

Identification and Occurrence of Heterophilic Rumen Bacteria and Fungi Isolated from Selected Nigerian Breeds of Cattle

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Abstract The kinetics of fermentative activities in the rumen justified the need for microbial assessment of autochthonous members of rumen community before prolonged fermentation in the selected breeds of cattle commonly adapted to Nigerian environment. A total of four breeds of cattle were selected for this study comprising of both male and female sexes of Bunaji (White Fulani), Futumi (Keteku), Bokolo (N'dama) and Djeli (Sokoto Gudali) respectively. Total heterophilic bacteria (THB) on Nutrient agar (NA) ranged between 9.1×10^8 cfu/g - 125×10^{11} cfu/g with the highest count recorded for male Djeli (Dm) and the least count for female Djeli (Df) respectively. Total heterophilic fungi (THF) on Malt Extract (ME) agar recorded the highest count (6.0×10^5 spores/g) for female Bunaji (Bf) and no growth was observed for female Bokolo (Bkf). The rumen pH ranged between 5.65 and 6.90 for female Futumi (Ff) and male Bunaji (Bm) respectively. Significant differences ($p < 0.05$) were observed for pH and total microbial counts based on sex. Standard methods of Colonial and biochemical assessments led to the isolation, characterization and identification of bacterial species of the genera; *Klebsiella*, *Proteus*, *Pseudomonas* and *Shigella*. *Klebsiella edwardsii* occurred highest (24.24%) while *Proteus morganella*, *Shigella dysenteriae* and *Shigella sonnei* occurred least (3.03%). Fungal species of the group; *Aspergillus*, *Botrytis*, *Cladosporium*, *Cephalosporium*, *Paecilomyces*, *Penicillium*, *Pullularia*, *Rhizoctonia* and *Trichophyton* were also isolated and identified. *Aspergillus glaucus* and *Pullularia pullulans* both occurred highest (17%) while *A. fumigatus*, *A. niger*, *Botrytis spp.*, *Cladosporium herbarium*, *Penicillium camemberti*, *Trichophyton mentagrophytes*, *T. rubrum* and *Rhizoctonia solani* occurred least at the level of 5%. In conclusion, breed as a factor had significant effects on the type, population and percentage occurrence of rumen bacteria and fungi studied in this work. The use of fistulated animals is recommended for microbial screening at different stages of fermentation without the need to sacrifice the animals.

Keywords: standard methods, rumen fermentation, cattle breeds, rumen microbes, percentage occurrence

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1. Introduction

1.1. Cattle

Cattle are a group of ungulate livestock called ruminants with a complex stomach system comprising four compartments namely; rumen (front gut), omasum (manypiles), reticulum (honey comb) and abomasum (true stomach) which are modified for rumination [1]; they are commonly raised as livestock for meat except in major parts of India and Nepal [2], as dairy animals for milk production and as draft animals for

pulling carts. Their by-products also serve as valuable raw materials for manufacturing industries. For instance; horns and hoofs are useful in ceramics, bones are a valuable source of calcium in livestock feed, skin for leather production and dung for organic manure and substrate for biogas production [3].

1.2. Zoologica Classification of Cattle

According to [4], cattle were scientifically classified as follows;

Kingdom: *Animalia*

Phylum: *Chordata*

Class: *Mammalian*

Order: *Artiodactyla*
 Sub-order: *Ruminantia*
 Family: *Bovidae*
 Sub-family: *Bovinae*
 Genus: *Bos*
 Specie: *taurus*

It should however be noted that *Bos taurus* is a sub-specie of *Bos primigenus*.

1.3. Nigeria

Nigeria is located in the tropical zone of West Africa between latitudes 4°N and 14°N and longitudes 2°21'E and 14°30'E and has a total area of 923,768 km². The country is bounded by Cameroon to the East, Chad to the northeast, Niger to the north, Benin to the West, and the Gulf of Guinea on the Atlantic Ocean forms the Southern limits of Nigerian territory.

Land cover ranges from thick mangrove forests and dense rain forests in the south to a near-desert condition in the northeastern corner of the country. Nigeria is by far the most populous country in Africa, accounting for about one-seventh of the total population of Africa's 53 countries [5].

