EVALUATION OF RUMEN MICROBIOTA AND THE ASSOCIATED MILK COMPOSITION PROFILES OF CROSSBRED DAIRY CATTLE USING NOVEL MOLECULAR GENETIC MARKERS

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DECLARATION

I declare that this thesis is my original work and has not been previously presented for an award of degree in any other University or institution of higher learning.

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DEDICATION

This thesis is dedicated to my beloved family!

Sincerely appreciate your support and encouragement all the time!

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LIST OF ABBREVIATIONS

AOAC Association of Official Analytical Chemists

ANOVA Analysis of Variance

BLAST Basic Loci Alignment Search Tool

Cfu/ml colony –forming units per milliliter

cP Centipoise

DNA Deoxyribonucleic Acid

FAO Food and Agriculture Organization

GDP Gross Domestic Product

IACUC Institutional Animal Care and Use Committee

KEBS Kenya Bureau of Standards

NRC National Research Council

VFAs Volatile Fatty Acid

NGS Next generation sequencing

OTUs Operational Taxonomic Unit

PCA Principle component analysis

PCR Polymerase chain reaction

QIIME Quantitative Insights Into Microbial Ecology

TMR Total Mixed Ration

USAID United States Agency for International Development

ABSTRACT

Dairy cow milk is both economically and nutritionally important to humans. Diet has been termed as the main driving force toward differences in milk production and composition. The effectiveness with which ruminants convert fibrous plant material into usable energy and human utilizable derivatives like milk is dependent upon the existing consortium of rumen microbes. However, little is known about the relationship of these microbes with milk composition constituents towards producing quality milk. Therefore, this study profiled the rumen microbes after subjecting the crossbred dairy cows to diets containing different concentrate levels and later assessed the correlation between the profiled bacterial taxa and milk composition parameters. A 4X4 Latin square design was conducted within 80 experimental days. The experimental four diets were formulated to contain 10%, 20%, 30%, and 40% commercial dairy concentrate and to meet the nutritional requirements of dairy cattle yielding 12 kg of milk per day. Rumen liquor and milk samples were collected after every ten days. Microbial composition was assessed using R software V4.1.2; then Spearman correlation was used to determine the relationship between bacterial taxa communities and milk production and composition constituents.

The results obtained showed that an increase in concentrate ratio across the diets led to an increase in the abundance of Bacteroidetes ($P \leq 0.05$) and Proteobacteria while Firmicutes and Fibrobacter decreased. Bacteroidetes and Firmicutes were the dominant bacteria, making up 83.7% of the total rumen bacteria. Further, indicating that dietary changes significantly affected some rumen bacterial community composition and diversity. On the other hand, a positive and significant correlation was exhibited between *Prevotella* ($P \leq 0.05$), Lentispaerae ($P \leq 0.01$), Synergistetes ($P \leq 0.01$) with milk protein. *BF311* was also positively and significantly correlated with milk fat ($P \leq 0.05$). Phylum Fusobacteria showed a negative

correlation with milk lactose ($P \leq 0.01$) as well as Tenericutes with milk protein ($P \leq 0.01$). These associations between the rumen microbiota and host phenotypes revealed utilization relationships and productive associations between dietary nutrients, affected bacterial groups, and milk composition constituents.

CHAPTER ONE

1.1 Introduction

Many intrinsic and extrinsic factors affect animal production (Byerly, 1967). However, the cost and the availability of feed has arguably been the most constraining element, with cost accounting for 50-70% of the total expenses in animal production (Spring, 2013). Several researchers have suggested arrays of alternative feedstuffs as a way to minimize the feed cost; nonetheless, the reduced dietary value of some alternative feeds cannot supply the nutrients needed for optimum ruminant production. For decades, ruminant nutrition has been researched and gradually refined, focusing on feed intake, fermentation parameters, metabolism, and digestibility. However, each of these measures is inextricably linked to the composition of the microbial community of rumen. Therefore, its crucial to consider microbial organisms while researching ruminants. Studies on rumen microbe have been made possible by utilizing nucleic acid-based, high-throughput approaches, which allow intense microbial understanding, thus laying a foundation for novel ruminant production technologies.

The effectiveness with which ruminants convert fibrous plant material into usable energy and human utilizable protein derivatives like meat and milk is the quintessential function of a ruminant forestomach, and this differentiates them from monogastric animals. The rumen efficacy is also a rising area of interest, and more awareness of rumen efficacy may be helpful in developing better feeds and feeding strategies. The rumen houses a vast number of microbial species that are in a symbiotic relationship with the host animal. The microbes break down fibrous plant materials and converting them to by-products like Volatile Fatty Acids (VFAs). Bacteria are the most dominant, comprising approximately 95% of the

population (Kibegwa *et al.,* 2020; Yang *et al.,* 2018). Other organisms include archaea, fungi, and viruses in different proportions.

Rumen microbial diversity is influenced by a wide range of factors, including genetics, diet, and animal management strategies (Jewell *et al.,* 2015; Petri *et al.,* 2013a). These differences in rumen microbial communities affect the VFAs ratio (propionate to butyrate to acetate), which could have direct effects on the quantity and quality of milk and meat. Milk has both economic and nutritional importance to humans. The financial implication is of interest to farmers, while nutrition is associated with milk consumption. Differences in milk composition have been attributed to genetic differences within species, breed difference, stage of lactation and parity, season variations, management: diseases and nutrition (Gajbhiye *et al.,* 2019; Kala *et al.,* 2017; Roessler *et al.,* 2019; Sandrucci *et al.,* 2019; Wint *et al.,* 2019), with diet showing profound effects (Krehbiel, 2014).

Arrays of research conducted on ruminants have primarily aimed at improving milk production and quality, and emphasis has been laid on the 'microbial organ' of the dairy cows. For example, Jami *et al.* (2014) demonstrated the potentiality of modulating milk quality parameters by studying the bovine rumen microbiome roles and identified a connection between milk fat to Firmicutes and Bacteroidetes. The Firmicutes to Bacteroidetes ratio relationship to milk fat poses a challenge to other milk constituents and how much of the variations in these milk compositions can be explained by bovine rumen microbial variations.

Thus, a critical approach to further improving the quality of milk in livestock is to identify gaps in our current knowledge on the influence of rumen microbial composition on various milk constituents in crossbred dairy cattle by evaluating the shifts and stability of the bovine rumen microbiome when subjected to different diets. Therefore, this study aimed to assess

the effect of varying concentrate inclusion levels on the microbial diversity in dairy cows and the subsequent variations in milk constituents.

1.2 Statement of the problem

Due to the increasing health conscience among dairy products consumers globally, there is a rising demand for better milk quality, drawing it back to intrinsic and extrinsic factors affecting milk quantity and quality. Among them genetics, parity, diet, stage of lactation, the physiological condition of the animal (Gajbhiye *et al.*, 2019; Kala *et al.,* 2017; Roessler *et al.,* 2019), with diet showing profound effects (Krehbiel, 2014). These feeds consumed by ruminants are fermented/degraded by rumen microbes to produce the human consumables. Still, little is known on the profiles of the rumen microbial population of crossbred dairy cattle in Kenya and Africa in general. There is ample evidence that these microbes affect the quality of milk produced by dairy cattle. The milk pricing system is also changing from just quantity to include quality, therefore directly influencing farmers' incomes. Therefore, it is essential to have a database of the profile of the ruminal microbes for future reference and to document the relationship between these microbes and milk component consumers currently favor.

1.3 Justification

Nutrition-sensitive interventions such as food-based either from plant or animal, remain the primary target towards solving nutrition insecurities with the emphasis being laid on culturally acceptable and locally available food products. LDF, that is, milk, egg, and meat, have the potential to foster nutritional adequacy of plant-based diets due to the high biological value of their protein and bioavailable macronutrients (Bruyn *et al.,* 2016). Milk and milk products were identified as markers of high nutritional diets because of their

potential to meet dietary recommendations, therefore, reducing the risk of chronic health diseases (Thorning *et al.,* 2016).

Consumption of livestock derived protein substantially contributes to assuring adequate nutrition, promote growth, and improve individual health at large (Delia *et al.,* 2018). Tang *et al.,* (2018) reported that consumption of dairy-based complementary foods could result in an infant's prominent growth pattern. As the Kenyan population continues to increase, so will the demand for animal food products. The rumen efficacy is also a rising area of interest, and more awareness of rumen efficacy may be useful in developing better feeds and feeding strategies. Furthermore, studying the link between bovine rumen microbes and milk quality provides useful information on the manipulation of microbes for better milk qualities. Better milk qualities will translate to increased competitiveness of the dairy industry in Kenya, thus contributing to GDP.

1.4 Research objectives

1.4.1 General objective

To evaluate the association between diet, rumen microbial profile, and milk composition of crossbred dairy cattle

1.4.2 Specific objectives

I. To investigate the linear effects of increasing the dietary concentrate proportions on dynamics of rumen microbes, in terms of diversity and abundance, in crossbred dairy animals
II. To evaluate the association between rumen microbes and milk composition profiles of crossbred dairy cattle fed on four different diets

1.5 Research hypotheses

I. There is no effect of increasing the dietary concentrate proportions on the rumen microbial profile of a crossbred dairy cattle

II. There is no relationship between changes in rumen microbes to changes in milk constituents of crossbred dairy cattle

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

Domesticated animals can be either ruminants or monogastrics, the anatomical distinction being the four compartments of the stomach possessed by ruminants, unlike monogastrics with one stomach. The four compartments include omasum, abomasum, reticulum, and rumen, with the latter being the largest. The size and epithelial structure of these four "sac-like" structures vary, implying specific activities such as nutrient absorption (Van Soest, 2018). Ruminants, such as goats, cattle, sheep, provides products which are consumed by human being adding to their nutritional wellbeing (Bettencourt *et al.,* 2015). The ruminant's unique ability to use recalcitrant materials extracted from plants that would otherwise be agricultural waste is a crucial factor in their function in agricultural production. The ruminants ferment these insoluble plant materials in the rumen, which is inhabited by a consortium of microorganisms that work symbiotically with their host (Cammack *et al*., 2018). The rumen is a diverse microbial ecosystem, with a slightly stable temperature (39°C) (Antanaitis *et al.,* 2016; Moran, 2005), acidity (pH range 5.8 - 6.8), and oxygen-deprived, though these values may vary with diet (Zhao *et al.*, 2018) and sickness.

Once the ruminant ingests feed, it first flows to rumen where it is temporarily stored before it's regurgitated, masticated, and re-swallowed into the rumen where the feed is exposed to rumen microbes for fermentation (Figure 1). This mastication helps in the digestion of feed by reducing the particle size, thus making them susceptible to microbial degradation. Rumen microorganisms then, use a significant amount of different enzymes to degrade lignocellulosic plant material into sugars that are eventually transformed into volatile fatty acids (VFAs), that is acetate, butyrate, propionate and, lactate, as well as ethanol and hydrogen gas through the fermentation process (Lombard *et al.,* 2014; Marvin-Sikkema *et al.,* 1993). These VFAs are then absorbed by the host, which converts them into human

utilizable products like milk and meat. The finely processed cud can flow to the next chambers (reticulum and omasum) before getting into the final compartment, the abomasum. In the abomasum, gastric juice and enzymes are produced to further degrade the feed as well as breakdown of flashed bacteria from omasum to nutrients (Figure 1).

However, the microbial composition of these forestomachs' and their relationship to diet and animal productivity is currently not well understood (Xue *et al.*, 2018) hence, making the forestomach's as microbial ecosystems increasingly attracting research attention every day. The study of these reticulo-rumen microbiota's compositional and functional characteristics may, therefore, be refining strategies to increase microbial fermentation and ruminant productivity.

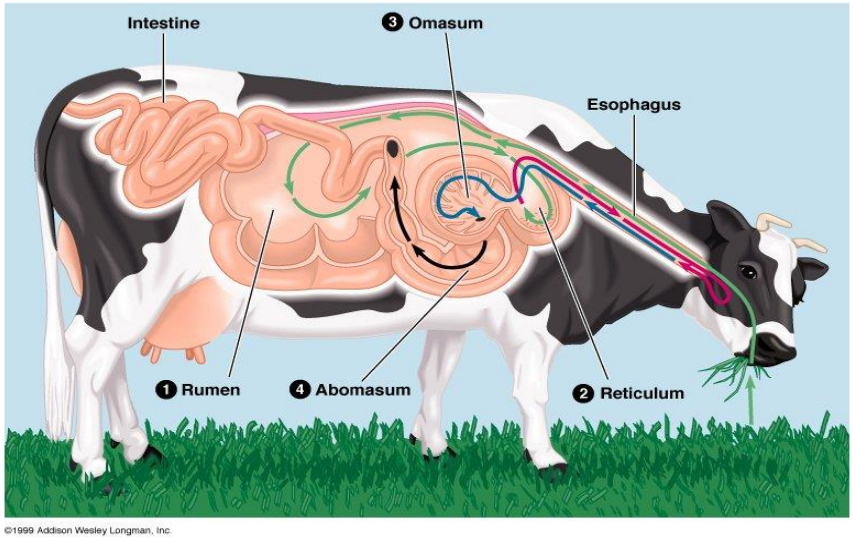

Figure 1: The ruminant digestive tract showing the different compartments and digesta flow. (Addison Wesley Longman Inc 1999).

2.2 Rumen microbes

2.2.1 Rumen microbial profile

The rumen is an anaerobic stomach chamber housing a consortium of microbial species that are in a symbiotic relationship with the animal. The bionetworks of microbes include bacteria, archaea, fungi, protozoa, and phages (Faniyi *et al.*, 2019). These microbes were

reported to be 10^{11}, 10^{9}, 10^{6}, 10^{6},10^{10} (per gram of digesta or per ml of fluid), respectively, by Morgavi *et al.*, (2013). Sirohi *et al.*, (2012), in their study of molecular tools for investigating the rumen microbial community structure and diversity, reported rumen microbial numbers to be 10^{10}-10^{11}, 10^{7}-10^{9}, 10^{3}-10^{6}, and 10^{4}-10^{6} cells/ml of bacteria, archaea, fungi, and protozoa, respectively. Based on the two studies, bacteria are the most dormant class of microorganism in the ruminant gut. Similar results of bacterial dominating, comprising approximately 95% of the population, were reported by Yang *et al.*, (2018) and Kibegwa *et al.*, (2020). These microbes can break down fibrous plant materials by converting them to nutrients like short chain fatty acids. These volatile fatty acids formed through ruminal fermentation of fibrous and non-fibrous carbohydrates are used for growth, maintenance, and production (Figure 2) (Bauman *et al.*, 2011).

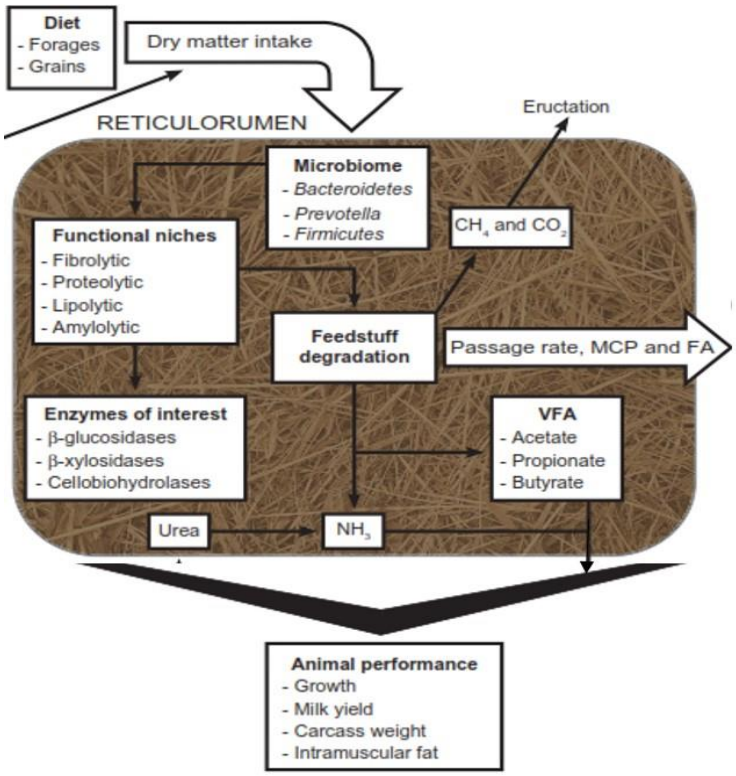

Figure 2; A Schematic representation of diet degradation process in the reticulorumen. (Mccann *et al.*, 2014).

The bacterial community is categorized into different classes based on the substrate they degrade. This classification includes; cellulolytic, proteolytic, amylolytic, lipolytic, fibrolytic, acetogenic, and ureolytic bacteria (Krause *et al.,* 2003; Lourenço *et al.,* 2010; McSweeney *et al.,* 2006; Patra & Yu, 2014; Russell *et al.,* 2009; Wright & Klieve, 2011). A study done by Kim *et al.,* (2011), while assessing the ruminal microbiome's diversity, reported Firmicutes and Bacteroidetes phyla as the predominant bacterial group in terms of abundance and richness of Operational Taxonomic Units (OTUs) after the bacterial population was estimated using16S rDNA-based methods. Petri *et al.,* (2013) reported similar results where the most rumen bacterial phyla to be Bacteroidetes, Firmicutes, and Proteobacteria, at levels of 33%, 43%, and 14% respectively. Kittelmann *et al.,* (2013) explored the co-occurrence of the microbial taxa that is archaeal, bacterial, and eukaryotic by amplicon sequencing. In their study, they analyzed rumen bacteria at the family level and recorded *Lachnospiraceae* and *Prevotellaceae* to be the most prevalent family belonging to *Firmicutes* and *Bacteroidetes*, respectively. While at the genus level, the most abundant microbe across a wide scope of geographical range in many ruminants was *Prevotella* (Henderson *et al.,* 2015). Therefore, irrespective of the diet, Firmicutes and Bacteroidetes were the predominant bacteria, while Proteobacteria and Fibrobacteres proportion fluctuated depending on the diet consumed (Firkins, 2010).

Anaerobic fungi found in the digestive tract of most herbivores mainly belong to the Neocallimastigomycota; Neocallimastigomycetes; Neocallimastigales Phylum, class, and order, respectively (Hibbett *et al.,* 2007). Tapio *et al.,* (2017) studied rumen microbes related to methane emission and documented the Fungi family to constitute of 8 to 20% of the total rumen microbial biomass depending on the host species. Moreover, six rumen fungal genera, that is *Anaeromyces*, *Cyllamyces*, *Orpinomyces* (polycentric), *Caecomyces*, *Neocallimastix*, ,

and *Piromyces* (monocentric) were isolated and characterized by Firkins and Yu, (2015) and Gruninger *et al.,* (2014).

The archaeal domain mainly belongs to phylum *Euryarchaeota*. Rumen methanogens were studied using 16S rDNA-based methods and identified the most dominant genus to be *Methanobrevibacter* (61.6%). Within this genus were two main clades *Methanobrevibacter gottschalkii* clade (33.6%) and *Methanobrevibacter ruminantium* clade (27.3%) (Janssen & Kirs, 2008; Seedorf *et al.,* 2014). Due to their ability to produce methane, these microbes possess a unique characteristic (coenzyme F420), acting as a cofactor for formate dehydrogenase and hydrogenase enzymes (Sirohi *et al.,* 2012).

Approximately 42 ciliated protozoa genera in the rumen have been identified with genera Entodinium and Epidinium dominating (Shin *et al.,* 2004). Entodiniomorphid (Entodinium, Metadinium, Eudiplodinium, Enoploplastron, Diplodinium, Polyplastron, Epidinium, Ostracodinium, and Ophryoscolex) and holotrich (Dasytricha, Isotricha) groups are the main classification of protozoa based on morphological features (Firkins *et al.,* 2020; Williams & Coleman, 2012). Although this group of microbes has the lowest number as mentioned earlier, (10^4-10^6 cells/ml), they represent the largest number of viable rumen biomass (Sirohi *et al.,* 2012). Additionally, these microbes have established a close symbiotic relationship with methanogens as methane gas emission is reduced by 9–40 % following protozoa elimination from the rumen.

