ANALYSIS OF THE GUT MICROBIOME SHEDS INSIGHTS INTO BREED RESILIENCE AND THE CHALLENGES OF ANTIMICROBIAL RESISTANCE IN DOHNE MERINO SHEEP

by

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PREFACE

The research contained in this dissertation was completed by the candidate from May 2021 to November 2023 while based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by the National Research Foundation (NRF) and the Agricultural Research Council.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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DECLARATION OF PLAGIARISM

I, Andiswa Mgaga, declare that

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ABSTRACT

This study focused on analysing the gut microbiome of Dohne Merino, one of South Africa's leading sheep breeds, that is also reared in Australia, New Zealand, and other European countries. Dohne Merino is of high economic importance in South Africa as it maintains livelihoods in many small rural communities. In South Africa, sheep and other livestock are exposed to multiple diseases and parasites. The efforts to manage these diseases and infections while keeping high productivity have led to an increased usage of antimicrobials in agriculture. This has resulted in high prevalence of antimicrobial resistance (AMR), that is a primary global concern demanding surveillance and action. Some sheep breeds and populations are known to be resilient to diseases, harsh production, and environmental conditions and have low AMR prevalence, which could be due to the defense provided by their gut microbiome. Studying the gut microbiome is essential because the gut microbiome contributes to animal nutrition and health. The study investigated the gut microbial environment of South African Dohne merino sheep. Metagenomic sequencing of the rumen, reticulum, omasum, and abomasum of six mature Dohne Merino ewes that were raised on open pasture was performed. Illumina HiSeq2500 was used to generate a total of 15million reads of 150bp illumina base pairs. The members of the microbial population were fully characterised, and the resistome of the gut was analysed. The microbial population was studied at phylum, class, order, genus, and species level, with bacteria being the most abundant and diverse domain. Other present domains included archaea and viruses. Majority of the microbial population was Bacteroides (53,9%) and Firmicutes (25,4%). Two illness-related bacterial phyla were also identified, Actinobacteria and Cyanobacteria. Identified archaea belonged to the phyla Candidatus Asgardarchaeota, Candidatus Thermoplasmatota, and Euryarchaeota. The observed viral population was very diverse with a total of 5 realms, 8 kingdoms,13 phyla, and 21 classes. Many of the viruses were dsDNA phages belonging to the Duplodnaviria realm. Disease-linked RNA viruses belonging to the Riboviria realm were also observed. Riboviria members have been associated with diseases such as influenzae, rabies and the corona virus. The relationships between microbiome composition and AMR prevalence across the four gut compartments were assessed. A total of 12 AMR genes were identified in the gut and were found to confer resistance to 15 antimicrobials. The observed antimicrobial resistance profiles were low compared to those reported for other breeds and species implying breed resilience of the Dohne Merino sheep. The analysis of the resistance profiles within the compartments revealed that, the higher the diversity, the lower the prevalence of AMR. The overall high diversity of the gut microbiome is the probable cause of breed resilience in Dohne Merino sheep. Additionally, the

observed AMR high prevalence with no clinical symptoms of AMR in the animals is also indicative of breed resilience. More studies that will investigate the relationships between the gut microbiome and AMR prevalence are required and the knowledge generated can then be applied to overcome the challenges of AMR in livestock species.

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1 GENERAL INTRODUCTION

1.1 Rationale

Ruminants are of particular interest, as they are major global sources of milk and meat (Pulina et al., 2017).Sheep, as members of the ruminant family, are valuable to farmers in many countries, including South Africa (Mahashi et al., 2019). They supply wool, meat, sheep's skin (used to make clothing, shoes, and rugs), milk, and items made from it, such as cheese and butter (Kilgour et al., 2008, Doyle et al., 2021). South Africa is known for its production of apparel wool. The wool produced in Namibia and Lesotho, two neighboring republics, has historically been soldin South Africa and was previously regarded as a component of South African production (Mahashi et al., 2019). In many small rural communities in South Africa, sheep are the source of livelihood maintenance. (Schoeman et al., 2010). However, small-scale sheep farmers in South African rural areas often face various challenges, such as limited access to veterinaryservices, unavailability of clean water for sheep to drink and suitable vegetation to feed sheep. Animals in such deprived environments often graze on open pastures and drink polluted water; this exposes them to both beneficial and diseases-causing microbes (Oduniyi et al., 2020, Mbatha, 2021). Which leads to additional challenges of diseases, parasitism and antimicrobial resistance(AMR) which are challenges common problems in many livestock species (Clifford et al., 2018, Tomley and Shirley, 2009) Microorganisms (including parasitic and pathogenicmicrobes) and AMR genes are often found in the gut microbiome of livestock. The gastrointestinal tract of livestock is home to various microbes both advantageous and detrimental to the health of the livestock host, as a collective these microbes are referred to as the gut microbiome (Nicholson et al., 2005) (Nicholson et al., 2005). The gut microbiome is a vital element in ruminant livestock research. A balanced microbiome has been found to benefit livestock in many ways such as improved digestion, immune system development and growth amongst other things. However, an overgrowth or imbalance in the gut microbiome can impact livestock health negatively (Round and Mazmanian, 2009).

Gut microbiomes differ across various host species; thus, studies focusing on specific host microbiotas are crucial. The microbiota can also vary significantly depending on the genetic makeup, diet, host age, sex, season, and geographic region (Tanca et al., 2017). Important issues for ruminant research include our knowledge and comprehension of the gut microbial populations, their interactions with one another, and their hos. as it has influences on health, immune response, behavior, and stress in farm animals therefore the gut microbiome a good indicator of animal welfare (Chen et al., 2021a).

The most understood component of the ruminant gut microbiome is the rumen microbiome. The rumen is the first of four compartments that feed passes through after ingestion. At a temperature between of 37–39 °C and pH of between 5.5 and 7.0, coordinated contractions mix the rumen content. (Öztürk and Gursel, 2021). Apart from these physiological features that aid fermentation in the rumen, the rumen is home to a dynamic and intricate ecology made up of phages, anaerobic bacteria, protozoa, anaerobic fungus, and methanogenic archaea (Huws et al., 2018). These rumen microbe's main function includes breaking down cellulose from plant cell walls, digesting complicated starch, producing volatile fatty acids, which are a significant source of energy for the host, and synthesizing protein, B vitamins and vitamin K (Medjekal and Ghadbane, 2021). Currently, little is knownon the microbial populations colonising gut of small ruminant such sheep and goats (Zhang etal., 2021). Studies examining the complete gut microbial makeup and diversity of small ruminants are essential due to their significance in the global food system. In the many cattle focused studies done in the past Firmicutes and Bacteroidetes are the dominantbacterial phyla in the gut. Firmicutes and Bacteroidetes are then typically succeeded in abundance by Proteobacteria, Tenericutes, and Actinobacteria. This is then followed by low abundances of Archaea and varying abundances of viruses. Euryarchaeota is the most prevalent archaeal phylum in ruminants (Zhu et al., 2021). When it comes to viruses, comparatively speaking, much less research has been done to understand viral populations than other microbial populations. However, they might play a major role in preserving the equilibrium of the microbial population, intra-ruminal microbial lysis, fiber breakdown, nutrition cycling, and genetic transmission (Gilbert et al., 2020). All in all it is important to have an understanding of the full microbiome complement because these co-exist and impact the host collectively, additionally species and breed specific studies are crucial as microbiomes differ in different breeds, species, ages, sex and are affected by environmental conditions, diet, medication usage, etc (Clemmons et al., 2019, Cholewińska et al., 2021)

This study is going to make use of next generation sequencing and bioinformatics to explore the gut microbiome of Dohne Merino sheep. Dohne Merino is a South African (S.A) native sheep breed that was developed in the 1940s. This breed was developed from S.A merino and the S.A Mutton merino (formerly known as the German Merino (De Klerk and Heydenrych, 1990). Today the Dohne Merino is one of the top breeds in the sheep farming industry in South Africa and is also reared in Australia, New Zealand, and other European countries (Van der Merwe et al., 2020). Like most sheep breeds, it is a good investment because it adapts well to harsh conditions, has high productivity, good fertility, and short generation interval (Schoeman et al., 2010)

In S.A, many rural communities rely on sheep for their existence and livelihood maintenance. Without their sheep products and animal trading, they would cease to exist (Schoeman et al., 2010). Sheep contribute products like meat (mutton and lamb), wool and sometimes milk to the market. Many meat products are consumed locally, while the bulk of the wool is exported (Davies et al., 2019). These contributions by sheep in general are very significant to the country's (S.A) economy, and they are even higher for dual-purpose sheep like the Dohne Merino breed which is bred for both meat and wool (Van der Merwe et al., 2020). Understanding the complete microbiome of Dohne Merino sheep could prove to be of great benefit in the continuous improvement of the breed and the sheep farming industry as whole. In addition, the study could yield insights that can be applied in other species like cattle.

1.2 Problem statement

Ruminants have four different stomach compartments (rumen, reticulum, omasum and abomasum) with unique "environmental" conditions. However, of the four compartments, only the rumen has received much attention in studies (Creevey et al., 2014, Gilbert et al., 2020a, Russell, 2002).This has led to the understanding that the rumen is themost numerous and diversly populated of the four chambers. That understanding could perhapsbe biased as the other compartments have not been well studied. The current study will performa comparative analysis of the microbial compositions of the four compartments of Dohne Merino sheep from the Eastern Cape province in South Africa, this is expected reveal great diversity even in the other stomach compartments. Sheep production is the most practiced livestock operation in the Eastern Cape province and increasing livestock production while achieving sustainable improvement is a goal of many livestock farmers in the area (Ngqulana and Obi, 2019). But the main obstacles to the growth of sheep productivity and output include issues like illness, a lack of feed, subpar housing, a lack of veterinary support services, and a restricted amount of grazing land. (Bettencourt et al., 2013). A lack of knowledge in the gut microbiome of sheep means that our perspective of the impact that these challenges have on sheep livestock might be narrow. A study of the gut microbiome of sheep in the Eastern Cape is expected to reveal how the issues faced by the farmers are affecting the sheep farming industry and if the gut microbiome can be manipulated to improve livestock production not only in sheep but in other livestock species as well.

Previous studies have found a correlation between the microbial population and antimicrobial resistance genes (ARG) in the environment(Strange et al., 2021). This a particularly a critical area of research because antimicrobial resistance (AMR) is a major global concern that is highly associated with livestock agricultural (Clifford et al., 2018). The World Health Organization (WHO) reports that antibiotics are increasingly becoming ineffective against treatment of diseases and infections as AMR spreads globally, which means we are slowly approaching an era with no effective antibiotics (WHO, 2021). In order to define the extent of the resistance issue, provide interventions that enhance the proper administration of antimicrobial drugs, and lessen the selection pressure for resistance, it is imperative to collect AMR surveillance data (Fuhrmeister and Jones, 2019). This study is expected to greatly contribute towards the AMR surveillance global action plan. Phage therapy holds great potential as an alternative to antibiotics in the efforts of overcoming the AMR challenge (Giri, 2021). For those reasons, it is empirical that gut microbiome (including virome) of ruminants be studied and well understood. This is expected to yield insights that could reduce the need for heavy usage of antimicrobials in livestock which will contribute towards reducing the prevalence of AMR.

1.3 Justification

The chosen farm for sampling in this study, the Wauldby farm was part of the action group that launched the Dohne Merino Breed Society (DMBS) in 1966. This farm has faced tremendous challenges of exceptionally heavy loads of roundworms (Haemonchus contortus). This was combated by dosing their sheep flock approximately every six weeks, however this led to an even bigger challenge of the wireworm to developing resistance to the anthelmintics being used. The Wauldby sheep gradually also started to acquire an inbuilt resistance and resilience to roundworms. Despite having roundworms in their bodies and roundworm eggs in their feces, the sheep do not exhibit any symptoms. (Burgess, 2016). It can be expected that the gut microbiome ofWauldby Dohne Merino is playing a key role in this observed resistance and resilience as roundworms grow inside the gut as well. Additionally, despite being the exposed to pathogensand parasites in the grazing field, the Wauldby Dohne Merino show no symptoms of AMR. This study of the gut microbiome of Wauldby Dohne Merino sheep is anticipated to yield greatinsights that could be useful to other farms that are challenged with parasitism as well.

Technological developments in sequencing and computational biology have significantly expanded our capacity to investigate the functional and taxonomic makeup of microbial communities, including viruses (Van den Bogert et al., 2019). Large volumes of biological data are analyzed and explored by bioinformatics tools, which also assist in the storing, organizing, systematizing, annotating, visualizing, querying, mining, understanding, and interpreting of complicated data volumes (Sharma et al., 2020).

The study of bulk samples' entire genetic makeup is known as metagenomics. Using metagenomics, direct sequencing of samples is performed without the need to cultivate a pure culture of organisms in the laboratory. Metagenomics provides the ability to study genes of organisms that were previously uncultivable and those that are challenging to grow in a lab. This is advantageous for microbial studies especially gut microbial studies as numerous members of the community cannot be cultivatedoutside of the gut, while other are found in very minute prevalences such that they can easily be missed. The DNA sequences of viruses, bacteria, fungus, and other microbes have been studied using metagenomics. This method has made it possible to identify novel microbes, investigate the evolutionary history of previously identified bacteria, and comprehend the role of microbial genes. Understanding microbial ecology—the roles that microorganisms play in their surroundings—through the use of metagenomics data can help create novel treatments for infectious diseases, improve food production, and protect the environment. These factors led to the determination that the metagenomics and bioinformaticsapproach was the most appropriate strategy for the planned investigation.

1.4 Aim and Objectives

Aim: This study aims to investigate and fully characterise the gut microbial environment of Dohne Merino sheep.

Objectives

- Characterise the microbial composition of the gut (four gut compartments) of Dohne Merino sheep.
- Analyse and classify the gut virome of Dohne Merino sheep.
- Assess the gut resistome of Dohne merino sheep through identification antimicrobial resistance genes (ARG).

1.5 Dissertation outline

This dissertation uses the metagenomics approach to analyse the gut microbiome of small ruminants Dohne Merino sheep from Eastern Cape, South Africa. Chapter one is an introductory chapter that provides background information regarding the research question, problem statement, justification (for the used methods, breed, and population study) and hypotheses. Chapter two highlights relevant literature including the role gut microbiome in ruminant livestock, currently known members of the gut microbial community of ruminants and the relevance of the gut microbiome to the antimicrobial resistance. Chapter three explores the diversity of the gut microbiome by analysing and comparing the microbial communities found in the four gut compartments of Dohne Merino sheep. Chapter four specifically looks at the viral population and its significance in the gut of sheep. Chapter five analyses the antimicrobial resistance genes present in the gut. Chapter six is the concluding chapter which discusses the overall results and recommendations for future studies.

2 LITERATURE REVIEW

2.1 Abstract

The gastrointestinal tract of ruminants is home to a large number and variety of microbes. These microbes are of crucial importance to the host's productivity and health. They play essential roles in biological processes like digestion and the immune system development. Thegut of ruminants consists of different components or compartments, with the major ones beingfour fermentation chambers, small intestines, and large intestines. The four fermentation chambers (rumen, reticulum, omasum and abomasum) are where most of the digestion work occurs and where most of the microbes are found. Microbes that have been found to inhabit the gut include bacteria, archaea, fungi, protozoa and viruses, with bacteria being the most abundant. The population dynamics of the microbes differs in each of the four fermentation chambers according to the conditions in the chamber. The rumen is considered the most biologically important of the four compartments. This compartment has received much attention from studies, and therefore its microbial population composition is the most understood of the four. For the host to maintain its' gains from the microbiome, there needs tobe a balance and stability in the microbial population. Though the impact of the gut microbiomeis mostly beneficial on the host, there are some concerns, especially regarding microbes harbouring antimicrobial resistance genes. Therefore, strategies for maintaining and possibly manipulating the gut microbiome as desired are important. The development of these strategiesstarts with a complete understanding of the entire gut microbiome.

2.2 Introduction

Formerly all microorganisms were considered as pathogens that cause infectious diseases to the host; however, with science advancement and microbial studies we have come to know that association between eukaryotic hosts and their microorganisms is far more complex and has essential biological functions. There are various forms of association between the host and its microbiome such as symbiotic and pathogenic associations (Sweet and Bulling, 2017). In ruminants, the symbiotic associations between the microorganisms in the gut and their host has allowed for ruminants to evolve the ability to digest fibrous feed that other mammals are unable to digest (Castillo-González et al., 2014). This means that these microbes are of crucial importance as the ruminant diet is plant-based. Improved health, production performance, feed and drug metabolism, immune system development and resistance to pathogen colonization are other benefits that ruminants gain from the microbes their gut microbiome (Cholewińska et al., 2020). The gut microbiome of ruminants has also been identified in other research as a critical factor in the development of mucosal and lymphoid structure, intestinal homeostasis maintenance, and host immune cell repertoire activation (O'Hara et al., 2020).

Livestock is positioned at the interface of the world's natural and human systems, according to the Food and Agriculture Organization of the United Nations (FAO). This idea has served as the foundation for the Sustainable Development Goals (SDGs) Global Agenda. Though all SDGs are seen as important and relevant to livestock, 6 out of 17 are significantly of higher relevance: SDG 1 (no poverty), SDG 2 (zero hunger), prioritized SDG 12 (responsible consumption and production), SDG 13 (climate action), SDG 15 (life on land) and SDG 17 (partnership for the goals). (FAO, n.d). Ruminant animals are the biggest contributors to the livestock sector, and they play very important roles in the world's economy and global food security. Many countries, including S.A, heavily rely on ruminants for livelihood maintenance (Thamaga-Chitja and Morojele, 2014). Without these animals, the economy and populations of many countries would suffer great consequences (Oltjen and Beckett, 1996). Given the importance of livestock in global economy and food security, efforts for improved and sustainable animal production are crucial, especially if we are to overcome two of the most significant current global challenges: increased antimicrobial resistance microbes and feeding the continuously growing human population (Alexander and Plaizier, 2016).

The diverse microbial community that lives in the ruminant gastrointestinal tract, known as the gut microbiome, is essential to the health and productivity of these animals. (O'Hara et al., 2020a). Novel and cost-effective strategies for balance conservation and modulation of the microbiomeare required as the gut microbiome must be diverse yet balanced and stable so that the gut canfunction efficiently and the host ruminant be healthy and fit (Belanche et al., 2020). Understanding the microbiota of ruminants and possibly manipulating it presents critical areas of exploration in science that can be valuable in reducing AMR and improving the overall health and production of the ruminants, which will contribute towards the SDGs. Through research, it has become apparent that the host animal and its resident gut microbiome have intricate and critical relations (Chen et al., 2021b). For the past few decades, research has been conducted on these microbial communities in an effort to increase the efficiency of cattle production while lowering or eliminating its negative effects. (Bahrndorff et al., 2016). These fundamental studies also help to understand, and therefore avoid, nutritional disorders. Though much research has been done towards understanding the gut microbiome of research, Presently, limited information exists regarding the microbial communities inhabiting the tiny ruminant's gut. (Zhang et al., 2021).

2.2.1 Physiology of the ruminant gut

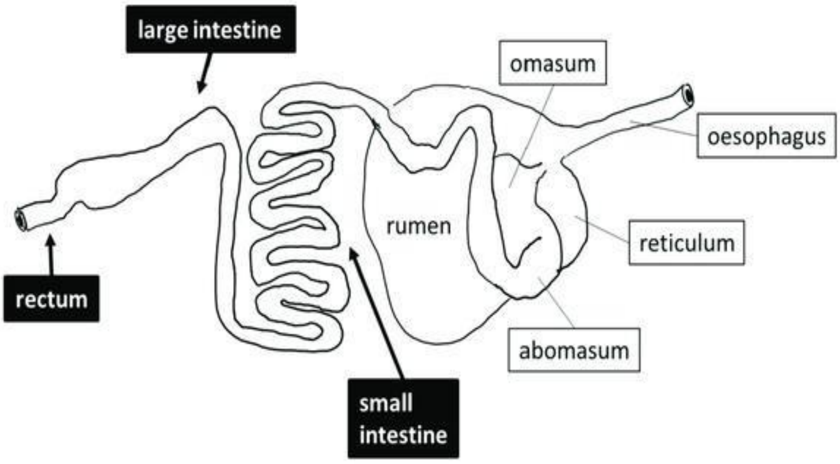


Figure 2.1 Schematic drawing of the gastrointestinal tract of ruminants (Al-Masaudi, Saad, et al. 2017:66).

The ruminants are different from other mammals in that their food passes through the fermentation chambers before it reaches the "true stomach", where normal digestion occurs in mammals (Liu et al., 2020). Four fermentation chambers or compartments (rumen, reticulum, omasum, and abomasum) make up the normal ruminant gastrointestinal system, which is then followed by the small and large intestines (Figure 2.1). These four fermentation chambers serve the purpose of breaking down and fermenting roughage (Al-Masaudi et al., 2017). The largest stomach compartment, the rumen, is located on the animal's left side and is made up of many sacs. The rumen stores feed because of its large size (Steele et al., 2016). The reticulum is a pouch-like structure located near the heart in the anterior region of the body. The reticulum's tissues organize into a network resembling a honeycomb (Malmuthuge and Guan, 2016). The reticulum and rumen are separated by a little tissue fold, but they are not totally apart. Together they are sometimes referred to as a rumeno-reticulum (Steele et al., 2016). The omasum is a globe-shaped structure with tissue folds inside of it. Its primary function is absorption. Lastly, the abomasum, which resembles a non-ruminant stomach and is the only chamber lined with glands (Owens and Hanson, 1992).

2.3 Acquiring the microbiome

The major job of the digestive system, which is made up of several tubular organs and related glands, is to break down food particles that are swallowed so that the body can absorb and use them to stay alive (Swanson, 2019). However, as we have established, thisis not possible without the gut microbiome. The GIT is home to a diversity of millions of microbial cells. The colonisation begins from when the host is born (Chen et al., 2021). Within a few days of birth, the stomach becomes colonized by bacteria and methanogenic archaea, and between two and eight weeks of age, distinct anaerobic, fungal, and protozoal communities establish themselves (Liu et al., 2020). The greatest increase in microbial diversity occurs between 2and 4 weeks of age (Belanche et al., 2020). The immediate environment, especially the mother, and diet both have an impact on the early assembly of various microbial phylotypes in the gut. (Liu et al., 2020). Direct contact with the mother offers a constant source of microbes through the mouth,, skin and milk (Huws et al., 2018b). It was noted in research by Morgavi et al., (2015) that the isolated lamb groups' rumen microbiota composition differed significantly from that of lambs raised in close proximity to adult ruminants.

2.4 The ruminal ecosystem

The rumen is considered the most biologically important of the four compartments and has received much attention from many studies while the other compartments remain relatively underexplored (Alexander and Plaizier, 2016). Interest in the rumen microbiome's role in animal production has grown as a result of the rumen's involvement in microbial metabolism for the host's health (O'Hara et al., 2020a). As mentioned above, the rumen is thelargest compartment of the stomach; it is described as a "black box" due to the diverse microbesthat inhabit it (Guo et al., 2021). Its population consists of trillions of microbes, and its gene content is hundreds of times that of host cells (Morgavi et al., 2015). The environment in the rumen is well adapted for the maintenance of a large and diverse microbial population, there isa constant supply of food and water, the temperature is kept at about 39°C, and the optimum pH is 6-6.9 (Bryant, 1959, Common et al., 2014).

The ruminants depend on the microbiome composed of prokaryotic (bacteria, archaea and viruses) and eukaryotic (fungi and protozoa) microorganisms to ferment feed in the rumen (Yáñez-Ruiz et al., 2015). The main end-products of that fermentation are volatile fatty acids

(VFAs), proteins and vitamins. Animal performance depends on ruminally generated VFAs because they can supply up to 70% of the host's energy needs (McLoughlin et al.,2020). Also, keeping the microbial community diverse in the rumen is necessary for milk production and composition (Chang et al., 2020).

There is adequate evidence suggesting that the developing rumen in new-borns presents a window for sustainable manipulation of the gut microbiome, since manipulations of the rumen microbiome in mature animals is rather limited by high host specificity. Once established, the ruminal ecosystem becomes resilient in the mature rumen, such that the composition of the microbiome goes back to its pre-alteration state once treatment ceases (Yáñez-Ruiz et al., 2015). Therefore early actions have a more lasting impact than thoseoccurring later in life (Distel et al., 1994). Furthermore, while the rumen microbiome is formed prior to the intake of solid feeds, the kind of feed that is ingested has a major influence on the composition of the rumen microbiome. Therefore, the first stages of solid feed intake also fall within the window of opportunity to change the initial colonizers' composition (Belanche et al., 2020). According to Li et. al., (2012), the growing rumen of 14-day-old calves has a total of 170 bacterial species, and the microbiota is sensitive to both food changes and host physiological changes.

2.4.1 Rumen bacteria

The most prevalent and varied microorganisms in the rumen environment are the rumen bacteria (Xue et al., 2018b). Their wide range of enzymatic activity (such as lipases, cellulases, proteases, and amylases) allow them to break down proteins, lipids, starch, and plant cell walls. Most bacteria in the rumen are anaerobic or obligate anaerobic bacteria and therefore, can survive and strive in the rumen because there are minimal amounts of oxygen present in the rumen (Russell, 2002). Research on rumen bacteria has revealed that, independent of diet, the core bacterial community is composed of three primary phyla: Bacteroidetes, Firmicutes and Proteobacteria (Castillo- González et al., 2014). Bacteroidetes are mostly found in the rumen and progressively become less common in the other three lower stomach compartments. Firmicutes and Euryarchaeota gradually increase in this trend: rumen having the lowest, followed by the reticulum and lastly the omasum (Xue et al., 2018b). Examples of bacterial species that have been found in the rumen in different studies include *Fibrobacter succinogenes*, *Ruminococcus albus*, *Butyrivibriofibrisolvens*, *Ruminobacter amylophilus* and *Treponema saccharophilum* (Guilloteau et al., 2010, Hernáez et al., 2018, Miller and Jenesel, 1979, Wolin et al., 1997, Christopherson et al., 2014, Xie et al., 2018). Beyond just the taxonomic sorting, the rumen bacteria can be divided according to their functions, i.e. lactate degrading bacteria, cellulose-degrading bacteria, pectin degrading bacteria and proteolytic and lipolytic bacteria (Wanapat, 2000). The type of bacteria present or dominant in the rumen depends on factors like forage, pH, and temperature. For instance, for cellulolytic bacteria growth and maintenance, the ideal pH range is between 6 and 9, and anything lower than that will negatively impact the microorganisms that break down fibre, temperatures of 39°C. and above will affect bacteria’s ability to adhere to feed particles, meanwhile, the presence of extracellular cellulase enzymes allows the biopolymer's β-glycosidic linkages (1–4) to be broken, releasing sugars that microorganisms can use (Castillo-González et al., 2014).