1.4. Breeds of Cattle

About twelve breeds (Plate 1) are reported for Nigeria but extensive studies on origin, adaptation and management have been conducted on Kuri, N'dama, Muturu, Gudali and Red bororo/Fulani [6].

The rumen (Plate 2b) as shown in the internal morphology of an adult cow (Plate 2a) is the first and the largest compartment where continuous anaerobic fermentation takes place by a complex consortium of microorganisms thus, characterized as the World's largest fermentation process. Ruminants chew cud by the process known as regurgitation, the modification of the alimentary is necessary for physiological, biochemical and nutritional purposes.

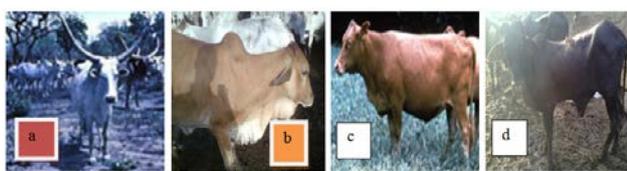


Plate 1. Selected Nigerian Breeds of Cattle; a: Bunaji, b: Bokolo, c: Djeli, d: Keteku

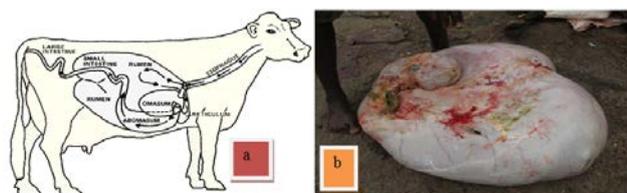


Plate 2. Internal Morphology (a) and Rumen of a Cow (b)

1.5. Objectives

This study aimed at enumerating, characterizing, identifying and determining the percentage occurrence of the total heterophilic bacteria and fungi autochthonous to the rumen of cattle with respect to different breeds commonly raised in Nigeria.

1.6. Rumen Microbes

Ruminants are borne with germ-free rumen; however, unique flora as well as fauna starts to colonize after birth. The new born ruminant is exposed to many different sources of microorganisms adhering to dam's vagina, udder, milk and saliva. Other sources of contamination are; manure, bedding and environmental flora present in air, water and feeds [7]. Therefore, the kinetics of fermentative activities in the rumen as a result of conventional feed in-take that are principally composed of lignocelluloses which make them a substrate of enormous biotechnological value [8] necessitated the need for assessment of micro-flora associated with rumen in this study.

2. Materials and Methods

2.1. Identification of Breeds of Cattle

The commonest available breeds (Plate 1) identified for this work were both male and female sexes of Bunaji (white Fulani), Keteku (Futumi), Bokolo (N'dama) and Djeli (Sokoto Gudali) according to the description of [6].

2.2. Location of the Study

Breeds identification was done at the Bodija abattoir situated at the extreme end of Bodija International market located in Ibadan North Local Government Area. There are four large sized slaughter halls for cattle, goats, sheep and pigs butchering respectively, the largest two of the four are strictly for cattle butchering [9].

2.3. Sampling

Following butchering of the animals, a kitchen-spoonful of rumen content was aseptically scooped immediately after opening the rumen, into pre-labeled sterile empty bottles from 4 different portions of each rumen from 8 skinned animals per week over a period of 12 weeks, with the aid of a sterile spoon. In this way, four breeds (male and female) were examined weekly.

2.4. Sterilization of Materials

Sterilization at 160°C for two hours in the hot air oven (Uniscope SM9023), steaming under pressure using an autoclave (Microfield Instrument, England, SM280-A) at a temperature of 121°C under a pressure of 15 psi (100 Kpa, 1.05 kg/cm³, 151 b/sq inch) for 20 minutes according to the method of Chamberland modified by [10], surface-disinfection with 70% ethyl alcohol (BDH AnalaR England grade) and incineration to red hot using Bunsen flame, were employed where applicable.