An earlier analysis by Morgavi *et al.,* (2013) on (meta)genomics and its application to ruminant production reported (10^{10} per g of digesta or per ml of fluid) of rumen viruses. Although this number was higher than that for fungi and archaea, the composition and functions are barely known; therefore, more studies are required to describe its function well. Genomics and Transcriptomics techniques were used to study the composition of rumen viruses by Berg Miller *et al.,* (2012). They conducted the first metagenomics-based study

using dairy cattle and reported *Siphoviridae* (36%), *Myoviridae* (28%), and *Podoviridae* (14%) families as the most dominant. Besides, they reported that the rumen virome was enriched for phages and transposable elements. Finally, the findings indicated, rumen virome to not only exhibit huge numbers but also diversity in abundance. Later, Ross *et al.,* (2013) metagenomically identified 14 purported viral sequences from rumen of dairy cattle. Amongst the individual dairy cows, the rumen viruses were taxonomically distinct yet preserved functionally.

2.2.2 Function of rumen microbes

Rumen microbes have a different preference for feed structures and therefore perform distinct roles in the rumen. They utilize different plant biomass to produce different fermentation substrates, although it has been reported that some microbes produce better products when they are symbiotic. For example, *Bacteroides succinogenes,* a cellulolytic species, and a non-cellulolytic species, *Selenomonas ruminantium* were reported to yield a significant amount of propionate following the breakdown of cellulose when cultured together. This increase was attributed to the degradation of cellulose to succinate by *Bacteroides succinogenes,* which is an energy source for *Selenomonas ruminantium* to produce propionate (Scheifinger & Wolin, 1973).

Rumen Bacteria

Bacteria represent the most considerable portion of microbes in the rumen. They are actively engaged in the degradation of lignocellulosic feed material through diverse enzymatic processes by converting them to volatile fatty acids and microbial protein in the rumen. These rumen microbes are vast and therefore perform different metabolic activities (Zhu, 2016). For example, *Fibrobacter succinogenes, Ruminococcus flavefaciens,* and *Ruminococcus albus* are the chief fiber digesters species, focusing mainly on cellulose and hemicellulose (Russell *et*

al., 2009; Joblin *et al.,* 2010 ; Wanapat & Cherdthong, 2009). Acetogens which are bacteria species harbored in the rumen, made up of a small number and varying from undetectable to $10^5 ml^{-1}$of rumen liquor, act as hydrogen sink producing acetate.

In studies done by Naas *et al.,* (2014) and Petri *et al.,* (2013b) they reported that bacterial phyla *Firmicutes* and *Fibrobacteres* were highly linked with the breakdown of cellulose in the host rumen. Further, they suggested *Fibrobacter succinogenes* correlated with succinate production by degrading cellulose. However, *Bacteroidetes* phylum was not considered to host populations of cellulolytics but as a potential shelter for saccharolytic species, that is., *Bacteroidetes* and *Prevotella*. These species were considered to have the ability to degrade a wide spectrum of hemicellulosic and pectin substrates. Four years after the 2014 study, Naas and a few other scientists delved on specific gram-positive Firmicutes. They reported *Ruminococcus albus* and *Ruminococcus flavefaciens* as responsible genera for the production of cellulosomes for lignocellulosic rumen degradation (Naas *et al.,* 2018).

Rumen Archaea

Rumen archaeal members contribute approximately 0.3 to 3.3% of the small microbial subunit (16S and 18S) rRNA in the rumen (Ziemer *et al.,* 2000). Members of this domain have a variety of diverse metabolisms and are present in several ecosystems (Delong & Pace, 2001); however, those considered to be in the rumen are purely anaerobic methanogens. This domain is divided into phylum *Crenarchaeota* and *Euryarchaeota.*

Methanogens, one of the rumen archaea, are obligate anaerobes surviving and growing in strict oxygen-deprived environments (Janssen & Kirs, 2008). They belong to phylum euryarchaeota (Janssen & Kirs, 2008; Wright & Klieve, 2011; Zhou *et al.,* 2011), and they do better in environment on substrates like methanol, acetate, hydrogen, formate, methylamine, among others (Sirohi *et al.,* 2010). They reflect a substantial loss of metabolizable energy in

ruminants (Hook *et al.,* 2010). Besides, they partially maintain rumen hydrogen pressure (Janssen & Kirs, 2008; Hook *et al.,* 2010; Wright & Klieve, 2011; Zhou *et al.,* 2011). Eight species have been isolated and identified into pure culture; *Methanobacterium formicicum, Methanobacterium bryantii, Methanobrevibacter ruminantium, Methanobrevibacter millerae, Methanobrevibacter olleyae, Methanomicrobium mobile, Methanoculleus olentangyi, and Methanosarcina barkeri* (Janssen & Kirs, 2008).

Rumen Protozoa

Protozoa are single-celled organisms whose function in the rumen is to ingest and stock minute particles of starch (Patel, 2018). Through this they can modify the rate of ruminal fermentation and prevent ruminal acidosis caused by high lactic acid production and drastic drop in rumen pH. Newbold *et al.,* (2015) documented that some protozoa can breakdown cellulose that could account for up to 33% of the digestive capacity of rumen fibre. In addition, protozoa heavily feed on bacteria, thus playing a critical role in the lysis and recycling of substantial amounts of protein in the rumen. Moreover, protozoa in the rumen helps in scavenging oxygen, benefiting obligate anaerobic bacteria (Zhu, 2016). Protozoa have been intimated to be associated with CH_4 production and emissions due to hydrogenosome's ability to produce high H_2 (Embley *et al.,* 1997; Newbold *et al.,* 2015; Tapio *et al.,* 2017). Finally, as aforementioned, protozoa, through predation of bacteria, are responsible for the bacterial protein turnover, and therefore, reducing protozoa number in the rumen may increase the supply of rumen microbial protein (Williams & Coleman, 1992).

Rumen Fungi and Viruses

Fungi, as documented by Qi *et al.,* (2011), are the incipient colonizers of lignocellulosic substrates. The study findings further indicted that rumen fungi possess special structures, rhizoids, which are capable of penetrating and breaking plant tissues. Furthermore, they produce fibrolytic enzymes that enable them to chemically breakdown plant cell walls.

Through degrading the cellulose and hemicellulose in the plant materials, rumen fungi can produce metabolites such as acetate, propionate, butyrate, as well as other end products hydrogen, carbon dioxide, formate, among others (Kittelmann *et al.,* 2012; Gruninger *et al.,* 2014).

Lastly are the rumen viruses whose composition and roles in the host are still incomprehensible. A metagenomic survey study on phage-bacteria relationships and Clustered Regularly Interspaced Palindromic Repeats(CRISPR) elements by Berg Miller *et al.,* (2012) reported that a virus could regulate microorganism quantity, select phage-resistant microbes, and foster Horizontal Gene Transfer (HGTs) in the environment.

2.2.3 Molecular techniques of assaying rumen microbes

Several techniques can be used to identify and quantify specific microbiota in the rumen ecosystem, varying in different scientific fields such as, from clinical studies to industries to environmental communities' studies. Before the development of these new technologies, the rumen microbes were quantified numerically and phenotypically using culture-dependent methods.

2.2.3.1 Culture-Dependent Techniques

Understanding of rumen microbes was initially based on conventional culturing techniques pioneered by Robert Hungate (Hungate, 1969). Since then, these methods have been used in the characterization of microbial communities. Bacterial isolation and cultivation in pure culture was the first step before quantifying; this allowed scientist to metabolically study the functions of isolates and define its environmental role within the rumen (Riesenfeld *et al.,* 2004). However, these cultivation-based techniques constituted less than 1% of the bacterial diversity in most samples (Riesenfeld *et al.,* 2004). Additionally, many microorganisms in the

host rumen were unable to grow through cultivation (Kim *et al.,* 2011) becoming reasonably difficult to phenotypically differentiate the isolates.

Even so, these techniques offered the current information on microbial roles in rumen as knowledge gleaned through the use of cultivation-based methods is the basis for constructing reference data sets and promoting culture-independent data analysis. Creevey *et al.,* (2014) assessed the culturability of rumen bacterial. They reported that through use of culture-dependent methods, it was possible to sequence and isolate the entire genome, predict its role, and further confirm the roles using physiologic studies. In regard to the aforementioned study and studies by Pham and Kim,(2012), it's clear that these methods allow researchers to gain further insight into metabolism, roles, and growth requirements of specific microbes, allowing more study into individual microbial genomes, thus enhancing knowledge of uncharacterized genes, and eventually offering microbial DNA manipulation capabilities

Though these methods allow bacterial species to be characterized and may serve as markers for uncultured bacteria, they are limited to disclosing the composition of complex microbial communities in the environment (Margesin & Miteva, 2011; Rosling *et al.,* 2011). For example, Mccann *et al.,* (2014), reported that in fiber-adherent rumen fractions, genus *Ruminococcus,* one of the most described cellulolytic bacteria, was not observed in the quantities above 2%, indicating a lesser role in the degradation of cellulose than initially thought. Epstein, (2013) documented that while using culture-dependent methods, just a fraction of the bacteria, archaea, fungi, and protists was defined.

Recently, the interest has been on complex anaerobic microbes due to their influence on the environment (Ze *et al.,* 2013), but its study remains challenging while using the culture based techniques. Difficulties in culturing most of the anaerobic organisms have been closely related to lack of knowledge in 1) understanding the optimal ecosystem and physiochemical conditions necessary for individuals development, as well as 2) symbiotic relationships

required to survive (Puspita *et al.,* 2012). Besides, these methods are limited to a small number of microorganisms that can be cultivable at the same time; this renders the method arduous and inapplicable when characterizing the functions of complex microbial populations.

The challenges above prompted scientists to move from time–consuming and cumbersome classical methods to rapid and effective techniques. These techniques, cumulatively referred to as nucleic-acid based molecular techniques, were unbiased by the culturing aptitude of microbial species. The primary merit of nucleic-acid based molecular techniques is that they are capable of assessing both qualitative and quantitative microbial diversity (Sirohi *et al.,* 2012).

2.2.3.2 Culture-Independent Techniques

Culture-independent techniques were developed and applied at the start of the 1990's (Deng *et al.,* 2008; Kobayashi & Onodera, 1999; White *et al.,* 1999) as a result of emerging DNA sequencing technologies. The initial methods were termed as pre-Next Generation Sequencing (NGS), which entailed fingerprinting. Pre-NGS was done following analysis of a region of amplified DNA for inferring the genetic composition or microorganism strata. The techniques include clone library (Lodish *et al.,* 2000), Terminal restriction fragment length polymorphism (T-RFLP) (Blackwood *et al.,* 2003; De Vrieze *et al.,* 2018; Ding *et al.,* 2013; Pandey *et al.,* 2007), Amplified ribosomal DNA restriction Analysis (ARDRA) (Alves *et al.,* 2005; Błaszczyk *et al.,* 2011; De Baere *et al.,* 2002), Real time Quantitative PCR (Paul *et al.,* 2020; Pinheiro & Siegfried, 2020), Ribosomal intergenic spacer analysis (RISA) (Srivastava *et al.,* 2016) and automated RISA (ARISA) (Ciesielski *et al.,* 2013), PCR amplification (Aird *et al.,* 2011), Amplified fragment length polymorphism (AFLP) (Paun & Schönswetter, 2012), Random Amplified Polymorphic DNA (RAPD) (Mbwana *et al.,* 2006), Fluorescence *in Situ* Hybridisation (FISH) (Grieb *et al.,* 2020; Zwirglmaier, 2005), Denaturing gradient gel

electrophoresis (DGGE) (Srivastava *et al.,* 2016) and temperature gradient gel electrophoresis (TGGE) (gadanho & sampaio, 2004). A comprehensive review of these methods with pros and cons has been done by Zhou *et al.,* (2011). A study done to compare rumen microbial diversity estimates using cultivation techniques and culture free techniques (clone libraries and sanger based sequencing) reported increased estimates of the species from 20 predominant species per ruminant host to over 300 species respectively (Krause & Russell, 1996; Edwards *et al.,* 2004).

Later, 40 years since sanger methods had been introduced, the world of molecular biology was advanced by the creation of the new method referred to as next-generation sequencing (NGS) or high throughput next-generation sequencing technology (Sogin *et al.,* 2006). This method, together with bioinformatics tools, has revealed existing microbial communities in greater diversity, thus, paving a way in studying complex rumen microbial ecology. The number of species per bovine host has increased to several thousand when sequenced using NGS, enabling more in-depth rumen microbial analysis (Creevey *et al.,* 2014; Fouts *et al.,* 2012; Elie Jami & Mizrahi, 2012). Moreover, the use of culture-independent methods revealed a few new microbes that play a crucial role in carbohydrate degradation (Hess *et al.,* 2011; Stewart *et al.,* 2018). Therefore, with the ability to evaluate the taxonomic composition of the rumen microbes, it is possible to monitor the variations in the rumen microbial population throughout the animal life cycle as a result of different treatment parameters (Henderson *et al.,* 2015).

2.2.3.3 Next-generation sequencing

Next Generation Sequencing (NGS) is a culture- free molecular tool based on high-throughput sequencing (HTS). This tool provides a greater opportunity for biologists to uncover a substantial part of the microorganisms (Siegwald *et al.,* 2017). In addition, these high -throughput sequencing have made it easier to study the often referred to as "-omics".

The biological study of these ‘omics’ include the analysis of genes (genomics), transcripts (transcriptomics), proteins (proteomics), and metabolites (metabolomics) (Heyer *et al.*, 2017). The –omics approach enables researchers to execute a sequence and functional-based analysis of microbial genomes obtained from an environmental sample (Morgavi *et al.*, 2013). Multiple microbial community analysis has been conducted in cattle GIT using NGS such as (Firkins *et al.*, , 2008; Hess *et al.*, 2011; Morgavi *et al.*, 2013; Jose *et al.*, 2017; Ogunade *et al.*, 2018).

2.2.3.4 Metagenomics

Metagenomics, also termed as functional metagenomics, community genomics, environmental genomics, or microbial ecogenomics (Panigrahi *et al.*, 2019), was first introduced by Jo Handelsman *et al*. (1998), when they framed the approach in which genomes from multiple microorganisms in a specified environment are analyzed through extraction and cloning of DNA (Andersen, 2019; Handelsman *et al.*, 1998). Primary metagenomics studies began with the traditional sanger sequencing method. Although sanger method tried to solve some of the questions that researchers wanted to answer, such as who is there, how many of them are there, and what are they doing? it had drawbacks, some like for culture-dependent methods since some organisms were underrepresented.

Due to the drawbacks associated with sanger, it resulted in the demand for genomic approaches that would provide a more precise microbial diversity and phylogenetic distribution evaluation at relatively low sequencing costs. This triggered the emergence of the next-generation sequencing (NGS) technologies. These techniques differ from the sanger method in aspects of less sequencing cost and high throughput due to their significantly parallel sequencing system. It produces longer and more reads with few errors and at a faster rate (Salipante *et al.*, 2014). As a result, these high-throughput sequencing platforms are widely being used while performing in-depth sequencing and data analysis on environmental

samples of researcher choice, including the rumen microbiome at a deeper and more accurate level than previously executed (Fouts *et al.*, 2012; Loman *et al.*, 2012; Indugu *et al.*, 2016).

As aforementioned, traditional metagenomic analyses entailed the extraction of whole genomic DNA, the use of restriction enzymes to digest the entire DNA into DNA fragments, ligating the fragments using vector, and finally propagating in a host. But currently, metagenomics analyses can either be classified into shotgun or targeted metagenomics. These two are used in assessing complex microbial communities in compositional structure, function, and diversity aspects. The use of this technique, in combination with other high throughput sequencing techniques, has contributed highly to the understanding of microbial community by providing phylogenic diversity information that was reasonably not attainable with culture-dependent methods (Andersen, 2019). This technique, therefore, offers scientists a chance to achieve more in-depth understanding and insights into uncultivable microbial diversity. In addition, they have started a novel era of microbial ecology alongside environmental ribosomal RNA genes sequencing

Available sequencing platforms such as the Roche 454 and Illumina, have intensely impacted the growth of the metagenome in recent years. This is due to the ease at which researchers can produce high quality, comparatively low-cost sequence data gigabases when using these second-generation sequencing platforms. Initially, the field was focused on targeted sequencing of specific marker genes, but with high throughput technologies, whole genome shotgun sequencing has become common. With these, large and complex data sets there is ease of assessment of the taxon's composition, potentiality, and functionality of study community.

Metagenomics empowered by the next-generation sequencing (NGS) has earned a lot of popularity as a result of its ability to characterize unevenly distributed GI microbiome. Studies have been done using the metagenome technology. These studies vary from cattle

(Brulc *et al.,* 2009; Callaway *et al.,* 2010; Qin *et al.,* 2010), to the human microbiome (Foster & Neufeld, 2013), seawater (Baker *et al.,* 2013; Martinez, 2013), ecology soil (Fierer *et al.,* 2012), glaciers (Choudhari *et al.,* 2013), and air (Scher & Abramson, 2011; Cao *et al.,* 2014), soil (Urich *et al.,* 2008; Tveit *et al.* , 2014), plant rhizosphere (Mendes *et al.,*2014), to metagenome in coprolites and teeth while studying ancient and tracing communities change over time respectively (Tito *et al.,* 2012; Adler *et al.,* 2013) . In addition, a study was done to explore virophages diversity using metagenome, and a new family of virophages was identified (Yutin *et al.,* 2015). From the studies above, metagenomics has been suggested to be a powerful tool in investigating complex microbial ecology and discovering novel microbes, therefore, easing the study of microbes' profile, functions, and their interaction with each other in the rumen.

2.2.3.4.1 **Shotgun metagenomics**

Shotgun metagenomics, also named whole genome sequencing (Kothari *et al.,* 2017), usually considers sequencing the entire genome by extracting and sequencing the total DNA of the sample (Siegwald *et al.,* 2017). Before the invention of the NGS, the shotgun method involved the preparation of libraries followed by an extended cloning cycle; however, presently, whole-genome sequencing helps us to achieve high-throughput data for microbiome research. This approach has therefore presented a whole understanding of a microbiota community due to its ability to sequence the whole genome, unlike targeted sequencing, which only sequences a region (Segata *et al.,* 2013). As a result, scientists can look beyond microbial profiling, that is, by use of this approach, understanding the metabolically active profile of the rumen niche has been made easier (Kothari *et al.,* 2017).

Although Shotgun metagenomic sequencing has almost negative PCR biases of the 16S rRNA gene-based NGS method (Poretsky *et al.,* 2014), it can only predict the functional potential, not the actual functional capability of ruminal microbes (Mccann *et al.,* 2014).

Additionally, it is still costly. Moreover, it produces a massive and complex structure of data; this makes storage and analysis a challenge(Lindgreen *et al.,* 2016).

In 2007, the first bioinformatics tool to analyze metagenome data was introduced. Since then, several computational algorithms have been developed, updated, and adopted by newly developed tools. The choice of which device to use has remained a challenge, especially to the new users. However, for the past five years, MG-RAST, EBI metagenomics, and iMicrobe (earlier CAMERA) have been the widely used methods in reviewing data generated through shotgun (Nathani *et al.,* 2013).

Besides that, Shotgun metagenomic sequencing is one of the leading technologies in researching functional microbiome profiles (Nayfach & Pollard, 2016). Being empowered by NGS, it can potentially identify all GI microbiome genes in cattle and construct genome of isolated microorganisms (Mccann *et al.,*2014). This sequencing technology has been used in several studies to investigate ruminal microbial diversity and functions in cattle, including cattle-fed diets to reduce methane (Ross *et al.,* 2013), cattle fed various forages (Patel *et al.,* 2014), and cattle with frothy bloat induced by wheat (Pitta *et al.,* 2016). Additionally, the approach was used to describe the functional profiles in buffalo and cattle fecal microbiome (Zhang *et al.,* 2017).