2.4.2 Rumen protozoa

Protozoa were first microbes to be discovered in the rumen, and together with bacteria, they do most of the digestion work (Kamra, 2005). Butyrate and acetate are the byproducts of protozoan digestion, which produces hydrogen molecules that methanogenic archaea and bacteria then absorb and transform into methane (Williams et al., 2020). Some rumen ciliates scavenge oxygen entering the rumen, which benefits other microbes (Williams et al., 1997). The largest and most important protozoa are the ciliates. The two known ciliates are classified into the entodinomorphs and holotrichs, which belong to the subclass Trichostomatia(Newbold et al., 2015). In terms of classification into functional groups, protozoa can only be divided into two functional groups: cellulolytic and lactate degrading protozoa (Castillo- González et al., 2014). Elimination of protozoa (defaunation) has been recognized to have thefollowing impact: a drop in pH due to lack of pH stabilization by protozoa, increase of lactic acid and propionic acid in the rumen, decrease in Ammonia nitrogen and reduced methanogenesis (Kamra, 2005)

2.4.3 Rumen archaea

Many archaea are methanogens, and these archaea in the rumen use hydrogen, oxygen and carbon dioxide to produce methane (Li et al., 2012). Methanogens are present in the rumen in large numbers. The most commonly abundant phyla in the rumen is Euryarchaeota which is a diverse phylum that includes methanogens (Angelidaki et al., 2011). Genera that have been reported to be found in the rumen include *Methanobacterium formicicum, Methanobacterium bryanti, Methanonobrevibacter ruminantium, Methanonobrevibacter smithii,* *Methanomicrobium* mobile, *Methanosarcina barkeri* and *Methanocelleus Olentangyi* (Kamra, 2005). It has been noted that an increase in methanogen population does not always result in an increase in methane production since rumen conditions might influence the expression of genes involved in methane synthesis. (Russell, 2002). Methanogenic archaea contribute significantly to animal nutrition and rumen function, although making up a very minor portion of the rumen microbial biomass. Effective hydrogen removal by methanogens promote an accelerated rate of fermentation and a nutritionally more favorable pattern of volatile fatty acid production. (Janssen et al., 2008).

2.4.4 Ruminal fungi

Though they represent a small proportion of approximately 8% of the biomass in the ruminal ecosystem (Castillo-González et al., 2014), fungi are the largest of the rumen microbes, with the longest generation time and have a role in the digestion of food consumed by the ruminant (Tanca et al., 2017). The gut fungi differ in many characteristics from other known members of the kingdom; the most acknowledge and obvious distinction is the anaerobiosis. Gut fungi are the only fungi that are known to be anaerobic. In the gut, these fungi synthesize various hydrolytic enzymes, including cellulases, xylanases, mannases, esterases, glucosidases, and glucanases which all play different roles in the degradation of plant fibre (Ivarsson et al., 2016, Fliegerová, 2015). In vitro studies suggest that due to the broad range of enzymes produced by anaerobic fungi, the anaerobic fungi's contribution to plant material degradation could be more significant than that of cellulolytic bacteria (Hess et al., 2020). Due to their superior ability to pierce plant tissue compared to bacteria and protozoa, the rhizoids of vegetative thalli of fungi are able to access plant material that other rumen microbes cannot (Wubah and methods, 2004).

2.4.5 Rumen viruses

Viruses remain the least explored microorganisms in the gut. The role and the impact of gut viruses have been an area of interest in the past few decades. Still, inadequate studies have been done to provide significant data and information compared with the other microorganisms. Viruses from the order Caudovirales have been shown to dominate the rumen viral community and are, therefore, the most studied and understood (Gilbert et al., 2020b). These viruses are tailed bacteriophages; they grow and replicate through infecting bacteria. Bacteriophages are the most abundant form of life on earth. Rumen bacteriophages and archaeal viruses play an important role in modulating the population dynamics in the rumen. They keep the bacterial numbers below the carrying capacity and prevent blooms in the ecosystem through lysis

(Fernando, 2012). The final stage of bacteriophage replication, lysis, leads to the death of bacterial host cells, preventing dysbiosis, an imbalanced microbial community. Furthermore, lysis releases remnants of the microbial host cell, such as proteins and nucleic acids and pieces of the cell wall, which can subsequently be absorbed and used by other rumen microorganisms; this process is known as intra-ruminal recycling (Gilbert and Klieve, 2015). However, the biological reason for the lysis is the release of viral progeny particles replicated within the bacterial host cell, which can become detrimental to the ruminants' wellbeing if theyreplicate uncontrollably. Rumen viral populations have also been shown to have an impact due to their ability to function as mobile genetic elements (MGE). Viral material can be picked up and transferred between the microorganisms they infect and reproduce in this way. (Gilbert et al., 2020b). But the question that remains is: is the rumen viral population more beneficial or detrimental for the ruminant? More research on the role and functions of viruses in the gut needs to be performed to answer this question.

2.5 Beyond the rumen

Though the gut consists of four compartments, only the rumen is well understood. Many studies have employed various methods such as culture-based analysis, 16S rDNA sequencing and shotgun metagenomics to report on the microbial composition of the rumen (Chen et al., 2015, Creevey et al., 2014, Guo et al., 2015). However, very little effort has been devoted into understanding the other compartments. Though reticulum, omasum and abomasum are recognised as relevant parts of the GIT of ruminants, their microbial community composition has hardly been explored. Therefore the microbial composition and its overall contribution to the digestion that takes place in these chambers is not well understood (Xue et al., 2018a). Upon reviewing the literature available for these compartments very little was found on the microorganisms that inhabit them, the most readily available data focuses on their physiology. This is why then the present study is relevant as it will bridge this knowledge gap.

2.5.1 The reticulum

The reticulum's lining has a honeycomb-like appearance, earning it its common title "honeycomb". It is situated in front of and beneath the rumen, with only a muscle tissue fold separating it from the rumen (Parish et al., 2009). The primary function of the reticulum is to collect and separate digesta particles and move them into the omasum or rumen, depending on their size. The larger particles are returned to the rumen for further digestion while the smaller particles are passed into the omasum. Phyla Firmicutes and Euryarchaeota have been found in the reticulum (Xue et al., 2018b).

2.5.2 Omasum

The ingesta presented to the omasum is subjected to extensive bacterial and protozoa alterations in the rumen and reticulum. However, there is evidence that the omasum produces additional changes in the character and composition of the ingesta (Xue et al., 2018b). According to Okine et al. (1998), the omasum functions as a two-stage pump based on research conducted on sheep and dairy cattle. More fluid is pumped into the omasum's body during the first stage, which draws substances from the reticulum into the omasum. In the second stage of the pump the contents of the omasum's body are expressed into the abomasum by contraction. The omasum's ability to decrease the size of passing particles is at least one of its recognized uses. it (Becker et al.,1963). The omasum has also been discovered to play significant roles in absorption. Various compounds contained within the ingesta that enter the omasum are absorbed. The first being water as 60-70% of the water that enters the omasum is absorbed (Troutt and Amstutz, 1972). This can amount to about 100 litres of water per day in big ruminants like cows. Substances, including short-chain fatty acids, salt, potassium, carbon dioxide, and ammonia are also absorbed. About 70% of the fatty acids in sheep pass through the omasum; however, an only appreciable amount of short-chain fatty acids flows out and enter the abomasum (Troutt and Amstutz, 1972). As with the reticulum, phyla Firmicutes and Euryarchaeota are prominent in the omasum (Xue et al., 2018b).

2.5.3 Abomasum

The constant nature of the abomasum's secretory activity is what distinguishes it most in adult ruminants. Larger ruminants' abomasum likely release 30 to 100 liters of gastric juice per day (Troutt and Amstutz, 1972). That is the needed amount of stomach fluids to handle the constant inflow of digesta into the abomasum. The digesta that are already in the abomasum are always highly acidic, even though relatively high pH materials are frequently introduced. (Trent, 1990). These acidic gastric juices furtherbreak down ingested feed in the abomasum before passing it into the small intestine. The highlyacidic “environment” in the abomasum may help explain its relatively low prokaryotic diversity (Xue et al., 2018b).

It is usually assumed that most microbes carried over into the abomasum with the ingesta are destroyed in the acidic environment and act as a source of protein for the animal as they are digested (Zeng et al., 2015).

Table 2. 1 Summary of all the microbes that have been found in each of the four compartments

Domain	**Phylum/Class/species**	**Compartment**	**Function/ Pathogenesis**
Bacteria	Fibrobacter	Rumen	Cellulose digestion
	Actinobacillus succinogenes	Rumen	Succinic acid production
	Ruminococcus albus	Rumen	Fibrous feed degradation
	Butyrivibrio fibrisolvens	Rumen	Fibre degradation, protein breakdown, biohydrogenation of lipids and butyrate production
	Ruminobacter amylophilus	Rumen, Abomasum	Starch degradation
	Treponema saccharophilum	Rumen	Unknown
	Escherichia coli	Rumen, Abomasum	Class/ strain dependent i.e. enterotoxigenic, enteroinvasive, enterohemorrhagic, enteropathogenic and enteroaggregative are cause diarrhoea while other E.coli can assist with preventing dysbiosis
	Lactobacilli	Rumen , Abomasum	Carbohydrate metabolism with lactic acid as end product
Eukarya (Protozoa)	Entodinomorphs	Rumen	Plant carbohydrate degradation
	Holotrichs	Rumen	Plant carbohydrate degradation
Archaea	*Methanobacterium*	Rumen	Methanogenesis
Bacteriophage (Viruses)	Caudovirales	Rumen	Prevents dysbiosis
Fungi	*Neocallimastix frontalis*	Rumen	produce high levels of cellulases and hemicellulases and are particularly proficient in producing xylanases
Bacteria	Firmicutes	Reticulum , Omasum	Carbohydrate fermentation

Archaea	Euryarchaeota	Reticulum, Omasum	Methanogenesis
Bacteria	*Mycoplasma*	Abomasum	mastitis, arthritis, pink eye bronchopneumonia and abortion
	Anaerostipes	Abomasum	Butyric acid production
	Gluconacetobacter, *Acetobacter*	Abomasum	Acetic acid production
	Anaerofustis	Abomasum	Unknown
	Pyramidobacter	Abomasum	Unknown

2.6 Interaction between the microbial populations

The several strategies that the gut microbes uses to engage with one another are mutualism, commensalism, parasitism, and competition. Mutualism involves benefits for both bacteria, commensalism involves benefits for one without influencing the other, and competition involves multiple microbes competing for substrates or space. The presence of protozoal populations is beneficial to bacteria and archaea. Protozoa scavenge oxygen and yield hydrogen molecules in their metabolic functions; this favours anaerobic bacteria and methanogens (Williams et al., 2020).As mentioned above, phage helps maintain population dynamics by keeping bacterial numbersin control (Fernando, 2012). The reduction of bacterial numbers has been shown to positive impact the fungal population with which they are often in competition for nutrients (Hess et al., 2020).

2.7 The gut microbiomes influence on the development of the immune system

So far, most benefits discussed in the sections above pertain to digestion. However, the microbiome has also been shown to play other vital roles, such as influencing the development of the mucosal immune system. Reduced growth of the intestinal epithelium and the mucosal immune system were found in ruminants raised in artificial environments as compared to those raised conventionally. The findings was consistent with a decrease in the number and diversity of gut microbiota. Furthermore, the rate of epithelial cell generation in ruminants raised artificially is lower than in ruminants raised conventionally, indicating the significance of gut microbiota in promoting intestinal epithelial cell proliferation and guaranteeing mucosal barrier healing after injuries.

Bacterial diversity is also suspected to benefit the resistance to enteric infections in young ruminants. Oikonomou et al., (2013) reported: that there is an association between the development of the gut microbiome within seven weeks of life and calves/lambs' health and growth; and that bacterial diversity tends to be lower in lambs with pneumonia and neonatal diarrhoea compared to healthy ones.

2.8 The Virome

The term virome can be described as the collection of all viruses that are found in a particular environment. In ruminants' bodies can be said that there exist several viromes as different parts of the body harbour distinct viral communities(Zárate et al., 2017a). For the context of this study text only the gut virome will be reviewed. Ruminants' gut consists of a diverse population of viruses, these can be viruses infecting the ruminant or other microbial organisms in the gut such as bacteria and archaea (Markiewicz et al., 2021). Although research on the intricate relationships between the virome and other microbiome components in the context of host genetics and their impact on the host's health state is still in its early stages, the results thus far suggest that viruses and other microbiome components can influence the course of infections by controlling the host's immune response (Gilbert andKlieve, 2015). According to research, the ruminant virome is an important source of genes through lysogenic conversion and plays important roles in the development of immunity, and cycles nutrients and energy (Malmuthuge et al., 2015). Metagenomic next-generation sequencing (mNGS) has been used to identify rare and novel infectious microbes and virus species(Malmuthuge and Guan, 2016). Besides the common diarrhea-causing viral pathogen *Rotavirus*, other viruses such as *Coronavirus*, *Torovirus*, *Norovirus*, *Nebovirus*, *Astrovirus*, *Kobuvirus* and *Enterovirus* have been detected from ruminant faeces by mNGS (Gilbert et al., 2020b). Very little work has been done to study the gut virome of ruminants andthe overall impact that viral infections have on the microbiome. A reduction in the diversity of bacteria in the stomach has been linked to diarrhea caused by rotavirus and coronavirus infections. (Oikonomou et al., 2013).

2.9 The resistome

The resistome refers to the total collection of resistant genes found within an environment (Zárate et al., 2017b). Antimicrobial resistance is a global health concern that has received much attention over the past decade as more and more antimicrobial agents are becoming ineffective in the treatments against microbial infections (Alian et al., 2012). A considerable part of this global health issue is compounded by the use of antimicrobial agents in the agricultural sector (Davies et al., 2019). The microbiome of livestock acts as reservoirs for AMR genes of pathogens (Hardy, 2002). All of the AMR genes found in pathogenic and non-pathogenic bacteria make up the antibiotic resistome of the gastrointestinal tract (GIT) (Levy, 2014). Antimicrobial veterinary medicine is often used in the following ways: therapeutic purposes (treatment of sick animals), prophylaxis (administration of antimicrobials to a herd or flock that is at risk of a disease outbreak) and meta-phylaxis (administration of antimicrobials to healthy animals belonging to the same flock with animals showing clinical symptoms of a disease). These usages of antimicrobials are often all labelled under therapeutic usage (Rushton et al., 2014). However, AMR genes in the ruminant gut are not entirely due to 'therapeutic usage' only, but also due to the non-medicated usage of antimicrobials as well. With the high demand of the fast-growing human population, farmers have had to resort to incorporating antimicrobials into animal feed to enhance animal growth and optimize food production by decreasing the amount of feed and time required by an animal to reach market weight (Marshall and Levy, 2011). The Department of Agriculture, Land Reform and Rural Development (DALRRD) reports antimicrobial consumption in animals to the WHO for animal health. Reports from 2014-2015 showed that the prominent antibiotic group used in animal health are growth promoters (62%).

The use of antibiotics can also lead to a decrease in the diversity of gut microbiota species overall, including the extinction of certain significant taxa, which alters metabolism and makes the gut more vulnerable to colonization. (Aarestrup et al., 2010). Reduced diversity does not necessarily mean a reduced number of bacteria overall. As the antibiotic-susceptible bacteria are eliminated, antibiotic- resistant bacteria multiply (Hennessey et al., 2020). Research points to a substantial degree ofantibiotic resistance in livestock with many resistance genes and resistant microbes harbouredin the gut.

2.10 Conclusions

Studies in the past have revealed that the gut of ruminants is home to a vast number and diversity of microbes. However, these findings are mainly from studying large ruminants like cattle; the gut of small ruminants like goats and sheep have not been adequately studied and could have essential novel findings if studied adequately. Although the composition of the rumen bacterial community has been extensively researched there is still lack of information on other important microbial entities such as viruses and there is also still a lack of information on the microbial communities of the other three stomach compartments. This, therefore, underlines critical scientific gaps that need to be filled in the research and understanding of the gut of ruminants. Additionally, the lack of sufficient knowledge on the prevalence of AMR in sheep , specifically Dohne Merino sheep highlights the need for AMR studies in this breed since AMR prevalence differs even within species and is highly influenced by the environment.

3 CHARACTERISATION OF THE GUT MICROBIAL POPULATION OF SOUTH AFRICAN DOHNE MERINO SHEEP

3.1 Abstract

The gut of sheep is characterised by a diverse microbial ecosystem that is vital for the host to perform important biological processes. These processes include metabolism, homeostasis, immunity and overall host health. Using a metagenomic approach the gut microbiome of South African Dohne Merino sheep was analysed. DNA extracted from the four gut compartments of six mature Dohne Merino ewes was sequenced on an Illumina HiSeq2500 platform which generated 15 million reads of 150 reads of illumina base pairs. Using a metagenomics approach in this study allowed for identification of low abundance and uncultured microorganisms that are difficult to detect with other approaches. To characterise and taxonomically classify the gut microbial population, bioinformatics tools such as the genomic database toolkit (GTDB-Tk) were utilised. GTDB-Tk serves as a convenient means to classify the increasing numbers of microbial genomes recovered from metagenomic sample using the reference genome trees. This study focused on the prokaryotes (bacteria and archaea). Additionally, the *k*-mer-based approach was also utilised to confirm the taxonomic assignment provided by GTDB-Tk while also statistically computing the abundances for the classified data. This was done with Kraken2 and Bracken respectively. The microbial population was analysed at phylum, class, order, genus, and species level. A total of 18 phyla were detected with Bacteroidetes (54%) and Firmicutes (25%) being the most abundant. Members of the archaeal domain made up 16,7% of the overall population. A total of 1, 762 genomes were detected in all the samples at a species level, with majority of the genomes assigned to uncultured species (82,7%). Compared to the bacterial population (15 of the 18 phyla), the archaeal population was less diverse (3 of 16 phyla). The Beta(β) and Gamma(γ) diversity indices were computed; the values of the β index ranged from 0.62-0,85, and the γ value was 1,769. Overall, 107 known species were identified many being bacterial species (n = 100). Most of the archaea identified were methanogens that produce methane gas as a product of fermentation. The majority of microbes found have a variety of properties and functions that are beneficial to the host. These include fermentation of complex fibre, antibiotic properties and maintaining homeostasis, with the most represented biological function being breaking down of complex fibre and carbohydrates. However, some members of Actinobacteria, Cyanobacteria and Bacteroidetes identified are associated with diseases. Overall, this analysis revealed a great diversity in the gut microbial composition of

Dohne Merino sheep which is considered to provide greater stability and flexibility to withstand pathogens.

3.2 Introduction

The importance of the gut microbiome has been recognized for its essential contrubution in the health and well-being of the host (Round and Mazmanian, 2009). The gastrointestinal tract harbours complex populations that, through studies, have been recognised to play fundamental roles in digestion and even the immune system development and overall animal health (Schuijt et al., 2013). Microbes are the most abundant and diverse form of life on earth. The microbiota found in the adult mammalian gastrointestinal tract (GIT) is around ten times greater than the host cells (Bäckhed et al., 2005).

Ruminants are the biggest contributors to the livestock industry worldwide. The health and well-being of ruminants is greatly impacted by the gut microbiome. (Li et al., 2012). Despite the range of ruminants with different diets and feeding strategies, similar gut microbes are found to be abundant worldwide with some degree of variation in the overall composition of the communities due to differences in diet, climate, and farming practices (Henderson et al., 2012). Most bacterial communities consists of a core bacterial taxa, that is dominated by *Bacteroidetes*, *Butyrivibrio* and *Ruminococcus* (both *Firmicutes*), as well as unclassified *Lachnospiraceae, Ruminococcaceae, Clostridiales (*all *Firmicutes) and Bacteroidales* is often found to be common amongst ruminants (Henderson et al., 2015).

Other gut microbes have effects that are not favourable to the host. This includes microbes like *Escherichia* that produce potentially toxic micro metabolites that are harmful to the host (Nicholson et al., 2005). Data suggests that the desirable “healthy” gut microbial community is one in which diversity is accompanied by a high degree of functional redundancy; this results in better overall health and better outcomes in several disease states (Wargo, 2020).

In the past, culture-based techniques were used to isolate and characterise microbes; this method was very useful, and key representative microbes, especially bacteria, were discovered through it. However, these technologies cannot be considered adequate, as most (50%) of the gut microorganisms cannot be cultured outside of the gut (Zhang et al., 2018a). This tremendously limited the study of gut microorganisms until the development of high throughput DNA sequencing at the onset of the 21st century (Barba et al., 2014). DNA sequencing approaches offer fast and convenient tools for describing these unculturable microbes (Marx, 2013). These high throughput DNA sequencing techniques have allowed a considerable increase in knowledge of ruminants' gut microbiome (Lettat et al., 2012).

The gut microbiome of small ruminants such as sheep and goats remain considerably underexplored. Therefore, more studies profiling the gut microbiome of such small ruminants are required. Dohne Merino is one of the most abundant sheep breeds in South Africa and is responsible for the upkeep of livelihoods in many small communities (Kotzé, 1951). The breed is a synthetic South African breed, now bred internationally for wool and meat (De Klerk and Heydenrych, 1990). Dohne Merino sheep from the Wauldby farm have displayed significant resilience to gut pathogens such as roundworms that has been attributed to high gut microbial diversity as a diverse microbiome has been associated with increased resilience to pathogens (Mafuna et al., 2021, Burgess, 2016, Lozupone et al., 2012). The objective of this investigation is to profile the gut microbial composition of Dohne Merino sheep from the Wauldby farm and to assess the level of diversity within the gut in order to gain a better understanding of the presumed breed resilience.

3.3 Methods and Materials

3.3.1 Animals and sampling

Experimental animals were sampled from the Wauldby farm located in the Mahlathi local municipality in the Eastern Cape Province, South Africa (Appendix Figure A1 for map location). Six Dohne Merino sheep were randomly selected from a herd of about 450 ewes. These ewes were raised on a sourveld and belong to a flock that has been participating in a selection and breeding program for resistance to Heamonchus corntortus parasites(Burgess, 2016), and has been utilised in other studies by Dlamini et al., (2019) and Mafuna et. al., (2019). Meta data such as age, sex and health conditions of the selected animals are as depictedin Table 3.1. The GIT of the sheep was inspected, and the animals were humanely slaughtered at the nearby slaughterhouse (Tandala Farm Abattoir). The four GIT compartmentsof interest were identified (rumen, reticulum, omasum, and abomasum) and each of the four stomach compartments was then opened along the greater curvature. Roughly 5g of contents were collected from each of the compartments using a sterile sampling spoon into a sterile collection tube. The samples were preserved in liquid nitrogen and transported to the

Agricultural Research Council-Biotechnology Platform (ARC-BTP) Pretoria, Gauteng, for laboratory analysis.

Table 3.1 Sampled Dohne Merino sheep from the Wauldbly farm

Animal	**ID**	**Age(years)**	**Additional Health conditions**
Dohne Merino sheep 2	B2.187	6	Resistant to *Haemonchus contortus*, to with no worm
Dohne Merino sheep 5	B1.219	7	Susceptible to H.*contortus* with worm present
Dohne Merino sheep 6	B0.515	8	Susceptible to H.*contortus* with worm present
Dohne Merino sheep 7	B9.181	9	Susceptible to H.*contortus* with worm present
Dohne Merino sheep 8	B9.233	9	Resistance to H.*contortus* with worm present
Dohne Merino sheep 9	B9.053	9	Resistance to H.*contortus* with no worm

3.3.2 DNA extraction

DNA was extracted from the samples according to the instructions in the ZymoBIOMICS DNA miniprep kit (Catalog Nos. D4300T, D4300, 4303). The final DNA concentration was determined by the Implen NanoPhotometer N60/N50 | UV-Vis Spectrophotometer. The purity calculation, which divides the absorbance at 260 nm by the reading at 280 nm, was used to evaluate the DNA's quality.

3.3.3 Next generation Illumina sequencing

Using Illumina® TruSeq® Nano DNA Library Prep kits, the extracted DNA was prepared into uniquely indexed paired-end genomic DNA libraries. Briefly, the first step in the library preparation was the fragmentation of DNA. DNA of 100ng per sample was used to reach a goal of 350bp fragment (per protocol). After fragmentation, the ends of the DNA fragments were repaired by employing an end-repair mix to remove overhangs. The 5' to 3' polymerase activity fills the 5' overhangs while the exonuclease activity eliminates the 3' overhangs. Then, in order to stop the blunt pieces from ligating to one another during adapter ligation, an adenine nucleotide was added to their 3' ends. To prepare the DNA fragments for hybridization onto a flow cell during the sequencing process, many indexing adapters were ligated to their ends in the following step.

As part of the preparation stage the DNA had to be enriched through Polymerase Chain Reaction (PCR). The TruSeq PCR primer cocktail was utilised for primers since the samples in this study are metagenomic samples with a variety of microbial organisms. The primers anneal to the ends of the adapters, and the DNA was amplified. Bio-Rad Laboratories PCR machine was set as follows:

- 95°C for 3 minutes,
- Eight cycles of:

— 98°C For 20 seconds

— 60°C for 15 seconds

— 72°C for 30 seconds

- 72°C for 5 minutes
- Hold at 4°C

The resulting DNA library was then quality checked and quantified following the steps in the library preparation manual. All the specific steps, reagents and parameters used in the library preparation stage followed the TruSeq® Nano DNA Library Prep reference guide as per manufacturer’s instructions. The PCR products were sequenced using an Illumina HiSeq 2500 platform.

3.3.4 Bioinformatics data analysis

The raw sequenced data were processed using bioinformatics software and tools. In the in-silico analysis stage, the first step was decontaminating and filtering the DNA by removing host (sheep) DNA and trimming the adapter sequences with Trimmomatic *v0.36* (Bolger et al., 2014) using the following parameters LLUMINACLIP:TruSeq3-PE.fa:2:30:10, LEADING:3, TRAILING:3 SLIDINGWINDOW:4:15, MINLEN:36. Which performed the
following:

- Remove adapters (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10)
- Remove leading low quality or N bases (below quality 3) (LEADING:3)
- Remove trailing low quality or N bases (below quality 3) (TRAILING:3)
- Scan the read with a 4-base wide sliding window, cutting when the average quality per base drops below 15 (SLIDINGWINDOW:4:15)
- Drop reads below the 36 bases long (MINLEN:36)

Quality controlled sequenced data was then assembled into contigs using Spades (Bankevich et al., 2012) v3.12.0. The contigs were assembled using Spades (Bankevich et al., 2012) *v3.12.0*. The resulting metagenomic assembled genomes(MAGs) were taxonomically classified with The Genome Taxonomy Database Toolkit (GTDB-Tk) *v1.7.0 (Chaumeil et al., 2020)* to describe the microbial composition of the gastrointestinal microbiota of sheep. GTDB-Tk classified archaeal and bacterial genomes by placing them in domain-specific, concatenated protein reference trees. In addition to GTDB-Tk, Kraken2 used verify the taxonomy assigned by GTDB-Tk. Kraken2 is a memory-intensive algorithm software that associates short genomic substrings (k-mers) with the lowest common ancestor (LCA) taxa. The outcomes from GTDB-Tk and Kraken2 were then converted into interpretable visuals with R-studio (R Core Team,2022) and Microsoft Excel (https://www.microsoft.com/en-za/microsoft-365/excel). Stacked bar plots were created with R-studio to visualise and compare the diversity of the four gut compartments. Excel was used to create other visuals such as donut charts and heatmaps.