2.5. Culturing Technique for Total Heterophilic Bacteria (THB) and Fungi (THF)

The modified methods prescribed by [11] were carefully adopted. One ml each of 100-fold serially diluted suspension was plated out with nutrient agar (NA) (LAB M™ UK) and incubated, using (Uniscope SM9023) at 37°C for 36-48 hrs to estimate the bacterial colonies formed [12].

The procedure for the THF was similar to that of THB used except that malt extract (ME) agar (LAB M™ UK) containing antibiotics was employed as the culture medium, also, the plates were incubated uninvertedly at 30°C for 5-7 days [13].

2.6. Isolation, Characterization and Identification of Rumen Bacteria and Fungi

Physical Colonial characteristics, cell morphology through Gram's staining procedure, spore formation through spore-staining technique and biochemical reactions led to the identification of bacteria and fungi that are primary colonizers of cattle rumen [14].

2.7. Statistical Analysis

Statistical separation by analysis of variance with differences was determined by the method of least significant differences (LSD) at the level of 5% ($p < 0.05$) as described by [15].

3. Results and Discussion

Rumen pH and Total Viable Microbial Counts: The pH measurement ranged between 5.65 for female Keteku (Kf) and 6.90 for male Bunaji (Bm) (Table 1). No significant difference ($p > 0.05$) was observed between male Djeli (Dm) and female Djeli (Df), male Keteku (Km) and male Bokolo (Bkm) exhibited the same pH-value (6.60). However, the standard error based on sex of the selected animals revealed a significant difference ($p < 0.05$) in pH between male (6.71) and female (6.10) cattle.

Table 1. Rumen Ph and Total Viable Counts for Both Heterophilic Bacteria And Fungi

Breed of Animal	THB	THF	pH Value
Bm	$5.1 \times 10^{10c} \pm 2.0 \times 10^9$	$1.5 \times 10^{3c} \pm 5.0 \times 10^2$	$6.90^a \pm 0.1$
Bf	$2.25 \times 10^{10f} \pm 5.0 \times 10^8$	$6.0 \times 10^{5a} \pm 1.0 \times 10^5$	$5.90^e \pm 0.1$
Fm	$3.4 \times 10^{10e} \pm 1.5 \times 10^9$	$9.5 \times 10^{4c} \pm 5.0 \times 10^3$	$6.60^c \pm 0.1$
Ff	$5.3 \times 10^{10b} \pm 1.0 \times 10^9$	$3.0 \times 10^{5b} \pm 2.9 \times 10^5$	$5.65^f \pm 0.05$
Bklm	$3.6 \times 10^{10d} \pm 1.5 \times 10^9$	$1.5 \times 10^{3c} \pm 5.0 \times 10^2$	$6.60^c \pm 0.1$
Bklf	$2.3 \times 10^{10f} \pm 1.5 \times 10^9$	NIL ^c	$6.10^d \pm 0.1$
Dm	$1.1 \times 10^{11a} \pm 1.5 \times 10^9$	$1.5 \times 10^{3c} \pm 5.0 \times 10^2$	$6.75^b \pm 0.05$
Df	$9.1 \times 10^{8f} \pm 1.0 \times 10^7$	$5.5 \times 10^{3c} \pm 5.0 \times 10^2$	$6.75^b \pm 0.15$

Values are means \pm Standard deviation of duplicate readings. Values on the same column with different superscripts are significantly different ($p < 0.05$). THB: Total Heterophilic Bacteria, THF: Total Heterophilic Fungi, pH: hydrogen ion concentration, Bm: male Bunaji, Bf: female Bunaji, Fm: male Futumi, Ff: female Futumi, Bklm: male Bokolo, Bklf: female Bokolo, Dm: male Djeli, Df: female Djeli, E8: $\times 10^8$, E10: $\times 10^{10}$, E11: $\times 10^{11}$.

3.1. THB

Dm and Df recorded both the highest (1.125×10^{11}) and the lowest (9.1×10^8) populations of THB respectively with a significant dissimilarity ($p < 0.05$) existing between them (Table 1). Bm, Bf, Km, Kf, Bkm and Bkf recorded THB

in the range of 2.25×10^{10} - 5.3×10^{10} . Although, Bf (2.25×10^{10}), Bkf (2.25×10^{10}) and Df (9.1×10^8) were significantly indifferent ($p > 0.05$). Significant difference ($p < 0.05$), however, existed between male (5.8125×10^{10}) and female (2.4728×10^{10}) cattle selected for this study, as revealed by the standard error based on sex.