2.2.3.4.2 **Amplicon-Based Metagenomics**

Amplicon based metagenomics also referred to as targeted metagenomics or metagenetics (Esposito & Kirschberg, 2014), is a highly targeted approach sequencing which allows analysis of genetic variation in specific genome regions. It uses oligonucleotides probes that are specifically designed to target and capture an area of interest, followed by next-generation sequencing. The discriminative region is amplified prior to the sequencing (Siegwald *et al.,* 2017). Amplicon sequencing is a commonly used approach to estimate the rumen microbiota

compositional profiles (Kittelmann *et al.,* 2013; Henderson *et al.,* 2015). The target of single-gene marker use is mainly gaining a deeper understanding of rumen microbial communities by providing solutions to questions pertaining to their composition and diversity. The single gene markers include 16S rRNA, 18S rRNA, 22S rRNA, and 28S rRNA.

Among all the targeted metagenomics methods, 16S rRNA, present in all prokaryotes, is the commonly used marker gene (Johnson *et al.,* 2019; Lan *et al.,* 2016), together with its eukaryotic equal, 18S ribosomal RNA unit (Klindworth *et al.,* 2013), due to their low sequencing cost and reasonable accuracy (López-garcía *et al.,* 2018). Under several studies, the 16s rRNA method has been used, for example, Kim *et al.,* (2011) on the evaluation of ruminal phylogenetic diversity of the microbes, documented Firmicutes and Bacteroidetes as the most predominant bacterial phyla after estimating their population using 16S rDNA-based method. Rumen methanogens were studied using 16S rDNA-based methods and identified the most dominant genus to be *Methanobrevibacter* (61.6%). Within this genus were two main clades *Methanobrevibacter gottschalkii* clade (33.6%) and *Methanobrevibacter ruminantium* clade (27.3%) (Janssen & Kirs, 2008; Seedorf *et al.,* 2014). Lastly, PCR amplicons of 18S rDNA from protozoa were sequenced, and two genera *Entodinium* and *Epidinium,* were revealed as the most abundant rumen protozoa groups (representing 38% and 17% of protozoal sequences, respectively) (Kittelmann *et al.,* 2013; Henderson *et al.,* 2015).

16S rRNA gene

The study of the metagenome would be broader if the whole genome of all microorganisms residing in the rumen were sequenced. However, owing to technology limitations and cost constraints related to this approach, universal single-gene markers deliver a greater full phylogenetic appraisal (Morgavi *et al.,* 2013). First outlined by Lane *et al.,* (1985), the 16S

rRNA is the popular phylogenetic marker used in the identification and classification of prokaryotes (Makkar & Cameotra, 2002). It is a small ribosomal subunit of the 30s ribosomes in prokaryotes. The 16S rRNA gene sequence is composed of ten conserved and nine hypervariable regions. The hypervariables are found between the conserved regions (Figure 3) (Yu & Morrison, 2004; Yu *et al.,* 2008). The conserved regions (C1-C10) of the 16S rRNA gene are employed in designing polymerase chain reaction (PCR) primers, which are used to amplify a specific portion of the gene. The 16SrRNA gene possesses four unique qualities that make it the prevalent phylogenetic marker method. These qualities include; 1) being an essential gene for the synthesis of protein and therefore not changing the function of the gene over time, 2) its ubiquity in all prokaryotes (Makkar & Cameotra, 2002), 3) the presence of conserved regions that tend to show limited sequence variations across vast phylogenetic distances which can be used for primer targeting, and 4) possession of the hypervariable regions (V1-V9) that allows distinction amongst distinct taxa (Patel, 2001). The conserved regions of the 16SrRNA gene have similar sequences in both bacterial and archaeal species, therefore able to identify most bacterial or archaea. On the other hand, the hypervariable regions differ for bacterial and archaeal species, therefore key to classifying either bacteria or archaea (Vinje *et al.,* 2014), by use of PCR for 16S rRNA gene with species-specific primers (Kim *et al.,* 2017).

Figure 3: 16S rRNA gene diagram showing the alternate ten conserved regions(C1-C10) and the nine hypervariable regions (V1-V9) (Kim *et al.,* 2017)

Initially, acquiring knowledge of the rumen bacterial community was based on the culturing approach. Still, it was challenging for scientists to mimic the rumen ecosystem in the

laboratory, that is, in vitro culturing technique. However, with advancements in molecular tools, use of molecular markers like the 16S gene and ITS regions have shown to be a conventional method for bacterial diversity elucidation, thus surmounting the challenges within vitro culturing approach. Recently, the use of next-generation sequencing techniques in analyzing PCR amplicons of marker genes/transcripts (called amplicon sequencing), such as, 16S rRNA gene or the *rpoB* gene, is a well-acknowledged approach in studies related to phylogenetic diversity in generating compositional profiles of rumen microbes, and has been used in several major studies (Henderson *et al.,* 2015; Kim & Yu, 2014; Kittelmann *et al.,* 2012).

While whole length sequencing of the 16SrRNA gene tends to provide a higher phylogenetic assignment accuracy in 16SrRNA based community analysis, recently, partial sequencing of the 16SrRNA gene has been done (Myer *et al*., 2016). Use of these partial are promising to be the most thorough approach when assessing phylogeny of poorly characterized members of the microbial community, even though short DNA fragments may not taxonomically be assigned as precisely as longer fragments (Patil *et al.,* 2011). Myer *et al.,* (2016) conducted a study to compare the V1–V8 full length of 16S rRNA gene and V1–V3 regions together with differences in sequencing platforms. The research also evaluated the effect of technical selections on the microbial profile, and the results indicated that the two gave similar output in terms of microbial diversity, that is, statistical indices. They also demostrated that the longer reads showed the significant diversifying group to be Proteobacteria and Verrucomicrobia phyla.

Many studies have been done using this method. One such study by Li *et al.,* (2012) used metagenomics tools (V3-V5 regions of 16s rRNA) to characterize pre- ruminant calves' rumen microbiota. In this study, the authors reported that there were 15 identified phyla, with Bacteroidetes dominating: 78% in the group of 42-day-old calves. Similar results indicating

the dominance of Bacteroidetes was recorded by (Ozutsumi *et al.,* 2006) in a study of the impact of protozoa on rumen bacterial by real-time PCR detection in cattle and observation of isolated and inoculated calves ruminal microorganism. The analysis of the operational taxonomic unit of the rumen microbiome was based on the 16S rRNA gene sequence. They reported that; regardless of the animal or its breed, Bacteroidetes phylum dominated, but the proportion of the bacteria fluctuated uncharacteristically due to diet type and composition influence. Further, Kim *et al.,* (2011) reported Firmicutes and Bacteroidetes as the most predominant bacterial phyla after estimating their population using the 16S rDNA-based method in terms of abundance and number of species-level OTUs. Finally, on the same note, Zhu (2016) used the 16S rRNA gene amplicon sequencing approach and reported Bacteroidetes, Firmicutes, and Proteobacteria phyla to be dominating the rumen bacterial community.

Despite this method's significant contribution to assessment and understanding of rumen and GI microbiome diversity (Kim *et al.,* 2017), several limitations have been associated with the 16S rRNA gene sequencing method. Which include PCR biases due to choice of the primer (Hong *et al.,* 2009), cycling conditions during amplification (Huber *et al.,* 2009), amplification efficiency differences, nonspecific annealing, the formation of PCR artifacts (Wintzingerode *et al.,* 1997; Poretsky *et al.,* 2014; Firkins & Yu, 2015), and inability to reveal new phylotypes, as PCR primers are precisely designed in line with known sequences (Ross *et al.,* 2012; Urich *et al.,* 2008), and that unless matched, it cannot be used to deduce function for a known species (Case *et al.,* 2007; Von Mering *et al.,* 2007).

2.2.3.4.3 **Analysis of 16s rRNA gene data**

Reliable bioinformatic pipelines are needed to analyze the massive datasets generated from NGS platforms. Few software packages are available for analyzing 16S rRNA gene sequences, among them is QIIME and Mothur (Schloss *et al.,* 2009). These two are the

commonly and highly used and cited bioinformatic software packages, respectively (Siegwald *et al.,* 2017). It is worth noting that different pipelines can yield different outputs because of the changes in strategy for OTU-picking, taxonomy allocation, and reference database used.

While comparing the clustering –first approach pipelines, MOTHUR was reported to be less effective (Siegwald *et al.,* 2017). In this study, they observed that MOTHUR wasn't effective, probably because it was developed when high-quality sequencing results are assumed. So it is not well suited for technologies that are prone to errors like Ion Torrent sequencing. Kopylova *et al.,* (2016) noted that, among the clustering –first pipelines, QIIME had the best performance but with integrations of algorithms such as SortMeRna, SWARM, and Sumaclust. These novel algorithms wane the well-acknowledged overestimation richness problems of OTU-based methods when richness is assessed before the taxonomic assignment. The QIIME was used in this study.

QIIME

QIIME (Quantitative Insights into Microbial Ecology) is a bioinformatics pipeline with various use for processing raw NGS data (Caporaso *et al.,* 2010). Although it's an open-source package, QIIME version 1.9 requires other dependencies, which help it to run effectively like USEARCH. The USEARCH is a unique sequence analysis tool offering high-throughput search and clustering algorithms to analyze data, with a free version containing 32-bits and a maximum of 4 GB RAM usage limitation, and the other is a 64-bit binary, with the latter requiring a paid license upon usage. In 2001, a standard protocol was published on the use of QIIME to analyze 16S rRNA gene sequences from microbial communities (Kuczynski *et al.,* 2011).

Briefly, the demultiplexed reads from the targeted sequencing are first subjected to preprocessing, which entails pairing reads, merging the paired reads, quality check, and

chimera detection before OTU –picking. As previously noted, QIIME entails several scripts that are geared towards achieving the OTU assignment; therefore, different commands are used. The paired-end reads are merged using USERCH (Edgar *et al.,* 2011). Following this is the quality check, sequences with low quality (often Q<20) and reads with length less than 450 bp are removed and truncated, respectively. Potential chimeras are then detected and removed from the sequences using either ChimeraSlayer (Haas *et al.,* 2011) or UCHIME (Edgar *et al.,* 2011), against the latest chimera Ribosomal Database Project (RDP) gold database. Operational taxonomic units (OTUs) are clustered using the cleaned sequences. The clustering is based on a 97% similarity threshold for 16S rDNA sequences. Usually, UPARSE and UCLUST are the commonly used algorithm (Caporaso *et al.,* 2010; Edgar, 2010, 2013). The clustering is done using the following OTU picking methods; closed-reference, denovo, and open-reference techniques (Navas-Molina *et al.,* 2013).

When closed- reference method is used, the sequences are clustered first, a representative sequence picked, and OTU assigned by blasting against a pre-defined sequence reference, which is set based on the similarity value of the chosen sequences. However, it is worth noting that the closed-reference method cannot establish new phylotypes due to the exclusion of sequences that don't match the reference sequence. Therefore, in addition to improving the reference databases, aligning sequences with sequence similarity value chosen based on OTUs (typically 97% similarity for 16S rDNA sequences); Denovo method (Rideout *et al.,* 2014) is a possible choice to avoid the closed- reference method problem. Although this similarity of 97% was deemed enough for delineating species (Koeppel & Wu, 2013), it is only an approximation. Sometimes various species such as *Bacillus globisporus* and *Bacillus psychrophilus* (Fox *et al.,* 1992), as well as *Clostridium botulinum* and *Clostridium sporogenes* (Rossi-Tamisier *et al.,* 2015) have similar 16S rDNA sequences with high identity (= 99%). Conversely, multiple copies of 16S rDNA from one species may be less

than 97 percent similar (Vetrovsky & Baldrian, 2013). Lastly, the method does not use a reference sequence.

To complement this limitation, open -reference method (Rideout *et al.,* 2014), which combines both closed and denovo OTU picking methods, resolves it. First, the sequences are subjected to sequence reference with those do not matching a reference sequence set being grouped into denovo OTUs. While using amplicon sequencing for taxonomic analysis, this method is highly recommended because microbial diversity accuracy estimation is not based on arbitrarily OTUs defined methods. Furthermore, microbial compositions 'true' and 'biological meaning' maybe reflected when the analysis is done at the OTU level (Li *et al.,* 2018). The QIIME pipeline uses Greengenes as a default reference database (Figure 4) (Zhu, 2016).

After microbial identification, microbial diversity should be estimated. Microbial diversity lays down basic principles in microbial ecology to detect changes in the composition of the community. This diversity comprises of richness and evenness. While richness refers to the number of species or OTUs observed, evenness indicates the similarity in population sizes of species in a community. Alpha diversity exhibits the diversity within a sample, and its analysis includes observed richness (number of detected OTUs) and predicted richness (Chao1, Shannon, Simpson, and ACE estimates of maximum species richness). Beta diversity measurements allow for a comparison of samples diversity and are usually done using multivariate analysis like principal coordinates analysis (Lozupone *et al.,* 2011; Mccann *et al.,* 2014)

Siegwald *et al.,* (2017), in their study of assessing the bioinformatics pipelines (common and emerging) for amplicon-based- metagenomics, computed richness indexes after taxonomic assignment using clustering-first and assignment-first pipelines. They also compared the performance of the two pipelines. Results indicated that, at the family level, the only

pipelines able to overestimate richness (8-10 % chao1 error) without errors were QIIME and BMP. In contrast, mothur, Kraken, and One Codex both underestimate richness at the equal ratio (-10% F Chao1 error). Interestingly, at the genus level, QIIME became extremely close to ground truth and is the only pipeline for a better estimation of genus richness than the family level, because the overestimation of richness is offset by resolution drop at the genus level. Similar observations were made in a study by D'Argenio *et al.*, (2014) on comparison of taxonomic and diversity profiles created using human Gut microbiome samples by MG-RAST and QIIME which indicated that there were no statistically considerable differences in assignments or alpha-diversity measures; nevertheless, the beta diversity measures were significantly different between the two pipelines. The researchers also noted QIIME generated more precise assignments, mainly because of the large number of reads MG-RAST was not able to classify.

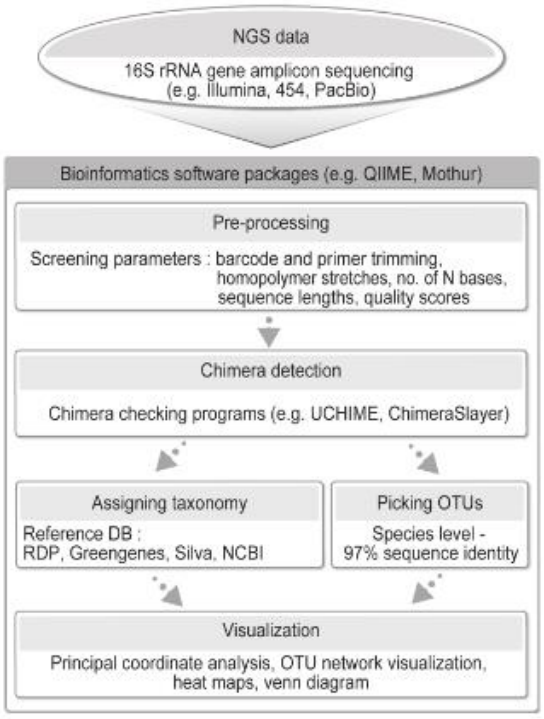

Figure 4: A flowchart outlining 16S rRNA gene bioinformatic data analysis generated by NGS using clustering – first methods (QIIME and MOTHUR) (Kim *et al.*, 2017).

2.2.4 Factors affecting rumen microbiota

Rumen microbial community composition varies depending on several factors including diet, physiological status, host genotype, age, geographical location, feeding regime, antibiotic treatment and season (Golder *et al.,* 2014; Guan *et al.,* 2008; Hook *et al.,* 2011; Kim *et al.,* 2014; Kim & Yu, 2014; Pitta *et al.,* 2010), health status (Plaizier *et al.,* 2017), stress (Deng *et al.,* 2017), feed intake (Derakhshani *et al.,* 2017). As early as in a one day old ruminant, some rumen bacteria may be detected with the diversity and similarity within the group increasing with age (Jami *et al.,* 2013). Among the factors affecting ruminal microbes, the diet has been reported as the significant factor in modifying the microbial communities (Ellison *et al.,* 2013; Henderson *et al.,* 2015; Petri *et al.,* 2013; Sun *et al.,* 2010) and has been the most investigated (Carberry *et al.,* 2012; Zhang *et al.,* 2014a). Due to this, diet becomes the focus, although other few factors are discussed briefly.

2.2.4.1 Host genetics

Host genetics influences the population of ruminal communities. Genetics contributes to variations of rumen microbes between animals (Franzolin & Dehority, 2010; Kala *et al.,* 2017). In a study by Hernandez-Sanabria *et al.,* (2010), it was observed that the breed of the sire influenced bacterial communities in progeny rumen. Differences between species have also been studied; for example, bacterial rumen composition was reported to vary between cattle and buffalo (Wanapat & Rowlinson, 2007). Nevertheless, genetic composition or breed of ruminants has shown little bacterial composition variation in other previous studies (Kothari *et al.,* 2017).

2.2.4.2 Age and Environmental factors

Previous studies have suggested that age could be a possible factor affecting rumen microbes. Jami *et al.,* (2013) conducted a study in dairy cows where they linked age to the rumen microbes. In their study, they reported that all three predominant bacterial phyla

(Bacteroidetes, Firmicutes, and Proteobacteria) abundances varied with age. Besides, they also noted that the diversity of microbes increased, and the microbial community became similar as the dairy animal aged. Geographical locations (Ishaq *et al.*,2015), day length condition, ambient temperature (McEwan *et al.,* 2005), change in seasons (Noel *et al.,* 2017b), are some of the environmental factors that have been investigated and shown a partial influence on rumen microbial communities.

2.2.4.3 Diet

Microbial populations are not stable but dynamic and fluctuate with changes in the ruminal environment and diet (Krehbiel, 2014). As mentioned earlier, the diet has been reported as the major factor influencing the rumen microbial community and has been the most investigated factor because diet determines which substrate is available for rumen microorganism. Manipulation of these microbial communities, therefore, paves a way to optimize their functionality in the rumen, resulting in desired product quality. Numerous studies investigating diet have been conducted. These include study by Henderson *et al.*,(2015) revealed that the major factor that influenced rumen bacterial community abundance was diet. Ruminal microbial communities were found to be distinct in animals fed forage compared to those fed concentrates (Ellison *et al.*, 2013; Petri *et al.*, 2013a).

Besides that, there is substantial evidence in the literature on the dietary impact on rumen microbiome (Agle *et al.*, 2010; Aguerre *et al.*, 2011; Berthiaume *et al.*, 2010; Bi *et al.*, 2018; Faniyi, *et al.*, 2019; Machado, 2014; Neubauer *et al.*, 2018; Ngu *et al.*, 2019; Noel *et al.*, 2017a; Petri *et al.*, 2012; Wang *et al.*, 2019; Zhang *et al.*, 2014). Two primary dietary methods used for feeding dairy cattle: a seasonal diet based on forage plus concentrate supplementation, or a Total Mixed Ration (TMR) based on mixing both forage and concentrate. Various rumen taxa elevate than the others when animals are subjected to

different dietary ingredients. Maintaining a safe and productive rumen microbiome is, therefore, a key to maximizing feed usage and increasing milk output (Mccann *et al.*, 2014).