3.4 Results

3.4.1 Data Profile

Raw sequences of 15 million reads of 150bp of illumina base were generated from the HiSeq -X sequencing platform. After trimming sequences and assembling into contigs with Spades a total of 2 000 159 contigs were assembled. Divided into 19 phyla, 24 classes and 107 species. Core phyla were identified as Bacteroides, Firmicutes and Candidatus Thermoplamatota and the sum relative abundance of Bacteroides and Firmicutes totalled 79,37%.

3.4.2 Combined composition of the gut microbial population

GTDB-Tk classified the members of the microbial population placing them into domain-specific, concatenated protein reference trees. The results in Figure 3.1 show the abundance of the genomes found across all the four gut compartments at the phylum level. A total of 19 phyla were identified across the sheep gut compartments. The most abundant phyla were Bacteroidota and Firmicutes, making up 53,9% and 25,4%, respectively (Firmicutes composed of the Firmicutes phylum and two sub phyla Firmicutes A and Firmicutes C which add up to 25%. The phyla with the lowest abundance were Actinobacteria (0,62%), UBP6 (0,17%), Euryarchaeota (0,57%), Cyanobacteria (0,45%), Campylobacteria, Candidatus Asgardarchaeta, Myxococcota and each making up 0,06% of the population. All the identified phyla belong to the bacterial and archaeal domains.

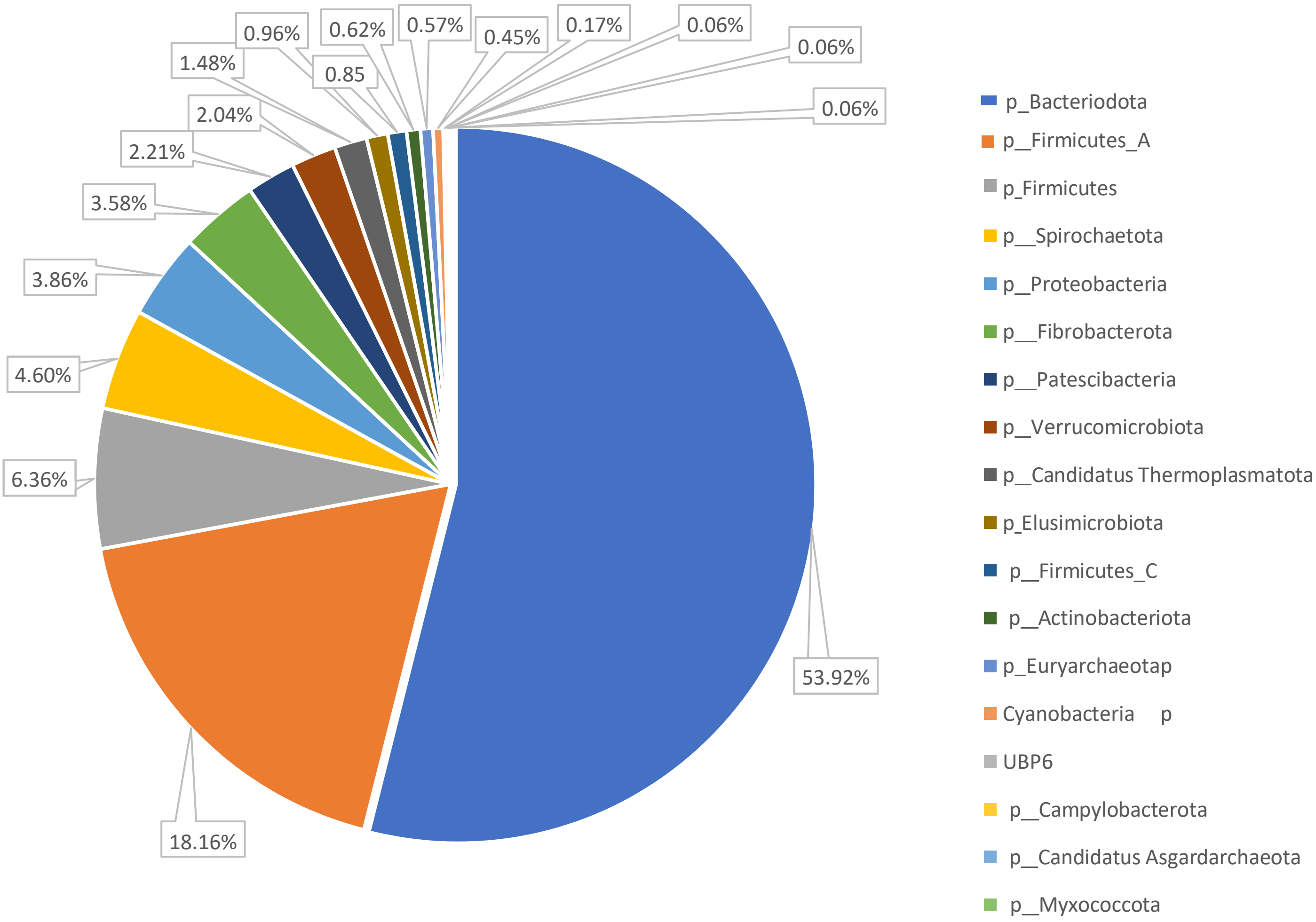


Figure 3.1 Overall composition of the gut microbial population of Dohne Merino sheep. The population was composed of mainly members from the bacterial domain with Bacteroidota(blue) and Firmicutes(orange, grey and royal blue) being the majority, a small percentage of the archaea was also identified (Candidatus Asgardarchaeta, Candidatus Thermoplasmatota and Euryarchaeota

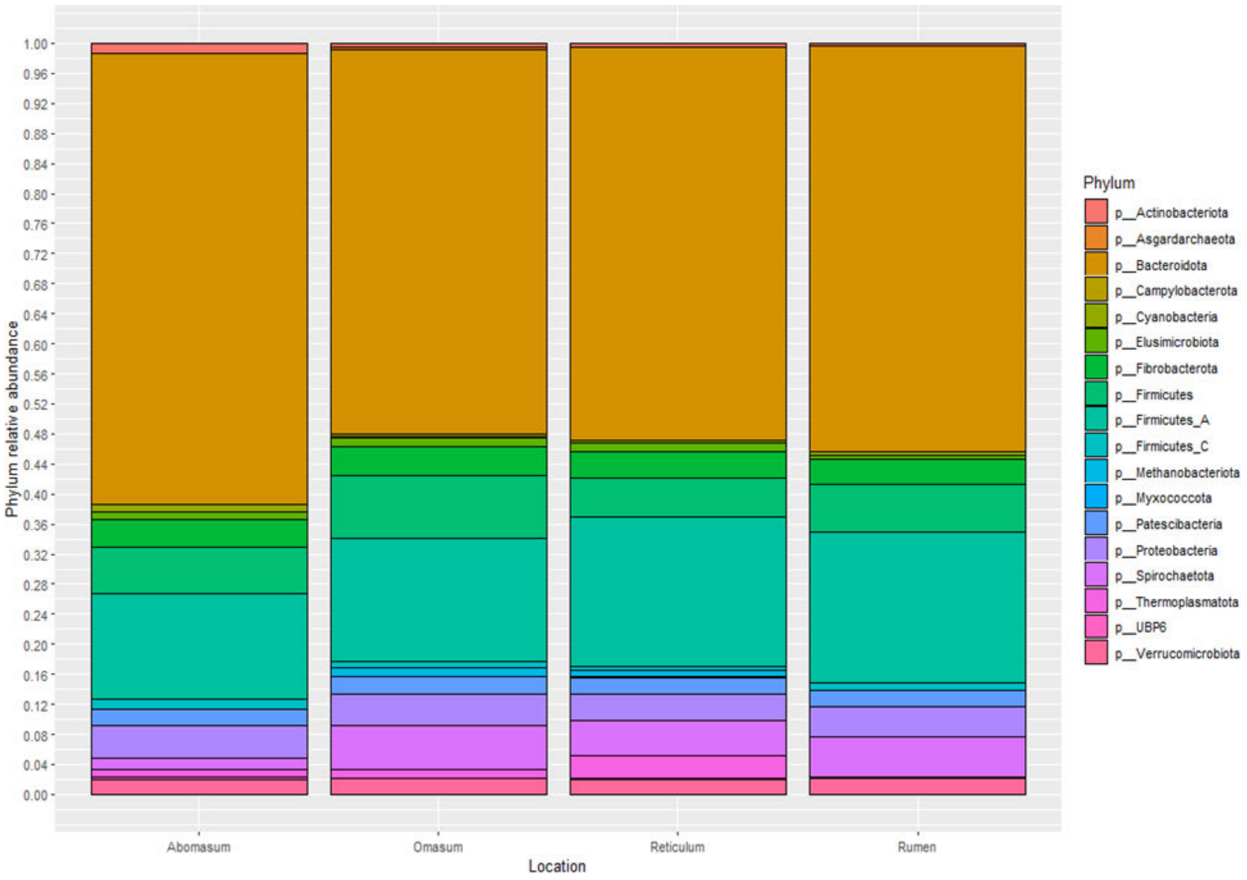


Figure 3.2 Overall composition of the gut microbial population of Dohne Merino sheep. The population was composed of mainly members form the bacterial domain with Bacteroidota and Firmicutes and it also contained a small percentage of the archaea

The composition for each of the gut compartments was further analysed and viewed separately in Figure 3.2. The Bacteroidetes phylum was the most abundant across all the compartments followed by Firmicutes. The following abundances were observed in each of the compartments for Bacteroidetes: 59% in the abomasum 50% in the omasum, 51% in the reticulum and 54% in the rumen. Firmicutes abundance percentages in the following order 31% of the rumen, 26% of reticulum, 25% of omasum and 22% in the abomasum. The phylum Cyanobacteria was only found in the rumen (2%) and abomasum (4%). The omasum is the only compartment that contained Asgardarchaeta (0,06%). Actinobacteria was found at a low abundance across all the four compartments, with the highest abundance being 5% in the abomasum and the lowest of0,5% in the rumen. Both the reticulum, and omasum had 1% constituted with actinobacteria. Overall, the reticulum was the most diverse compartment containing 15 of the 18 phyla whilst the rumen was the least diverse, containing 12 phyla. The abomasum, and the omasum both had 14 phyla.

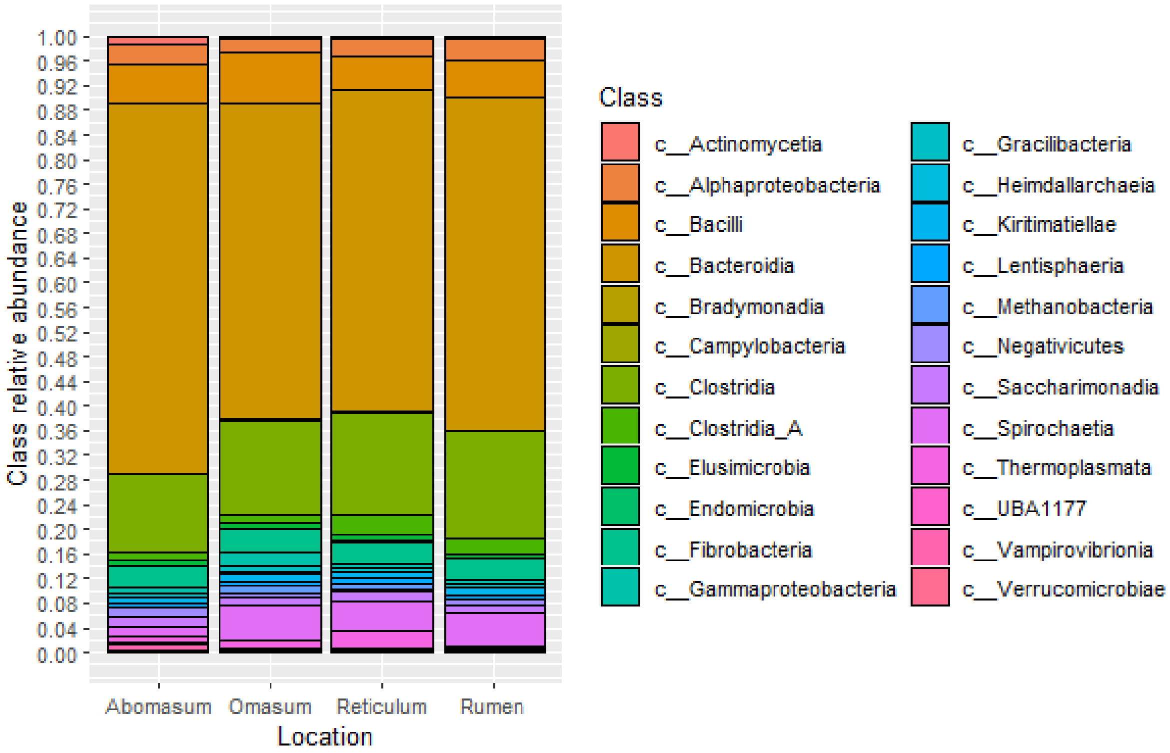
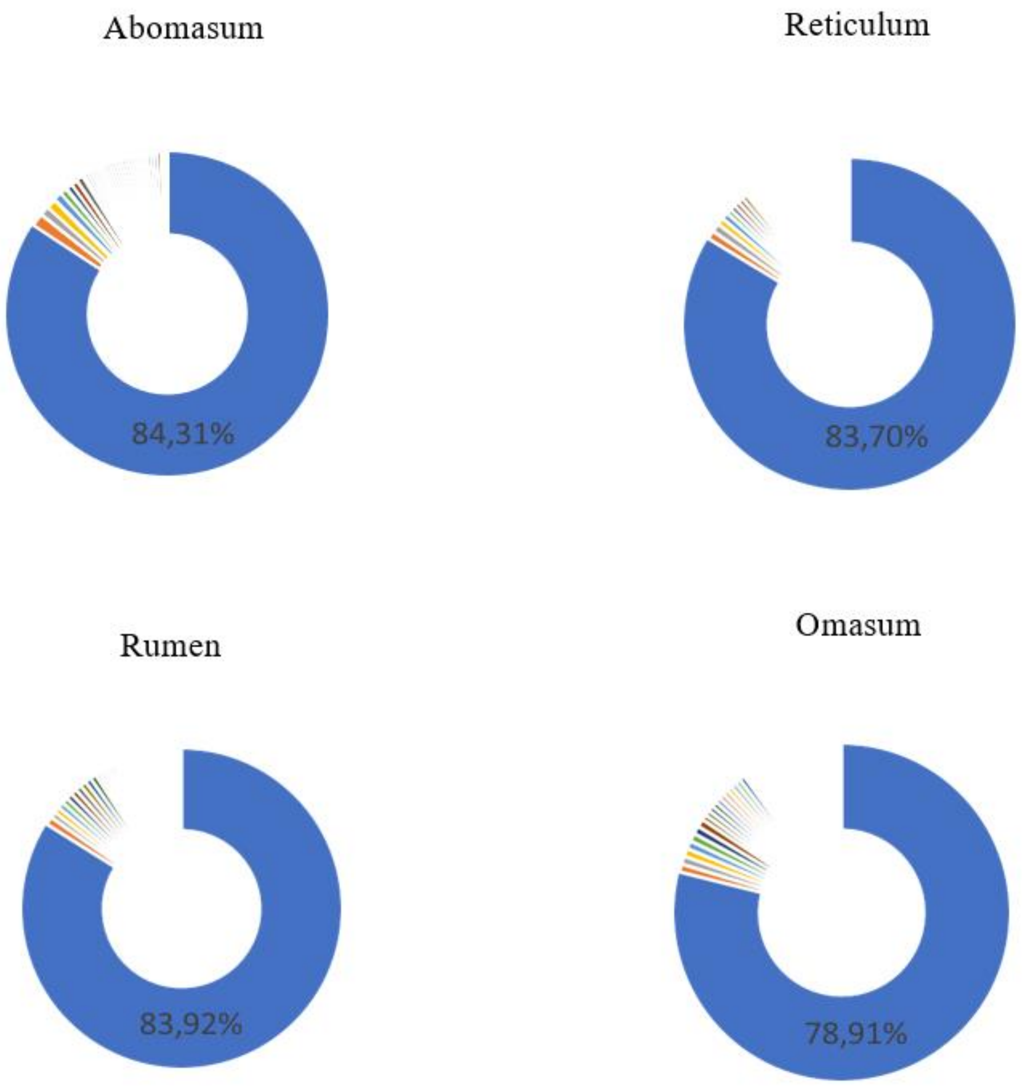


Figure 3.3 Classification and relative abundance of microbial classes in gut sheep. The total number of classes was 24 across the entire gut. The classes with the highest abundances included Bacteroida, Clostridia and Bacilli.

A total of 24 classes were identified and 16 of these 24 were common across all four compartments. The reticulum and the omasum had 21 classes, and the abomasum and rumen had 19 classes. The most abundant classes were Bacteroidia and Clostridia. Bacteroidia abundance was in the following order: abomasum (60%), reticulum (57%), rumen (53%), and omasum (51%). Clostridia was found in the following percentages: rumen (24%), reticulum (26%), omasum (18%) and abomasum (15%). The Clostridia percentages were composed of Clostridia and one other sub-phylum Clostridia_A. Other classes that were present in significant percentages across all the compartments include Bacilli (7-10%), Alphaproteobacteria (6-9%) and Spirochaete (2-5%). Methanobacteria was found in all the compartments except the abomasum. Though found in low abundance, Fibrobacteria was found in all compartments at proportions of 2% in the rumen and 3% . The Campylobacteria and Heimdallarchaeia classes were found only in the omasum. The Endomicrobia and Bradymonadia were present in the reticulum only (Figure 3.3).



Abomasum

Reticulum

Rumen

Omasum

Figure 3.4 Abundance of unknown species in each of the compartments. The blue component represents uncultured species which make up the majority of the population in each of the compartments (78,9%-84,4%). Cultured which are represented by the coloured components make up 21% or below in each of the four compartments.

Beta (β), and gamma (γ) diversities were estimated for the whole population at species level. Gamma diversity was 1,769 and dominated by uncultured species. As depicted in Figure 3.4, unknown species made up between 78,9% to 84,3% of each compartments' population. Cultured species made up ≤ 21% in each of the compartments. The total number of known cultured species was 107 across the entire gut, these species are identified and further discussed in sections 3.4.3 and 3.4.4 below. Figure 3.5 below is a matrix that results from utilising the Jaccard distance to perform pairwise analysis of the compartments for the measurement of β diversity. The gut compartments were found to generally have large distances between them the β diversity index values for each pair were as follows: reticulum and omasum - 0.85, omasum and rumen -0.84, rumen and reticulum - 0.71, rumen and abomasum - 0.69, omasum and abomasum - 0.65, reticulum and abomasum - 0.62 as illustrated in Figure 3.4.

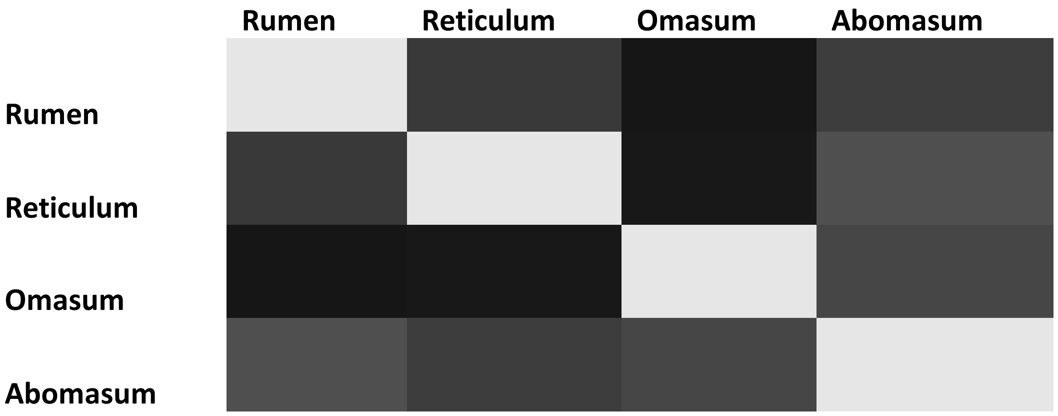


Figure 3.5 Beta diversity measurement using the Jaccard distance measurement. The intensity of the shading is proportional to the measure of diversity between each pair. The darker the shade the larger the diversity. The pair with the highest diversity is the reticulum and abomasum.

3.4.3 Diversity of the archaeal population at different taxonomic levels

Since bacteria and Archaea were the point of focus in this study, these domains were then analysed separately to get more detail on the diversity and composition of each. Figure 3.6 is the diversity of the archaeal population at a phylum level.

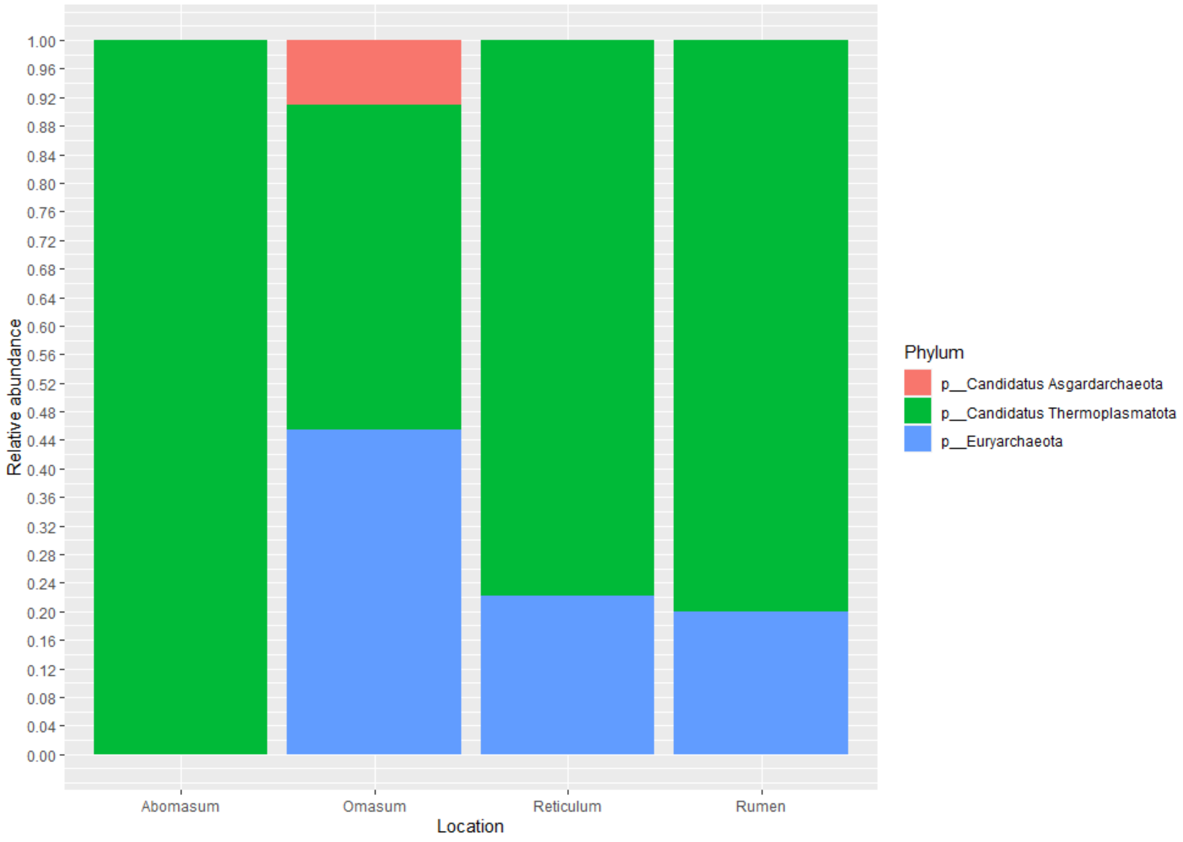


Figure 3.6 Phylum diversity of the archaeal population in the four digestion chambers of sheep. The diversity of the archaea is very low at a phylum level, with only 3 phyla identified.

The archaeal domain comprised 16.7% of the total combined bacterial and archaeal population. Figure 3.6 reveals three archaeal phyla found in the studied population. The most abundant phylum of archaea was *Thermoplasmatota* making up 100% of the abomasum, 46% omasum, 78% reticulum, and 81% rumen populations. The omasum was the only compartment that contained the Asgardarchaeota phylum (9%), Euyarchaeota was the second most abundant archaeal phylum making up 45%, 22% and 19% of the omasum, reticulum, and rumen populations respectively.

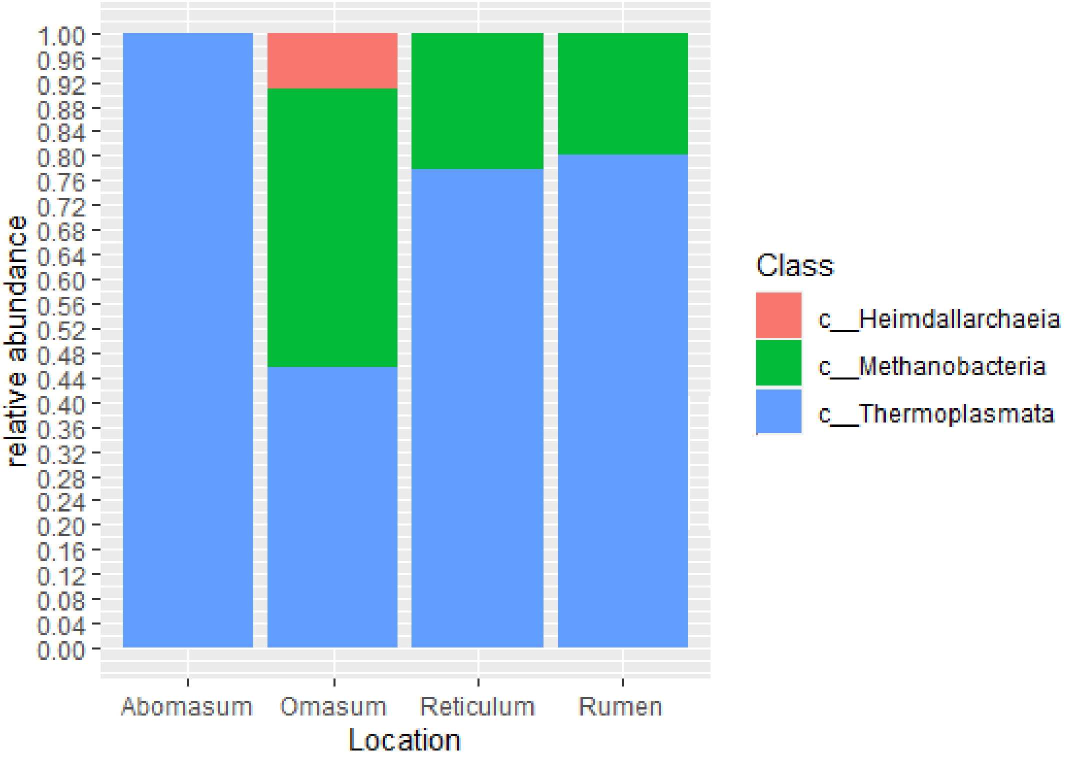


Figure 3.7 Diversity of the archaeal population at a class level of classification. Three Archaeal classes could be identified with majority of the population belonging to Thermoplasmata.