Table 2. Colonial And Morphological Characteristics of the Fungal Isolates

Isolate	Macroscopic Examination	Microscopic Examination
<i>Aspergillus fumigates</i>	White colony turned greyish-green	single conidia in chain, arising from the terminal bulb of the conidiophores, septate mycelium
<i>Aspergillus glaucus</i>	White colony turned bluish-green	"
<i>Aspergillus niger</i>	White colony turned smoky-green	"
<i>Botrytis sp.</i>	Loose grey colony	Spores appeared like a bunch of grapes
<i>Cladosporium herbarium</i>	Deep greenish-black small heaped colonies	Spores occur in large tree-like clusters, septate hyphae
<i>Cephalosporium sp.</i>	Tough white colony turning pale-pink	Sliming spores borne in small balls on the end of slender hyphae
<i>Paecilomyces variotii</i>	White woolly colony with a pink tint turned dirty brown	Long and slender phialides, mycelium appeared disintegrated
<i>Penicillium camemberti</i>	Matured colony showed grayish-green colouration	Single spores in chain developing from the branching conidiophores emanating from the septate mycelium
<i>Pullularia pullulans</i>	Shiny white colony turned black and became leathery	Dark hyphae bearing conidia as lateral buds
<i>Trichophyton interdigitale</i>	Granular form produced flat wing with spores covering the surface, brownish pigmentation on the reverse side of the culture	Spiral hyphae, abundant roundish micro-conidia that resembled a bunch of grapes along the sides of the hyphae
<i>Trichophyton metagrophytes</i>	The fluffy specie grew like cotton-wool with the same sort of pigmentation	"
<i>Trichophyton rubrum</i>	Slow fluffy growth, red pigmentation on the reverse side of the culture,	Elongated micro-conidia borne along the sides of the hyphae, resembled barb wire under a low power objective
<i>Rhizoctonia solani</i>	Colony appeared in several successive white and black rings, the reverse side of the culture appeared yellow	No aggregation of hyphae, hyphae assumed several distinguishing turns of Y-shape with spores appearing like elongated yeast buds

3.2. THF

The morphological description of the fungal isolates is as shown in Table 2. The fungal load (Table 1) ranged between 1.5×10^3 (value enumerated for Bm, Bkm and Dm) and 6.0×10^5 (count recorded for Bf) with no growth

observed for Bkf), thus, the counts obtained in this study are in agreement with the fungal load obtained by [16]. However, standard error based on sex revealed a significant difference ($p < 0.05$) male (2.4875×10^4) and female (2.26376×10^5) cattle selected for this study, implying that cows generally, are more loaded with fungi than bulls.

Colonial and microscopic characteristics of fungal Isolates are as shown in Table 2.

Anaerobic fermentation of forages into cellulose and hemicelluloses is carried out in the rumen through microbial degradation [17]. Majority of rumen bacteria are also described as obligate anaerobes, having an optimal pH range of 6.0-6.9 at an optimum temperature of 39°C. The bacteria can tolerate a considerably higher level of organic acids without affecting adversely their metabolism.

The pH conditions of the sampled rumens could be said to be in agreement with the optimal range (6.0-6.9) described by [16]. Bf and Kf that had slightly acidic rumen pH (5.90 and 5.65 respectively), This could have resulted from the feed ingredients they were fed with, as feed rich in soluble sugars are capable of lowering the rumen pH when they are finally broken down [18].

The bacterial load obtained in this work (10^{10} - 10^{11} cells/ml) agrees with the result obtained by [16] in which the efficiency of ruminants to utilize a wide variety of feeds was based on a highly diversified rumen microbial ecosystem consisting of bacteria [19].

Obligate anaerobic fungi found in the rumen have an active and positive role to play in fibre degradation [20]. Fungi have additional advantage of better penetration of the lignocellulosic feeds over the cellulose-degrading bacteria due to the presence of different enzymes like *proteases* and *esterases* in addition to *cellulases* and *hemicellulases*.