2.2.4.3.1 Effect of concentrate on rumen microbes

Different levels of concentrates have been reported to affect rumen bacteria richness and diversity. Sun *et al.*, (2010) noted that when animals were fed 0% to 50% concentrates, the rumen bacteria were relatively stable, but the diversity decreased when the concentrate level increased to 70%. Petri *et al.*, (2012) assessed the effects of withdrawing forage from a high concentrate diet and reported that *Fibrobacter succinogenes* was lower in animals fed a diet with high concentrate. Studies have reported that feeding animals a high concentrate diet decreases the ruminal pH, lowers the rumen microbial diversity, and increases the amount of VFAs produced (Sato, 2016).

A recent study on effects on high concentrate diets on microbial composition, use and short chain acids process in the rumen of dairy cows by Zhang *et al*., (2020) reported that increasing concentrate level from 40% to 70% increased the relative abundance of *Clostridium* spp., *Eubacterium* spp., *Ruminococcus* spp. While investigating changes of goat ruminal microbiota and their metabolites , Mao *et al*., (2016), reported that the *Thalassospira, Papillibacter, Succiniclasticum, Prevotella, Lysinibacillus* decrease in abundance as the dietary grain increased from 0% ,25% to 50% while *Mogibacterium, Acetitomaculum* and *Butyrivibrio* increased.

Fernando *et al*., (2010) reported increased abundance in the populations of *Prevotella bryantii, Megasphaera elsdenii, Selenomonas ruminantium* and *Streptococcus bovis* following an adaptation to high concentrate diets. Additionally, the real time PCR analysis also detected a decrease in the abundance of *Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* populations. Increasing concentrate level in a corn based diet increased the

number of members belonging to family Prevotellaceae while members of family S24-7 decreased during the concentrate adaptation period (Anderson *et al*., 2016).

2.2.4.3.2 Effect of forage on the microbiome

Changes in dietary ingredients results in change the composition of the microbial population in the rumen content. Most of these dietary manipulations can change from forage/fibre to concentrate based. Forage is an essential constituent of most ruminants' diet, and various forage levels in TMR affect the abundance and diversity of rumen microbiome. An experiment was conducted to assess the effects of feeding a high fiber diet to animals by Thoetkiattikul *et al.,* (2013). In their findings, they reported that *Fibrobacteraceae, Ruminococcaceae,* and *Lachnospiraceae,* cellulolytic and fibrolytic bacteria, were higher compared with animals consuming a diet with low fiber. Distinct forage sources favor further bacterial growth, as stated by Zhang *et al.,*(2014), where genera *Prevotella* and *Selenomonas* proportions increased under alfalfa hay diet compared with the diet containing cornstalks.

The high number of OTUs in the forage-based diet was reported by Li *et al*., (2019) which indicated that more bacterial strains were needed to cooperate in the degradation of fiber (NDF and ADF) than in breaking down starch and crude protein (Li *et al*., 2019). Recently, Wang *et al*.,(2020) reported similar results and argued that more rumen microbial diversity is observable in a high forage diet as a concentrate diet tends to lower ruminal pH and this may hinder the growth of rumen bacteria with acid sensitivity. In studies done by Li *et al*., (2012) and Wang *et al*., (2012), the abundance of Firmicutes phylum reduced as concentrate level increased. This is because the Firmicutes are fiber degrading microbes.

In another study conducted to assess the effect of TMR with 45:55 of forage: concentrate ratio diet; in each of the diets, at least 20% consisted of maize stover or alfalfa. Results indicated that 2,690 and 2,523 OTUs were observed in corn stover and alfalfa diets, respectively. Accordingly, when the Shannon alpha diversity index was performed, the predominant phyla were Bacteroidetes followed by Firmicutes; this was supported by a previous work where ruminal pH was observed to be about 6.5 (Jami & Mizrahi, 2012; McCann, 2013; Thoetkiattikul *et al*., 2013). Moreover, in alfalfa diet, genera *Prevotella* and *Selenomonas* were observed at a greater relative abundance, and this further explained the greater concentrations of propionate and butyrate observed. Alternatively, in corn stover diet, unclassified *Ruminococcaceae*, *Paraprevotella*, *Anaerotruncus*, unclassified *Rikenellaceae*, and *Papillibacter* genera were greater (Zhang *et al*., 2014). In conclusion, animals fed pasture recorded the prevalent genera to be *Prevotellaceae* regardless of fraction, and higher propionate has been shown in the presence of these strains(Strobel, 1992).

2.2.4.3.3 Carbohydrate hydrolysis in the rumen

Ruminant diets comprise of carbohydrates, protein, lipids, vitamins, and minerals. Ruminants can breakdown fibrous plant materials into human-edible products, such as milk. However, this is made possible due to the presence of the symbiotic rumen microbes (Loor *et al.,* 2016; Xue *et al.,* 2018) in the ruminants gut. The metabolic breakdown of the essential substrates can be categorized into four major classes: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Angelidaki *et al.,* 2011; Thauer *et al.,* 2008). Briefly, the microbial communities with hydrolytic capabilities convert carbohydrates, lipids, and protein polymers into small subunits, monomers, which can further be metabolized through acidogenesis to form organic acids. These organic acids are additionally utilized to form absorbable compounds, including acetate, carbon dioxide formate, and hydrogen gas, by acetogenic

microbes. Finally, ending products: carbon dioxide and hydrogen gas, are used to form a fully reduced compound called methane. This is made possible by a specialized group of microbes, archaeal, through methanogenesis (Ferry, 2010; Thauer *et al.*, 2008).

Carbohydrate hydrolysis starts with mastication, where feed particles are reduced in size in the mouth. By adding saliva, helps to balance the rumen condition and improve the digestion of fiber. The main plant cell walls contain 90% complex polysaccharide and 10% glycoprotein (McN eil *et al.*, 1984). These complex plant cell wall components include hemicellulose, lignin, cellulose, pectin, polyphenol, phytic acid, toxic compounds, and fermentable sugars. Of all the polysaccharides used in plant structures, cellulose is an essential and abundant component.

Cellulose, a glucose homo-polymer linked by ß-4-linked D-glucosyl residues, forms approximately 20-30 percent of primary cell walls (Zhu, 2016). Its initial fermenters are *Ruminococcus* and *Fibrobacter* species (cellulolytic species). These primary degraders make cellulose available for secondary degraders for fermentation into the essential carbohydrate degradation end products (James Flint et al., 2008). Ruminants do not possess enzymes required in the breakdown of complex structural plant materials; thus, digestive processes are primarily dependent on the inherent microbial community of the rumen. However, these microbes can produce enzymes; for example, fungi and protozoa have fibrolytic enzymes that aid in cellulose breakdown. Precisely, during fiber degradation, enzymes like cellulase, xylanase, and ß-glucannase are present in the rumen. Many enzymes used in cellulose and hemicellulose hydrolysis belong to the glucosyl hydrolases (GH) family. Enzymes in this family hydrolyses glycosidic bond between carbohydrates molecules (Henrissat & Bairoch, 1993). However, for effective degradation of cellulose to take place in the rumen, the cooperation of these three main GH enzymes is pertinent, endoglucanase, exoglucanase, and beta-glucosidases, with the end product being glucose.

In a previous analysis, other microbial species involved in fiber degradation were described systematically by Krause *et al.*, (2003). For example, *Butyrivibrio fibrisolvens* with xylanolytic capability play a critical role in the digestion of fibres, and *Prevotella* though not known to be highly cellulolytic but does contain several xylanases. These species, with the help of cellulolytic species, can convert cellulose to simple sugars then to short chain fatty acids (mainly butyrate, propionate, and acetate), H_2 and succinate. Ultimately, the volatile fatty acids are eventually transmitted via the rumen cell wall into the bloodstream and used by the host for energy source for metabolism, growth, and milk production (James Flint *et al.*, 2008) (Figure 5).

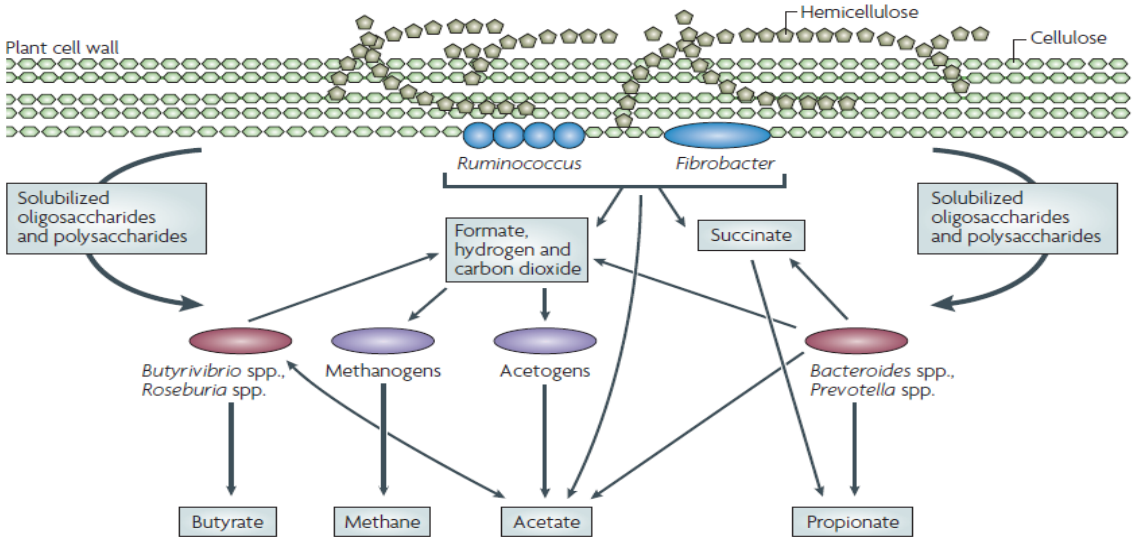

Figure 5: *Ruminococcus* and *Fibrobacter* cellulolytic species acting as insoluble plant fiber (James Flint *et al.*, 2008).

2.3 Milk quantity and quality

2.3.1 Milk constituents

The use of bovine milk and dairy products in human nutrition has a long history (Haug *et al.*, 2007). Milk, man's oldest food, is described as fresh and clean secretion in the alveoli cells of

mammary tissue (Shah & Alhawaj, 2020). Milk is made up of complex constituent's mixture of fats, proteins, carbohydrates, minerals, vitamins, and other miscellaneous constituents dispersed in water (chloride, sodium, and urea) (Table 1). The composition of the significant nutritional milk constituents varies depending on genetics (species and breed) and feeding regime. Despite milk having nutritional value to both young and adults, it may have both negative or positive health effects on human health (Haug *et al*., 2007).

Table 1: Gross composition of bovine milk in grams per 100 ml

Milk constituents	Maximum	Minimum	Average
Water	90.5	80.5	87.2
Fat	8.2	1.7	3.7
Proteins	5.5	1.4	3.5
Lactose	6.1	2.5	4.9
Ash	1.2	0.4	0.7

Adapted from Park (2009)

2.3.2 Milk production and consumption and marketing in Kenya

The livestock production sector contributes 4.1% to agricultural GDP in Kenya (KNBS, 2019). Kenya has the largest dairy herd and industry in Sub-Saharan Africa, with an approximation of 4.5 million heads (FAO, 2018). The country's annual milk consumption per capita is also among the highest in Africa and the highest in sub-Saharan Africa at 110 liters, which is equivalent to 5.2 billion liters a year (Rademaker *et al.,* 2016). The milk consumption has been estimated to rise at an annual rate of about 3% for the next decade, totaling 139 liters by 2022 (USAID-KAVES 2015) and 220 liters by 2030 (KDB, 2019), owing to the strong tradition of integrating milk in diets, increasing urbanization, growing middle class and regional export opportunities. Moreover, milk production in the country has also elevated by 5.3% from (KNBS, 2020).

Differences in milk production have resulted in fluctuations in production costs. These differences are attributed to the livestock production system used, the animal genotype (breed), and the quality/availability of feeds (Muia *et al.,* 2011). Incomes, therefore, vary with the season, accessibility of the farm, total farmer yield, milk selling ways (formal and informal), and the value of by-products, such as manure. This implies that profit varies with different areas of the country. The informal milk market accounts for nearly 80% of the total milk produced, and its price is 22% higher than the formal market (Odero-Waitituh, 2017). However, it is disadvantaged because of its low-quality standards, as there are no tests done on delivery (Muriuki, 2011).

2.3.3 Milk quality and payment methods in Kenya

Milk quality can be judged based on nutritional constituents' percentage, color, bacteriological count, smell, somatic cell count, presence of antibiotic residues, adulteration,(Teresiah *et al*., 2016). The changes in the above aspects may be due to the health status of animal, breed, species, diet, milk handling practices both during milking, storage, transport, and processing (Teresiah *et al*., 2016). According to Njiru, (2018), either way, the formal or informal milk market doesn't meet the set quality standard requirements by Kenya's Dairy Board. In most European countries, milk payment is based on milk composition or hygienic quality. In Kenya, Quality Based Milk Payment System (QBMPS) is still at infancy with only a small portion of dairy sector players, such as, Happy Cow limited buying milk on quality, this translates to a high number of Kenyan dairy farmers receiving payments based on quantity rather than quality (Foreman and Leeuw, 2016).

2.3.4 Factors affecting milk quality

Several factors affect milk quality along the milk value chain. These can be broadly classified into animal parameters (endogenous) and non-animal parameters (exogenous) factors.

Animal parameters include; genetic differences within species, breed difference, stage of lactation and parity (Gajbhiye et al., 2019; Roessler et al., 2019). Non-animal parameters include; season variations, management: diseases and nutrition (plane of nutrition, forage; concentrate ratio, forage quality (particle size) and level and type of dietary fat) (Roessler *et al.,* 2019; Sandrucci *et al.,* 2019) and exogenous microbiological quality factors; environment, collection, and processing equipment and human milk handlers from the farm to the factory (Naing *et al.,* 2019).

2.3.4.1 Effect of diets on milk quality

A study by Li *et al.,* (2017), reported that prolonged feeding of high concentrates diet had both beneficial and detrimental effect on animal health and productivity. Animals fed on high concentrates produced milk with low fat and protein content compared to those provided low concentrates diets due to increased amount of NEFA (Nonesterified fatty acid) and TG (Triglycerides) in the liver, which are substrate precursors of milk fat (Li *et al*., 2017). The dietary effect on milk quality is as a result of various substrates which were produced when the feed is degraded in the ruminant GIT.

Gabbi *et al*., (2013), Aguerre *et al.* (2011), and Neveu *et al*. (2013) reported that increased concentrate consumption results in increased amounts of lactose and protein in milk. This was attributed to the fact that higher concentrate to roughage levels increases the consumption and synthesis of propionic acid and glucose in the intermediate metabolism of non-fibrous carbohydrates, thereby raising the lactose and protein content in the milk. Other studies have also reported a decrease in the amount of lactose and protein when the concentrate to fiber ratio in the diet increased (Agle *et al.* 2010, Machado 2014). The reduction in milk protein and lactose in the latter study was attributed to the slight increment in total digestible nutrients.

Increasing the amount of concentrate in the diet lowers the amount of butterfat in milk. According to Louis *et al*., (2015), this is because concentrate it supports propionic acid production which promotes redirection of energy towards secretion of body fat (fattening metabolism) instead of milk fat. Moreover, higher concentrate to roughage levels has been mostly associated with decreased rumen pH, partial biohydrogenation, and with the dilution effect of increasing milk production to a greater extent than fat production (Agle *et al*., 2010; Aguerre *et al*., 2011; Neveu *et al*., 2013). Harris and Bachman, (2012) assessed the factors affecting milk Solids-Not-Fat, freezing point of milk with main focus been on management and nutritional factors and documented that high intake of roughage reduces the milk SNF and milk production as energy levels or dry matter intake is reduced in the dairy animal.

CHAPTER THREE: METAGENOME PROFILING OF CROSSBRED DAIRY CATTLE FED ON DIFFERENT LEVELS OF DIETARY CONCENTRATE

3.1 Abstract

Elucidation of existing diverse rumen microbiota is of interest due to its implication on ruminant productivity. Previous studies have reported diet to highly affect the stability of these rumen microbes, however the microbial composition of the forestomach and their relationship to diet in crossbred animals reared in the tropics is still open to scientific research. A 4X4 Latin square experimental design to investigate the linear effects of increasing the dietary concentrate proportions on dynamics of rumen microbes, in terms of diversity and abundance, in admixed dairy cows was conducted. Four diets were formulated to contain 10%, 20%, 30%, and 40% commercial dairy concentrate and to meet the nutritional requirements of dairy cattle yielding 12 kg of milk per day. Rumen liquor samples were collected after every 10 days, and microbial composition assessed using R software.

The results showed that Bacteroidetes and Firmicutes were the dominant bacteria making up to a total of 83.7% of the total rumen bacteria. Further, the findings indicated that increasing the amount of concentrate in the diet significantly impacted on several rumen bacterial communities. Significantly, only Bacteroidetes increased ($P \leq 0.05$) with increase in concentrate proportion in the diet. The proportion of Firmicutes on the other hand reduced from 16.95% to15.35% as the concentrate level increased. The results of this study evidenced that increasing levels of concentrate in the diet, affects the rumen bacterial composition.

3.2 Introduction

Domesticated ruminants (examples goats, cattle, sheep, buffaloes) provide products consumed by human beings, adding to their nutritional well-being (Bettencourt *et al.,* 2015). The ruminant digestive tract is complex, comprising four compartments with the rumen being the largest: others being reticulum, omasum, and abomasum. The ruminant's unique ability to use recalcitrant materials extracted from plants that would otherwise be agricultural waste is crucial in their function in agricultural production.

Cattle, being herbivores, feed on complex structural plant materials rich in cell wall polymers (complex carbohydrates) such as cellulose, hemicellulose, lignin, pectins, and xylans. The effectiveness with which cattle convert these fibrous plant material into usable energy to produce human utilizable derivatives like meat and milk is the quintessential function of a ruminant forestomach, precisely rumen (Opdahl, 2017). This is because of the vital symbiotic relationship between the rumen and the vast number of microbial species. These microbes enable the host to utilize feeds rich in cellulose by converting them to a wide spectrum of metabolites, like Volatile Fatty Acids (VFAs), used for nutrient supply by the host animal and microbes for their proliferation. Bacteria are the most dominant, comprising approximately 95% of the population (Kibegwa *et al.,* 2020; Yang *et al.,* 2018). Other organisms include archaea, fungi, and viruses in different proportions.

The diverse microbial population adapts to various dietary feedstuffs and management strategies (Knapp *et al.,* 2014). Elucidation of these rumen microbiota compositions is of interest due to its implication in ruminant productivity. Moreover, the complex associated with rumen microbial organ has made rumen an important part of research, raising more interest. Therefore, more awareness of the microbial ecosystem stability may help develop strategies for feeding ruminants efficiently and sustainably. The microbial composition of the bovine forestomach and their relationship to diet in crossbred animals reared in the tropics is

not fully understood and is open for more exploration. The effects of diets with different forage to concentrate ratio on dairy animals has been studied. However these studies used only few rations and as not yielded a dataset wide enough to explore the variation of the microbiota (Hua *et al.,* 2017; Mao *et al.,* 2016; Saleem *et al.,* 2012). As a result, it is unclear how concentrate level affects crossbred rumen microbes. Therefore, this study was carried out to investigate the linear effects of increasing the dietary concentrate proportions on dynamics of rumen microbes, in terms of diversity and abundance, in admixed dairy animals. Finally, as a sub-objective, this study also aimed at determining the bacterial community shift and stability following dietary treatment by creating two sampling points (day 10 and day 20) in a way to examine the duration required for rumen bacterial to attain or restore stability.

3.3 Materials and methods

3.3.1 Ethics statement

This experiment was approved by the Faculty of Veterinary Medicine, University of Nairobi's Institutional Animal Care and Use Committee (IACUC), Reference number: FVM BAUEC/2020/268.

3.3.2 Study area

The research was carried out at the University of Nairobi Veterinary Teaching Farm. The farm lies between latitudes 1° 14' S and 33° 4' S and longitudes 36° 42' E and 36° 3' E in Kiambu County.