Figure 3.7 represents the archaeal population at a class level. Only three archaeal classes were found. The omasum is the only compartment that contains all three classes, the rumen and reticulum have two *Methanobacteriota* and *Thermoplasmata*. The abomasum only had one class, the Thermoplasmata, making the omasum the only compartment that had the class Heimadallarchaeia.

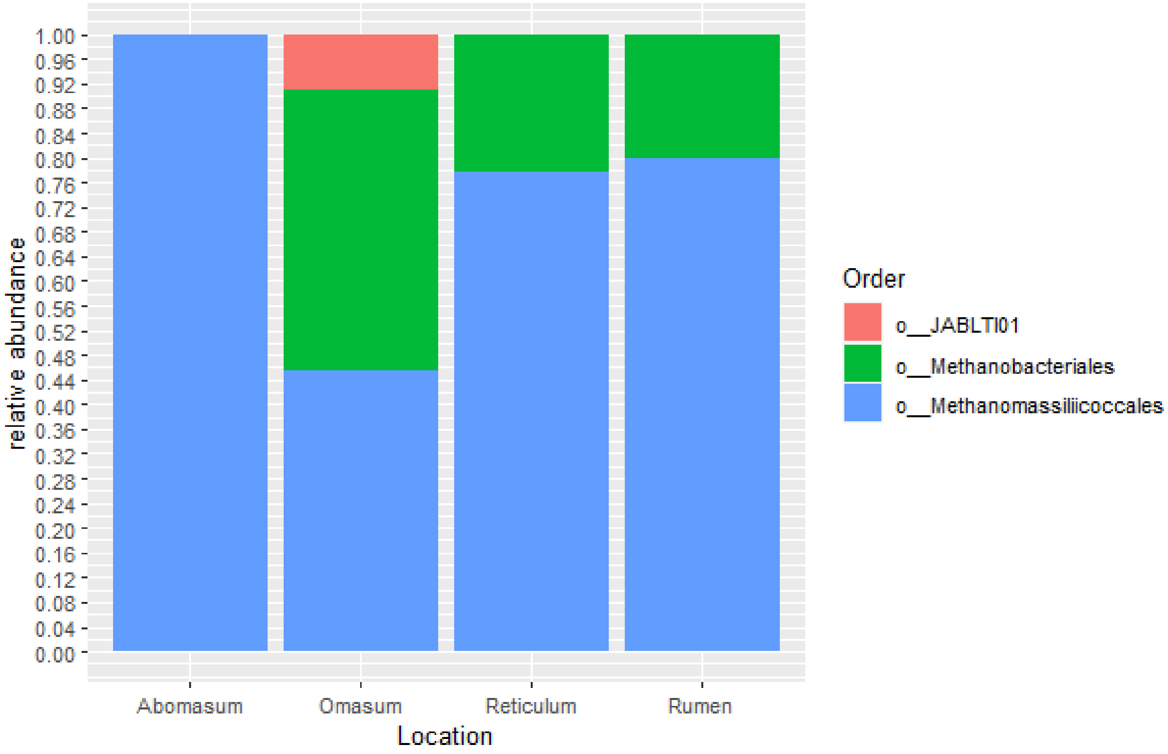


Figure 3.8 Diversity of the archaeal population at an order level of classification. The Methanomassilicocales was the most abundant, followed by Methanobacteriales and lastly JABLTI01 which was found only the omasum.

Orders Methanomassilicocales, Methanobacteriales and JBLTI01 (in that order of abundance) were found for archaea, as seen in Figure 3.8. The abomasum had only one class Methanomassilicocales, the reticulum and the rumen had two Methanomassilicocales and Methanobacteriales, which the Methanomassilicocales class was in abundance (± 80%) in both chambers. The omasum had all of three orders making it the only compartment with the order JBLTI01. In Figure 3.9 the abundance of the archaeal population is displayed at a family level. Three families in total are present (Methanomethylophilaceae, Methanobacteriaceae and N/A). The third family(N/A), whose members were only discovered in the omasum (8%), is unknown. Most of the archaea belongs to the Methanomethylophilaceae and 100% of these were observed in the abomasum, 54% in the omasum, 76% in the reticulum and 80% in the rumen. Thirty-eight percent (38%) of these methanobacteriaceae were in the omasum, 24% in the reticulum and 20% in the rumen.

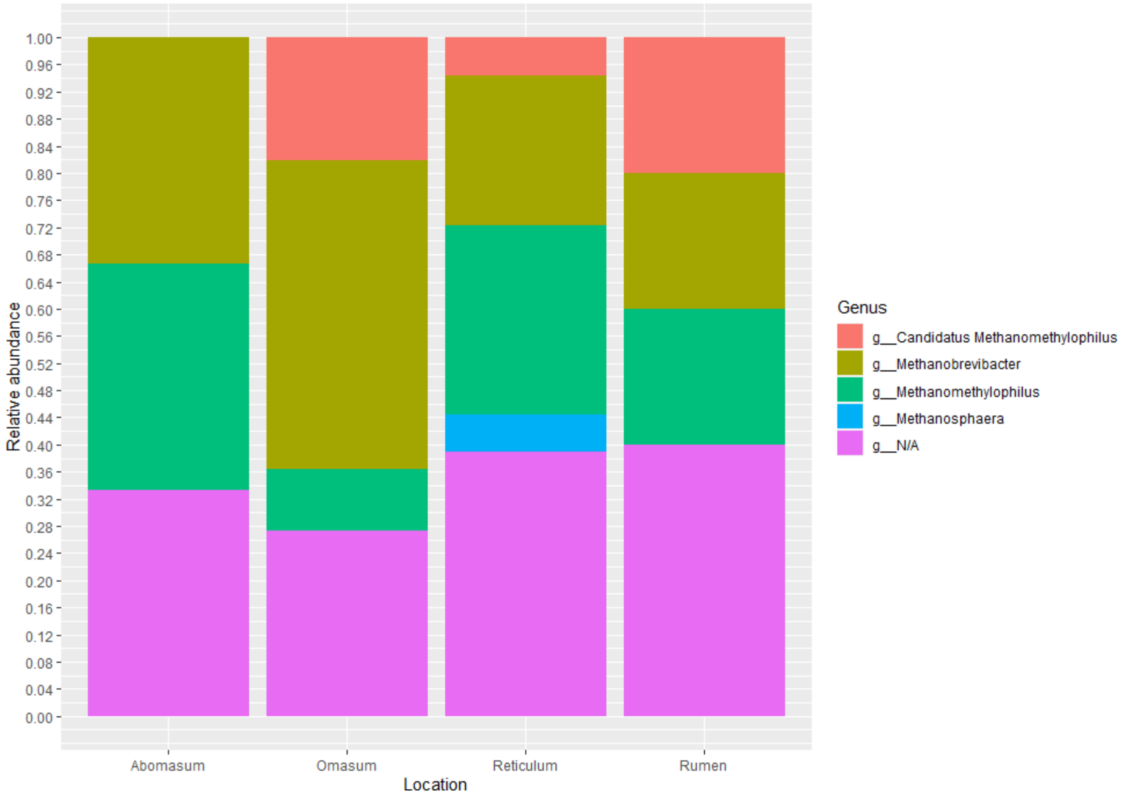


Figure 3.9 Diversity of Archaea in the gut of Dohne Merino sheep at a genus classification level. Most Archaea is unclassified at a genus level with N/A abundances ranging between 27% and 39% across the compartments.

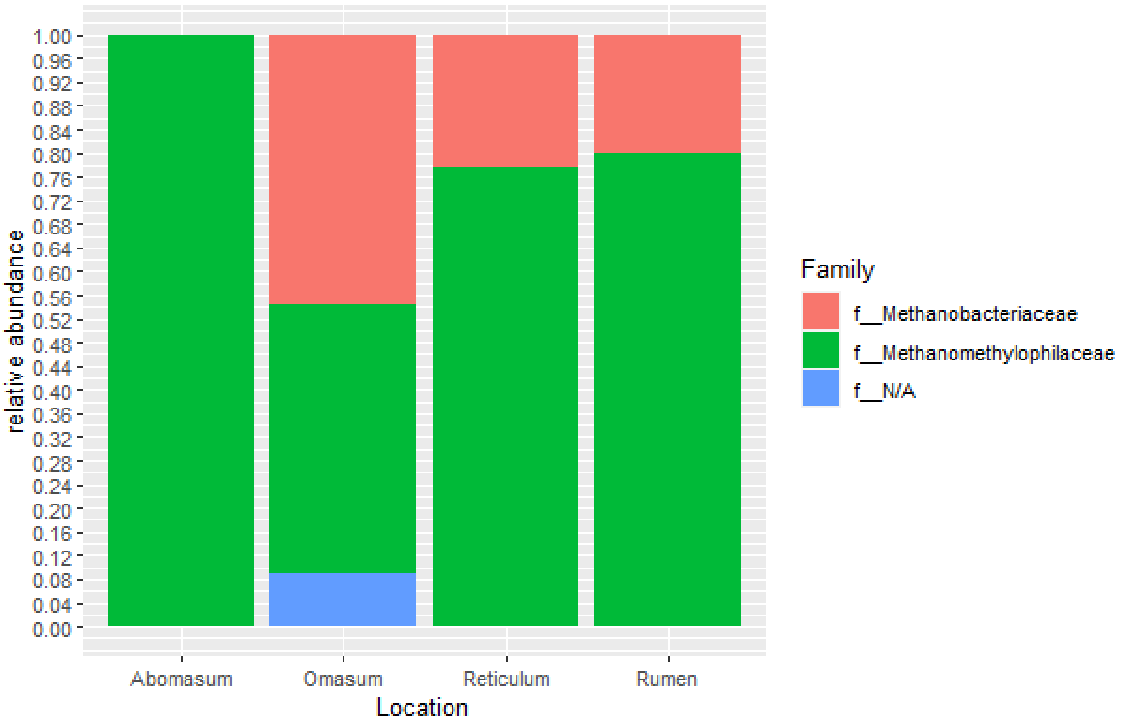


Figure 3.10 Family classification of archaea found in the gut of sheep. At a family level, only two known families were identified. About 8% of the omasum population was of an unidentified family.

In Figure 3.10 a large proportion of the overall archaeal population belong to unknown genera (N/A). 32% in the abomasum, 27% omasum, 38% reticulum and 39% rumen. The most abundant known genus *Methanobrevibacter* constituted 35% of the abomasum population, 44% of the omasum, 20% of the rumen and reticulum. The second most abundant genus in the known genera is *Methanomethylophilus* which was found in percentages of 35%, 8%, 27% and 20% in the abomasum, omasum, reticulum, and rumen respectively. The omasum is the only compartment where *Candidatus Methanomethylophilus* was not found, while it was found at varying percentages in the other compartments: 18% in the omasum, 6% in the reticulum and 20% in the rumen. The least abundant genus was *Methanosphaeara*, making 7% up of the reticulum population.

Table 3.2 Archaeal species found four digestion chambers of Dohne Merino sheep

Species	Percentage
Candidatus methanomethylophilus alvus	0,4%
Thermoplasmatales archaeon BRNA1	0,9%
Methanobrevibacter millerae	1,5%
Methanobrevibacter ruminantium	1,3%
Methanobrevibacter sp. YE315	0,1%
Methanobrevibacter alleyae	0,04%
Methanosphaere sp. BMS	0,5%
Methanogenic archaeon	96,5%

A total of seven known archaeal species were found in the Dohne Merino sheep gut in this study. Most of the archaeal population (96,5%) were of uncultured species and were generically classified as Methanogenic archaeon. Four of the seven known species were of *Methanobrevibacter* genus: *Methanobrevibacter millerae* (1,5%), *Methanobrevibacter ruminantium* (1,3%), *Methanobrevibacter* sp. YE315 (0,1%) and *Methanobrevibacter alleyae* (0,04%). Other known species found were *Thermoplasmatales* archaeon BRNA1 at 0,9%, *Methanosphaere* sp. BMS at 0,5% and Candidatus *methanomethylophilus alvIus* at 0,4.

3.4.4 Diversity of the Bacterial population at various taxonomic levels

Figure 3.11 displays the components of the bacterial phyla of the gut population. From this figure, it is observable that the composition of the omasum, reticulum and rumen were very similar. 13 phyla were observed. The most abundant bacteria phylum was Bacteroidota, and the least abundant were *Actinobacteria* and *Cyanobacteria* across all four chambers. The ratio of the archaea to bacteria was 1:6 in this population.

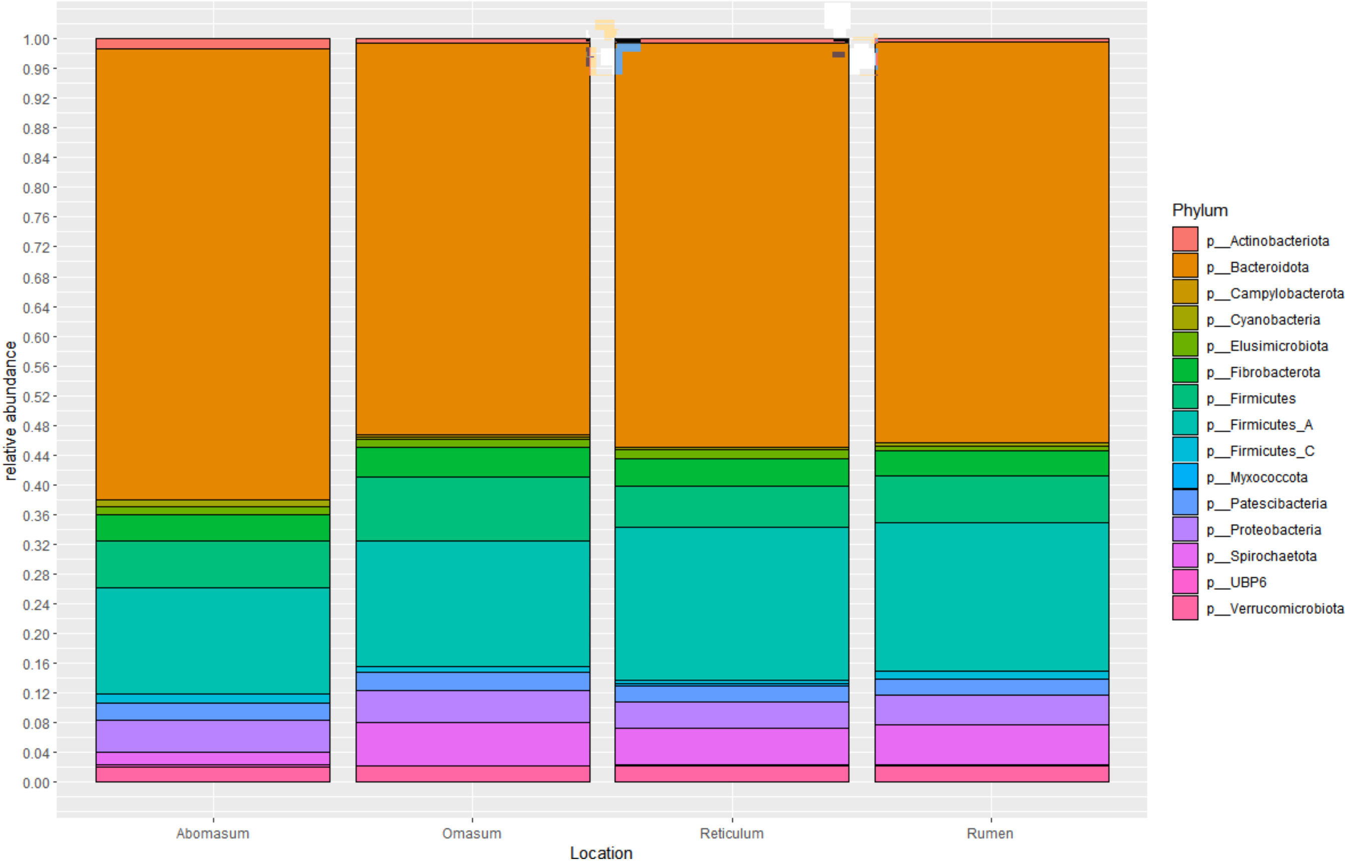


Figure 3.11 Diversity of sheep gut bacteria at a Phylum level. The bacterial population in the gut of sheep is very diverse with varying abundances of different phyla in the different gut compartments. The omasum ,reticulum and rumen have similar population structures.

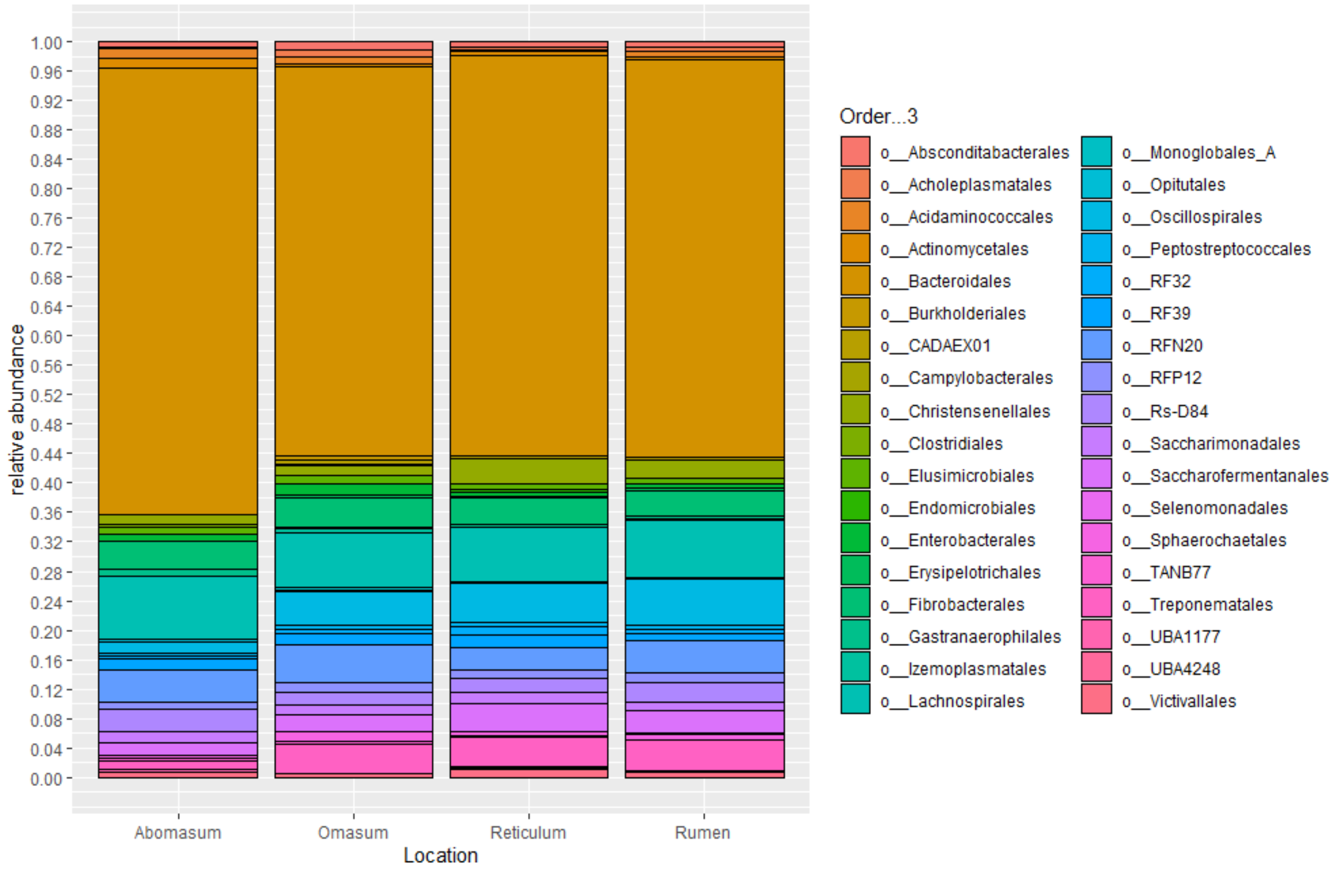


Figure 3.12 Diversity of the bacterial population in the four fermentation chambers at an order level of classification. A high diversity of bacterial orders is observed with a total of 36 orders identified.

The bacterial population was further analysed at the order level, Bacteroidales, *Monoglobales_A*, *Lanchnospirales*, *Campylobacterales* were some of the bacterial orders found in abundance. The bacterial order composition of the rumen, reticulum and omasum were still very similar with observations of ± 54 % *Bacteroidales*, 8% *Lanchnospirales*, 6% *Monoglobales*, 4% *Campylobacterales* and 2% Fibrobacterales. The bacterial order composition of the omasum was different from the other three other compartments with 60% *Bacteroidales*, 11% *Lanchnospirales* and 4% *Fibrobacterales*. The bacterial population was very diverse albeit only few of the members of the population were observed at high relative abundances. Orders such as *Actinomycetes*, *Enterobacterales*, *Victavallales*, *Peptostrecocalles* and *Endomicrobiales* account for the diversity but observed in very low abundances. There were other bacteria observed that have not been taxonomically classified but have NCBI taxonomy identification numbers because they were reported in previous studies These included RF32, RF39, RFN20, Rs-D84, TANB77, UBA1177, UBA4248 and CADAEX01.

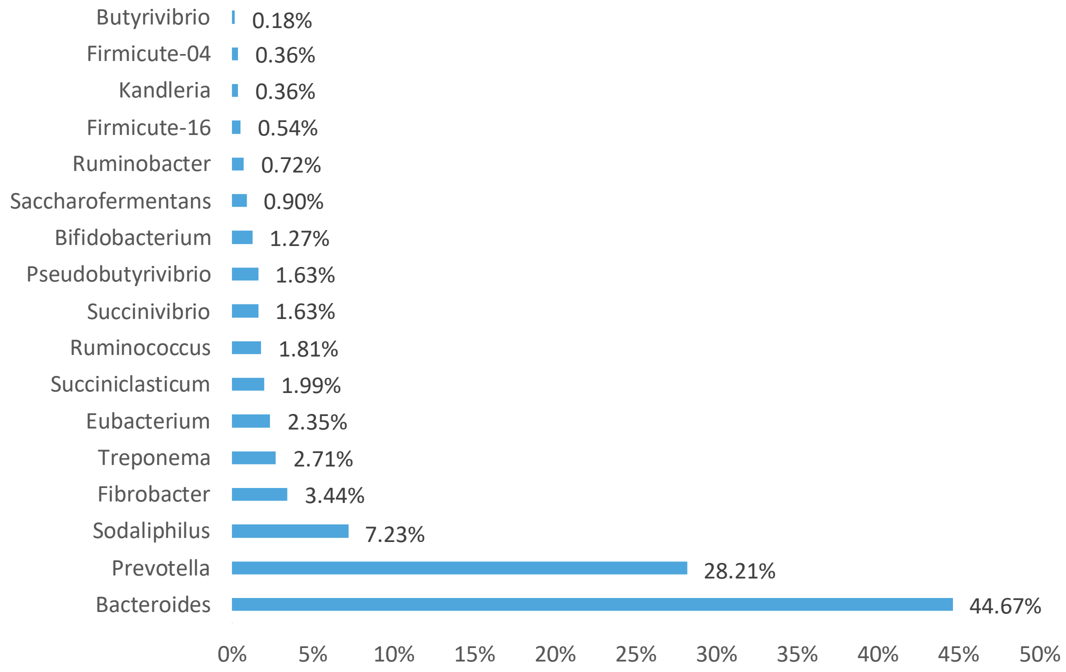


Figure 3.13 Abundance of cultured bacteria genera identified in the gut of sheep. Bacteroides and Prevotella were found to be the most abundant genera.

Figure 3.13 represent the abundance of the bacterial genera across the whole gut. The relative percentages of the genera presented in the figure were quantified with the exception of bacteria of unknown taxonomy, and those with an abundance under the chosen cut off mark of value 0,15%. Of the unidentified bacteria that were excluded, more than 40% of the remaining population belonged to the *Bacteroides* (44,67%) genus. After *Bacteroides*, *Prevotella* made up 28,2% of the population second to the *Bacteroides*. The remainder of population members was made up of the following genera: *Sodaliphilus*(7,2%), *Fibrobacter*(3,4%), *Treponema* (2,7%), *Eubacterium* (2,3%), *Succiniclasticum*(1,99%), *Ruminococcus*(1,8%), *Succinovibri* and *Pseudobutyrivibrio* (1,6%) *Bifidobacterium*(1,3%) *Ruminobacter*(1%) *Saccharofermentans* (0,9%) *Firmicute*-16 (0,54%), *Kandleria* and *Firmicute*-04 (0,36%) and *Butyvibrio* (0,18%).

At higher levels of classification most of the bacterial population could be classified. However, more bacteria could not be classified further down the taxonomic level of classification and majority of the microbial population was of unknown species. A total of 100 known cultured bacterial species were found in this study and are as listed in Appendix Table A1. Table 3.3 below only list species that have been identified as biologically important in literature. Of these species, *Prevotella* genus had a total of 7 species, which is more species than any of the other genera. Six species of the *Bacteroides* genus were classified as biologically important. In the *Ruminococcus* and *Butyrivibrio* genera 4 and 3 species were reported respectively. Only one species was reported in the *Fibrobacter* genus.