Reference [1] attributed population variation between bacteria and fungi to the competitive and unfavourable conditions created around fungi, such include; variation in pH resulting from C/N ratio [18], nutrient availability [22], the toxic effect of phytochemicals in feeds [23,24] and the parasitic effect of *Cladosporium herbarium* on other fungi.

Fungi found in rumen are involved in the early part of degradation (hydrolysis and acidogenesis) by means of extra-cellular digestion of the substrates, depending on the nature of substrate. Reference [25] reported that the removal of fungi from bovine stomachs had significantly reduced effect on the amount of fibrous feed breakdown within rumen. Ruminal obligate anaerobic fungi make up only 5-10% of microbes but are absent on diets poor in fibre.

Despite their low numbers, the fungi still occupy an important niche in the rumen because they hydrolyze some ester linkages between lignin and hemicellulose or cellulose, and help break down digesta particles [20]. The partial digestion of microbial carcasses releases high quality protein to cattle and allows them to thrive on grasses and other vegetation. The digestion of these microbes in the small intestine is a major source of nutrient, as microbes usually supply about 60 to 90% of the total amount of amino acids absorbed [17]. The efficiency of utilization of 'feed energy' is the amounts lost as heat (45%), faeces (40%), and urine together with combustible gases (10%). Whereas, the energy converted to products (tissue accretion) is as low as 5% [26].

3.3. Percentage Occurrence of the Isolates

3.3.1. Bacteria

Highest bacterial occurrence (24.2%) (Figure 1) was recorded for *Klebsiella edwardsii* which was isolated from rumen of both male and female breeds of cattle studied.

Pseudomonas aeruginosa was found in all the breeds studied except Dm, thereby occurring at 21.2% while *Shigella sonnei*, *Proteus morganella* and *Shigella dysentery* had the least percentage occurrence.

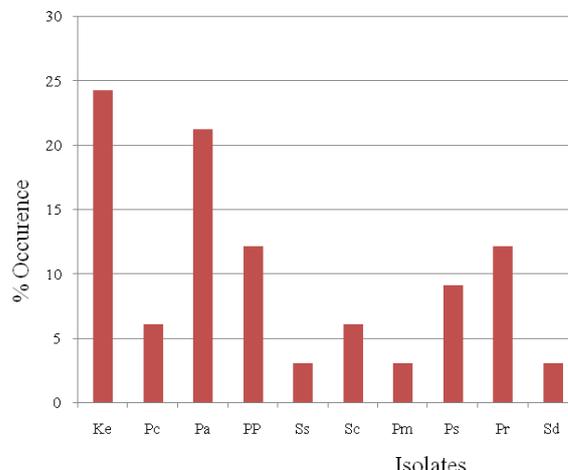


Figure 1. % Occurrence of Bacterial Isolates in the Rumens of selected Breeds

Ke: *Klebsiella edwardsii*, Pc: *Pseudomonas cepacia*, Pa: *Pseudomonas aeruginosa*, Pp: *Pseudomonas pseudomallei*, Ss: *Shigella sonnei*, Sc: *Shigella ceylonensis*, Pm: *Proteus morganella*, Ps: *Pseudomonas stutzeri*, Pr: *Proteus rettgeri*, Sd: *Shigella dysenteriae*.

3.3.2. Fungi

Both *Pullularia pullulans* and *Aspergillus glaucus* occurred highest (16%) (Figure 2). All other isolates had the least occurrence (5-6%) except *Cephalosporium s.* and *Trichophyton interdigitale* which occurred at 11%.