3.3.3 Experimental Animals and diets

Four dairy cows (n=4), numbered AN 1, AN 2, AN 3 and AN 4, were purposefully selected from the farms milking herd. The animals were selected based on the following parameters: 1st parity, early lactation and an approximate body weight of 350±50kg. Following a ten-day

acclimatization period, the animals were fed on four experimental diets in four consecutive 20-day periods. The four different diets were formulated to contain 10%(D1), 20%(D2), 30%(D3), and 40%(D4) commercial dairy concentrate and to meet the nutritional requirements of dairy cattle yielding 12 kg of milk per day using the NRC - Nutrient Requirements of Dairy Cattle Software v 1.9 (NRC, 2001) (Appendix Table 1). The dietary components for the roughage portion were Boma Rhodes grass hay and Lucerne hay. These roughages were mixed with a commercial dairy concentrate, into a total mixed ration (TMR), to avoid the selection by the cows.

The nutrient composition of the dietary components was assessed through proximate analysis (Crude Protein, Crude Fiber, Ether Extract, Dry Matter and Ash) procedures outlined in AOAC (1998). The Neutral Detergent Fiber, Acid Detergent Fiber and Acid Detergent Lignin were analyzed using Van soest method (Van Soest *et al.*, 1991) at the University of Nairobi's Department of Animal Production, Animal Nutrition Laboratory.

3.3.4 Experimental design and Feeding management

A 4 X 4 Latin square cross over experimental design with four lactating dairy cattle and four diets, with each dietary period lasting 20 days (Table 2) was used. Throughout the experimental period, the animals were housed in individual cages (Figure 6), only being let out to go for milking before being brought back to the pens. The animals were offered feeds twice daily in the morning (8 am) and evening (6 pm), half the daily allowable feed being given during each feeding. Besides, freshwater and mineral supplement were offered *ad libitum.*

Table 2: Experimental layout of crossbred dairy cattle fed four diets in four periods

Periods/Animals	An 1	An 2	An 3	An 4	Total
Period 1 (Day 1- 20)	D_1	D_2	D_3	D_4	4
Period 2 (Day 21- 40)	D_2	D_3	D_4	D_1	4
Period 3 (Day 41- 60)	D_3	D_4	D_1	D_2	4
Period 4 (Day 61- 80)	D_4	D_1	D_2	D_3	4

An1- animal number 1; An2- animal number 2; An3- animal number 3; An4- animal number 4; D1- diet 1; D2- diet 2; D3- diet 3; D4- diet 4

Figure 6: Animals housed in individual cages

3.3.5 Sample collection

Rumen fluid samples were collected after every ten days. The samples were collected in the morning before feeding. During sample collection, animals were taken into a cattle crush and restrained manually (Figure 7). An unfractionated rumen sample was then withdrawn after insertion of a flexible oral-gastric tube as described by Lodge-Ivey *et al.*, (2009). The first 200-mL of rumen fluid was discarded to minimize chances of saliva contamination and prevent any cross-contamination. Then 40-mL of rumen fluid was collected and placed in

labelled sterile 50ml polypropylene centrifuge tube. To minimize carry-over of rumen sample from one cow to another, the sample collection device was cleaned thoroughly using running water before sampling the next cow. Samples were then placed in a cool box full of ice and transported to the Department of Animal Production for storage at -20℃ awaiting DNA extraction and further analysis.

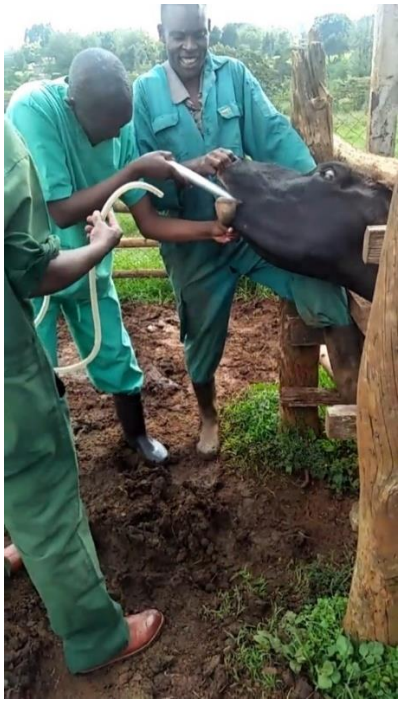

Figure 7: Animals restrained manually and in a crush during sample collection

3.3.6 DNA extraction

The frozen samples were left to thaw at room temperature for 1 hour and homogenized by mixing thoroughly (vortexing for 30 seconds). Total genomic DNA was extracted from a representative subsample of rumen liquor using the QIAamp DNA stool mini kit (Qiagen) manufacturer's guidelines. However, few alterations from the manufacturer's guidelines were done to increase the DNA yield while reducing the amount of RNA recovered. These modifications were (i) doubling the recommended sample amount and (ii) adding 2 µl of RNAse mixture to the sample. UV light Trans illuminator on 1% agarose gel electrophoresis

was used to visualize the extracted DNA, and its quality and quantity assessed using Nanodrop spectrophotometry (Nanodrop Technologies).

3.3.7 Libraries preparation and sequencing

Library preparation and sequencing was performed at Admera Health Limited (USA) using the Nextera DNA Preparation Kit and the Nextera Index Kit (Illumina, San Diego, CA, USA). The library preparation followed the Illumina 16s Protocol for the amplification of V3-V4 regions. Briefly, the extracted DNA underwent a two-stage PCR protocol that targeted the 16S Ribosomal RNA V3-V4 region. The first PCR used specific primers, 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG' as forward primer and, 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACH VGGGTATCTAATCC' as reverse primer, that was geared towards amplifying the V3-V4 region while the second PCR was for assigning indices to respective samples in preparation for multiplexing. After the PCRs, the amplified DNA template was cleaned to purify it away from free primers using AMPure XP beads and freshly prepared 80% ethanol. The quantity and fragment size of the libraries was estimated using Qubit spectrophotometry and Agilent 2200 bioanalyzer, respectively. This was followed by library normalization and pooling. Finally, following libraries preparation, libraries denaturing, and sample loading, a paired-end (250 bp reads per end) sequencing was done on a MiSeq sequencer using v3 reagents (Quick *et al.,* 2017).

3.3.8 Bioinformatics analysis and Operational Taxonomic Unit assignment

The paired-end raw fastq sequencing reads were analyzed using the QIIME v 1.91 (Caporaso *et al.,* 2010). The analysis involved several scripts within QIIME that were geared towards achieving OTU assignment. Briefly, paired-end reads were merged using USERCH (Edgar *et*

al., 2011). Next, the quality of the data was assessed, and poor-quality reads removed. Specifically reads with average base quality drop below 20 and those with less than 450 bp were trimmed and truncated as this is the size of the target region (V3-V4).

After quality control, chimeras were detected and removed via UCHIME (Edgar *et al.,* 2011) against the latest chimera rdp gold database https://www.drive5.com/uchime/uchime_download.html . Operational taxonomic units were then clustered based on 97% similarity threshold using the UPARSE algorithm (Edgar,2013). An open -reference method was then used to pick the representative sequence for normalization and taxonomy assignment for each OTUs using RDP classifier by a Blasting against the GreenGenes database version 13.8 (http://www.metagenomics.wiki/tools/16s/qiime/install/greengenes).

3.3.9 Diversity and Statistical analysis

Data obtained from QIIME 1 analysis was imported into R software where alpha diversity analysis was conducted to assess the taxonomic richness, dominance, and evenness using phyloseq package in R (McMurdie & Holmes, 2013). The alpha diversity indices were: Chao1 minimal richness index (Chao & Shen, 2003), inverse Simpson diversity index (Hill, 1973; Simpson, 1949), and Shannon diversity index (Shannon & Weaver, 1949). Rarefaction analysis followed with plotting the rarefaction curve was done to assess within and between sample microbial community coverage. To reveal the similarity and the dissimilarity between the communities (beta diversity), weighted unifrac metrics was done (Hamady *et al.,* 2010)and multivariate analysis; principal component analysis (PCA) and Non-metric Multidimensional Scaling (NMDS) was conducted to visualize the data using R software.

Finally, One–way ANOVA was performed to assess differences in the microbial relative abundance of OTUs between the four diets using R software. Results were reported as means with standard errors and a significant level at $P \leq 0.05$.

3.4 Results

3.4.1 Diet composition

The results for the chemical composition analysis of the dietary components are shown in (Table 3). Rhodes grass and Lucerne chemical composition was reported to be similar to those documented in previous studies by Bresson *et al.,* (2009) and Ondiek *et al.,* (2010).

Table 3: Chemical composition (%DM) of the diet ingredients

		Dietary ingredients		
		Lucerne	Bomas Rhodes	Concentrates
proximate	Dry matter (DM)	95.58	95.44	91.39
	Ash	12.86	10.32	7.09
	Crude protein (CP)	17.03	6.41	14.41
	Ether extract (EE)	1.76	1.67	7.72
	Crude fiber (CF)	37.77	39.98	13.5
	Nitrogen free extract (NFE)	30.58	41.62	57.28
van soest	Neutral detergent fiber	58.32	68.95	-
	Acid detergent fiber	40.61	43.86	-
	Acid detergent lignin	10.91	5.37	-
	Cellulose	29.7	38.49	-
	Hemicellulose	17.71	25.09	-

3.4.2 Rumen bacterial microbiome changes across the four dietary treatments

3.4.2.1 Quality control and diversity indices

The results of the raw reads were 11,159,803 from 32 rumen samples (4 animals for each of 8 sampling weeks). Once the reads were merged, they reduced from 4.14% minimum to 4.92% maximum, both in diet 4. Subsequently, quality control reported a reduction of 47.32% (diet

4), minimum; 51.05% (diet 1), maximum. Diet, sampling days, and the interaction between diet and sampling days did not significantly affect the initial number of reads or the read reduction at the joining or quality control phase (Table 4).

Table 4: 16S rRNA gene (V3-V4) bacterial sequence details

Diet	Sampling time	Initial Sequences	% Reduction after joining	%Reduction after QC
Diet 1	Day 10	346878.75±7567.79	4.21±0.18	47.54±0.17
	Day 20	332273.25±34140.76	4.27±0.09	51.05±2.62
Diet 2	Day 10	410317±22550.49	4.29±0.17	48.25±0.64
	Day 20	342615.5±27805.38	4.29±0.15	48.44±0.18
Diet 3	Day 10	374284.75±45717.45	4.16±0.08	48.03±0.52
	Day 20	363765.75±45672.46	4.33±0.06	47.81±0.33
Diet 4	Day 10	318799.75±51044.03	4.92±0.46	48.78±0.35
	Day 20	301016±48931.75	4.14±0.07	47.32±0.37
P-VALUES	**Diets**	0.31	0.44	0.52
	Sampling Days	0.32	0.34	0.48
	Diets*Sampling Days	0.86	0.1	0.11

Data for 16s rRNA gene bacterial sequences are presented as mean ± SE, % - percentage, QC- quality control

After blasting the representative OTUs on the greengenes database at 97% similarity level, a total of 631 OTUs were identified. Among those 423 OTUs were shared across the diets and upon classifying a total of 26 bacterial phyla, 65 classes, 103 orders, 156 families, and 273 genera were detected. The alpha diversity indices did not report any significant difference among the diets, sampling days, and with the interactive effects of both diet and sampling days. Interestingly, all the indices reduced as the concentrate level increased from diet 1 to diet 4 (Table 5). The samples were rarified to an even depth of 90% of the minimum sample before conducting beta diversity. To assess the degree of similarity between the microbes across the diets, a NMDS plot was traced which revealed that the bacterial communities were similar between the four diets with the Firmicutes, Bacteroidetes, and Fibrobacteres

contributing the most to the Principal Component Analysis (PCA) plot respectively as shown in Figure 8, with the longer the distance, the higher the contribution.

Table 5: Metagenomic analysis of bacteria community in dairy cows within the different diets (alpha diversity)

			OTU richness			OTU diversity		
		Sampling days	**Observed**	**Chao1**	**ACE**	**Shannon**	**Simpson**	**Fisher**
Diet		Day 10	31322±1510.865	100095.408±5553.33	121190.408±7256.033	8.554±0.112	0.998±0	15396.499±1501.601
	Diet 1	Day 20	27761.5±2464.813	89013.893±6075.656	107576.882±8524.518	8.439±0.171	0.998±0.001	13051.437±1522.004
		Day 10	37126.25±3610.707	115523.007±8850.013	141580.084±12229.031	8.733±0.124	0.999±0	18137.499±2387.466
	Diet 2	Day 20	28398.25±3075.438	92841.617±9743.89	110875.435±13355.086	8.499±0.135	0.999±0	13279.967±1976.666
		Day 10	31287±2484.31	99796.973±7034.71	120319.738±8454.956	8.531±0.108	0.999±0	14197.845±1234.815
	Diet 3	Day 20	29704±3296.87	93542.219±9817.91	112071.255±12664.543	8.425±0.121	0.998±0	13347.69±1562.239
		Day 10	28073.75±5453.088	85645.192±16278.97	103183.212±20129.113	8.433±0.249	0.998±0	13625.944±2887.427
	Diet 4	Day 20	27257.5±5088.882	85081.078±14662.064	101541.782±18573.974	8.378±0.215	0.997±0.001	13003.944±2729.774
P-value	**Diet**		0.56	0.37	0.38	0.63	0.18	0.68
	Sampling days		0.16	0.18	0.17	0.27	0.32	0.15
	Diet * Sampling Days		0.69	0.75	0.73	0.95	0.55	0.72

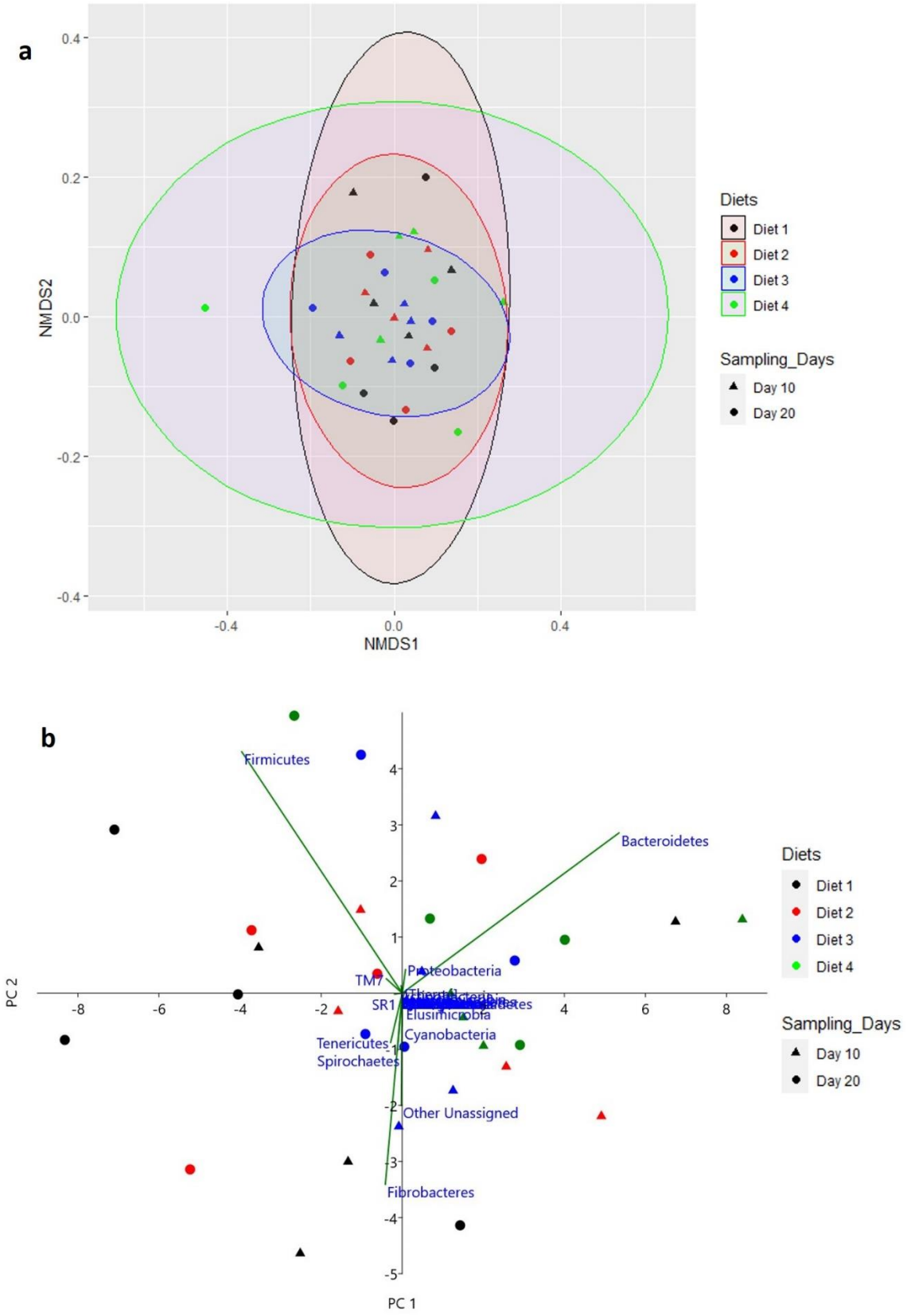

Figure 8: (a) NMDS illustrating the similarity of bacterial communities across the four diets and (b) BIPLOT showing the contribution of each phylum into the principal component analysis (PCA).

3.4.2.2 **Effects of diets on bacterial community structure at the levels of phylum and genus**

The taxonomic analysis revealed that majority of bacteria from animals irrespective of diet to be Bacteroidetes, Firmicutes phyla, followed by Tenericutes, Fibrobacteres, Spirochaetes, TM7, Proteobacteria, Cyanobacteria, SRI, Elusimicrobia, and Actinobacteria respectively. These, together with those represented in Table 6 had a relative abundance mean > 0.001. The relative abundance of the Bacteroidetes and Firmicutes ranged from 65.29% – 69.70% and 15.36% - 16.96% presenting an average of about 83.71% of the total bacterial population (Figure 9).

At the genera level, a total of 273 genera were identified. Out of 273 genera, Bacteroidetes represented 39, Fibrobacteres 1, Firmicutes 75, and Proteobacteria 99. However, this study only focused on genera of four phyla (Bacteroidetes, Firmicutes, Fibrobacteres, and Proteobacteria) on the ground of relative abundance mean of ≥ 0.001 as shown in Table 7. *Prevotella* belonging to Bacteroidetes phylum was the most abundant genus, others being *CF231, YRC22, BF 311, Bacteroides,* and *Paludibacter*. Fibrobacteres only had one genus, *Fibrobacter*. *Succiniclasticum* was the dominant genus at the Firmicutes phylum, followed by *RFN20, Butyrivibrio, Streptococcus, Ruminococcus,* and *Clostridium,* respectively. The proteobacteria phylum had 5 genera that passed the relative abundance cutoff of ≥ 0.001, among those 5 genera, *Desulfovibrio* was the dominant genus followed by *Stenotrophomonas*, *Acinetobacter*, *Hyphomonas,* and *Ruminobacter*.

Diet had a significant effect on phylum Bacteroidetes (P≤0.05) only. Sampling days on the other hand significantly affected two phyla TM7 (P≤0.05) and Chloroflexi (P≤0.05), and one genus *Ruminococcus* (P≤0.01) belonging to Firmicute phylum. The interactive effect of diet and sampling days did not significantly affect both phylum and genus taxa. As animals were transitioned from a forage-based diet (D1) to a diet containing more concentrate, 40% (D4), a noticeable increase in the abundance of Bacteroidetes was observed. Additionally, the

Proteobacteria number also slightly increased, though not significantly. The proportion of Firmicutes reduced from 16.95% to15.35% as the concentrate level increased. The Fibrobacteres showed a decrease, though not significant (see Figure 9). Of interest was the trend which the genus for Bacteroidetes, Proteobacteria and Firmicutes phyla showed in response to dietary change. As the phyla Bacteroidetes and Proteobacteria proportion increased with an increase in concentrate ratio, the genera number in both phyla also increased. However, genera in Firmicutes remained relatively unaffected as the concentrate level shifted from 10% to 40% (see Table 8).

