reason, sulphur improves the microbial digestion of cellulose, contributing to the synthesis of amino acids, especially methionine and cysteine. Therefore, studies of ruminal microbes represent a key area of nutrition research in ruminants, and it is important to improve our understanding of these complex microbial populations and their interactions. Elucidating the composition of the gut bacterial community and its changes is essential to improve the health management and productivity of this important ruminant. To our knowledge, there has been no report of using ammonium sulphate-fortified diets to study developmental changes in the ruminal microbial flora in WAD rams. Thus, this study was aimed at characterizing the colonization process of ruminal microbes in WAD rams using ammonium sulphate-fortified diets.

Materials and methods

Experimental site

The study was conducted in sheep unit of

the Teaching and Research Farm, University of Ibadan, Nigeria. The location is 7° 27'N and 3° 45'E and at 200-300m above sea level. The climate was humid tropical with mean temperature of 25-29°C and the average annual rainfall of about 1250mm.

Animals and management

Sixteen West African Dwarf (WAD) rams aged between 8-10 months, weighing 12.20±0.12kg were randomly allotted to

one of four groups with 4 rams per group in a completely randomised design. Each group was assigned to diets shown in Table 10 and ammonium sulphate at inclusion level of 0, 2.5, 5.0, 7.5g/kg (NH₄)₂SO₄ were added as T1, T2, T3 and T4 respectively. Prior to the experiment, all rams were given prophylactic treatment of Oxytetracycline Long Acting (L.A.) at 1mL/10kg Body Weight (BW) and dewormed with Ivomectin

Table 1: Gross composition of experimental diet (% DM)

Ingredients (kg)	T1	T2	T3	T4
	0.00	2.50	5.00	7.50
Dry Cassava Peel	60.00	60.00	60.00	60.00
Brewers Dry Grain (BDG)	23.00	23.00	23.00	23.00
Palm Kernel Cake (PKC)	10.00	10.00	1.00	10.00
Urea (NH ₂) ₂ CO ₂	1.00	1.00	1.00	1.00
Dicalcium Phosphate (DCP)	1.00	1.00	1.00	1.00
Oyster Shell (OS)	2.00	2.00	2.00	2.00
Salt (NaCl)	2.00	2.00	2.00	2.00
Premix (Growers)	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00
<i>Calculated</i>				
CP	10.00	10.00	10.00	10.00
GE	3200.00	3200.00	3200.00	3200.00

T: Treatment, T1: 0g/kg (NH₄)₂SO₄, T2: 2.5g/kg (NH₄)₂SO₄, T3: 5.0g/kg (NH₄)₂SO₄, T4: 7.5g/kg (NH₄)₂SO₄, CP: Crude Protein, GE: Gross Energy. Growers premix: Vitamin A, 100,000 i.u; Vitamin D₃, 20,000 i.u; Vitamin E, 200mg; Vitamin K₃, 20mg; Vitamin B₁, 30mg; Vitamin B₂, 50mg; Niacin, 450mg; Calcium Pantothenate, 100mg; Vitamin B₆, 40mg; Vitamin B₁₂, 0.2mg; Folic acid, 10mg; Biotin, 0.5mg; Chlorine chloride, 3000mg; Manganese, 12,000mg; Iron, 1000mg; Zinc, 800mg; Copper, 85mg; Iodine, 15mg; Cobalt, 3mg; Selenium, 1.2mg; Anti-oxidant, 1.2mg

Table 2: Chemical analysis of Ammonium sulphate-fortified diets

Parameters (%)	T1	T2	T3	T4	SEM
	0.00	2.50	5.00	7.50	
Dry Matter	94.90	94.73	94.05	94.91	0.25
Crude Protein	11.01 ^c	11.95 ^c	12.28 ^b	14.91 ^a	0.37
Ether Extract	0.62	0.70	0.63	0.78	0.01
Ash	12.04	12.30	12.19	12.80	0.02
NDF	30.35	31.71	31.67	30.06	0.02
ADF	25.20	26.58	25.96	25.34	0.01
ADL	6.16	6.18	6.12	6.14	0.03
Gross Energy (Kcal/kg)	3211.60	3249.20	3233.30	3399.90	41.42

a,b,c: Means within rows with unlike superscripts are significantly different from each other (P<0.05). T: Treatment, T1: 0g/kg (NH₄)₂SO₄, T2: 2.5g/kg (NH₄)₂SO₄, T3: 5.0g/kg (NH₄)₂SO₄, T4: 7.5g/kg (NH₄)₂SO₄, SEM: Standard Error Mean, NDF: Neutral Detergent Fibre, ADF: Acid Detergent Fibre, ADL: Acid Detergent Lignin