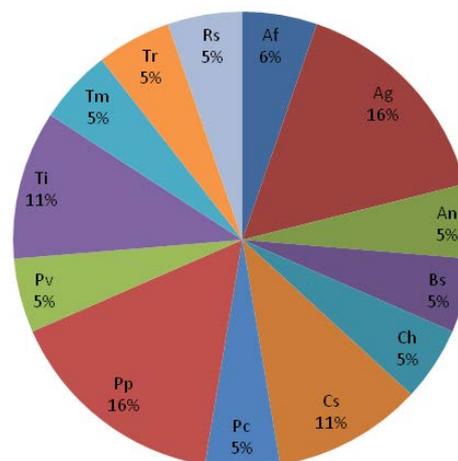


Figure 2. Percentage Occurrence of Fungal Isolates in the Rumens of selected Breeds

Af: *Aspergillus fumigatus*, Ag: *Aspergillus glaucus*, An: *Aspergillus niger*, Bs: *Botrytis sp.*, Ch: *Cladosporium herbarium*, Cs: *Cephalosporium sp.*, Pc: *Penicillium camemberti*, Pp: *Pullularia pullulans*, Pv: *Paecilomyces variotii*, Ti: *Trichophyton interdigitale*, Tm: *Trichophyton mentagrophytes*, Tr: *Trichophyton rubrum*, Rs: *Rhizoctonia solani*.

Bacterial especially species of *Proteus*, *Klebsiella* and *Citrobacter* are peculiar to cow dung [27], however, *Klebsiella edwardsii* was isolated from rumen of each of all the breeds of cattle studied. *Pseudomonas aeruginosa* was found in all the breeds studied except Dm, the presence of *P. aeruginosa* is as a result of its ability to

form acid from simple sugars [28] and its protease enzyme helps in protein decomposition in the rumen.

Proteus morganella has been reported to have a commensally relationship within the intestinal tracts of mammals and reptiles as normal flora [29] while some strains carry antibiotic-resistant plasmids and have been associated with nosocomial outbreaks of infections such as sepsis, ecthyma, endophthalmitis, chorioamnionitis, urinary tract infections, septic arthritis, meningitis and bacteremia as reported by [30].

Pseudomonas stutzeri, a denitrifying bacterium resident in soil is potentially useful in bioremediation [31].

Shigella dysenteriae which is closely related to *Salmonella*, is a Gram negative, rod-shaped, non spore-forming and non-motile facultative anaerobe, causes shigellosis (bacillary dysentery), *S. dysenteriae* spreads by contaminated water and food, severe pathogenicity is displayed by its potent and deadly 'shiga' toxin [32].

Reference [33] classified *Proteus rettgeri* as one of the five species of genus *Providencia*, (other species include; *P. alcalifaciens*, *P. heimbachae*, *P. rustigianii* and *P. stuartii*). It resembles *Shigella paradysenteriae* in biochemical characteristics but was antigenically distinct from the well-recognized type of *Shigella*.

Pseudomonas cepacia according to [34], causes onion skin rot, attacks tobacco and capable of biodegrading oil. It is an important human pathogen which causes pneumonia in immunocompromised patients with underlying lung diseases such as cystic fibrosis or chronic granulomatous disease [35].

Among fungi isolated, *Aspergillus glaucus* is a fungus primarily found in Arctic marine environment [36,37]. *Pullularia pullulans* is a ubiquitous black yeast-like fungus and well known as naturally occurring epiphyte or endophyte of a wide range of plant species such as apple, grape, cucumber, green beans and cabbage without any disease symptoms. It produces useful enzymes such as siderophores and pullans and has been known to be a potent biological control agent against storage disease. *Rhizoctonia solani* is a saprophytic soil-borne microbe that is best known to cause various plant diseases such as collar rot, root rot, damping off and wire stem.

4. Conclusions and Recommendations

It can be concluded from this study that microorganisms as normal microbiota of rumen, play a vital role in sustaining and maintaining a balance in the rumen ecosystem. Symbiotic relationship that exists between rumen microbes and cattle enables the animals to enjoy adequate supply of nutrients released by the microbial activities, which eventually results in biodegradation of fibrous feedstuff abundantly available in Nigeria.

Breed as a factor, was found to have significant effects on the percentage occurrence, type and load of rumen bacteria and fungi at the early stage of rumen fermentation.

The use of fistulated animals is recommended for microbial screening at different stages of fermentation without the need to sacrifice the animals. Further investigations on rumen microbes from Nigerian breed of cattle should be directed towards molecular assessment of the prospective isolates.

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