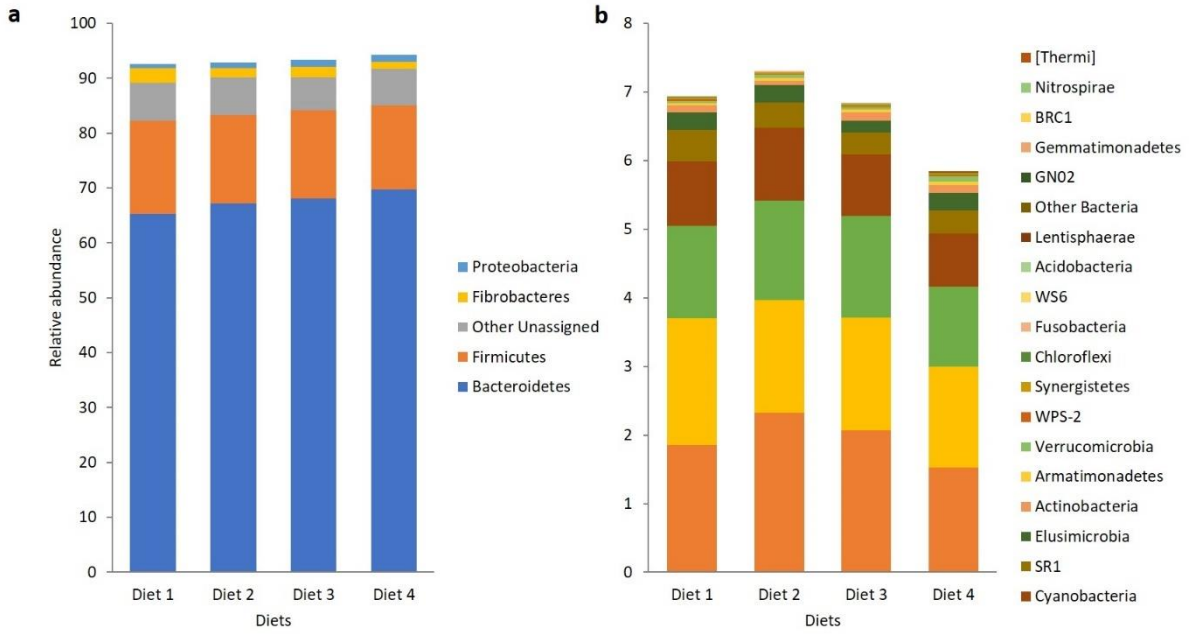

Figure 9: Stacked bar graph showing the bacterial phyla: (a) illustrating the main phyla and (b) showing all the other bacterial phyla identified.

Table 6: Percentage Average abundance of Bacterial composition community at phylum level across all the diets

Bacterial phyla	DIETS								P-value		
	Diet 1		Diet 2		Diet 3		Diet 4		D	T	D*T
	Day 10	Day 20	Day 10	Day 20	Day 10	Day 20	Day 10	Day 20			
Bacteroidetes	65.773±2.713	64.813±0.684	68.285±0.923	66.061±1.778	68.019±0.575	68.162±0.856	69.976±1.731	69.439±0.616	0.04	0.38	0.86
Firmicutes	16.92±1.798	16.991±2.256	15.087±1.416	17.268±0.411	15.66±0.738	16.58±0.993	15.676±1.75	15.036±0.641	0.73	0.53	0.77
Unassigned; Other	7.444±0.882	6.508±0.258	7.285±0.759	6.313±0.693	6.106±0.609	5.797±0.496	6.676±0.902	6.55±0.464	0.45	0.23	0.89
Tenericutes	1.667±0.152	2.043±0.201	2.157±0.284	2.491±0.326	2.8±0.787	1.34±0.299	1.244±0.415	1.801±0.324	0.24	0.86	0.06
Fibrobacteres	2.626±1.268	2.528±0.874	1.665±0.389	1.688±0.675	1.923±0.661	2.006±0.496	1.158±0.435	1.39±0.324	0.33	0.9	0.99
Spirochaetes	1.353±0.298	2.335±0.544	1.69±0.309	1.589±0.218	1.755±0.164	1.523±0.223	1.274±0.38	1.673±0.223	0.71	0.25	0.23
TM7	1.186±0.178	1.515±0.261	1.156±0.146	1.746±0.352	1.316±0.133	1.645±0.268	1.047±0.165	1.282±0.131	0.48	0.02	0.86
Other	0±0	0±0	0.001±0	0.001±0.001	0.001±0	0.001±0.001	0.001±0.001	0.001±0.001	0.77	0.66	0.99
Fusobacteria	0.001±0	0.007±0.007	0.001±0.001	0.004±0.004	0.001±0	0.003±0.002	0.006±0.005	0.001±0.001	0.92	0.43	0.34
Acidobacteria	0±0	0.002±0.001	0.001±0.001	0.004±0.002	0.002±0.001	0.001±0	0.005±0.005	0.001±0.001	0.77	0.98	0.42
Proteobacteria	0.868±0.169	0.879±0.059	1.087±0.379	1.015±0.225	1.399±0.742	1.033±0.092	1.251±0.234	1.28±0.578	0.73	0.72	0.95
Cyanobacteria	0.892±0.059	0.986±0.239	1.124±0.308	1.01±0.226	0.712±0.103	1.091±0.317	0.644±0.236	0.901±0.196	0.64	0.35	0.72
SR1	0.41±0.18	0.521±0.265	0.274±0.107	0.462±0.176	0.194±0.057	0.447±0.137	0.35±0.151	0.324±0.153	0.81	0.27	0.85
Elusimicrobia	0.243±0.056	0.255±0.095	0.251±0.056	0.247±0.085	0.162±0.021	0.182±0.036	0.185±0.06	0.335±0.105	0.58	0.38	0.67
Actinobacteria	0.105±0.023	0.099±0.034	0.044±0.003	0.087±0.014	0.137±0.04	0.106±0.035	0.124±0.076	0.109±0.01	0.43	0.93	0.77
Verrucomicrobia	0.023±0.007	0.037±0.007	0.03±0.003	0.064±0.033	0.032±0.003	0.028±0.008	0.132±0.101	0.044±0.012	0.39	0.68	0.41
Armatimonadetes	0.031±0.005	0.041±0.011	0.044±0.015	0.045±0.018	0.053±0.011	0.034±0.011	0.048±0.029	0.04±0.009	0.94	0.71	0.81
Synergistetes	0.023±0.006	0.011±0.001	0.019±0.004	0.009±0.003	0.019±0.009	0.022±0.012	0.025±0.017	0.018±0.003	0.83	0.32	0.82
Chloroflexi	0.007±0	0.014±0	0.004±0.001	0.013±0.005	0.01±0.002	0.01±0.004	0.007±0.002	0.009±0.003	0.74	0.03	0.43
WPS-2	0.042±0.027	0.01±0.003	0.009±0.001	0.015±0.004	0.008±0.003	0.005±0.003	0.019±0.013	0.01±0.007	0.39	0.23	0.38
Lentisphaerae	0.001±0.001	0±0	0.001±0.001	0.001±0	0.001±0	0.001±0	0.001±0	0.001±0.001	0.79	0.56	0.59
Gemmatimonadetes	0±0	0±0	0±0	0.001±0.001	0±0	0±0	0.001±0.001	0.001±0.001	0.49	0.23	0.96
WS6	0.001±0	0.002±0.001	0.001±0.001	0.001±0	0.001±0	0±0	0.001±0.001	0±0	0.27	0.85	0.19

Data for 16s rRNA gene bacterial phyla presented as mean ± SE, D -diets, T-sampling days, D*T – the interaction between diet and sampling days

Table 7: Average abundance of Bacterial composition community at genus level across all the diets

phylum/Genus	DIETS								P-value		
	Diet 1		Diet 2		Diet 3		Diet 4		D	T	D*T
	Day 10	Day 20	Day 10	Day 20	Day 10	Day 20	Day 10	Day 20			
Bacteroidetes											
Prevotella	0.286±0.028	0.282±0.018	0.303±0.022	0.273±0.015	0.29±0.026	0.305±0.029	0.301±0.021	0.272±0.013	0.94	0.46	0.70
CF231	0.034±0.004	0.033±0.006	0.029±0.005	0.023±0.005	0.027±0.006	0.032±0.003	0.026±0.007	0.027±0.006	0.44	0.98	0.82
YRC22	0.024±0.004	0.025±0.004	0.027±0.002	0.026±0.002	0.029±0.002	0.031±0.004	0.031±0.003	0.025±0.002	0.34	0.67	0.56
BF311	0.008±0.001	0.006±0.002	0.006±0.001	0.008±0.001	0.006±0.001	0.009±0.005	0.005±0.001	0.006±0	0.84	0.47	0.85
Bacteroides	0.001±0	0.001±0	0.001±0	0±0	0±0	0±0	0.001±0	0.001±0	0.30	0.74	0.21
Paludibacter	0±0	0.001±0	0.001±0	0.001±0	0±0	0.001±0	0.001±0	0±0	0.94	0.42	0.33
Fibrobacteres											
Fibrobacter	0.026±0.013	0.025±0.009	0.017±0.004	0.017±0.007	0.019±0.007	0.02±0.005	0.012±0.004	0.014±0.003	0.33	0.9	1
Firmicutes											
Succiniclasticum	0.058±0.018	0.023±0.003	0.034±0.009	0.035±0.009	0.019±0.003	0.03±0.006	0.036±0.01	0.023±0.01	0.41	0.2	0.12
RFN20	0.009±0.001	0.018±0.002	0.015±0.003	0.016±0.002	0.016±0.004	0.016±0.006	0.013±0.006	0.016±0.002	0.91	0.22	0.54
Butyrivibrio	0.011±0.002	0.014±0.003	0.011±0.002	0.014±0.002	0.015±0.003	0.016±0.003	0.015±0.008	0.016±0.002	0.71	0.50	0.99
Streptococcus	0.001±0.001	0.016±0.016	0.001±0	0.001±0	0.003±0.003	0.002±0.001	0.001±0.001	0.004±0.002	0.53	0.30	0.45
Ruminococcus	0.003±0	0.004±0	0.003±0	0.004±0	0.003±0	0.004±0.001	0.003±0	0.003±0	0.4	0.01	0.08
Clostridium	0.001±0	0.002±0	0.001±0	0.001±0	0.002±0.001	0.002±0.001	0.002±0.001	0.001±0	0.56	0.82	0.55
Proteobacteria											
Desulfovibrio	0±0	0.001±0	0.001±0	0.001±0	0.001±0	0.001±0	0.002±0.001	0.001±0	0.68	0.94	0.09
Stenotrophomonas	0±0	0±0	0±0	0±0	0±0	0.001±0	0.001±0	0±0	0.78	0.46	0.37
Acinetobacter	0±0	0±0	0.001±0.001	0.001±0	0±0	0±0	0±0	0±0	0.25	0.85	0.83
Hyphomonas	0±0	0±0	0±0	0±0	0.002±0.002	0±0	0±0	0±0	0.42	0.33	0.41
Ruminobacter	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0.001±0	0.55	0.61	0.44

Data for 16s rRNA gene bacterial genus in four main phyla presented as mean ± SE, D -diets, T-sampling days, D*T – the interaction between diet and sampling day

Table 8: Genus taxonomic count across the diets in the three main phyla

	GENUS TAXONOMIC COUNTS			
MICROBES	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**
Bacteroidetes	22	27	30	31
Firmicutes	61	58	61	60
Proteobacteria	66	64	73	75

3.5 Discussion

The dairy cow being a ruminant depends on rumen microbes to convert the indigestible lignocellulosic plant residues into easily absorbable nutrients. This conversion is however dictated by the existence of a reservoir of enzymes secreted by extremely diversified rumen microbes. The most extensively researched and probably most significant element influencing the composition of rumen microbes is the host diet (Noel *et al.,* 2017a). The inherent nature of the feed and changes in its physicochemical properties provoked by its fermentation have been documented to favor the growth of certain rumen microbial ecotypes in both rumen liquor and solid feed material in the rumen (Kim *et al.,* 2011).

However, to create a stable microbial ecosystem in the rumen that can suitably, efficiently, and optimally extract nutrients from the poor-quality feedstuff, several questions still need to be answered. This study was therefore carried out to find solutions to two main questions; how do rumen bacterial communities shift (qualitatively and quantitatively) upon diet change and does a certain core group of bacteria ultimately persist regardless of the diets given to the animals.

A switch from a predominantly roughage-based diet to a diet incorporated with concentrate causes a change in the bacterial taxa, probably due to the change in the type of substrate (Fernando *et al*., 2010). The results of alpha indices did not show any significant change due change in diets. This was in agreement with findings reported by Fernando *et al.,* (2010), who

stated that the diets or any other environmental factors may change the composition of the bacterial genera, but the bacterial density relatively remains the same in the rumen. This finding is further supported by Bainbridge *et al.*, (2016). It is worth noting that, as the concentrate level increased in the diet in this study, bacterial species richness reduced. Similar results have been reported (Zhang *et al.,* 2017) in dairy cow and sheep (Li *et al.,* 2019). The high number of OTUs in the forage-based diet indicates that more bacterial strains are needed to cooperate in the degradation of fiber (NDF and ADF) than in breaking down starch and crude protein (Li *et al.*, 2019). Recently, Wang *et al.,* (2020) reported similar results and argued that more rumen microbial diversity is observable in a high forage diet as concentrate diet tends to lower ruminal pH and this may hinder the growth of rumen bacteria with acid sensitivity.

The relative abundance data revealed Bacteroidetes and Firmicutes were the dominant bacterial phyla, regardless of the diet and sampling day. This was consistent with recent findings by Li *et al.*, (2019), Li *et al.*, (2020), and Zhang *et al.*, (2020). Similarly, Stergiadis and Mora-Ortiz, (2021), when using omics approaches to unravel the role of microbial communities in the rumen of dairy animals reported Firmicutes, Bacteroidetes, and Proteobacteria as the main rumen microbes. However, similar to findings reported by Jami *et al.*, (2014), Li *et al.*, (2019), and Zhang *et al.,* (2020), the relative abundance of Bacteroidetes was significantly higher compared to that of Firmicutes. In this study, only Bacteroidetes were significantly affected by the dietary changes ($P \leq 0.05$) whereas Firmicutes, Proteobacteria, and Fibrobacter were not significantly affected by the dietary changes. Same results of diet having no effect on Firmicutes, Proteobacteria, and Fibrobacter were reported by Jami & Mizrahi (2012), Kala *et al.*, (2017), and Singh *et al.*, (2012).

At the lower taxa, Bacteroidetes phylum had 6 genera with a relative abundance of ≥ 0.001, with *Prevotella* being the most abundant. Previous studies have also reported *Prevotella* as

the dominant genus in the Bacteroidetes phylum (Wang *et al.,* 2020; Zhang *et al.,* 2020). Contrary to the number reported by Guo *et al.,* (2020), where *Prevotella* had a relative abundance of up to 41.5%, in this study, the relative abundance percentage ranged from 28.3 - 29.7. Additionally, the predominance of this genera remained relatively stable, unaffected by diet or sampling days. Despite this genus not being considered among the cellulolytic bacteria (Krause *et al.,* 2003), its members have been described to be highly involved in starch, hemicellulose (Dias *et al.,* 2018), protein (Mhuanthong *et al.,* 2014), and pectin degradation. The recurrent predominance of this genus has been associated with two reasons; (1) their ability to occupy a wide metabolic niche despite having same metabolic capabilities and (2) members of the genus exhibiting an exceptional amount of genetic variability or relatedness (Purushe *et al.,* 2010; Ramšak *et al.,* 2000). These two possibilities, nevertheless, need to be explored further.

Despite remaining unaffected by diet or sampling days, the abundance of Firmicutes phylum reduced as concentrate level increased. This finding was similar to findings by Li *et al.,* (2012) and Wang *et al.,* (2012). At the genera level, it was predominantly comprised of *Succiniclasticum*. This was in concordance with findings reported by Wang *et al.,*(2020). In contrast to findings reported by Bi *et al.,* (2018), where the abundance of *Succiniclasticum* increased in dairy cows fed elevated concentrate concentrations, our study reported a decrease, however not significant. The predominance of this genus was considered as a result of a large quantity of fiber defined by the mean of NDF compared to carbohydrate given by mean of non-fibrous carbohydrate level in the diets (Zhang *et al.,* 2017).

Ruminococcus genus, one of the important fibrolytic microorganisms in Firmicutes phylum displayed a decrease with adaptation to a grain diet. A gradual decrease in abundance of this genus was also reported by Fernando *et al.,*(2010) and Tajima *et al.,*(2001). In this study, *Ruminococcus* abundance was not impacted by the four diets but by sampling days ($P \leq 0.01$).

Species of this genus were reported to have a positive correlation with fiber content indicating their function as key fiber users in the rumen (Kala *et al.,* 2017). However, very minimal representation of fiber-based bacteria may be the reason for no effect of dietary variation on these bacteria in many studies (Kala *et al.,* 2017), including the current study.

Butyrivibrio, which is also a main genus in the Firmicutes phylum is able to utilize both starch (Fernando, Purvis, et al., 2010) and hemicellulose (Freetly *et al.,* 2020) in the rumen. Due to their ability to breakdown sugars, their number is expected to increase as the animals are subjected to a grain-based diet, as exhibited in this study, however, the increase was not significant. Contrary, some studies have reported a reduced abundance of this genus with concentrate elevation in the diet (Fernando *et al.,* 2010; Li *et al.,* 2019).

Regardless of Proteobacteria phylum not being among the top three dominant phyla, this phylum reported the highest number of genera (99). Among the 99 genera, only 5 had a relative abundance of ≥ 0.001, with *Desulfovibrio* regarded as the most abundant. The abundance of this phylum, however, was not significantly affected by the diet, sampling days or the interaction effects of diet and sampling days. Proteobacteria phylum displayed an insignificant trend of increase with an increase in concentrate proportions which was in concordance with Bi *et al.,* (2018),whose findings reported an increase, although not significant, in Proteobacteria when animals were offered 50% concentrate. This study reported low abundance of Proteobacteria of around 1% across all the diets. Several studies have also reported low number of this phylum (Bainbridge *et al.,* 2016; Patel *et al.,* 2014b; Pitta *et al.,* 2014a). Previous studies have theorized this low abundance to be associated with two reasons; (i) due to using the 16s metagenomic approach (Pitta *et al.,* 2015) and (ii) use of different diets in experiments (Parks *et al.,* 2013).

Proteobacteria phylum is primarily made up of Gram-negative bacterial members who are actively engaged and playing a significant role in extremely diversified metabolic pathways like carbohydrate, lipid, carbon, and nucleotide metabolism in the rumen, with the most prevailing function being degradation of readily fermentable carbohydrates (Fernando *et al.*, 2010; Petri *et al.*, 2013a; Pitta *et al.*, 2014b; Pitta *et al.*, 2010). Members of this phylum were reported to have a high colonization abilities and adaptability to new environment (Francino, 2012) , justifying the high number of the genus despite the low abundance. Its therefore suggestive that members of this phylum play a significant role in the host rumen as the animal transitions from roughage to concentrate diet (Chen et al., 2011; Fernando, Purvis, et al., 2010) and as result contributing significantly to differences in phenotypes of the ruminant host (Mukhopadhya *et al.*, 2012).