Table 3: Chemical composition of *Panicum maximum* (%DM)

Ingredients	<i>P. maximum</i>
Dry Matter	38.50
Crude Protein	7.81
Ether extract	0.70
Ash	8.94
Neutral detergent fibre (NDF)	60.00
Acid detergent fibre (ADF)	38.00
Acid detergent Lignin (ADL)	7.00

Diversity and physical properties of rumen microbial ecosystem

Fifty (50) mL of rumen contents were collected from 16 rams after 109 days of feeding them with ammonium sulphate-fortified diets representing the four diets. All collections were performed before the morning feeding (at 08:00 a.m.). Rumen content samples were collected using suction tube. Immediately after collection, the pH was measured using a pH meter as well as the temperature using the thermometer. Rumen contents were squeezed through four layers of cheesecloth to remove particulate matter.

Determination of bacteria and fungi load using pour plate method

Media preparation for bacteria count

28g of nutrient agar was weighed, dissolved and melted over hot plate, this was made up to mark in a 1 litre flask with distilled water and placed inside a water bath at 90°C and dissolved in 2 conical flasks that were autoclaved at 121°C for 15 minutes.

Media preparation for fungi count

mark with sterile water in 1 litre flask, autoclaved at 121°C for 15 minutes and 10mL of 10% lactic was added to inhibit the growth of bacteria. Potato dextrose agar was used because of the presence of starch in it that enhances fungus growth.

Method for fixing and counting rumen protozoa

5mL of liquor was added to 5mL of MFS stain; left it in a dark place to avoid colour fading. A drop of solution was placed into

each well on the slide and carefully covered with to avoid bubbles after which it was viewed under the microscope using x10 objective. This was counted using neubauer chamber on a microscope with the aid of a counting vice.

Experimental layout

Sixteen WAD rams were randomly allotted to four dietary treatments with 4 replicates per treatment in a completely randomised design.

Statistical design and model

Data were computed and subjected to a one-way analysis of variance using the procedure of Statistical Analysis System (SAS, 1999). Significant treatment means were compared using Least Significant Differences (LSD) option of the same software. Experimental model of the design is: $Y_{ij} = \mu + T_j + I_{ij}$. Where Y_{ij} =Individual observation, μ =General mean, T_j =Effect of j th treatment, I_{ij} = Experimental error.

Results

Table 4 shows the **rumen** physical properties **and diversity of microbes** of WAD ram fed ammonium sulphate-fortified diets. Rumen liquor temperature differed ($P < 0.05$) significantly across the treatments and was close to the range for sheep in the tropics (39 – 40°C). However, significant ($P < 0.05$) difference was observed in rumen liquor temperature and pH amongst ammonium sulphate fortified diets. Rams on D4 recorded the highest pH (6.82) while those on D1 had the lowest (6.27), but the values recorded were within

the level for optimum ruminal fermentation. Rumen microbial diversity of WAD rams fed ammonium sulphate-fortified diets are shown in Table 4. There was significant ($p<0.05$) difference in *E. coli* and *S. typhimurium* of rams fed ammonium sulphate-fortified diets. The *E. coli* ranged from 2.75 log₁₀cfu/mL in D1 to 4.33 log₁₀cfu/mL in D4. *S. typhimurium* of rams fed ammonium sulphate-fortified diets were significantly ($P< 0.05$) different across the diets with rams on D4 recording the highest (6.67 log₁₀cfu/mL) and the least was observed for those on D1 (5.6 log₁₀cfu/mL), increased inclusion of (NH₄)₂SO₄ enhances the growth of *S. typhimurium*. Rams fed D4 had the highest ($p<0.05$) bacterial population (4.86 log₁₀cfu/mL) while those on D1 recorded the least (4.82 log₁₀cfu/mL). Rams fed D4

had the highest ($P<0.05$) bacterial population (4.86log₁₀cfu/mL) while those on D1 recorded the least (4.82log₁₀cfu/mL). The rams on (NH₄)₂SO₄ fortified diets showed an increase in bacterial population as (NH₄)₂SO₄ inclusion increased over those on control (D1) diet. This is suggestive of (NH₄)₂SO₄ stimulating the proliferation of bacterial population.