Table 3.3 Genus and Species of biological important bacteria found in the gut of Dohne Merino sheep

Genus	**Species**
Prevotella	*Prevotella ruminicola* *Prevotella intermedia* *Prevotella melaninogenica* *Prevotella dentalis* *Prevotella fusca* *Prevotella sp. oral taxon 299* *Alloprevotella sp. E39*
Bacteroides	*Bacteroides coprosuis* *Bacteroides caecimuris* *Bacteroides cellulolystic* *Bacteroides zoogleformamans* *Bacteroides xylanolyticus* *Bacteroides uniformis* *Bacteroides fragilis* *Bacteroides heparinolyticus* *Bacteroides thetaiotaomicron*

Fibrobacter	*Fibrobacter succinogenes*
	Ruminococcus sp. JE7A12
Ruminococcus	*Ruminococcus albus*
	Ruminococcus bicirculans
	Ruminococcus champanellensis
Butyrivibrio	*Butyrivibrio fibrisolvens*
	Butyrivibrio hungatei
	Butyrivibrio proteoclasticus

3.5 Discussion

The diverse population of microorganisms that reside in the gut (gut microbiome) is considered of high importance to ruminant livestock production and heath due to its key roles in digestion, energy production, growth, immune defense, and host health. Furthermore, recent studies have demonstrated the connection between the composition and function of the rumen microbiome, animal productivity, and climate change-relevant traits including feed efficiency and methane emission (O'Hara et al., 2020b). The microbial population found in the stomach of ruminants often consists of cellulolytic, hemicellulolytic, amylolytic, proteolytic, and bio-hydrogenating (lipolytic) species. These species have a high degree of functional redundancy and can efficiently break down plant fiber that is indigestible to the host. (Firkins and Yu, 2015). However ruminant production systems face significant challenges such diseases, parasitism, poor hygiene and nutrition which may all cause a deviation in the expected and preferred gut microbial composition. (Rinninella et al., 2019). The sampled Dohne Merino sheep in this study serve as representatives of a sheep population that reared under marginalised production systems and has tremendously been affected by parasitism (*Haemonchus contortus* and the *Ehrlichia ruminantium*).

Many previous studies have identified Firmicutes and Bacteroidetes as the most abundant phyla in the gut microbiome of ruminants (Russell, 2002, Kamra, 2005, Li et al., 2012, Malmuthuge et al., 2015). Therefore, finding these phyla dominant in the gut microbiome analysed in this study is an expected outcome and aligns with previous studies. Bacteroidetes is a very diverse phylum; also known as Bacteroidota or the Cytophaga–Flexibacter–Bacteroides (CFB) group. This name is reflective of the the diversity of organisms in this group (Thomas et al., 2011). The Bacteroidetes phylum comprises four classes: Bacteroidia, Flavobacteria, Sphingobacteria, and Cytophagia. All the classes in Bacteriodetes are aerobic except for Bacteroida; therefore, that explains why Bacteriodia is the only class belonging to this phylumfound in this study as there are very limited amounts of oxygen in the gut (Thomas et al., 2011).The breakdown of complex biopolymers in the gut is Bacteroides' principal biological function, which is also the most researched. Furthermore, bile acid metabolism and the conversion of harmful and carcinogenic substances are processes carried out by these bacteria. (Thomas et al., 2011). Additional roles played by Bacteroidetes include enhancing host health by interacting with the immune system to trigger T-cell-mediated responses. (Mazmanian et al.,2008). Bacteroides have been identified as one of the first microorganism to appear in newly born animals (Wexler, 2007).

Conversely, certain Bacteroidetes species exhibit highly pathogenic behavior, and despite being a part of the normal GIT microbiota, they can nonetheless result in opportunistic infections if the intestinal mucosal barrier is compromised. (Smith et al., 2006). Members of the Bacteroidetes have also been identified to be noxious, as some strains possess virulence factors (Jotwani and Gupta, 1991). One of the Bacteroides species found in this study, Bacteroides fragilis, has been linked to polymicrobial diseases with a mortality rate of more than 19%. It can cause infection if it is displaced from the gut into the bloodstream or surrounding tissue after surgery, illness, or trauma (Wexler, 2007, Brook, 2008).

Firmicutes, the second most populous phylum, is composed primarily of Gram-positive bacteria, many of which are found in natural environments. According to current estimates, the phylum contains around 250 genera that are grouped into three primary classes: Clostridia (Genera: Pectinatus, Sporomusa, Acetobacterium, etc.) Mollicutes (Genera: Mycoplasma, Ureaplasma, and Spiroplasma, etc.) and Bacilli (Genera: Lactococcus, Listeria, and Pediococcus, etc.). Seven key classes make up certain taxonomic systems: Thermolithobacteria, Clostridia, Bacilli, Erysipelotrichia, Negativicutes, and Limnochordia (Rinninella et al., 2019). Firmicute classes found in the gut of sheep from the current study include *Bacilli, Clostridia and Negativicutes.* Many of the members of this phylum break down carbohydrates that can't be digested by the hosts' enzymes, such as dietary fibre and resistant starch (Rinninella et al., 2019). *Prevotella,* the second highest abundant genus in this study, forms part of the most dominant microbial enterotypes in the gut of many ruminants (Stojanov et al., 2020). Commensal *Prevotella* bacteria in the gut contribute to polysaccharide breakdown. However, studies also suggested some *Prevotella* species as potential intestinal pathobionts (Precup and Vodnar, 2019). Khalil et al., 2022 stated that further studies are still needed in order to reveal health- or disease-modulating properties of *Prevotella* species in the gut (Khalil et al., 2022).

Because much of the Wauldby farm is sourveld, the Wauldby sheep are particularly benefiting from Bacteroides and Firmicutes' remarkable capacity to breakdown complex polysaccharides. Sourveld forage has lower nutritional yields and is very difficult to digest in winter. Therefore, the large abundances of Firmicutes and Bacteroides is suggestive of the sheep's ability to digest sourveld forage effectively persists even in winter.

The genera *Bacillus and Clostridium* that were both identified in this study, constitute the family *Bacillaceae* family and order Bacillales. *Bacillus* has antimicrobial effects and is involved in the fermentation of foods. They've been discovered to assist with irritable bowel syndrome, healing underlying inflammation and disturbance in the gut microbiota (Manson et al., 2008, McCracken and Lorenz, 2001). Previous studies have proven that the environment is one of the biggest influencers to shaping the gut microbiome in livestock. (Song et al., 2020). Since livestock raised on open pasture are exposed to various pathogens and parasites. We therefore theorise that the observance of microbes with such defense mechanisms as the ones mentioned above (controlling inflammation, disturbances in the gut microbiome) was induced by the exposure to pathogens since Wauldby sheep are raised on an open pasture and are free grazing and the farm has faced significant challenges with parasites and pathogens such as Haemonchus *contortus* (wireworm) and *Eschrlichia ruminantium*. For a more conclusive assertion on of this theory, a study comparing the gut microbiome of pasture raised and confined Dohne Merino would have to be performed. Commensal *Clostridia* forms part of the microorganisms that start to colonise the intestine in breastfed animals during the first month of life (Lopetuso et al., 2013, McCracken and Lorenz, 2001). Butyrate is released as a fermentation byproduct during Clostridium digestion. (Pryde et al., 2002). The majority of butyrate generation in the gut occur among anaerobic bacteria, specifically those that are abundant in the Clostridial subphylum (Lopetuso et al., 2013), one of the subphylums found in abundance in this study. There is a variety of positive effects rendered by butyrate. Butyrate functions to regulate the transepithelial fluid transport, mucosal inflammation, oxidative status, reinforces the epithelial defense barrier, and modulating visceral motility (Canani et al., 2011, Comalada et al., 2006). Visceral motility is especially of importance to ruminants because the rumeno-reticulum contractions (forming part of visceral motility) functions to mix ingesta with digesta, inoculates ingesta with microbes and also assists in regurgitation for rumination (Waghorn and Reid, 1983). Whilst the defense properties offeredby the Clostridial subphylum reaffirms the theory mentioned above about the environment of pasture raised animals playing a role in the shaping of the gut microbiome by placing a need for defense against parasites and pathogens the animals are exposed to in the field (Lopetuso et al., 2013).

According to McGorum et al. (2015), cyanobacteria are a diverse group of common environmental bacteria that have the ability to produce a large variety of cyanotoxins. According to research, cyanotoxins from aquatic cyanobacterial blooms, scums, and mats are known to cause hepatic and neurological disorders in both birds and animals (Baldwin and Whitton, 1992). Nevertheless, there has never been a report of grazing cattle being exposed to terrestrial Cyanobacteria as opposed to aquatic ones. It has only been hypothesised that grazing livestock is exposed to *Cyanobacteria* that can grow on turf grasses can suffer diseases and illness related to such hepato- and neuro-diseases caused by dermotoxins released by

Cyanobacteria (Tredway et al.,2006, McGorum et al., 2015). *Cyanobacteria* have only been associated with negative effectsin the guts of animals; therefore, it is preferred that they be found in low abundances such as the ones observed in this study(0,45%). In addition to the already known parasites (*Haemonchus contortus* and the *Ehrlichia ruminantium,* Cyanobacteria may be one of the pathogenic microbes that induced the presence and abundance of health defense related microbes.

Actinobacteria are part of the phyla that are often found in very low abundances (Li et al., 2021). Despite making up a very small portion of the population, they play a significant role in maintaining gut homeostasis (Binda et al., 2018). Some of the biological functions of *Actinomycetes* (*Actinobacteria* order found in this study) include production of key metabolites such as formate, acetate, succinate, lactate, and various antibiotic and the metabolic compounds (Hanning and Diaz-Sanchez, 2015). These metabolites have a variety ofvital functions such as, carbohydrate digestion, body-weight modulation, oxidation reactions and inflammation control (Tretter et al., 2016, González Hernández et al., 2019, Wang et al., 2020). They have been detected as one of the early colonizers of the gastrointestinal and oral tract (Favier et al., 2002). However, it should be noted that Actinobacteria are associated withboth health and disease (Li et al., 2018). *Actinomyces* are members of the Actinobacteria community, that have been identified as a causative agent of actinomycosis, a disease that causes swelling in the area of the maxilla and mandible of livestock (Fitzgerald et al., 2007).Lesions in the tongue, intestines, lungs, and liver are sometimes caused by caused by Actinomyces overgrowth. Internal organ actinomycotic lesions are typically discovered after death (Becker et al.,1964). However, this disease-causing genus of the Actinobacteria phylum was not found in thisstudy, but rather the *Propionibacterium australiense* species belonging to this phylum was found, which the species is linked to Granulomatous Bovine Lesions (Bernard et al., 2002). This species has not been reported in sheep in previous studies.

The adaptability and hardiness of Dohne Merino sheep have often been cited as the key traits contributing to the breed's success over time (Dzomba et al., 2023 {Cloete, 2023 #288)}. It was reported that following repeated infections with the wireworm, the sheep on the participating farm (Wauldby) started to naturally select for resistance *to Haemonchus contortus.* The farm eventually became a part of a trial research to establish to establish practical and effective ways to select for sheep that are resilient and resistant to Haemonchus contortus (Dlamini et al., 2019 {Burgess, 2016 #269)}. These reports, along with the fact that this study found a highabundance of microbes that digest difficult, low-nutrition forage, both defense- and disease- related microorganisms while the sheep showed no signs of the associated diseases, reaffirms the concept the of breed resilience in Dohne Merino sheep.

The majority of the abundant genera in this study (Figure 3.13) was found to be the same as the abundant genera in the Henderson et al. (2015). This includes *Fibrobacter, Prevotella, Ruminococcus, Eubacterium, Succinivibrio, Pseudosuccinivibrio, Succiniclasticum and Treponema.* The threshold for significant relative abundance was > 0,15%, therefore the list of significantly abundant genera in this study included other genera that were not recognised as abundant in the Henderson et al. (2015). These include genera like *Ruminobacter, Saccharofermentans, Bifidobacterioum, Eubacterium and Sodaliphilus,* which most have not been recognised as biologically important in literature, as they do not play key roles in digestion, homeostasis and immune defense (La Reau and Suen, 2018, Arshad et al., 2021, Wang et al., 2021). But they are a good indicator of the high diversity in the gut of the sheep in this study.

In the current investigation, bacteria comprised more than 80% of the total microbial population. The overall bacterial population of this study is very diverse, with a total of 1000 known species across the different compartments. This reflects that the bacterial population was far more diverse and richer compared to the archaeal population. Archaea has been reported as the next most abundant phylum in the gut after bacteria, ranging between 0,1 -23%. (Kim et al., 2020, Levy and Jami, 2018, Roehe et al., 2016). This study's findings align with previous research as the total archaeal population falls within that range (16,7%). The archaeal domain has been expanding over the years with new branches being added at the different levels of classification (Adam et al., 2017). According to the List of Prokaryotic names with Standing in Nomenclature(LPSN), there are nineteen known archaeal phyla (Parte et al., 2020). Of these, only three were observed in this study, and included *Euryarchaeota, Candidatus Thermoplasmatota and Candidatus Asgardarchaeota*, which is considered as an archaeal superphylum not phylum. Only two known archaeal classes were identified, *Thermoplasmata* and *Methanobacteria*.

Functionally diversified metabolic pathways, including as the Wood-Ljungdahl route as a carbon-fixation approach, potential nucleotide salvage pathways, and novel methods of phototrophy, such as new rhodopsins, are encoded in the genomes of Asgard archaea (MacLeod et al., 2019). Asgard archaea are also active in nitrogen and Sulphur cycling (Liu et al., 2018, Seitz et al., 2016). In addition to several lineages of uncultivated archaea with unknown characteristics, the Euryarchaeota phylum includes both methanogenic and nonmethanogenic orders (Spang et al., 2017). *Methanobacteria* are part of the methanogens, a group microorganism that perform fermentation of feed and produce methane as an end-product of fermentation (Hook et al., 2010). In a study by Wright et al., (2004) *Thermoplasmata* have also proven to fall under methanogens that ferment feed and produce methane gas. Because few cultures and genomes are available, uncharacterized *Thermoplasmata* metabolisms remain unexplored (Huet al., 2021).

Methanogens utilise substrates made by other microbes, including acetate, formate, methanol, hydrogen (H2), and carbon dioxide (CO2) during fermentation. Methanogenesis pathways convert hydrogen into methane gas, which helps to maintain low hydrogen partial pressure and speed up the digestion of fiber in the rumen, this helps oxygen levels to not reach excessive and toxic levels. Despite the fact that this activity is an essential and normal aspect of gut function, it also has negative impacts because the methane released during this process accounts for a major amount of the agricultural greenhouse gas (GHG) emitted and represents a significant loss of dietary energy (6–10%) for the host animal. (Rust and Rust, 2013, Holman and Gzyl, 2019). GHG are the main drivers of the challenge ofglobal warming (Naqvi and Sejian, 2011). GHG from ruminant meat production are significant, it is suggested that regulations should be put to ruminant meat production in orderto achieve the goal of reducing global warming (Del Prado et al., 2023). However, those regulations might have adverse effects on the economy and food security globally as the humanpopulation continues to place a high demand on livestock meat production for the maintenanceof food security (Godber and Wall, 2014).

In an effort to create vaccinations against methanogens that would reduce the amount of methane released by ruminants, Wright et al. (2003, 2004) enriched, isolated, and conducted phylogenetic surveys on methanogens. Results from those studies revealed that most methanogens from the gut contents of sheep and cattle belonged to the genus *Methanobrevibacter* (Wright and Pimm, 2003, Wright et al., 2004). Findings from the current study are consistent with the findings by Wright et al., as *Methanobrevibacter* was found to be the most abundant archaeal genus. Four species of *Methanobrevibacter* were identified: *millerae, ruminantium, sp. YE315* and *alleyae* in this study. This lack of diversity was expected as earlier research has demonstrated that the methanogen community in other sheep, including Indian sheep, seems to be more diversified than that of Merino sheep, with Indian sheep having 9 genera and 20 species of methanogens. (Malik et al., 2022). Methanogen diversity was higher in pasture-grazed sheep compared to sheep given either oaten hay or lucerne hay in a study conducted by Wright et al. (2004). We believe that this study has provided valuable insight into the leveldiversity of methanogens in the gut of Dohne Merino sheep in South Africa. However, more studies are needed to compare methanogens diversity of both pasture grazing and kept hay feeders to better if reveal if free grazing will sheep have a greater diversity of methanogens inSouth African sheep and if that has any significant effect on the host.

Overall, the analysis of the archaeal population from this study reveals that the archaeal population of the sheep is made of mostly methanogens and only a minute proportion of the archaeal population was found to have other functions besides methanogenesis. One of the archaeal species found in this study, Candidatus Methanomethylophilus alvus, has strains that have been found to encode for Pyrrolysyl-tRNA synthetase (PyIRS). PyIRS is a member of the family of enzymatic proteins whose function involves the cellular process of tRNA aminoacylation required for protein translation in mammals. (Meineke et al., 2018). The relatively high abundance of methane producing archaea is a calamity to the environment considering that global warming continues to be a major threat to life on earth (Spang et al., 2017).

The use of metagenomics analysis in this study allowed for detection of low abundance members in the microbial community, such as *Thermoplamatota, Elusimicrobiota Actinobacteria, Cyanobacteria, UBP6, Campylobacteria, Asgardarchaeta and Myxococcota* in this study. This is the added advantage of the metagenomics approach for microbial population analysis, detecting uncultured and low abundance members which may be missed with other approaches (Escobar-Zepeda et al., 2015). However, the metagenomics approach also reveals that there is still a limitation on the existing databases used for taxonomic classification as a significant portion of the overall microorganisms could not be classified.

The different stomach compartments have comparable levels of diversity although the reticulum tended to have the most diverse population and the abomasum the least diverse. The small differences in the diversity and the richness of the stomach compartments are possibly influenced by the functions and physiological conditions of the different chambers. The rumen, reticulum, and omasum are where pregastric fermentation and feed breakdown takes place, whereas the abomasum—also referred to as the actual stomach—is comparable in structure and function to the stomach of a non-ruminant animal. This means that the abomasum environment is more acidic than the other compartments (Swanson, 2019). The reticulum and omasum have considerably higher prokaryotic diversity than the rumen and abomasum, according to a recent study by Xue et al. (2018) that examined the microbial diversity of the four stomach compartments in Yak. Furthermore, Xue and colleagues stated that the stomach compartments' physiological conditions such as pH, VFAs, redox potential and nutrient supply all strongly determine the composition in each compartment. Therefore, extremely low pH in the abomasum may help explain the reduced level of diversity observed in the abomasum.

3.6 Conclusions

The study population showed a significant degree of diversity with the majority of the organisms being bacteria, then followed by a lesser fraction of archaea. This is the anticipated typical microbial composition ratio in ruminants' guts, reflecting normal gut function and feed digestion. Many of the microbes had overlapping functions with the most represented biological function being the digestion/fermentation of complex feed material and plant-fibre, which expected and beneficial for pasture-grazing sheep especially in a sourveld. Other represented functions include homeostasis, immune defense, cell growth, antibiotic properties, and metabolism. Which we can conclude are as a result of the populations continued exposure to parasites such as *Haemonchus contortus* and Ehrlichia *ruminantium*. While the majority of the microorganisms are advantageous to the health and welfare of their host, others possess harmful qualities and have been linked to illnesses and diseases. However, the low abundance of disease-associated microbes (Actinobacteria and Cyanobacteria), absence of illness symptoms, presence of immune defense microbes related (*Bacillus* and *Clostridia*) and overall high degree of functional redundancy in the microbial population analysed in this study indicate a healthy gut microbial population and environment for the sheep. This is a sign of resilience of the Dohne Merino breed since the sheep are exposed to pathogens and parasites in the pasture but still maintain a relatively healthy gut microbial environment. Based on the findings of this study we agree with research that states that additional external factors such grazing environment, host species/breed and diet also influence the composition in each of the compartments. Additionally, we recommend that a study that compares pasture raised and kept grass-fed Dohne Merino be performed for a better understanding of the environmental effect on the Dohne Merino gut microbiome. In this this study, the reticulum was the most diverse compartment. The findings in this study are not consistent with claims in literature that identify rumen as the most diverse compartment we believe this is because the majority of research were rumen-focused anyway. We thus recommend more research that would examine all four compartments for a more accurate view of the whole gut microbial populations of all ruminants.

4 THE GUT VIROME OF DOHNE MERINO SHEEP

4.1 Abstract

Gut viruses are the least understood entity in the gut microbial population of ruminants. This is because viruses are typically more challenging to study than the other members of the microbial population. Additionally, bacteria are deemed of greater importance because of their role in breaking down complex material in the gut. However, viruses are equally important as they are vital in controlling the microbial population numbers and homeostasis. This section of the study was aimed at profiling the gut virome of Dohne Merino. Previously sequenced data (section 3.3 in chapter 3) was analysed using viral metagenomics tools. CheckV, GeNomad and Kaiju2 tools were utilised to detect viral genomes and assign taxonomy. The composition of the virome was found to consist of 5 realms, 8 kingdoms, 13 phyla, 21 classes and 37 other members classified either at an order or family level. This is a very diverse population of viruses. Majority of the viruses (70%) were phages of the Duplodnaviria realm. This was reported as a plausible finding because viruses depend on the host for survival, so if bacteria were the most abundant kingdom identified in the characterisation of the microbial population (chapter 3) the phages had more bacterial cells to be hosted in. The viruses were found to possess a range of viral nucleic acid structures (dsDNA, -ssRNA, +ssRNA, ssDNA and dsRNA), with double-stranded DNA being the most common at 65,7%. It was also observed that not all the viruses in the gut infect prokaryotes, but some, like the RNA viruses of the Riboviria realm have been linked to various diseases in eukaryotes. Horizontal gene transfer and cross-species interaction was also suspected to contribute to virome diversity, as some viruses were found to be naturally hosted in plants or marine species. A comparison of the different gut compartments revealed different viral diversities and compositions in the compartments. Based on the outcomes of this study and the microbial composition analysis study in Chapter 3 it was concluded that the gut viral population is influenced by the observed in the microbial hosts population of the compartments. It was concluded that there is a probable relationship between the functions of the compartments, the composition of the microbial population and the virome of the gut that needs to be researched further.

4.2 Introduction

The gut ecosystem of ruminants is a diverse and complex environment that hosts microbial organisms such as bacteria, archaea, fungi, protozoa, and viruses (Lobo and Faciola, 2021).

The variety and abundance of virus populations frequently exceeds that of the microbial communities they coexist with and prey upon (Gilbert et al., 2020a). However, viruses are still understudied in any given ecosystem, despite their ubiquity. (Parmar et al., 2016). The first identification of viruses as inhabitants of the rumen happened inthe 1960s (Hoogenraad et al., 1967, Paynter et al., 1969), closely aligned with the development of technologies to study viruses. Before that, viruses were considered temporary to gut intestinal tract (GIT) of ruminants, ingested with feed or water (Martha et al., 2011). With thedevelopment of new detection and taxonomic classification tools, the viral population of the ruminant gut has been hypothesised to play important roles in the maintenance of the microbialpopulation, intra-ruminal microbial lysis, nutrient cycling and genetic transfer (Klieve et al., 1991, Solden et al., 2018, Lobo and Faciola, 2021). However, the efforts and resources devotedto understanding the viral population associated with the gut microbial population of ruminantsare still considerably lower than that given to the other members of the microbial population (Gilbert et al., 2020a).

The gut viromes is significant in gaining a comprehensive understanding of the gut microbiome, additionally According to Kwok (2020), gaining a better understanding of livestock virome is essential for identifying possible zoonotic infections and preparing for One Health. Determining the characteristics of virus metagenomes particular to a species may be used to establish a monitoring baseline for early detection and to monitor the spread of infections among various hosts. (Mihara et al., 2016). While the combination of metagenomics with essential meta data such as age of livestock and farm types can provide better comprehension of what is the normal diversity, what is rare, novel, and infectious in the virome of livestock (Namonyo et al., 2018). Small ruminants (sheep and goat) were found to be the least investigated category in a review of viral metagenomics studies on farm animals, with just 6 out of 121 studies devoted to them. (Kwok et al., 2020). This indicates an information gap in the field of virome investigations of small ruminants, which this work intends to address.

Bacteriophages are recognised as the normal and most common viruses in the gut of ruminants. Viruses that infect bacteria and archaea are often grouped together and referred to as phages due to their similarity (Gilbert et al., 2017). Past research indicates that the most prevalent viruses in the ruminal environment are phages belonging to the families Myoviridae, Siphoviridae, Mimiviridae, and Podoviridae. (Bergman, 1990, Anderson et al., 2017). In the gut, phages play an essential role of keeping the gut microbiome abundance and diversity within the guts' carrying capacity (Friedersdorff et al.,2020). Due to phages' extreme host specificity, the abundance of phage populations frequently correlates with the abundance of the bacteria that serve as their hosts. (Payet and Suttle, 2013).

Since the gut bacterial and archaeal diversities differ in different species, diet types, ages and types of animal-keeping practices, the viral diversity is expected to differ and be shaped by these dynamics as well because viruses depend on their bacterial and archaeal host for survival in the gut. Studying different breeds in different environments is essential for a clear definition of what is the normal avian species virome. This study is the first to perform a gut virome analysis of Dohne Merino sheep in the Eastern Cape province, the place of origin where Dohne Merino was first developed to specifically withstand the harsh environmental conditions in the area. Sheep are the most profitable livestock in South Africa and Dohne Merino remains one of the most important breeds since its development in the country. It is on this rationale the Dohne Merino in the Eastern Cape province was the selected study population.

In addition to providing insights on one of the most significant sheep breeds in South Africa in its' original intended habitat, this study will aid in the extension of the currently limited ovine-specific virome data. Although advances in technology have allowed gut viruses to be better understood (especially the phages), very little has been uncovered about the resident viruses of the gut of sheep and there is more to the virome than phages. Therefore, this study aims to characterise the gut virome (phage and non-phage) of Dohne Merino sheep using the sequencing and metagenomics approach. This will be done through analysis of illumina sequenced data while also taking in consideration of meta data such as health status of the animals as well as farming practices.

4.3 Materials and Methods

Data that was previously sampled, sequenced and quality controlled as described in Chapter 3, section 3.3 was analysed through viral bioinformatics tools for the scanning and identification of viral genomes according to the steps below. The viral metagenomic analysis continues from the assembly step in Chapter 3 methodology where the DNA raw sequences were assembled into contigs with Spades.

4.3.1 Viral Metagenomics Analysis

CheckV (Nayfach et al., 2021) was used to for scanning contig data for viral genomes, assessment of viral genomes quality, through identification of host contamination for integrated proviruses, estimating completeness for genome fragments (report on this on results), and identification of closed genomes. Utilising GeNomad (Camargo et al., 2023), the output from CheckV was then used in geNomad to identify viruses and plasmid genomes by taxonomically classifying mobile genetic elements. Kaiju (Menzel et al., 2016) was also used to confirm the taxonomic profiles obtained from geNomad. Kaiju works by directly assigning reads to taxa using the NCBI taxonomy and reference database.