Besides Firmicutes, Fibrobacteres phylum is also an important rumen microbe for fiber degradation. Notably, this phylum only reported one genus, *Fibrobacter.* Neither the phylum nor the genus was significantly influenced by diet, sampling days, or their interactive effects, however, both exhibited a decrease with increase in the concentrate ratio in the diet. This phylum has been reported to be highly impacted by the diet fed to the animal with evidence seen in a metagenomic study where the phylum was reported completely absent from fiber-adherent bovine ruminal microbes (Brulc *et al.*, 2009). Functionally, members of this phylum have been linked with the breakdown of cellulose in the host rumen (Naas *et al.*, 2014; Petri *et al.*, 2013b).

Regarding microbial stability, this study did not report any change on the four phyla discussed due to different sampling days, however, TM7 and Chloroflexi were affected. At the genus level, only *Ruminococcus* was impacted, this may be due to animal variations. This study, therefore, theorizes that by the end of 10 days upon dietary treatment the rumen microbes had acclimatized to the new diet as many phyla and genera remained unaffected by

the different sampling days. This agrees with a study done by Fernando *et al.,* (2010) that demonstrated the potential of rumen bacteria to shift within seven days after changing the ratio of forage to concentrate in the diet (80:20 to 60:40).

These findings can be useful for cross-sectional/over studies, as single sampling at day 10 following change of diet can reasonably be considered as a representative for the microbial community. Nevertheless, more studies need to be conducted to give a deeper insight on the adequate time required to wash out the microbes for the previous diet, and as result clearly defining the factors influencing rumen microbial temporal stability.

3.6 Conclusion

This study aimed at gaining basic understanding the diversity of rumen bacterial community when the animals were subjected to different types of diets. The results showed that increasing the amount of concentrate in the diet significantly impacts on several rumen bacterial communities. There was significant increase of Bacteroidetes as the concentrate proportion increased, while other taxa showed non-significant changes that is Firmicutes and Fibrobacteres decreased as diet changed from forage based to concentrate based while Proteobacteria phylum increased, however insignificantly.

The results of microbial stability suggest that by day 10 following dietary change rumen microbiome have acclimatized, however re-evaluation in order to accommodate required rumen microbiome adaptation is recommended.

CHAPTER FOUR: ASSOCIATION BETWEEN DIET, RUMEN MICROBES AND MILK COMPOSITION PROFILES OF CROSSBRED DAIRY CATTLE

4.1 Abstract

Globally, there is an increased level of health consciousness among dairy product consumers leading to changing consumer preferences. To meet these changing preferences, a better comprehension of factors affecting milk composition is essential. Diet, among others, has been identified as the major driving forces but little is known about the how the interaction of diet and microbes can affect the quality of milk in crossbred dairy cows. This study was conducted to evaluate the effect of varying concentrate inclusion levels in the diet on milk parameters and the relationship between changes in rumen communities to changes in milk constituents of crossbred dairy cattle. An 80- days 4X4 Latin square experimental design with four diets formulated to contain 10%, 20%, 30%, and 40% commercial dairy concentrate and to meet the nutritional requirements of dairy cattle yielding 12 kg of milk per day using the NRC was conducted. Rumen liquor and milk samples were collected after every 10 days. Spearman correlation was then done to assess the relationship between bacterial taxa communities and milk production and composition constituents.

The findings of the study indicated that with an increase in concentrate level from 10% to 40%, milk fat decreased while milk lactose, protein, and solids not fat linearly increased. A positive and significant correlation was exhibited between *Prevotella* ($P \leq 0.05$), Lentispaerae ($P \leq 0.01$), Synergistetes ($P \leq 0.01$) with milk protein. *BF311* was also positively and significantly correlated with milk fat ($P \leq 0.05$). Phylum Fusobacteria showed a negative correlation with milk lactose ($P \leq 0.01$) as well as Tenericutes with milk protein ($P \leq 0.01$). Correlation analysis revealed an existing relationship between rumen bacterial community and the milk parameters.

4.2 Introduction

The composition of milk from dairy cattle is of major concern to farmers, milk processors and consumers. This is because the milk payment system in Kenya and Africa in general is gradually shifting towards a quality-based milk payment system, consequently having a direct impact on farmer's income and the cost of milk production (Louis *et al.,* 2015). Besides, globally there are increased levels of health consciousness and conscience among dairy product consumers, resulting in changing preferences. To meet this increased demand on healthy foods, a better comprehension of factors affecting milk composition is essential. Recent reports have indicated animal breed, nutrition, age, parity, stage of lactation, seasons, and physiological conditions of the animals to be the factors associated with changes in milk yield and composition (Gajbhiye *et al.,* 2019; Kala *et al.,* 2017; Roessler *et al.,* 2019). It is well documented that the diet fed to dairy cows is key determinant in milk yield and composition (Krehbiel, 2014). Once feed is consumed, it is broken down by a consortium of microbes residing in the rumen into absorbable nutrients like propionate, butyrate, acetate, and lactate (Volatile Fatty Acids, VFAs). These VFAs are then absorbed into the bloodstream and transported to liver, mammary tissues where they are utilized in the synthesis of milk (Mccann *et al.*, 2014).

Advancements in modifying the composition of milk by dietary manipulation derive from essential contributions of the entire animal system that is feeding, rumen fermentation processes, and cellular work at mammary tissues (Bauman & Griinari, 2003). However, of interest to this study is rumen microbes' influence on milk quality as a result of diet offered to the animal. Jami *et al.,* (2014) demonstrated the potential role of the bovine rumen microbiome in modulating milk composition and identified a connection between milk fat to Firmicutes and Bacteroidetes. The Firmicutes to Bacteroidetes ratio relationship to milk fat

poses an interesting challenge on other milk constituents and how much of the variations in these milk compositions can be explained by bovine rumen microbial variations. Due to scarcity of information on how the interaction of diet and microbes can improve the quality of milk in crossbred dairy cows, there a need to bridge the gap.

Given the foregoing, this study evaluated the effect of varying concentrate inclusion levels on milk parameters and the relationship between changes in rumen communities to changes in milk constituents of crossbred dairy cattle.

4.3 Materials and methods

4.3.1 The study area, experimental design, diets and feeding management, rumen sampling, DNA extraction, libraries preparation, and sequencing

The materials and methods are as described in Chapter 3, Sections 3.3.2 to 3.2.8

4.3.2 Milk sample collection and analysis

Milk samples were collected in the morning, before feeding, after every 10 days. Milk samples were collected using the aseptic technique procedure described by Metzger et al., (2018). The samples were placed in a labeled sterile 50ml polypropylene centrifuge tube. They were then placed in a cool box full of ice and transported to the Department of Animal Production, where the composition was determined immediately. The milk components, (that is, fat, protein, lactose, and SNF) were determined by an automatic ultrasonic milk analyzer device (Lactoscan MCC, SLP 60, V60), calibrated for cattle milk. Differences in the milk constituents were then assessed using SPSS Version 25.

4.3.3 Statistical analysis

The four main bacterial taxa, as described in section 3.4.2, were correlated with the dietary nutrients by use of linear Pearson correlation method. Non-parametric correlation matrix (Spearman's rank correlation) was used to illustrate the level of correlation of all 26 bacterial phyla obtained during blasting against the greengenes database and the milk averages that is, production, fat, lactose, protein, and SNF based on the sampling days. Significance was declared at $P \leq 0.05$. Due to the high number of genera in the four phyla, there was a need to select only a few that would be correlated with milk averages, therefore, to determine the OTUs that had the highest contribution to variation in milk parameters, a random forest regression was done in the R software using the 'random forest' package. Briefly, the input data was matched and then merged into a Phyloseq object. The OTUs were pruned to eliminate those with relative abundance below 0.001 and the tree built using 1000 bootstrap replicates. Based on 3- fold cross-validation value, a selection step was done to identify the number of genera taxa to be included in the prediction step. This was run several times and the one with the lowest validation error was selected at least 55.2 % variance. Finally, based on the predictive values, the most important 15 taxa were generated and plotted using ggplot in R.

The 15 genera taxa were then correlated with the milk averages using the spearman's rank correlation method and significance declared in values less or equal to 0.05. The two datasets (correlation coefficiencies for phyla and genera with milk averages) were combined in MS excel, then used to plot heatmap for visualization using corrplot in R software V4.1.2. Finally, the correlation networks between milk averages and bacterial taxa were visualized in Cytoscape package (Shannon *et al.,* 2003).

4.4 Results

4.4.1 Effect of concentrate level on milk production and composition

The results for the effects of level of concentrate inclusion in lactating crossbred diets on milk yield and composition is shown in Table 9. There was no significant difference in the milk production and milk composition with increase in concentrate level. However, there was an apparent increase in milk production, protein, lactose, and SNF while there was a slight decrease in the milk fat as the concentrate level increased from diet 1 to diet 4.

Table 9: Effects of concentrate inclusion level on milk yield and composition of crossbred dairy cattle

Parameters	Diets				P-value
	Diet 1	Diet 2	Diet 3	Diet 4	
Milk production	7.81±.99	7.87±.91	8.01±1	9.03±1.5	0.89
Fat (%)	4.52±.72	3.86±.68	3.44±.63	3.37±.39	0.55
Solids Not Fat (SNF - %)	4.33±.21	4.41±.27	4.31±.26	4.56±.27	0.9
Protein (%)	1.72±.08	1.71±.11	1.72±.11	1.82±.11	0.86
Lactose (%)	1.72±.11	1.7±.15	1.71±.13	1.84±.14	0.87

4.4.2 The association between diets, microbes and milk composition

The bacterial microbes were correlated with different dietary substrates that they have been shown to digest from previous studies. The nitrogen free extracts (NFE) quantity was used as an indicator of the amount of starch in the feed as stated by Naseri (2020). All four bacterial phyla fitted in linear Pearson correlation matrix showed a strong positive correlation ranging from 0.854 to0.993. All the correlations were significant except for the Fibrobacteres phyla which also had the lowest correlation. The highest correlation was identified with Bacteroidetes (r=0.993), which was also significant at $P \leq 0.01$(Table 10).

Table 10: Pearson correlation analysis of the four main phyla and dietary nutrients

Bacterial phyla abundance		Nutrients Utilized by The Bacterial Phyla		
		NFE, CP, Hemicellulose	Cellulose	NFE
Bacteroidetes	Pearson Correlation	**.993****	-.993**	.993**
Firmicutes	Pearson Correlation	-.960*	**.960***	-.960*
Proteobacteria	Pearson Correlation	.976*	-.976*	**.976***
Fibrobacteres	Pearson Correlation	-0.854	**0.854**	-0.854

**. Correlation is significant at P≤ 0.01 (2-tailed).
*. Correlation is significant at P≤ 0.05 (2-tailed).
Values in bold represents the correlation value. NFE- nitrogen free extracts, CP- crude protein

Further, Spearman correlation analysis based on abundant bacterial phyla and genera unveiled the relationship webs among bacterial taxa and milk-related traits. In total, 26 bacterial phyla and 15 bacterial genera (Figure 10) were utilized in generating the correlation matrix. Many bacterial taxa that showed a negative correlation or no correlation with milk production, showed a positive correlation with milk protein, lactose, and SNF. Milk yield had the most negative (22 OTUs) relationships with bacterial taxa. It was positively and significantly correlated with Bacteroidetes (r =0.55, P≤0.01). Other bacterial taxa that were positively correlated with milk yield included BRC1, Thermi, and GN02. Phylum Firmicutes and three of its genera showed a negative relationship with milk yield. Among those three, *Ruminococcus* was significant (r= -0.370, P≤0.05), others being *Succiniclasticum* and *Coprococcus*. Additionally, phylum Fibrobacteres and genus *Fibrobacter* were also negatively correlated with milk production. Other phyla like Chloroflexi, Cyanobacteria, WS6, and WPS-2, and genus *BF311* also displayed a negative relationship with milk yield.

Among the milk composition parameters, milk fat was positively and significantly correlated with genus *BF311* (r= 0.385, P≤0.05), belonging to Bacteroidetes phylum. In the same phylum, *Bacteroides* also positively correlated with milk fat. Moreover, Phylum Fibrobacteres and genus, *Fibrobacter*, positively correlated with milk fat. Other phyla that showed a positive relation with milk fat were Cyanobacteria, Elusimicrobia and Tenericutes.

Conversely, milk fat was negatively correlated with Actinobacteria, Firmicutes, *Ruminobacter*, TM7, and Gemmatimonadetes. Phylum Proteobacteria, a few of its genus, together with *Prevotella* also showed a weak negative correlation with milk fat.

As shown in Figure 11, milk protein, lactose and SNF shared many similar correlations. However, milk protein had the highest number of taxa that were positively correlated with it, 4 phyla and 7 genera. *Prevotella*, belonging to Bacteroidetes, had a positive and significant correlation (r=0.371, P≤0.05). Lentispaerae (r=0.446, P≤0.01) and Synergistetes (r= 0.443, P≤0.01) also exhibited a positive relationship with milk protein. Other taxa that had an association with milk protein included, *Bacteroides*, Chloroflexi, *Coprococcus, Pseudobutryivibrio, Clostridium, Ruminobacter*, SR1, WS6, and *Desulfovibrio.* Tenericutes had a negative correlation with milk protein which was significant at P≤0.01. Fusobacteria, Cyanobacteria, *Paraprevotella*, BRC1, and *BF311* also had a negative relationship with milk protein.

Further, the heatmap showed milk lactose to be positively correlated with Lentispaerae (P≤0.05), *Desulfovibrio, Prevotella, Butyrivibrio, Clostridium, Paraprevotella, Succiniclasticum,* Gemmatimonadetes, Chloroflexi, and Verrucomicrobia. A negative correlation existed between milk lactose and Fusobacteria (r=-0.421, P≤0.01), Spirochaetes, Tenericutes, and Nitrospirae. There was a positive correlation between Solids-Not- Fat with Lentispaerae, *Desulfovibrio*, Synergistetes, and *Prevotella* and negatively correlated with

Fusobacteria, Tenericutes, and BRC11. Interestingly, Spirochaetes, Fusobacteria, and *RFN20* showed a consistent negative correlation with all parameters in the matrix (Figure 12).

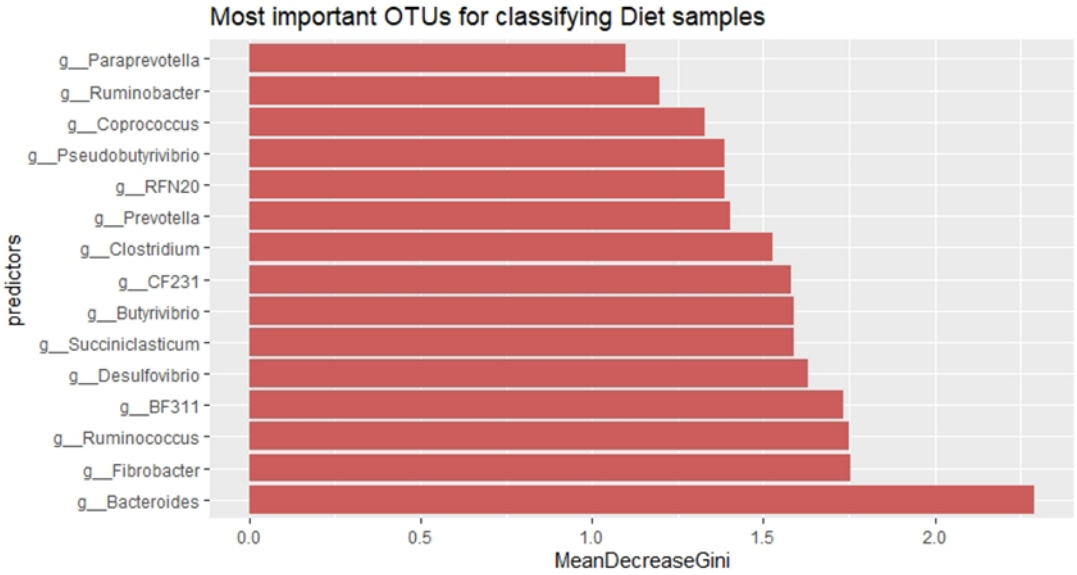

Figure 10: Random Forest illustrating the of 15 most important OTUs

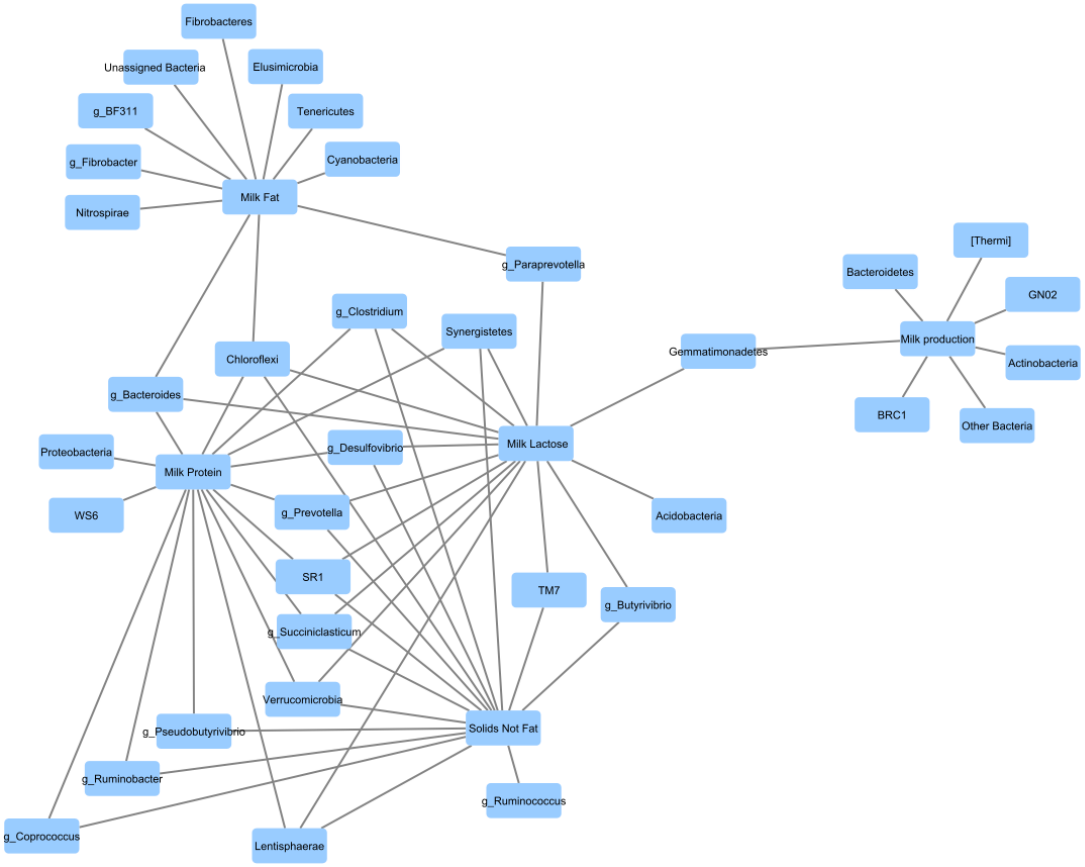

Figure 11: Correlation network illustrating association between milk composition parameters and rumen bacterial taxa. Only bacterial taxa with positive correlation were chosen to be displayed.