The fungi was highest ($P<0.05$) for rams on the D4 (2.66 log₁₀cfu/mL) while those fortified with (NH₄)₂SO₄ were higher compared to control. This may be attributed to the presence of sulphur in (NH₄)₂SO₄ that enhances the proliferation of fungi. The protozoa values was lower for (NH₄)₂SO₄ fortified diets over control because sulphate reducing bacteria compete with methanogens for hydrogen use by reducing methane production.

Table 4: Rumen physical properties and microbial load of WAD rams fed ammonium sulphate - fortified diets

Parameters	D1	D2	D3	D4	SEM
	0.00	2.50	5.00	7.50	
pH	6.27 ^d	6.42 ^c	6.68 ^b	6.82 ^a	0.06
RL Temp. (°C)	40.10 ^a	39.40 ^c	39.80 ^b	40.00 ^a	0.08
<i>E. coli</i>	2.75 ^d	3.00 ^c	3.75 ^b	4.33 ^a	0.05
<i>S. typhimurium</i>	5.67 ^d	5.95 ^c	6.28 ^b	6.67 ^a	0.07
Bacteria	4.82 ^b	4.83 ^b	4.85 ^a	4.86 ^a	0.09
Fungi	2.53 ^b	2.57 ^b	2.64 ^a	2.65 ^a	0.75
Protozoa (/mL)	5.95	5.80	5.62	5.39	0.06

a, b, c: means within rows with unlike superscripts are significantly different from each other ($p<0.05$). d

rumen liquor, d1: 0g/kg (nh₄)₂so₄, d2: 2.5g/kg (nh₄)₂so₄, d3: 5.0g/kg (nh₄)₂so₄, d4: 7.5g/kg (nh₄)₂so₄,

rl temp.: rumen liquor temperature, sem: standard error mean, *e. coli*: *escheriacoli coli*, *s. typhimurium*: *salmonella typhimurium*., unit of microbes: (log₁₀cfu/ml)

Discussion

Rumen pH is an important factor that measures the acidity and alkalinity of rumen contents Bowen (2009). Thus, for optimum rumen microbial fermentation, the rumen pH should lie between 6.00 and 7.00. However, the rumen pH values observed in this study lies within the range of values (6.00 - 7.20) for maximum microbial growth as reported by

Koutsoumanis *et al.*, (2007). The pH of ammonium sulphate-fortified diets was higher than the control (Tables 4), which may have favoured bacteria. Cellulolytic bacteria (e.g., *Butyrivibrio fibrisolvens*, *Bacteroides succinogenes*, *Ruminococcus albus* and *R. flavefasciens*) demonstrate sensitivity to low pH (Russell and Dombrowski, 1980). Mould *et al.* (1983) concluded that the growth of cellulolytic

bacteria is completely inhibited at pH values below 6.0. In this study, the pH was maintained above 6.08. The rumen liquor temperature ($^{\circ}\text{C}$) levels were different amongst treatments. This observed variation might be as a result of difference in evolution of heat from microbial fermentation activity in the diets. This is in consonance with the finding of (Adeyosoye *et al.*, 2010) who reported that the trends at which temperature rise in the rumen following ingestion of feeds is due to the evolution of heat in the fermentation process which has been used as a measure of microbial fermentation rate in the rumen. However, the rumen temperature values obtained in this study were within the relative constant range values ($38.00 - 40.00^{\circ}\text{C}$) for continuous microbial fermentation as reported by Okoruwa *et al.* (2014). Microbial load in the rumen is very important because, it is an index of the amount of microbial protein made available to the sheep daily (Kissada *et al.*, 2010). This is because sulphur is one of the important factors for fungi growth and proliferation (Gordon and Philips, 1998) that enhances microbial protein synthesis in the rumen. Low levels of sulphur supplementation in the diet containing cassava foliage could reduce microbial biomass in the rumen (Promkot *et al.*, 2007). Addition of sulphur sources to sulphur-deficient diets stimulates the total number of bacteria and stimulates rumen fermentation and microbial protein synthesis (Hegarty *et al.*, 1994). Therefore, rumen fermentation may have been stimulated by the additional sulphur from sulfate in the diet, resulting in increased microbial load as shown in table 4.