The tab-separated output files from Kaiju were converted into interactive HTML5 charts of hierarchical data using Krona (Ondov et al., 2011) for diversity analysis and visualisation . The tab-separated output files from CheckV were converted to xls files through Microsoft Excel for further visualisation and diversity analysis. The virus host database (Mihara et al., 2016) was mined for host identification of the viruses, and the International Committee on Taxonomyof Viruses database (ICTV) (Lefkowitz et al., 2018) (https://ictv.global/taxonomy) was used to find the genetic structures and characteristics of the viruses found in this study.

4.4 Results

A diverse viral population was identified in the gut of Dohne Merino sheep. Figure 4.1, provides the taxonomic tree of the overall viral population classified with GeNomad, depicting **5** realms, 8 kingdoms and 13 phyla found in sheep's gut. The most diverse realm is Monodnaviria, with 3 kingdoms identified; however, 1 phylum was identified in each kingdom. The kingdom with the most phyla was Orthornavirae, with 4 of its phyla present in this study. Overall, from figure 4.1 it can be observed that as the level of classification goes lower the diversity increases.

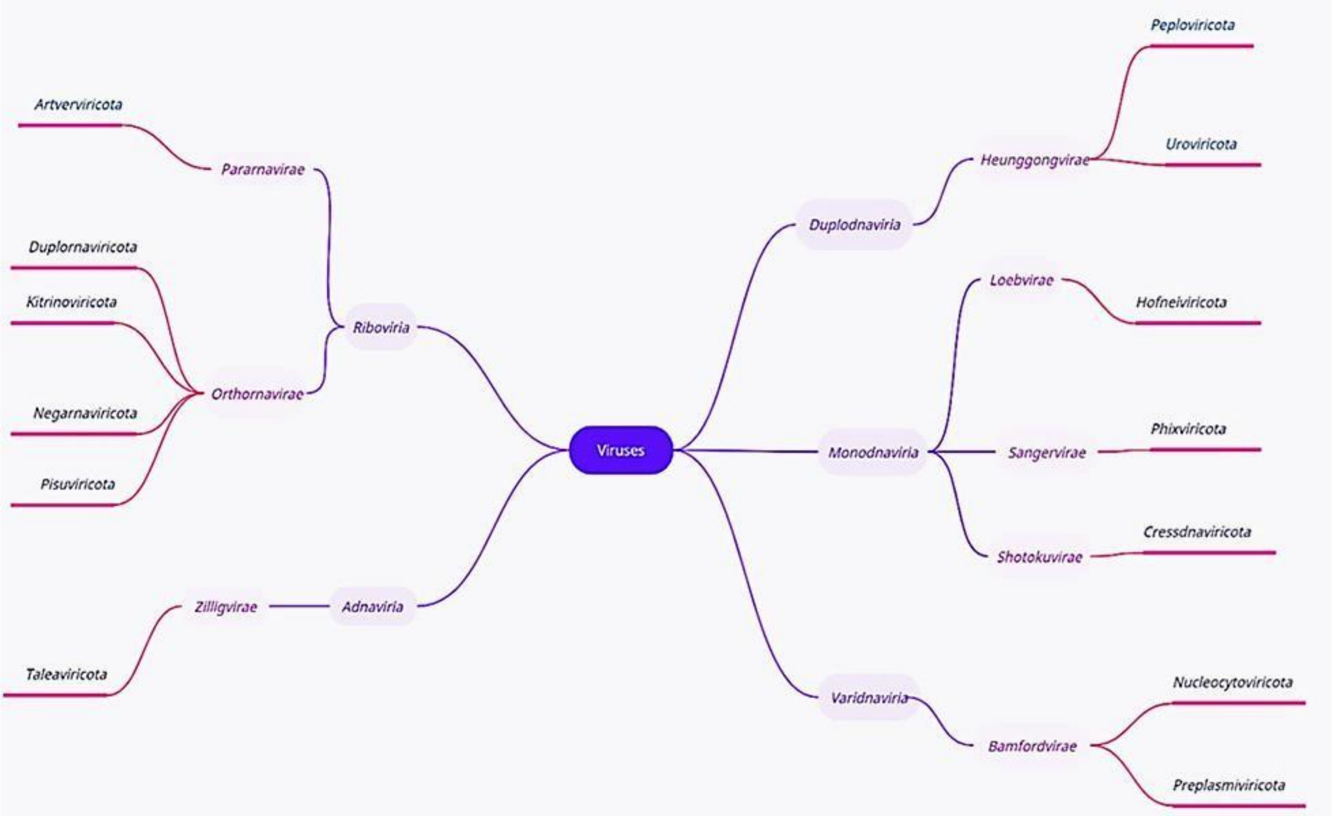


Figure 4.1 Taxonomic tree of the viral population in the gut of Dohne Merino sheep. The population was classified into 5 realms, 8 kingdoms and 13 phyla It is observed that diversity increases as the level of taxonomic classification deepens

Figure 4.2 is a Krona plot of the viral classification performed using Kaiju. A total of 85,981 sequences were classified, and only 162 (0.01%) of the sequences were unidentified at a realm level. The Kaiju classification revealed that many of the viruses belonged to the realms Duplodnaviria (77%) and Varidnaviria (21%); other realms that were present but at very low abundances include Riboviria, Monodnaviria and Adnaviria. These are the same realms that were identified through GeNomad, however some of the names that are shown in Figure 5.2 as being other present realms are not consistent with the currently acceptable viral classification system (i.e., Naldaviricetes is a viral class, not realm).

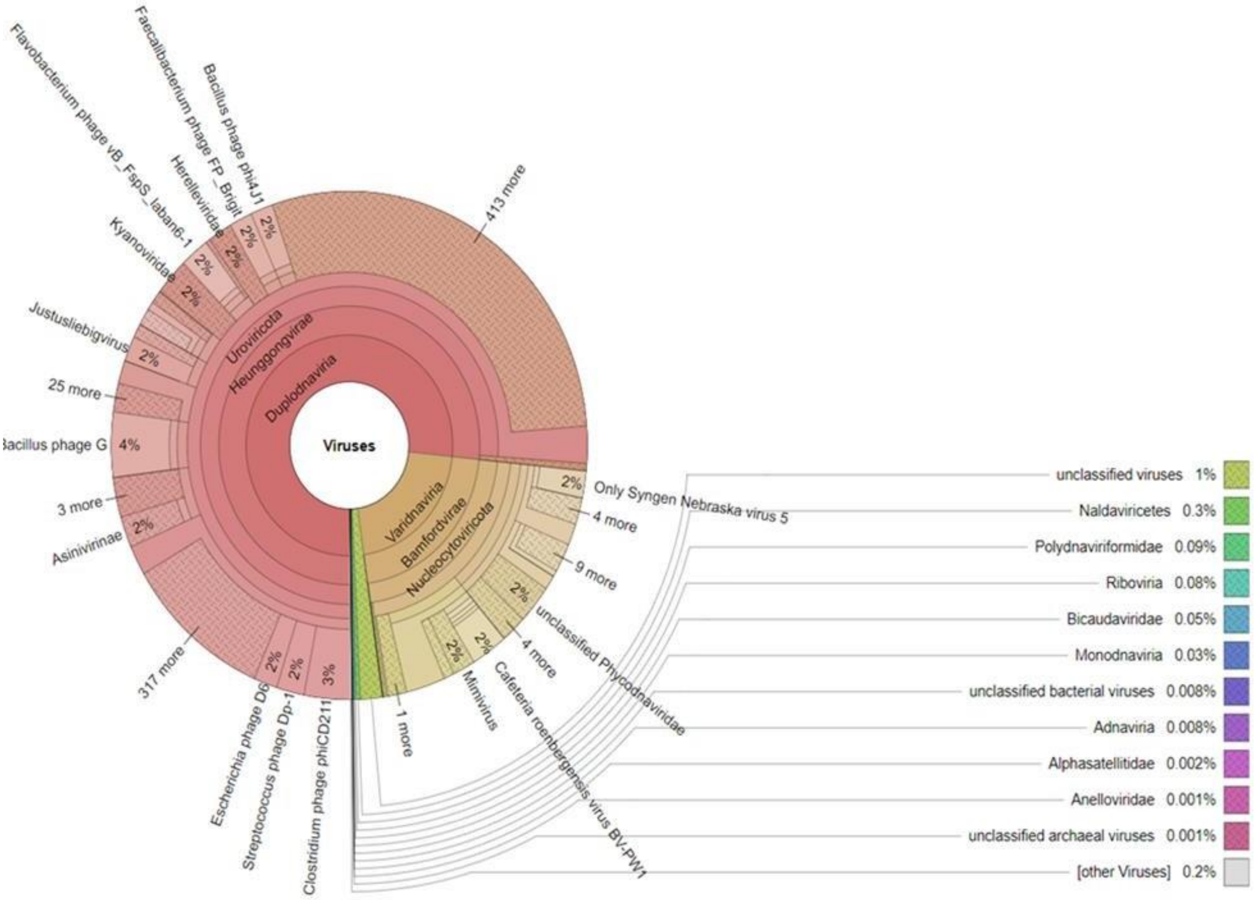


Figure 4.2 Krona plot of viruses in the gut of Dohne Merino sheep. More than half of the of the population belongs to realm Duplodnaviria. Varidnaviria is the second most abundant phyla (21%). Other present realms include Riboviria, Adnaviria and Monodnaviria.

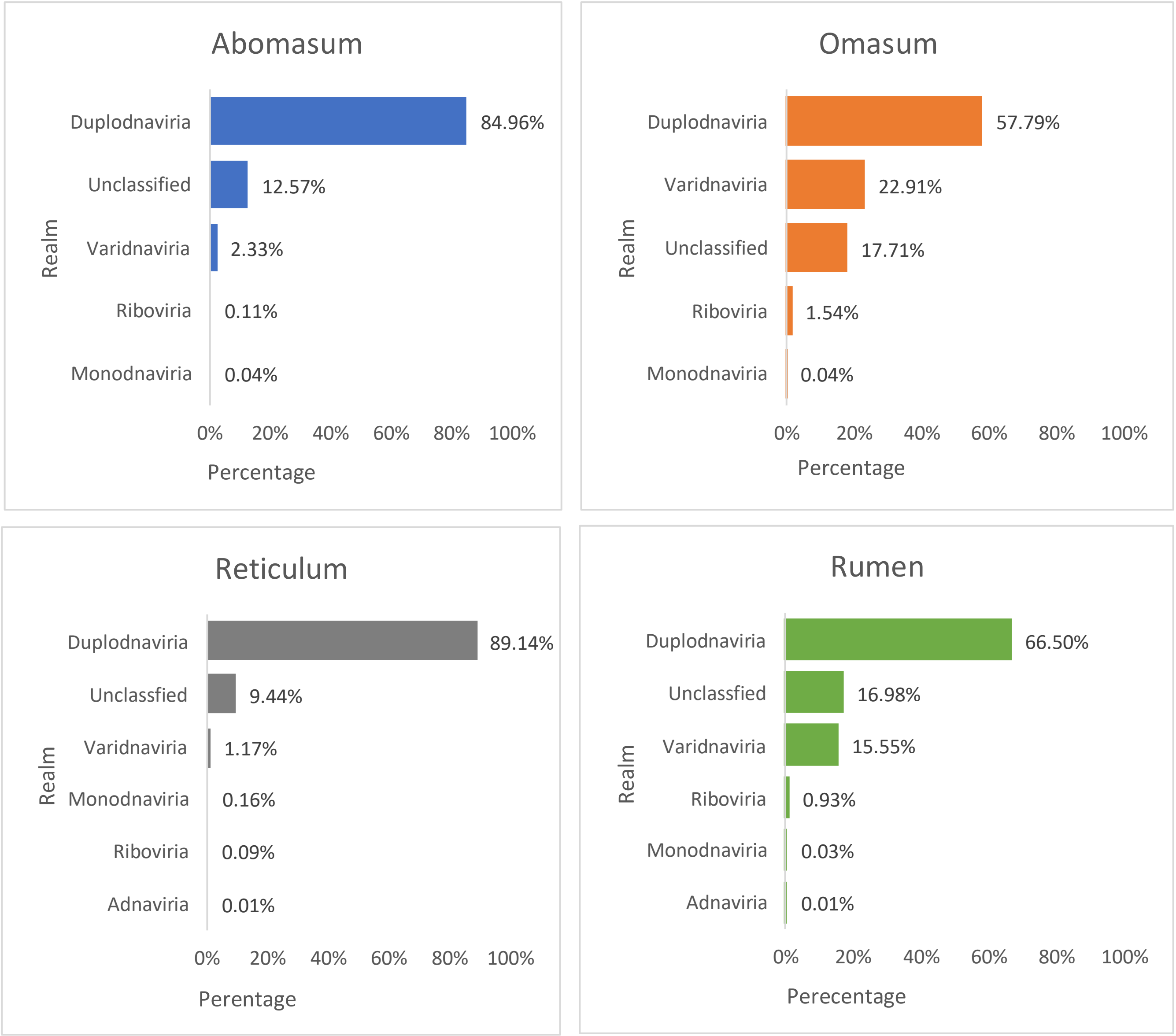


Figure 4.3 Viral composition of the four gut compartments of Dohne Merino. 1:Abomasum, 2:Omasum, 3: Reticulum, 4: Rumen. Duplodnaviria was found at a high prevalence across all four chambers.

In Figure 4.3, the viral realm compositions of the four compartments are depicted. In all the compartments, Duplodnaviria is the most abundant, with the reticulum having the highest abundance (89,14%), followed by the abomasum (84,96%), then the rumen (66,50%) and lastly the omasum (57,79%). Additionally, all the compartments had unclassified viruses ranging between 9,4% and 22,9%. The abomasum and the omasum were found to have 4 known realms, while the reticulum and rumen had 5. The additional realm that the rumen and reticulum had, which was not found in the other two compartments, was Adnaviria.

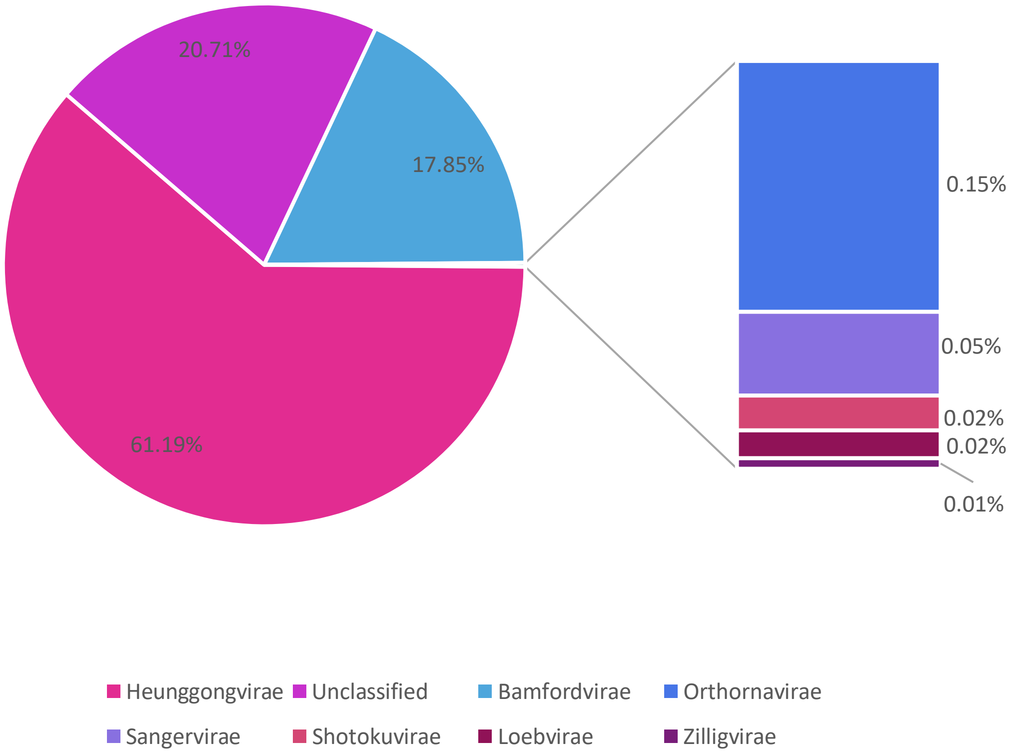


Figure 4.4 Pie chart depicting the overall kingdom diversity and abundance of the population of viruses in the gut of Dohne Merino sheep. Seven viral kingdoms were identified, with most of the viruses belonging to the Heunggongvirae kingdom(61%). . Approximately 21% of the population was unidentified viruses.

At a kingdom level, 7 kingdoms identified with Heunggongvirae making up about 61% of the total gut viral population, followed by unclassified viruses making up 20,7% and then Bamfordvirae at 17,9% (Figure 4.4). Other present kingdoms included Orthornavirae (0,15%), Sangervirae (0,05%), Shotokuvirae (0,02%), Loebvirae (0,02%) and Zilligvirae (0,01%).When the kingdoms are viewed by compartment, the following observations (illustrated in Figure 4.5 below) can be made: the Heunggongvirae kingdom was the most abundant in all four compartments, making up about 89% in the rumen, 86% in the reticulum, 66% in the rumen and 58% in the omasum. Other kingdoms found in all compartments included Bamfordvirae and Sangervirae in that order of abundance. Orthornavirae, Loebvirae and Shotokuvirae were absent only in the abomasum, while the Zilligvirae kingdom was exclusiveto the reticulum and rumen.

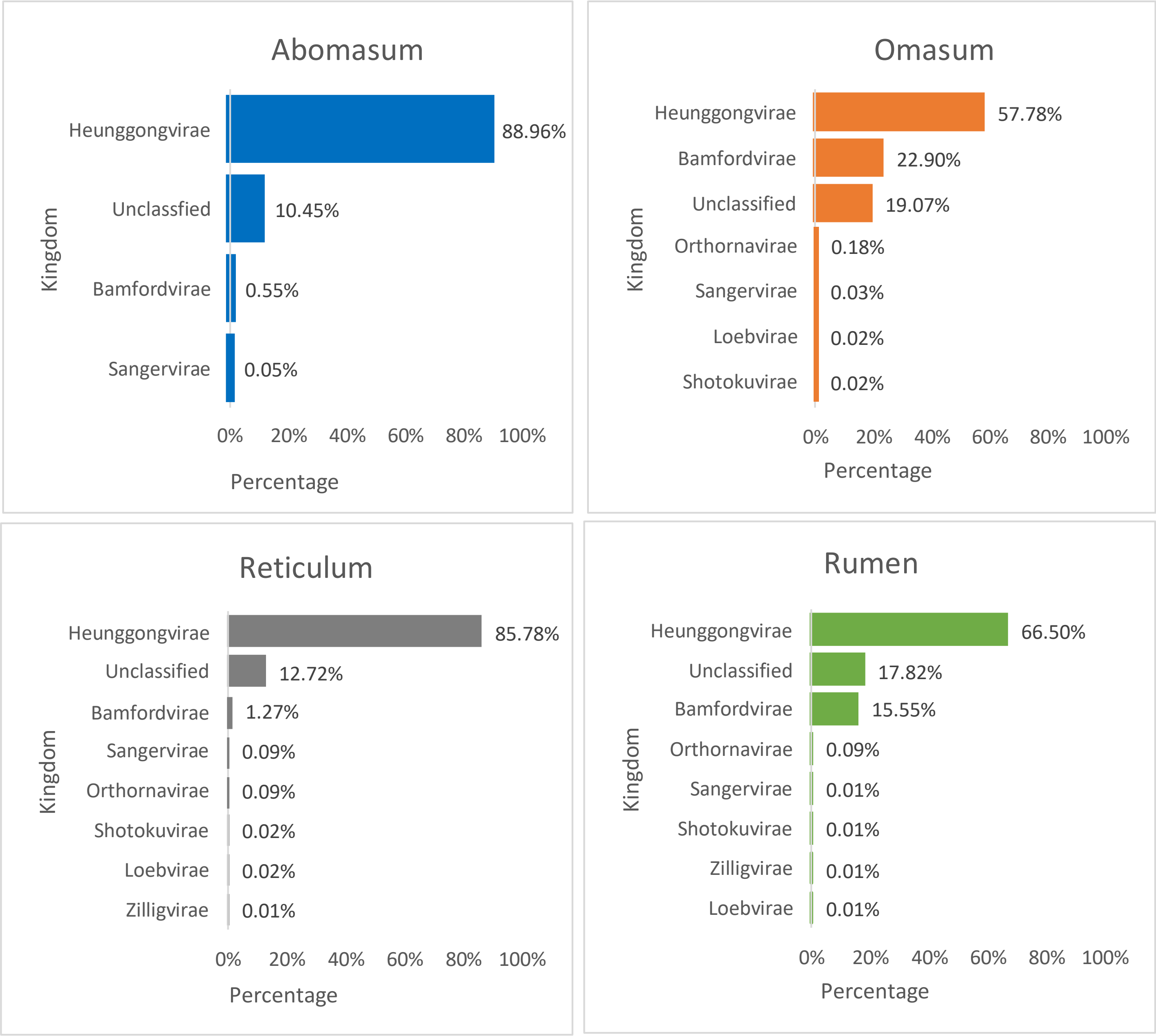


Figure 4.5 Abundance of viral kingdoms in the four stomach compartments of Dohne Merino sheep. The rumen and the reticulum had the highest diversity with 7 kingdoms each, followed by the omasum with 6. The omasum was the least diverse with only 3.

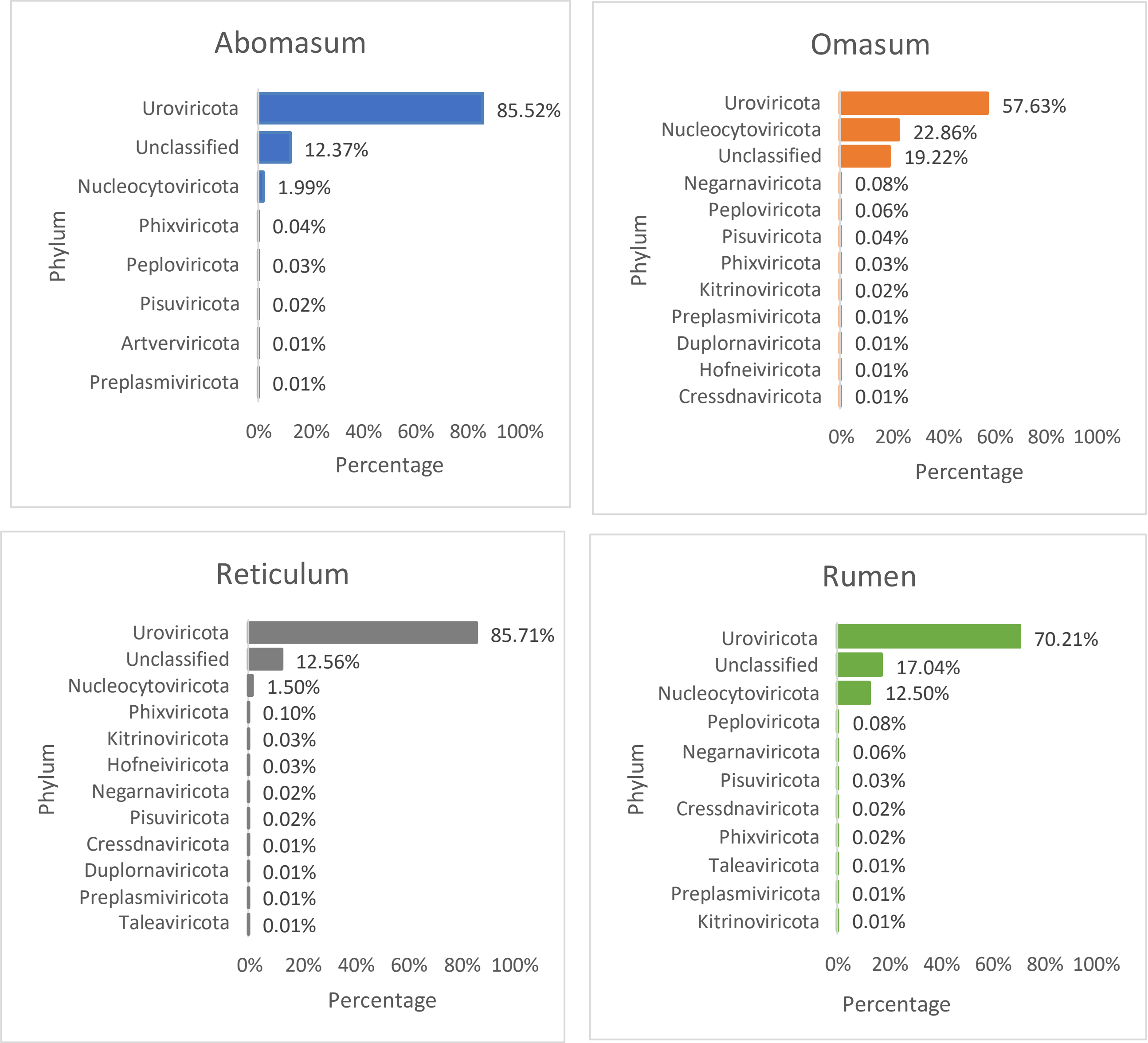


Figure 4.6 Phylum diversity of viral population in the four stomach compartments of Dohne Merino sheep. The phylum diversity analysis reveals a high diversity across all the four compartments with an overall total of 13 phyla across the entire gut.

The diversity of the viral phyla for the four compartments is seen in Figure 4.6 above. As briefly mentioned above, thirteen known phyla were present in the population. The omasum and the reticulum had the most significant number, with 11 phyla each; the rumen had 10, and the abomasum had 7. The Uroviricota phylum was found in a high abundance across all four compartments, with abundance percentages ranging from 57,63% to 85,72%. Nucleocytoviricota, Phixiviricota, Preplasmiviricota, and Pisuviricota are other phyla found in all four compartments. The rumen, reticulum and omasum had the following phyla in common: Negarnaviricota (0,02-0,08%), Kitrinoviricota (0,01-0,03%), and Cresssdnaviricota (0,01-

0,02%). The omasum and the reticulum had Duplornaviricota (0,01% in both) and Hofneiviricota (0,01% and 0,03% respectively) in common, whilst the omasum and the reticulum and the rumen had Teleaviricota (0,01% in both) in common. Peploviricota (0,03-0,08%) was found in all compartments except for the reticulum, and Artiveviricota was found in the abomasum only, making the abomasum the only compartment with a phylum that is unique to it. All the compartments had a certain percentage of unclassified viruses ranging from 19,2% to 12,4%.