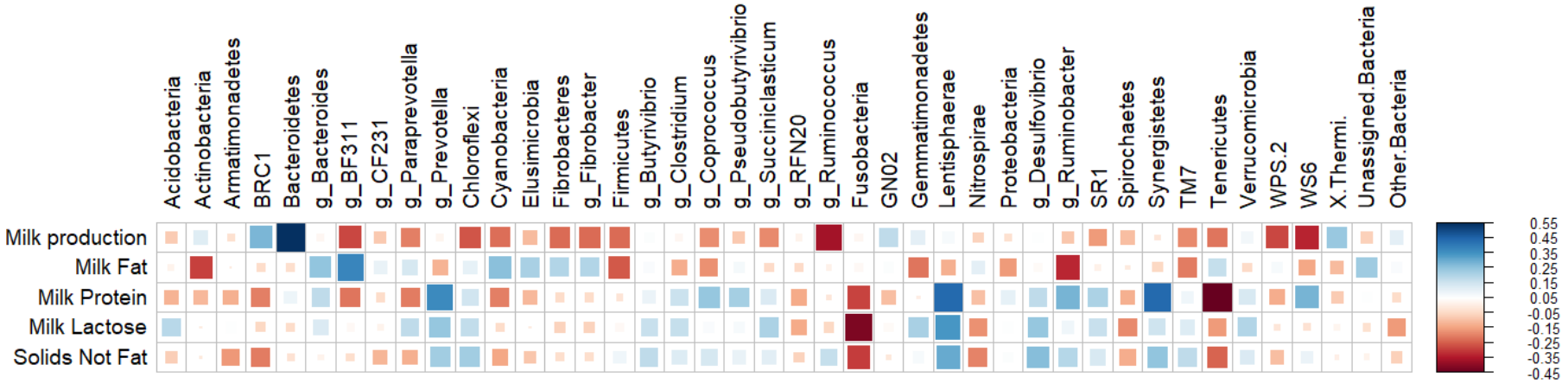

Figure 12: Spearman correlation between the bacterial taxa and milk averages. The scale color denotes how the taxa are correlated, i.e., either positive or negative. Blue denotes positively correlated ($r \geq 0.50$), brown denotes negatively correlated ($r \leq -0.40$) and white denotes no correlation. Strong correlations are indicated by large squares and darker color while weak correlations by small squares and faded color.

4.5 Discussion

Due to scarcity of information on the relationship between diet and microbes in enhancing milk quality of Kenyan crossbred dairy, this study evaluated the relationship between diets, rumen bacterial community, and milk constituents after subjecting dairy animals to diets having different inclusion levels of concentrates. The correlation results indicated that there exists a link between some bacterial taxa, both at phylum and genus level with milk composition constituents. This was an indication that many milk composition values are influenced by the bacterial community composition in the rumen. Pearson correlation also gave an affirmation that the bacterial community residing in the rumen are influenced by the type and proportion of the feed offered to the host animal.

Analysis of Spearman correlations between the bacterial taxa and milk yield parameter showed that several taxa are positively associated, and some significantly related in a moderate way. Specifically, a significant and positive correlation was exhibited between phylum Bacteroidetes and milk yield. Most members of this phylum are considered to have capability of utilizing starch, protein, and hemicellulose (Dias *et al.,* 2018; Mhuanthong *et al.,* 2014). A strong and significant correlation was also reported in this study between Bacteroidetes and the dietary nutrients. Notably, all the genera in this phylum used in the correlation matrix negatively correlated with milk yield. Similar results of some genera in this phylum like *BF311, CF231, Paraprevotella* having a negative correlation with milk yield was reported by Indugu *et al.,* (2017). Moreover, Jami *et al.,* (2014) and Bainbridge *et al.,* (2016) similarly documented a negative correlation between *Prevotella* and milk yield.

The relative abundance of Bacteroidetes phylum increased significantly as animals were fed more non-fibrous carbohydrates. Being starch-degrading bacteria, upon the breakdown of non-fibrous carbohydrate, propionate/ propionic acid is produced (Drackley *et al.,* 2001;

Kononoff & AJ Heinrichs, 2003). There exists a positive correlation between Bacteroidetes and propionate proportion as recently documented by Guo *et al.,* (2020). Furthermore, propionate correlated positively with milk production (Xue *et al.,* 2018). These positive correlations between Bacteroidetes and dietary nutrients, and between the product of starch decomposition with milk yield, provide a plausible reason for the positive relationship between this phylum and milk yield.

The correlation network showed milk fat to be positively correlated with *BF311, Bacteroides,* Fibrobacteres, *Fibrobacter,* Cyanobacteria, Elusimicrobia, *CF31,* and Tenericutes. Previous studies have reported some of these microbes to be associated with milk fat. Indugu *et al.,* (2017) reported *BF311* and members of *Bacteroides* to be positively correlated with milk fat. Xue *et al.,* (2018) also reported a positive relationship between the abundance of genus *CF31* and milk fat. Additionally, Proteobacteria phylum and *Prevotella* genus displayed a weak negative correlation as reported by Jami *et al.,* (2014) and Indugu *et al.,* (2017). However, contrary to the current study findings, Jami *et al.,* (2014) reported a positive correlation between Actinobacteria, Proteobacteria, and Firmicutes to milk fat.

The findings from this study that Fibrobacteres, one of the important fibrolytic bacteria in the bovine rumen, has a strong positive correlation with cellulose content in the diets. Similar to previous studies by Pitta *et al.,* (2014a); Pitta *et al.,* (2014b) and Pitta *et al.,* (2014c),this study showed that, high percentages of forage and relatively high NDF content was associated with the relative abundance of Fibrobacteres. This was clearly demonstrated by the relative abundance of this phylum decreasing with an increase in concentrate level inclusions as shown in Chapter Three. As reported by McCartney and Vaage, (1994) and Ruckebusch & Thivend, (2012), members of this phylum degrade fiber, producing acetate as the end product. Zhang *et al.*,(2017) were able to correlate positively the acetate proportions to *Fibrobacter* ($r > 0.62$, $q < 0.01$), and a higher proportion of acetate was reported to impact

positively milk fat content (Xue *et al.,* 2018) as acetate is a precursor to milk fat (Lima *et al.,* 2011). In this study, milk fat was reported to be highest in diet 1 which contained a high forage to concentrate level. This correlation, therefore, confirms the positive relationship between milk fat and this phylum as well as with the genus belonging to this phylum.

Despite genus *BF311* having a significant positive correlation with milk fat, knowledge about the feed type that the members of this genus utilizes and the product they produce is not clear. However, Bi *et al.,* (2018) suggested an existing correlation in the metabolic ability to metabolize short-chain fatty acids between this genus and *Prevotella,* which was unlike findings from this study as those two genera never displayed a similar correlation toward any of the milk yield and composition parameters.

Interestingly, milk lactose, protein, and SNF shared many similar correlations with the bacterial taxa. However, protein reported the highest number when compared to the components. Among the shared taxa with positive correlation are *Prevotella,* Lentispaerae, Synergistetes, *Desulfovibrio, clostridium Butyrivibrio,* SR1*,* chloroflexi, Verrucomicrobia, and *Succiniclasticum.* Jami *et al.,* (2014) reported a similar association between genus *Desulfovibrio,* belonging to phylum Proteobacteria, *Succiniclasticum,* and *Clostridium* with milk protein and lactose yield. In regards to milk protein, the study by Jami *et al.,* (2014) and Xue *et al.,* (2018) did not positively correlate its content with members of *Prevotella.* Similarly, *Prevotella* had a negative correlation with milk lactose, (Jami *et al.,* 2014), unlike findings from this study.

Genus *Prevotella* has been reported several times to be a dominant group in phylum Bacteroidetes. Members of this genus were positively associated with milk lactose, SNF, and significantly with milk protein. *Prevotella* is a non-cellulosic, and proteolytic microbe which is capable of utilizing easily fermentable carbohydrate (simple sugars like hemicellulose) and protein to produce succinate, a propionate precursor as the most significant end result of

fermentation (Attwood & Reilly, 1995; Purushe *et al.,* 2010; Zootechnie, 1996), as well as acetate (Stevenson & Weimer, 2007). Previous studies reported positive correlation between the abundance of genus *Prevotella* with butyrate, acetate, and NH_3-N concentrates (Bi et al., 2018; Chiquette & Allison, 2008). Furthermore, the amount of butyrate was positively associated with milk protein content in a study by Xue *et al.*, (2018). It is noteworthy that as genus *Prevotella* increased when the cows were fed on diets 1 to 4, milk protein, albeit non-significantly, also increased. This confirms the positive and significant association of this genus with milk protein.

Members of Proteobacteria have been associated with carbohydrate fermentation resulting in succinate, (Sun *et al.,* 2016; Wallace *et al.,* 2015), precisely those belonging to the family Succinivibrionaceae that is *Succinivibrio, Ruminobacter* (Xue *et al.,* 2018). This study, nevertheless, identified only *Ruminobacter* which was positively correlated with averages of milk protein, lactose, and SNF, however, the correlation was weak with milk lactose. A positive correlation between this genus and milk protein was reported by Indugu *et al.*,(2017). *Desulfovibrio,* the most dominant genus in this phylum had a positive correlation with milk lactose, protein, and SNF. Members of this phylum were reported to be more in high grain diets (Fernando, Purvis, *et al.*, 2010) as well as the amount of these three milk parameters. One plausible mechanism for the positive correlation between members of this phylum and the above milk averages could be due to their ability to convert succinate to propionate which is further metabolized in the liver during gluconeogenesis to produce glucose, which is a precursor to milk lactose (Liu *et al.*, 2013), and in absentia of enough oxygen, lactate, which was attributed with high yield milk protein (Xue *et al.*, 2019).

As illustrated by the heatmap analysis results, many genera in phylum Firmicutes were negatively correlated with milk production and milk fat. Members of this phylum are fibrolytic and therefore their abundance reduces as the concentrate ratio increase, as

presented in Chapter Three. This is supported by the strong positive correlation between Firmicutes and cellulose (r=0.960, P≤0.05). This phylum has been positively correlated with butyrate proportions (Guo *et al.,* 2020). And a substantial amount of butyrate is an indication of a higher rate of fiber fermentation (Bi *et al.,* 2018), justifying the positive correlation between five out of seven Firmicutes genera with milk protein, SNF, and at least three genera with milk lactose.

In line with the study by Indugu *et al.,* (2017), *Coprococcus*, belonging to the Firmicutes phylum had a positive correlation with milk protein. However, the study obtained a positive between the genus and milk fat which contrasted the findings of this study. Its positive correlation with milk protein could be due to the known functionality of this genus with productions of butyrate and propionate (Shabat *et al.,* 2016). *Butyrivibrio* and *Pseudobutyrivibrio* are fibrolytic and amylolytic bacteria that possess the ability to convert fiber and starch into utilizable nutrient, butyrate or butyric acid (Fernando, Purvis, et al., 2010; Goad, 1998). The abundance of *Butyrivibrio* has been positively correlated with butyrate concentration by Bi *et al.*,(2018) and Freetly *et al.,* (2020). As part of this analysis, the proportion of *Butyrivibrio* and *Pseudobutyrivibrio* positively correlated with milk protein and as their abundance increased with increase in the level of inclusion concentrate in the experimental diets, the amount of milk lactose, protein and SNF also increased insignificantly. However, *Pseudobutyrivibrio* had a strong correlation with milk protein compared to *Butyrivibrio*. The positive correlation reported between correlated butyrate proportion with milk protein content (Xue *et al.,* 2018), was evident in the current study.

Succiniclasticum genus is specialized in fermenting succinate through succinate decarboxylation process to produce propionate (Fernando, Purvis, *et al*., 2010; McCabe *et al.*, 2015; Pope *et al*., 2011; Shabat *et al*., 2016; Van Gylswyk *et al*., 1997). This genus was the most abundant in Firmicutes phylum as reported in this study and its abundance increased as

high concentrate level were fed to the animals, this collaborate what is reported by Li, *et al.,* (2012), Petri *et al.,* (2013a), and Zhang *et al.,* (2017). This genus had a positive correlation with milk lactose, protein, and SNF, but a moderate-higher correlation with milk lactose as stated by Jami *et al.,* (2014). Members of this genus were found to be consistent with the amount of propionate (Li, *et al.,* 2012). This propionate further is metabolized in the liver during gluconeogenesis to produce glucose which is supplied for lactogenesis (Liu *et al.,* 2013; Xue *et al.,* 2019).

Clostridium a member of Firmicutes phylum, also showed a positive correlation with milk SNF, lactose, and milk protein contents. According to Sirohi *et al.*,(2012), members of genus *Clostridium* are cellulolytic, amylolytic, and proteolytic bacteria and their main role are to produce acetate and butyrate. The current study findings are evidenced by the positive association of butyrate with milk protein yield (Xue *et al.,* 2018). The *Ruminococcus* genus was only positively correlated with milk SNF. Members of this genus were reported to possess the capability to convert fibrous plant materials into acetate, succinate among others (Henderson, *et al.,* 2015; Neubauer *et al.,* 2018). However, a lot remains unclear about the milk SNF and which metabolite positively correlates with its amount.

4.6 Conclusion

The findings of the study indicated that with an increase in concentrate level from 10% to 40%, milk fat slightly decreased while milk lactose, protein, and solids not fat, production displayed an insignificant increasing trend. Correlation analysis also revealed an existing potential utilization relationship or productive association between dietary nutrients, affected bacterial groups, and milk composition constituents as illustrated by the correlation network.

CHAPTER FIVE: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

The composition, structure, and function of the cattle rumen microbial community are inexpressibly important to the health and nutrition of the host, as these microbes are responsible for breaking down fibrous plant materials and converting them into energy substrates which can be used by the host. As research continues to illustrate the effects of specific ruminal microbe shifts or their abundance on ruminant productivity, rumen microbial composition continues to appear complex. Due to this, and its unclear relationship to diet in crossbred animals reared in the tropics, it has increasingly attracted research attention. Therefore, the primary objectives of this study were to; (i) evaluate the rumen bacterial community and its stability when the animals were subjected to four different diets and (ii) assess the relationship between rumen microbes and milk composition profiles of dairy cattle fed on four different diets.

5.1.1 Effects of diet on Rumen bacterial microbiome

The findings of objective 1 identified a total of 26 bacterial phyla, 65 classes, 103 orders, 156 families, and 273 genera. The overall rumen bacterial diversity as estimated by alpha diversity indexes suggested the total number of OTUs reduce as the diet shifted from fiber-based to concentrate-based. More diversity was seen in diet 1 which had a higher proportion of forage than concentrate. This findings were in agreement with a recent study in bovine animals (Wang et al., 2020).

As the dietary concentrate level increased across the diet, the predominance of Bacteroidetes and Firmicutes remain unaffected. Even though current results suggested a core group to be dominating across all the diets, the relative abundance of those taxa changed, some

significantly. Phylum Bacteroidetes increased significantly as concentrate level shifted from 10% to 40%, in agreement with Bi *et al.,* (2018). The relative abundance of Proteobacteria also increased while Firmicutes and Fibrobacter decreased, insignificantly as in concordance with Jami and Mizrahi, (2012) and Kala *et al.,* (2017).

At the genera level, dietary changes caused several OTUs to vary significantly. An overall evaluation was not conducted on all genera, only the ones in the four bacterial phyla that this study focused on. *Prevotella* dominated at the Bacteroidetes phylum. Several studies have reported the predominance of this genus (Wang *et al.,* 2020; Zhang *et al.,* 2020), further suggesting the dominancy of this genus, *Prevotella* in starch-based diets. Feeding animals with high forage to concentrate ratio resulted in a higher abundance of fibrolytic bacteria in dairy (Fernando *et al.,* 2010; Zhang *et al.,* 2017). In accordance with these findings, this study observed a higher abundance of *Fibrobacter* in Fibrobacteres phylum and *Succiniclasticum,* belonging to Firmicutes phylum in diet 1. Despite Proteobacteria phylum showing a lower abundance than documented by Zhu, (2016), this phylum had the highest number of genera (99) compared to the other three phyla and was dominated by genus *Desulfovibrio*. Analysis of data obtained in this study did not identify any diet-specific core bacteria group as the Bacteroidetes and Firmicutes dominated across the diet regardless of different forage to concentrate inclusion levels unlike (Zhu, 2016). This discrepancy could be a result of using different dietary composition and proportions in the present study and the studies in literature.

In agreement with the initial expectation of the rumen bacterial community attaining stability by day 10, except in the case of host perturbations, the bacterial community did not show significant shifts over the course of two samplings days. This finding is comparable to Fernando *et al.,* (2010), where the rumen microbial community was stated to be stable by day 7 following dietary change. This study, therefore, supports the assertion that diet is a key

factor governing the day-to-day variations in the diversity and dynamic shift of the dairy rumen bacterial community.

5.1.2 The correlation between diet, rumen microbes, and milk quality

Diet, an important rumen diversity driver, has become a primary link to connect the rumen microbes with the host phenotype. This study evaluated the correlation between dietary ingredients and the bacterial phyla reported in objective 1, which showed a strong positive and significant correlation. In line with the findings from previous studies by Bauman and Griinari, (2003), Gabbi *et al.*, (2013), Aguerre *et al.*, (2011), and Neveu *et al*., (2013), milk parameters were affected by the diet given to the animal. Even though the impact was insignificant, there was a decreasing trend with milk fat and an increase with milk lactose, protein, and production, as concentrate amount increased from 10% to 40%.

Further spearman correlation analysis between the bacterial phyla and milk composition parameter revealed there exists a relationship between bacterial community and host phenotype. A positive correlation was observed between Bacteroidetes and milk production. This was evidenced by the correlation reported between the phylum with the amount of propionate (Guo *et al.,* 2020) and the correlation between the amount of propionate with milk yield (Xue *et al.,* 2018). Correlation analysis also displayed an association with specific milk parameters, that is, milk fat, lactose, and milk SNF percentage. The correlations between bacterial taxa and these milk-related traits were evidenced by the reported relationship between them with volatile fatty acids and between fatty acids and the specific milk component. These correlations are indicative of the existing potential association between milk composition parameters and rumen bacterial taxa.

5.2 Conclusion

Dietary modifications significantly affected the composition of the rumen bacterial community and diversity. Precisely, an increase in concentrate ratio across the diets led to an increase in the abundance of Bacteroidetes and Proteobacteria while proportions of Firmicutes and Fibrobacteres reduced. Further, the existing associations between the rumen microbiota and host production traits revealed that there were some utilization and productive association between dietary nutrients, affected bacterial groups, and milk composition constituents.

5.3 Recommendation

1. Using the existing information on correlation from this study and actualizing it make probiotics that farmers can use to improve productivity and thus sustainable productivity.
2. A need for further research of genera with low abundance as well as the unclassified OTU groups that were not considered or missed in correlation analysis using NGS approaches, this may aid in understanding better the rumen ecosystem and improving host productivity.
3. It is more informative if an interdisciplinary approach integrating nutrition, metagenomics, metabolomics, and transcriptomics are utilized to analyze the functional impart of the ruminal microbial community to host physiological parameters. This could aid in developing a potential and manipulative strategies that farmers can use to improve productivity.

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APPENDIX

Appendix Table 1; Diet ingredients and amounts for 90 days feeding trial of crossbred dairy cattle.

	Morning amount (kg)	Evening amount (kg)	Totals(kg)
Acclimatization period of 10 days per animal			
Rhodes hay	3.68	3.68	7.36
Lucerne	2.45	2.45	4.90
Concentrates	0.00	0.00	0.00
Totals	**6.13**	**6.13**	**12.26**
Diet 1 for 20 days per animal			
Rhodes hay	3.68	3.68	7.36
Lucerne	1.84	1.84	3.68
Concentrates	0.61	0.61	1.22
Totals	**6.13**	**6.13**	**12.26**
Diet 2 for 20 days per animal			
Rhodes hay	3.68	3.68	7.36
Lucerne	1.22	1.22	2.44
Concentrates	1.23	1.23	2.46
Totals	**6.13**	**6.13**	**12.26**
Diet 3 for 20 days per animal			
Rhodes hay	3.68	3.68	7.36
Lucerne	0.61	0.61	1.22
Concentrates	1.84	1.84	3.68
Totals	**6.13**	**6.13**	**12.26**
Diet 4 for 20 days per animal			
Rhodes hay	3.68	3.68	7.36
Lucerne	0 .00	0.00	0.00
Concentrates	2.45	2.45	4.90
Totals	**6.13**	**6.13**	**12.26**