In addition to this, sulphur supplementation increased the concentration of all three microbial groups but the most dramatic increase was observed with the number of sporangial forms of rumen anaerobic fungi.

Fungi have an additional advantage of better penetration of the lignocellulosic feeds over the cellulose-degrading bacteria because of the presence of hyphae (thread-like) structure that penetrate into the substrate. Bacteria have different fibre-degrading enzymes (cellulases, hemicellulases, xylanases, avicelases, glucosidases *e.t.c.*) found to be associated with rhizomycelia which enable it to degrade fibres (Paul *et al.*, 2003) after it has been exposed by fungi for degradation by bacteria. Thus, ammonium sulphate was added to the treatments to provide adequate sulphur requirement for anaerobic fungi to maximally digest the components of dietary fibre leading to the production of energy to the animal (Okoruwa *et al.*, 2014). These results are consistent with the premise that increased rumen function due to addition of sulphur to the diet is due to increased microbial activity.

The reason for this increase can be adduced to promotion of the growth of rumen bacteria and optimization of rumen function (*i.e.* ruminal ecosystem in terms of structure of microbial populations and activities) by fortification with ammonium sulphate as shown in Table 4 and this is in agreement with Akin and Benner, (1988). Bacteria can use carbohydrates as carbon skeletons for proteins in combination with ammonia (Bach *et al.*, 2005,) and also utilize sulphur (organic and inorganic) to synthesize sulphur containing amino acids (Kandyliis, 1984) to produce microbial protein.

Sulphate reducing bacteria can compete against methanogens for hydrogen use by reducing methane production (Russel, 1998). Protozoa also have a negative impact on animal productivity in that the engulfment and digestion of bacteria by protozoa (Charles *et al.*, 2015) significantly lowers the flow of microbial protein leaving the rumen (Jouany and Ushida, 1999). Thus the use of defaunation to decrease methane

production from ruminants would have to be balanced against the effects on fibre and protein metabolism in the rumen. Nakamura *et al.* (1981) and Yoshida *et al.* (1982), using rumen fluid from sheep fed nitrate, showed that the protozoal fraction obtained by low-speed centrifugation stimulated the rate of nitrate and nitrite reduction by the bacterial fraction, suggesting that rumen protozoa might possess reductase activities. It seems unlikely, however, from their pathways of nitrogen and energy metabolism that ciliate protozoa produce either assimilatory or dissimilatory reductases. The activity associated with the protozoal fraction of the rumen microbiota may be due to stimulation of bacterial activity by a factor provided by the protozoa such as metabolic hydrogen. Hydrogen is a very effective reductant for nitrate *in vitro* (Jones 1972). Significant hydrogen production would be expected in a suspension of rumen ciliate protozoa containing fermentable carbohydrate (Hungate, 1966). This is why ammonium sulphate-fortified diets serve as anti-methanogenic compounds because sulphur reducing bacteria compete with methanogens which led to the reduction of hydrogen as well as ciliated protozoa numbers as shown in Table 4.

Also, microbes in the rumen play an important role in sustaining and maintaining a balance in the rumen ecosystem. Symbiotic relationship that exists between the rumen microbes and the ruminant animal enables the animal to enjoy adequate supply of nutrients released by the microbes' activities which eventually result in the biodegradation of fibrous feedstuff abundantly available in Nigeria.

Conclusion

The study showed that ammonium sulphate is a feed additive that serves as a ready

source of nitrogen and sulphur, which enhanced the proliferation of sulphate reducing bacteria which compete with methanogens for hydrogen use, by reducing methane production. The pH was affected by ammonium sulphate because it is basic in nature and it ranged from 6.82 (D4) to 6.27 (D1). Microbial population was significantly affected by ammonium sulphate fortification except for protozoa which decreased as the inclusion level of ammonium sulphate increased for all treatments. The bacterial and fungal population were higher for the ammonium sulphate-fortified diet over the control as a result of the influence of ammonium sulphate on their growth. Diets fortified with ammonium sulphate at 7.5g/kg helps in the proliferation of microbes', gives a better understanding of the role of rumen microbes and the establishment of the microbial population, which help to maintain the host's health and improve animal performance.

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