Table 4.1 Distribution of viral classes in the four stomach compartments of Dohne Merino

Class	**Compartments identified in**	**Percentage**
Caudoviricetes	Abomasum, Omasum, Reticulum, Rumen	69,90%
Unclassified	Abomasum, Omasum, Reticulum, Rumen	16,26%
Megaviricetes	Abomasum, Omasum, Reticulum, Rumen	12,24%
Pokkesviricetes	Abomasum, Omasum, Reticulum, Rumen	1,39%
Herviviricetes	Abomasum, Omasum, Reticulum, Rumen	0,06%
Malgrandaviricetes	Abomasum, Omasum, Reticulum, Rumen	0,04%
Yunchangviricetes	Omasum, Reticulum, Rumen	0,04%
Duplopiviricetes	Abomasum, Omasum, Reticulum, Rumen	0,02%
Alsuviricetes	Omasum, Reticulum, Rumen	0,02%
Arfiviricetes	Reticulum, Rumen	0,01%
Maveriviricetes	Abomasum, Omasum, Reticulum, Rumen	0,01%
Faserviricetes	Omasum, Reticulum, Rumen	0,01%
Tokiviricetes	Reticulum, Rumen	0,005%
Stelpaviricetes	Omasum, Reticulum	0,005%
Ellioviricetes	Omasum	0,003%
Insthoviricetes	Reticulum	0,002%
Vidaverviricetes	Reticulum	0,002%
Revtraviricetes	Abomasum	0,002%
Resentoviricetes	Omasum	0,002%
Chrymotiviricetes	Omasum	0,002%
Monjiviricetes	Rumen	0,002%

Table 4.1 shows the identity of the classes (21 in total) reported in this study including information on the compartment from which the classes were observed. The reticulum had the highest number of classes with 14 classes (two being unique to it); in the omasum, 13 classes were identified (two unique to omasum only), 12 were found in the rumen and 9 in the abomasum, each having only one unique class. This makes the reticulum the most diverse and

abomasum least diverse at a class level. The high viral diversity of the reticulum is also observable in other upper levels of classification, such as in the kingdom diversity and phylum diversity in Figures 4.5 and, 4.6 respectively, where the reticulum had the highest diversity with seven kingdoms and eleven phyla.

Table 4.2 Nucleic structures and known hosts of identified viral families and orders

Order/Family	Nucleic acid	Known host
Algavirales	dsDNA	Eukaryotic algae
Caudovirales	Linear dsDNA	A broad range of bacteria
Imitervirales	Large dsDNA	Protists
Crassvirales	dsDNA	Bacteroidetes
Ackermannviridae	dsDNA	Proteobacteria
Straboviridae	Linear dsDNA	Escherichia coli
Pimascovirales	Circular dsDNA	Insects
Chitovirales	Linear dsDNA	A diverse range of animal hosts
Microviridae	Linear dsDNA	Enterobacteria, intracellular parasitic bacteria
Schitoviridae	Linear dsDNA	Proteobacteria species: Vibrio algae nolyticus
Durnavirales	dsRNA	Eukaryotes in general
Herpesvirales	Linear dsDNA	Animal hosts dependent on species
Martellivirales	+ssRNA	Range of eukaryotic hosts
Kyanoviridae	dsDNA	Cyanobacteria
Priklausovirales	dsDNA	Co-dependent on other viruses
Herelleviridae	Linear dsDNA	Firmicutes
Cirlivirales	Circular ssDNA	Avian species
Zobellviridae	Linear dsDNA	Proteobacteria species: Klebsiella pneumoniae
Ligamenvirales	dsDNA	archaea of the phylum Thermoproteota
Tubulavirales	ssDNA	Gram-positive bacteria
Goujianvirales	-ssRNA	Crustacea
Thumleimavirales	dsDNA	Archaea in general
Rountreeviridae	Linear dsDNA	Gram-positive bacteria
Patatavirales	+ssRNA	Plants
Bunyavirales	-ssRNA	arthropods, plants, protozoans, and vertebrates
Methanobavirales	dsDNA	Methanogenic archaea
Mononegavirales	-ssRNA	A diverse range of animal hosts
Stellavirales	+ssRNA	Mammal and avian species
Ortervirales	-ssRNA	A diverse range of hosts dependent on family
Reovirales	dsRNA	A broad range of hosts
Vilmaviridae	Linear dsDNA	Mycobacteria
Ghabrivirales	dsRNA	Fungus: Metarhizium anisopliae.
Lefavirales	Circular dsDNA	Insects
Demerecviridae	Linear dsDNA	Bacteria in general
Drexlerviridae	Linear dsDNA	Bacteria in general
Articulavirales	-ssRNA	A broad range of vertebrates and invertebrates
Mesyanzhinovviridae	Linear dsDNA	Bacteria in general

Table 4.2 shows all viral order/families found in this study, along with nucleic structures and known hosts. Double-stranded DNA (dsDNA) viruses are the most dominant, with 63,89% of the population being dsDNA. Negative strand RNA (-ssRNA) made up 13,89% of the population, positive strand RNA (+ssRNA) and double stranded RNA (dsRNA) were 8,33% each and single stranded DNA (ssDNA) 5,56%. The viruses in this study span a wide range of hosts with those infecting bacteria are the most common. Other presented hosts include insects, plants and marine species.

4.5 Discussion

The gut microbiome of animals is highly diverse, populated by bacteria, archaea, eukaryote and viruses (Parmar et al., 2016). Gut viruses remain the least understood of these microbes and need to be a focal point in studies. (Sutton and Hill, 2019). Additionally, the recent COVID-19 pandemic has emphasised the relevance of viral studies (Harvey and Holmes, 2022). However, viruses are more challenging to study compared to other microbes in any ecosystem mainly because they are hard to culture and grow in a laboratory as they need a living host to reproduce. Furthermore, viruses are highly genetically diverse, rapidly developing creatures with a wide range of genome replication mechanisms, organisations, and virion structures (Sanjuán et al., 2021). According to Parmar et al. (2016), different viral analytical tools tend to produce varying results.

A more thorough examination of viruses has been made possible by the recent advancement of unbiased metagenomic next-generation sequencing, which has improved our understanding of the animal virome and provided new insights into the evolution of viruses (Lobo and Faciola, 2021). Metagenomic viral investigations have demonstrated that animals are home to an extensive array of viruses with different genome types, including retroviruses, single-stranded DNA, double-stranded DNA, and both segmented and unsegmented genomes. (Zhang et al., 2018b). Most of these types of viruses were also identified in the gut of the Dohne Merino sheep in this study. As of 2021, animal viruses could be placed into 5 (of 6) realms, 5 (of 10) kingdoms, 11 (of 17) phyla, 26 (of 39) classes, 36 (of 59) orders and 99 (of 189) families, according to the classification by the ICTV (Walker et al., 2021). All five realms, seven kingdoms, thirteen phyla, twenty classes, twenty-five orders and twelve families were observed in this study.

Because they can multiply and mutate quickly, bacteriophages are typically the most prevalent organisms in the biosphere. (Martha et al., 2011). Tail-tailed phages, head-tailed archaeal viruses, and evolutionarily similar herpesviruses are currently classified by the ICTV into the realm Duplodnaviria and kingdom Heunggongvirae (Evseev et al., 2023). This realm and kingdom were observed in highest abundance in this study, which aligns with many other studies that recognise the majority of the virome being phages (Martha et al., 2011,Zuppi et al., 2022, Haq et al., 2012). Most phages are tailed phages with dsDNA and are unifiedwithin the class *Caudoviricetes (Gulyaeva et al., 2022). Caudoviricetes* also encompasses tailed bacterial and archaeal viruses with capsids and a dsDNA genome, as well as viruses from the following abolished families: Myoviridae, Podoviridae, and Siphoviridae (Turner et al., 2023). This makes the Caudoviricetes class enormous and very diverse. It is therefore plausible that the virome in this study will be predominantly Caudoviricetes (70%), as most phages belong to Caudoviricetes. Caudoviricetes possess dsDNA(most abundant genome structure in this study) and are believed to be linear (Iranzo et al., 2016). Members of the class Caudoviricetes has a variety of replication processes. One can categorize a large number of Caudoviricetes phages as temperate or virulent. After inserting their genome into a host cell, virulent phages enter a lytic state, multiply, and subsequently lyse the cell to release viral progeny. Temperate phages can transition from lysogenic to lytic state at any time (Mäntynen et al., 2021, Gulyaeva et al., 2022). Because of their known hosts being bacteria and archaea, Caudovirales form part of the viruses that are presumed to play a vital role in themaintenance of favourable numbers of microbes in the gut (Gulyaeva et al., 2022).

The other phylum under the Heunggongvirae kingdom is Peploviricota, which has a sole class Herviviricetes (observed in this study) with a sole order Herpesvirales (Evseev et al., 2023). Herpesviruses are large enveloped viruses that possess a linear double-stranded DNA of 120–240 kb (Ryu, 2016). Herpesviruses are highly prevalent viruses that establish a persistent infection in animal hosts (Brunson et al., 2016). Ovine Herpesvirus-2 (OvHV-2) is the principal causative agent behind Malignant catarrhal fever (MCF) in ruminants worldwide, which is a significant source of economic loss (Li et al.,2004). MCF is a very unpredictable disease with no effective treatment currently. Sheep are carriers of (OvHV-2) but they are not clinically susceptible to MCF infection. Sheep often transmit the virus to cattle, which are dead-end hosts. Transmission from sheep to cattle has been demonstrated at distances of at least 70 m . The observance of Herpesvirus class, Herviviricetes(0,06%) does not necessarily have health implications on the sheep but it poses a threat to cattle since most farms (including the participating farm) are mixed livestock farms, keeping more than one species of livestock and exchange of microbes may occur in the grazing fields (Burgess, 2016). This finding emphasizes the need to give equal attention and study to all livestock species as the microbiome of one species might have adverse effects on another species in mixed livestock farming systems. Consequently, we recommend a thorough investigation of how livestock species in mixed livestock farming systems influence one another microbiome and what implications does that have on the health of the animals.

The Varidnaviria realm includes large DNA viruses of the phylum Nucleocytoviricota of the Bamfordvirae kingdom, as well as smaller viruses with tailless capsids (Gaïa et al., 2023). Varidnaviria is a diverse realm with 2 kingdoms, 3 phyla, seven classes, 15 orders and 25 families (Evseev et al., 2023). However, only 1 of the phyla, Bamfordvirae, was identified in this study. This phylum comprises the majority of the Varidnaviria and consists of two kingdoms, Nucleocytoviricota and Preplasmiviricota, both ofwhich were found present in the gut of Dohne Merino sheep (Kotsinyan et al., 2022). Membersof the Nucleocytoviricota phylum are characterised by their large size of their genomes and particles. Moreover, they differ from viruses with smaller genomes in that they have a large number of genes related to transcription, translation, DNA replication, and repair (Koonin et al., 2015). The majority of viruses in this family are also able to replicate in the cytoplasm and nucleus of their hosts, hence the term "nucleocytoplasmic" (Woo et al., 2021). The *Mimiviridae*family is the most studied family of the Nucleocytoviricota phylum, this family belongs to theorder Imitervirales, of class Megaviricetes, which was one of the classes found at a high level of abundance in this study (12,24%). The interest on the *Mimiviridae* observed in literature is likely due to the family possessing the virus species *Megavirus chiliensis*, the biggest the known virus (Lartigue et al., 2015).

Giant viruses are found throughout the biosphere, but they seem to be more prevalent and varied in marine environment (Ha et al., 2023). Their known natural hosts include algaeand amoeba. However, they can be hosted in protozoa and have been described as having the potential to infect animals (sheep and cattle) and humans (Tokarz-Deptuła et al., 2019). Findingsuch a significant abundance of giant viruses in the gut of Dohne Merino sheep in this study emphasises the need for more research on the significance and pathogenicity of these viruses on animals and humans as previously highlighted by Tokarz-Deptuła and colleagues (2019).

In contrast to DNA viruses, little is known about the diversity and function of RNA viruses in microbial communities. All RNA viruses and reverse-transcribing viruses (with either an RNA or DNA genome) fall under the realm of *Riboviria*. This realm was created in 2018 to support all viruses that encode dependent RNA polymerase (RdRp), and it was expanded to include RdDp-encoding retroviruses a year later. RdRp and RdDp viruses are classified into two different kingdoms: Orthornavirae and Paranrnavirae both identified in the present study. Most *Riboviria* viruses are eukaryotic viruses linked to well-known diseases such as coronaviruses, Ebola virus, HIV, influenza viruses, and the rabies virus that occur in animals and humans. *Orthornavirae*, though not the most abundant, is the most diverse kingdom in this study, with a total of 4 phyla detected. Three different genome types—positive-strand (+) ssRNA viruses, negative-strand (-) ssRNA viruses, and double-stranded dsRNA viruses—can be found in the Orthornavirae kingdom. These viruses have all been identified in the current virome. All of the RNA viruses in this study (Patatavirales, Durnavirales, Martellivirales, Goujanivirales, Stellavivirales and Articulavirates) were of the kingdom Orthornavirae except for the order Ortervirales of phylum Arteviricota under the kingdom Paranrnavirae. Some of the most common infections linked to the order Ortervirales include bovine leukaemia immunodeficiency virus (BIV) and Jembrana disease virus (JEV) (González-Méndez et al., 2023). The consensus in literature is that most RNA viruses are highly pathogenic and are responsible for zoonotic and epidemic diseases (Markiewicz et al., 2021, Poltronieri et al., 2015, Stern et al., 2017). It is preferred that DNA virome should be greater than RNA virome in the gut, as most RNA viruses infect the mammalian cells not the bacteria in the gut (Murcia et al., 2009, Hodgins et al., 2002). However, some RNA viruses are extraordinarily good inducers of cellular and humoral immune responses (Palese, 1998). Therefore, the observation that RNA viruses are outnumbered by the DNA viruses in this study is a sign of good health of the Dohne Merino sheep.

Monodnaviria is a realm of viruses that includes all single-stranded circular DNA viruses and a few circular double-stranded DNA (dsDNA) viruses (Dyson, 2023). Four kingdoms make up the Monodnaviria realm, which was founded in 2019: Loebvirae, Sangervirae, Trapavirae, and Shotokuvirae (Turner et al., 2023). Of these, only Trapavirae was not identified in the present study. For Loebvirae, Sangervirae and Shotokuvirae, one phylum was found per kingdom:

Hofneviricota, Phrixiviricota and Cressdnaviricota, respectively. Viruses in the Loebvirae and Sangervirae kingdoms infect prokaryotes (bacteria and archaea), and viruses in Shotokuvirae infect eukaryotes. Though found in low abundances, Loebvirae (0,02%) and Angervirae (0,05%) form part of phages and, therefore contribute to overall benefits provided to the host by phages in the gut, as discussed above.

The last of the realms encountered in the present study is Adnaviria that unifies archaeal filamentous viruses with linear A-form double-stranded DNA genome (Krupovic et al., 2021). Archaeal viruses that form filamentous virions are currently classified as follows: one Zilligvirae kingdom, one phylum: Taleaviricota, one class: Tokiviricetes and three orders Ligamenvirales, Maximonvirales, Primavirales. Only the Ligamenvirales order was identified in this study and was only present in the reticulum and rumen with a low 0,05% total abundance. Considering the low abundance of archaea compared to bacteria present in this study, it is reasonable that the abundance of archaeal only viruses is also low.

Upon mining the virus host database for previously published hosts for the viruses identified in this study, a diverse host range was found including the expected bacterial, archaeal, and animal host as well as some unexpected ones such as plant, algae and other marine species. Transfer of viruses across species through direct contact and through the environment is a naturally occurring phenomena that have been shown to play a role in strain mutation and therefore host diversification (Acar Kirit et al., 2022). These phenomena can account for the identification of viruses such as Algavirales, Goujianvirales and Lefavirales, whose known natural hosts are neither sheep nor the members of the gut microbiome. This should be a concern because if viruses infecting plant and marine species can be found in livestock, then it can be assumed that viral transfer between humans and livestock and across livestock species happens at a higher degree due to their close proximity to one another (Tomley and Shirley, 2009). The transfer of zoonotic viruses such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), poxvirus and influenza from animals to humans has led to disease breakouts and severe consequences to human health in the past (Haider et al., 2020, Rahman et al., 2020).

The composition and the diversity of viral population differ for the four stomach compartments. With the reticulum having the most diverse viral population and abomasum having the least diverse, which is likely influenced by the diversity of the hosts in each of the compartments, since the reticulum had the highest diversity of bacteria and archaea, and the abomasum had the lowest. This agrees with literature, where a correlation has been found between abundance of phages and their bacterial hosts in the gut (Strange et al., 2021). Currently there has not been any research that focused on the viral component of the other three compartments except the rumen. This makes it challenging to fully interpret the findings of the current study and draw conclusions since there is no premise of which viruses are expected the be part of normal virome with significant functions. However, this study effectively provides a census of the gut virome in Dohne Merino sheep. It is suggested that the significance, functions, and implications of the observed gut virome be researched further in other studies since it is beyond the scope and the study design of this work.

4.6 Conclusions

The gut of Dohne Merino sheep has a diverse population of viruses, which was expected in line with a diverse population of bacteria and archaea (especially bacteria) in which these viruses can be hosted. It can be concluded that the rich diversity of bacteria observed in reticulum translates to a richer diversity of viruses in the same compartment, as compared to the others. The varying virome diversities in the different gut compartments reinforce the conclusions made in chapter 3, that there is a relationship between gut microbial population (including viruses) and function of the gut compartments. Much like with bacteria, not every member of the microbiome is beneficial for the animal’s health, this hold true for viruses as some disease related viruses were identified. Though RNA viruses are often associated with eukaryotic diseases it is preferable that they are present in small doses (as observed in the current study) rather than completely absent in the gut as they are useful for immune system activation. This study suggests that the possibility of cross species transfer of viruses and host diversification should be considered in further research as they pose great threat to human health. Overall, this study reveals that there is a rich viral diversity in the entire gut (four compartments) of sheep and highlights the knowledge gap in the roles played by viruses in the gut microbiome and overall health of the animals.

5 PREVALENCE OF ANTIMICROBIAL RESISTANCE GENES IN THE GUT OF DOHNE MERINO SHEEP

5.1 Abstract

Sheep represent roughly 0.33% of the agriculture sector's 2.34% GDP contribution. Additionally, sheep contribute greatly to household food security in South Africa. In the efforts to treat diseases while maintaining high production antimicrobials are employed in livestock including sheep. The use of antimicrobials has become very common in the agricultural sector, which over the decades has resulted in a major global concern, namely antimicrobial resistance (AMR), phenomena where microorganisms mutate or adapt in ways that enable them to withstand antimicrobials, which renders antimicrobial treatments ineffective. Better understanding of AMR genes and the mechanism of resistance is the key for prevention of the spread resistance strains, genes, and ultimately improved management of diseases. In the present study, the metagenomics approach was used to mass screen for antimicrobial resistance genes in the gut of Dohne Merino sheep in South Africa, a country where more than 1 million people died from AMR in 2019. To acquire broad picture antibiotic resistance status in Dohne Merino sheep the ABRicate software was utilised to examine metagenomic illumina sequenced data for the presence of ARGs. Twelve antimicrobial resistance genes conferring resistance to fifteen antimicrobial drugs were identified in this study. The identified resistance genes were *NimJ* (30,07%), *lnu*(C) (12,59%), *tet*(W) (12,59%), *tet*(O) (11,19%) *tet*(40) (10,49%), *tet*(Q) (9,79%), *tet*(44) (4,90%) , *mef*(A) (2,80%), *ileS* (2,10%), *aadE*(2,10%), *aph(2")-If* (0,7%) and *rpoB* (0,70%). These genes translate to resistance to the following medicines: tetracycline, lincosamide, amikacin, gentamicin, kanamycin, tobramycin, mupirocin, streptomycin, rifamycin, lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, and streptogramin. Two of the identified genes were found to be multi-drug resistance (MDR) genes (*aph(2")-If* and *mef*(A)). This is a higher prevalence of AMR than you would expect from sheep since previous studies have found lower levels of resistance in sheep. We deduced this must be due to the dosing of the animals that the participating farms performs every six. These results were indicative of a high risk of AMR and MDR development in Dohne Merino sheep. The resistance genes found in this study employ various mechanism such as efflux pumps, ribosomal protection, inhibiting bacterial protein biosynthesis, and enzymatic inactivation to confer resistance. This knowledge about modes of actions utilised by the ARG is very relevant in the development new drugs. Overall, study revealed the need for the expansion of research on AMR in sheep as they could be harbours of many ARG.

5.2 Introduction

Artificial, semi-synthetic, and naturally occurring compounds with characteristics that stop the growth of microbes or eliminate them are known as antimicrobials. (Rushton et al., 2014). Antimicrobials are effective against bacteria, fungi, viruses, or protozoa. The development and application of antibiotics has not only saved or enhanced human life, but it has also improved animal production and health in the livestock industry, promoting food security, food safety, and the preservation of animal resources (Thamaga-Chitja and Morojele, 2014). However, the agricultural animal sector has become very reliant on the use of antimicrobials over the years, using antimicrobials for treatment of diseases and as feed additives (Wie et al., 1998). The following are common applications for antimicrobial veterinary medicine: prophylaxis (giving antibiotics to a flock or herd that is susceptible to a disease outbreak), meta-phylaxis (giving antibiotics to healthy animals in the same flock as animals exhibiting clinical symptoms of a disease), and therapeutic purposes (taking care of sick animals). Moreover, the high demand of the fast-growing human population has led farmers to resort to incorporating antimicrobials into animal feed and other supplements including water. This enhances animal growth and optimises livestock production by decreasing the amount of feed and time required by an animal to reach market weight (Marshall and Levy, 2011). The usage of antimicrobials as feed additives has been in the rise in the past decade (Van et al., 2020).

The inappropriate use of antibiotics in animals is thought to be a major driver of the rise in AMR bacteria in livestock as well humans (Reardon, 2023). There are multiple ways in which antimicrobials are misused in agriculture including using the overuse of growth promoters, using the same antimicrobial over and over without rotation and using wrong antimicrobial for a given disease or species, all these exacerbate the problem of AMR in livestock (Mupfunya et al., 2021a). AMR microbes evolve and develop to become tolerant or resistant to antimicrobials that were previously effective in treating or killing them(Clifford et al., 2018, Mupfunya et al., 2021b). This renders very important medications unsuccessful in the treatment of microbial diseases in both humans and animals and makes animals even more susceptible to infections (Marshall and Levy, 2011). AMR has been labelled as a major global challenge by the world health organisation (WHO), because not only does AMR threaten human and animal health, but by causing challenges to animal health it is also poses a threat to global food security as animals and animal products are a key part of food security globally. AMR is a greater cause of death in South Africa than enteric infections, chronic respiratory diseases, self-harm, and interpersonal violence (IHME, 2023). Drug-resistant illnesses claimed the lives of 4.95 million people in 2019. Of those fatalities, AMR was directly responsible for 1.27 million, and one in five of those deaths involved children under the age of five. (IHME, 2023). These statistics demonstrate the severity of the AMR issue in South Africa.

The South African AMR surveillance report from 2018, gave the following statistics for the usage of antimicrobial in the agricultural sector in South Africa: Growth promoters are a common class of antibiotics used in animal health, accounting for 62% of use, followed by macrolides (11%) and tetracyclines (17%) (DoH-SA, 2018). 2,08% of meat samples examined by the National Chemical Monitoring Program included antimicrobial residues, such as penicillins, tetracyclines, sulphonamides, and macrolides. (DoH-SA, 2018). The 23%-36% of all antimicrobials used in SA are used in animals. Mupfunya et al. (2021) notes that while South African cattle farmers lack understanding about antimicrobials and antimicrobial use practices, they still have access to some antimicrobials without veterinary supervision. In their study, Mupfunya and colleagues also found that only 1 out of 28 interviewed farmers in their study understood what AMR is, indicating that most South African farmers use antimicrobials without an understanding of the overall impact that mishandling of antimicrobials could have on both the health of the animals and their own health.

In the context of animal health, the failure of antibiotics makes it impossible to treat sick animals, causing suffering as well as higher rates of morbidity and death. Increased resistance onbacterial strains causes reduced growth, early culling, and greater mortality in livestock. The widespreadresistance to antibiotics in livestock has rendered traditional treatments useless, leaving veterinarians to rely on pleuromutilins, which the WHO has designated as medically important.The fact that pleuromutilin resistance has already been documented is concerning as it raises the possibility of serious livestock losses (Bartley, 2022). Strengthening the knowledge of AMR through research and surveillance forms part of the strategic bjectives of the global action plan against the spread of AMR. This work intends tocontribute towards the achievement of that goal through the enrichment of the knowledge of AMR in sheep, an understudied species in the research of AMR in livestock. Chapter 3 and 4 revealed a diverse population of microbes in the gut of Dohne Merino sheep and raised awareness of a possible transfer of microbes across species. Since microbes are generally carriers of AMR genes (ARGs), we thought it crucial to scan for ARGs that could be carried by the observed microbial population in this study. Additionally, AMR is influenced by multiple factors such as the microbiome, age of animal, the environment and farming practices (Fletcher, 2015, Soundararajan et al., 2022). The participating farm doses the sheep every six weeks to cope with the parasites and pathogens discussed in Chapter 3, therefore this increasesthe probability of AMR in these animals. We expect the findings of this study to differ from previous studies that have found low levels of AMR in sheep, because of the different farmingpractices.

Several methodologies are used to study AMR, many earlier studies depended on culture-based techniques, but more recent studies have adopted the metagenomics approach. Both approaches have benefits and draw backs. With the culture-based technique, there is certainty on the source of resistance since microbes are isolated and cultured before their genome is scanned for ARGs, however not all microbes can be cultured therefore ARGs on uncultured species are missed (Waskito et al., 2022). The resistome, which are factors that determine antibiotic resistance in the microbial population, are sought for via the metagenomics method. Utilizing the resistome can help forecast a microbial community's potential resistance pattern (Escobar-Zepeda et al., 2015). The main advantage of this method is that the resistome can be identified even if it is not present in the current disease-causing pathogens. However, because ARGs are mass scanned froman "environmental" sample it cannot be known specifically which microbe the source of which ARG. Metagenomics only offers a wholistic picture of the microbial community's resistance potential. This study already used metagenomics sequencing to analyse the gut microbial community of Dohne Merino sheep, hence the same approach will be used to unveil the antimicrobial potential of gut microbial community in question.

5.3 Materials and Methods

The analysis for the resistome in this chapter was conducted using the same sequenced data created in section 3.3 of Chapter 3. AMR screening techniques followed up from the chapter 3 methodology after the assembly of the DNA sequences into contigs.

5.3.1 AMR resistance gene screening and analysis

Fasta DNA contigs were submitted to ABRicate (Seemann, 2016) which performed a mass screening of ARGs on the contigs. The ABRicate software contains several antimicrobial resistance databases, including NCBI, AMRFinderPlus, CARD, and ResFinder, and each database contains data on thousands of ARGs. The NCBI and CARD databases produced the most hits for this investigation. Outputs from ABRicate were analysed and visualised with R-studio (R Core Team,2022) and Microsoft Excel. The Comprehensive Antibiotic Resistance Database (CARD) website (Alcock et al., 2023) was mined using the NCBI reference names of the identified genes to find further information on the genes, such as resistance phenotype, carrier species and mechanisms utilised to confer resistance.

5.4 Results

Figure 5.1 below depicts the abundance of contigs that contain resistance genes found by ABRicate in each of the four stomach compartments. The rumen had the highest level of resistance with 43 contigs carrying ARGs, omasum had the second highest resistance with 38 contigs carrying resistance, the reticulum had 32 and last the abomasum had 25. The identity of the genes carried by these contigs is revealed in Figure 5.2.

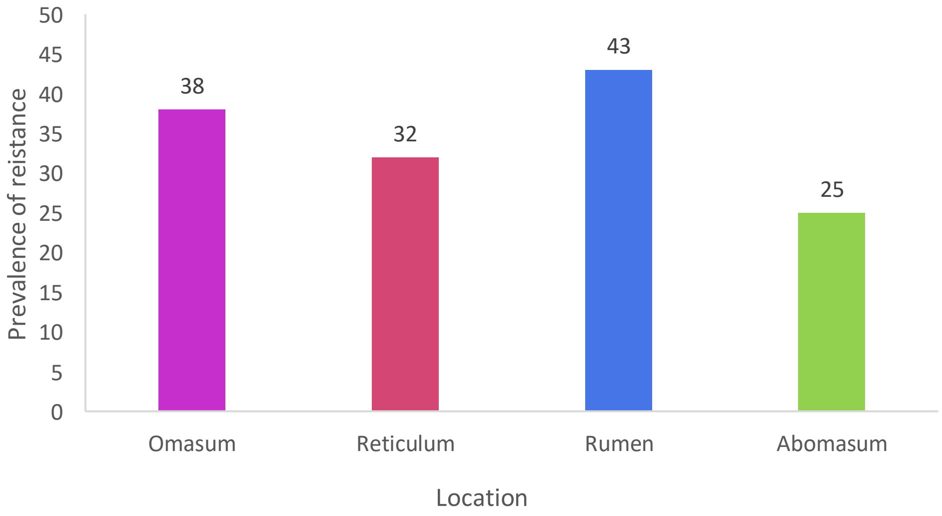


Figure 5.1 Prevalence of antimicrobial resistance in each of the four stomach compartments of sheep. Bar plot showing the number of DNA contigs carrying antimicrobial resistance genes amongst the sampled animals stratified by stomach compartment.

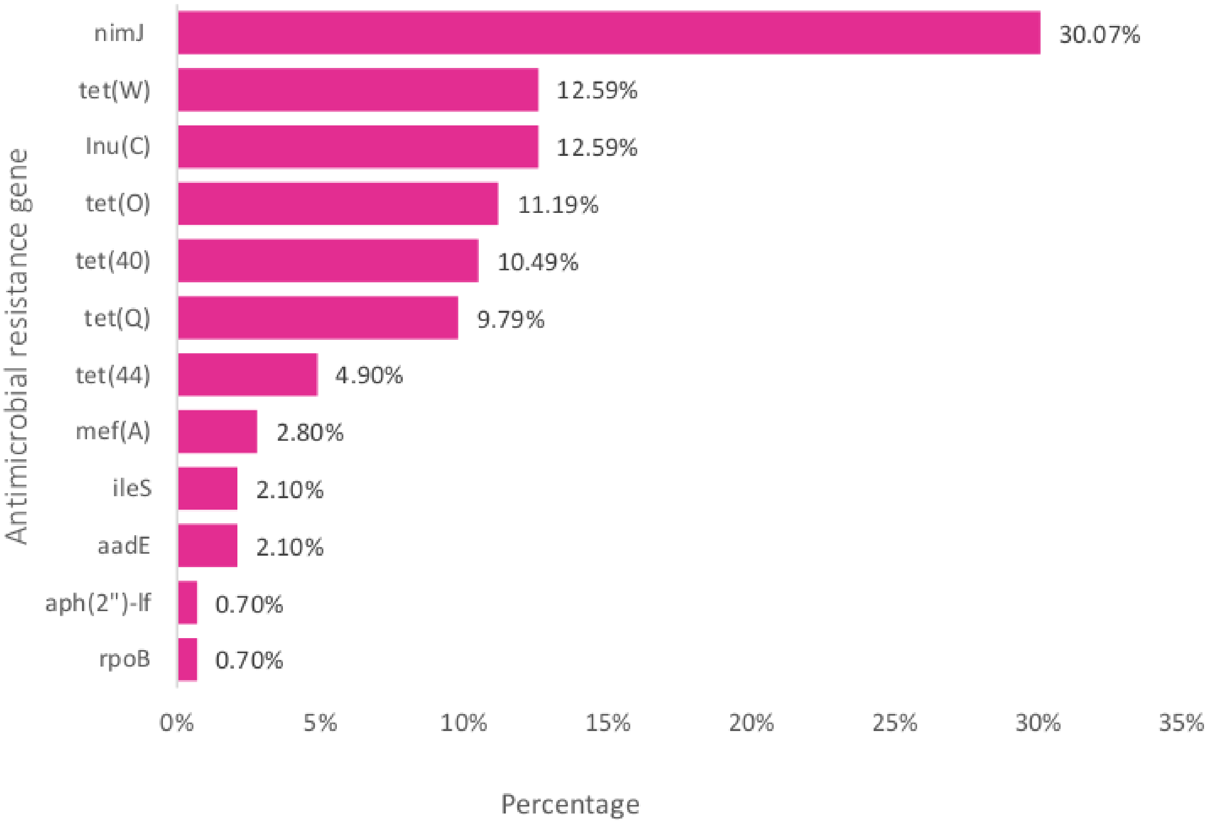


Figure 5.2 Abundance of antimicrobial genes detected in the gut of sheep. Bar plot showing the identity and percentage of the prevalence of each of the resistance gene identified in the gut.

A total of 12 resistance genes were found and identified in this study. The identity of the genes and the percentage contribution to the total antimicrobial resistance are as follows : *NimJ* gene presented the highest occurrence (30,07%), followed by *lnu*(C) (12,59%), *tet*(W) (12,59%), *tet*(O) (11,19%) *tet*(40) (10,49%), *tet*(Q) (9,79%), *tet*(44) (4,90%) , *mef*(A) (2,80%), *ileS* (2,10%), *aadE*(2,10%), *aph(2")-If* (0,7%) and *rpoB* (0,70%) (Figure 5.2). The confidence for each of the gene segments identified in this study was acceptable, all the gene segments had a coverage (proportion of gene covered by segment found in this study) and identity (proportion of exact nucleotide matches) above 80%.

Table 5.1 (below) gives the antimicrobial drug that each of the genes confer resistance to and the resistance mechanisms utilised by the microbes that carry the gene to confer resistance. The nimJ gene confers resistance to nitroimidazole. The 5 tet (O, Q, W, 44 and 40) genes carry resistance to tetracycline. These tetracycline resistance genes account roughly 49% of the total resistance genes detected in this study. The lnu(C) gene confers resistance to lincosamide, the aph(2”)-lf gene carries resistance to four antimicrobials amikacin, gentamicin, kanamycin, and tobramycin. IleS confers resistance Mupirocin, aadE: streptomycin, rpoB: rifamycin and mef(A) to lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin and tetracycline. This makes mef(A) the gene that results in the most resistance phenotypes, conferring resistance to a total of 7 antimicrobials, followed by aph (2")-If conferring resistance to 4 antimicrobials. The rest of the genes carry resistance to only one antimicrobial. Tetracycline resistance is the only resistance phenotype that is conferred by multiple genes.

Table 5.1 Antimicrobial resistance genes detected with their resistance phenotypes and mechanisms of resistance

GENE	RESISTANCE PHENOTYPES	MECHANISM OF RESISTANCE
NimJ	Nitroimidazole	Impaired drug activation, high expression level of multidrug efflux pumps or DNA repair systems
Tet (O,Q, W,40 and 44)	Tetracycline	Ribosomal protection proteins, Efflux pumps, enzyme inactivation
lnu (C)	Lincosamide	Target-site modification, efflux of the antibiotic, drug inactivation.
aph (2'')-lf	Amikacin; Gentamicin; Kanamycin; Tobramycin	Reduced uptake or decreased cell permeability, alterations at the ribosomal binding sites, target site modification.
ileS	Mupirocin	Target site modification
aadE	Streptomycin	Antibiotic inactivation
Mef(A)	Lincosamide; Macrolide;Oxazolidinone; Phenicol;Pleuromutilin;Streptogramin;Tetracycline	Efflux pumps, Ribosomal methylation.
rpoB	Rifamycin	Antibiotic target alteration, Antibiotic target replacement

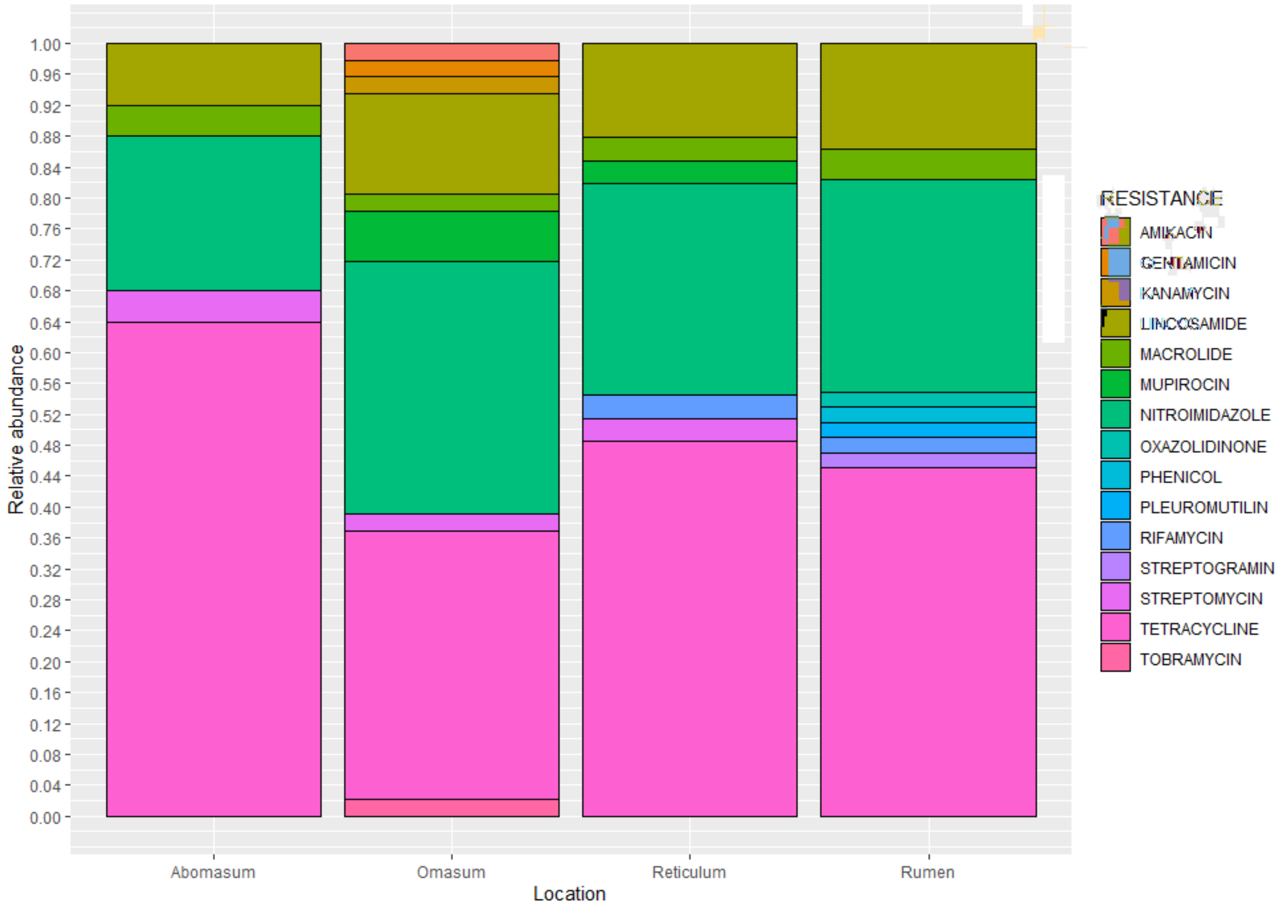


Figure 5.3 Resistance phenotypes found in each of the four stomach compartments. Stacked bar chart of AMR abundance per stomach compartment (y-axis) according to AMR phenotype (colours) proportional to the total AMR within each compartment.

In Figure 5.3, the overall resistance for each of the stomach compartments is depicted. Combined, the resistance genes found in this study gives rise to a total of 15 antimicrobial resistance phenotypes. Each of the compartments had multiple resistance phenotypes, with the omasum having the most and the abomasum having the least. The number of contigs carrying ARGs (Figure 5.1) is not directly proportional with the number of resistance phenotypes observed in Figure 5.3. As the rumen had the most contigs carrying ARG but the abomasum is the compartment with the most resistance phenotypes. The omasum had genes that conferred resistance to the following 10 antimicrobials: tetracycline, nitroimidazole, linconsamide, macrolide, amikacin, gentamicin, kanamycin, tobramycin, mupirocin, and streptomycin. The rumen had the second highest resistance phenotypes, with resistance to the following 9 antimicrobials: tetracycline, nitroimidazole, lincosamide, macrolide, rifamycin, oxazolidinone, phenicol, pleuromutilin and streptogramin. The reticulum carried resistance to 7 antimicrobials: tetracycline, nitroimidazole, lincosamide, macrolide, mupirocin, rifamycin, and streptomycin. The abomasum carried resistance to only 5 of microbials: tetracycline, nitroimidazole, lincosamide, macrolide and streptomycin. When considering the proportion of the total resistome by AMR phenotype, tetracycline AMR was the most common, followed by nitroimidazole AMR. Generally, resistance to tetracycline, macrolide, nitroimidazole and lincosamide were found in high prevalence and were present across all four compartments. Resistance to aminoglycosides (gentamicin, tobramycin, rifamycin amikacin, and streptomycin) followed by other kinds of resistance phenotypes including oxazolidinone, phenicol, pleuromutilin and streptogramin were rare.

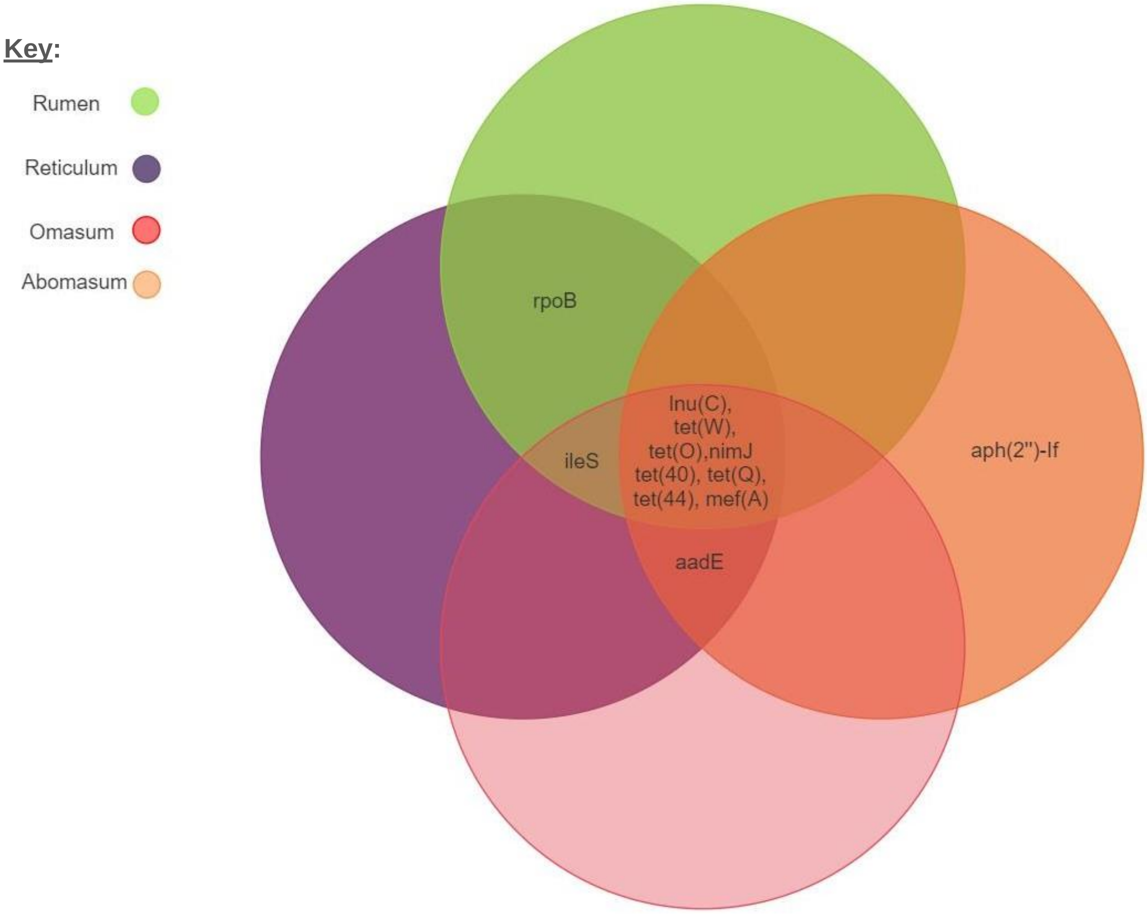


Figure 5.4 Core resistance genes present in the sheep gut. Venn diagram showing the eight core resistance genes in the four compartments of Dohne Merino sheep.

To determine whether core resistance genes in the study population, we observed AMR genes that were consistently present in all compartments and in all the animals (Figure 5.4). We identified eight core resistance genes, *tet*(O,W,Q,40,44), *Inu*(c), *nimJ* and *mef*(A).

Additionally, the analysis revealed that one gene was unique and specific to only one compartment *aph (2")-lf* gene in the omasum. Two genes were found in three of the four compartments, *aadE* (absent in the rumen only) and *ileS* (absent in the abomasum only). One gene was specific to two of the four compartments, rpoB (reticulum and rumen). Each of the compartments was found to carry a total of 10 genes.

5.5 Discussion

Resistance to antibiotics is a major burden worldwide, posing challenges to global health and economic sector (Waskito et al., 2022). AMR research is vital not only for AMR surveillance but also because information from these studies can be used by decision makers to inform development of new drugs or vaccines (IHME, 2023). As part of AMR surveillance, the Department of Agriculture, Land Reform and Rural Development (DALRRD) has been informing WHO about antimicrobial consumption in animals. Reports from 2014-2015 showed that the prominent form of antibiotic used in animal health are growth promoters (62%), which is usually a mix of antimicrobial agents that differ for different growth promoters (Al-Dobaib and Mousa, 2009). The report also showed that tetracyclines were reported as the second prominent antimicrobials (17%) followed by macrolides (11%). Penicillins, tetracyclines, sulphonamides, and macrolides are among the antimicrobial residues in meat products that have been found in 2,08% of meat samples examined as part of the national chemical monitoring program.

Tetracyclines are the most popular antimicrobials amongst farmers (Mupfunya et al., 2021a) because of their broad-spectrum activity, cost effectiveness and certain favourable pharmacokinetic properties (Patyra et al., 2019). Numerous Gram-positive and negative bacteria, spirochetes, obligatory intracellular bacteria, and protozoan parasites are among the many organisms for which tetracyclines are known to be effective against. (Grossman, 2016). Because tetracyclines are the most commonlyused antimicrobials, microbial populations are more likely to develop their resistance (Thakeret al., 2010), as evidenced by high tetracycline resistance and diversity of tetracycline resistance genes observed in this study. Tetracyclines are also used in cattle feed for growth promotion at subtherapeutic levels in many regions of the world. This practice regrettably enhances the selection for resistance. (Granados-Chinchilla andRodríguez, 2017). Because of popularity of Tetracyclines in veterinary medicine it is unsurprising that the findings of this study are in alignment with findings from many other studies where tetracycline resistance genes were found to be highly abundant in livestock (McKinney et al., 2010, Kazimierczak et al., 2009, Nogrado et al., 2021). Additionally, with tetracyclines making up 17% of the total antimicrobial usage in South Africa, it is expected that resistance to this group of antimicrobials will be high(DoH-SA, 2018).

Nitroimidazoles, also referred to as 5-nitroimidazoles is a class of antimicrobials with very strong antibacterial and antiprotozoal properties (Van Der Wouden et al., 2000). Nitroimidazoles started being utilised to treat infections caused by parasites in the late 1950s (Samuelson, 1999). By late 70s this family of antimicrobials had been studied enough to expand its' usage as an antibacterial to anaerobes (Alauzet et al., 2019) Since then, these antimicrobials has been extensively used in the treatment of infections caused by anaerobic bacteria in human and veterinary medicine (Alauzet et al., 2019). Like tetracycline genes, there are different variants of the nitroimidazole resistance genes. To date there are eleven nim genes(*nimA* to *nimK*) these share about 57.6% to 89.8% nucleotide sequence identities (Alcock et al., 2023). However only the *nimJ* gene was found in this study.

Nitroimidazole resistance was first described in *B. fragilis* in 1978. Since *B. fragilis* is one of the bacterial species found in the characterisation gut microbial population of the animals sampled in this study it is the probable source for the observed nitroimidazole resistance. Resistance to metronidazole has been low and steady over the years (>3%) in many parts of the world except for higher resistance rates reported in Spain (4.8%), South Africa (8.7%) and Pakistan (16%) (Kalach et al., 2001). Albendazole is the most common member of the nitroimidazole class, it is a synthetic nitroimidazole used for the treatment of a variety of intestinal parasite infections, including round worms that the Wauldby Dohne Merino are often being treated for. Wauldby farmers reported signs of resistance to the anthelmintics before the recent introduction of the active ingredients, Derquantel and Monopantel(Burgess, 2016) .

Macrolides have been known for less than decades, but resistance to macrolide and lincosamide has been increasingly reported in gram-positive bacteria (Leclercq, 2002). Tylosin and spiramycin belong to macrolides and they are used as feed additives (Civitareale et al., 2004). Liadlomycin is a member of lincosamide group and is also used in feed additives (Bauer et al., 1995). Though these are newly discovered drugs, their usage as feed additives increases the selection pressure (Castanon, 2007). Resistance microbes can travel in between individuals and across species through direct contact and other in-direct means such as feed, water and environment (Larsson and Flach, 2022) This is the probable reason macrolide and lincosamide resistance are amongst those with high resistance and present in all compartments in this in the study even though feed additives are not used on the sheep in the Wauldby farm.

The usage of tylosin and spiramycin in food animals has been withdrawn by the European Union due to safety concerns for humans as the transfer of these gene to human through the food chain could be very harmful health (Civitareale et al., 2004). However, these feed additives are still used in many parts of the world including South Africa. Macrolides constituted 11% of all the mostly used drugs in South Africa in the period of 2014-2015, with Tylosin making the majority of the in-feed antimicrobials sold, at 61% of this total (DoH-SA, 2018, Eagar et al., 2012). In a 2018 study, macrolides and lincosamides usage was reported to have increase from 6.9% to more than double in a period of 4 years , contributing 17.4% of the total antibiotics used in the Limpopo province of South Africa. This high usage of lincosamides and macrolide in South Africa explains their high prevalence of resistance observed in this study. Resistance to macrolide is translated by the Mef(A) gene (a multi-druggene resistance to observed in this study, discussed below). Resistance to lincosamide is translated by the *lnu*(C) also observed in this study. Both the *mef(A)* and *lnu(C)* are often foundin Streptococcus bacteria, a few Streptococcus species were identified in this study (*Streptococcus sp, HSISB, Streptococcus equinus, Streptococcus gallolyticus, Streptococcus infantarius, Streptococcus sp, CNU 77-61 and Streptococcus sp, CNU G3,* observable in Table A1). This diversity of Streptococcus bacteria that could be carriers of the *lnu*(C) gene could be used to explain the high abundance of the *lnu*(C) gene observed in Figure 5.2.

Aminoglycoside is a broad group of antimicrobials and is one of the very first antimicrobials to be developed in the 1940s (Vakulenko and Mobashery, 2003). Because of their origin from soil bacterial belonging to genera *Streptomyces* or *Micromonospora*, aminoglycosides are identified by suffix "-mycin" and "-micin" (Yao and Moellering Jr, 2011). Amikacin, gentamicin, kanamycin, rifamycin and streptomycin are all the aminoglycoside resistance phenotypes present in this study. Four of the five aminoglycoside resistance phenotypes are conferred by the same gene, *aph(2")-lf* (Caldwell et al., 2016). This gene is often found in *Campylobacter* conferring resistance to aminoglycoside (Yao et al., 2017). Seeing that *Campylobacteria* was identified in the gut of the sheep it is therefore the presumed carrier of the *aph*(*2")-lf* gene hence the carrier of aminoglycoside resistance in this study. The 4 aminoglycoside antimicrobials whose resistance is conferred by the *aph(2")-lf* were all found in the one compartment, the omasum, which means it is the only compartment where this gene was present. The *aph*(*2")-lf* gene is an MDR gene conferring resistance to more than one drug, the presence of this gene in this study indicates that the sheep are at risk of developing MDR, which would result in the inability to treat many microbial infections and therefore high rates of mortality.

Amikacin is a semisynthetic aminoglycoside that was developed as part of the solution to the enzymatic modification of most early aminoglycosides (Ramirez and Tolmasky, 2017). Amikacin, , has been effectively employed as protection against strains that would otherwise be resistant to other aminoglycosides due to its ability to withstand most aminoglycoside modifying enzymes (Victor et al., 1977, Pacifici and Marchini, 2017). There has been some level of resistance observed in microbes since the introduction of amikacin in the 70s, however amikacin remains the most defiant antimicrobialto the action of aminoglycoside modifying enzymes (Kawaguchi et al., 1972, Shaw et al., 1993). This renders the antibacterial efficacious and indispensable for combatting many illnesses resulting from organisms resistant to multiple drugs (Ramirez and Tolmasky, 2017).Therefore, the presence of amikacin resistance in this study is a cause for concern as this is anessential drug in the treatment of AMR related illnesses.

Pseudomonas fluorescens is the source of the topical antibiotic mupirocin, which was first made commercially available in 1985 (Perkins et al., 2008). Methicillin-resistant Staphylococcus aureus (MRSA) infections were frequently treated with mupirocin. Butover time bacteria have acquired resistance to mupirocin as well (Dadashi et al., 2020). Mupirocin resistance is known to be carried by *mupA, mupB* genes and the novel gene *ileS* (Godbeer et al., 2014). In the present study the *mup* genes were not found but the *ileS* gene waspresent, implying therefore that the mupirocin resistance observed in this study is conferred bythe *ileS* gene. The *ileS* gene is a novel mupirocin resistance gene that is often reported in multi-resistant *S. aureus* (Perkins et al., 2008). Besides *S. aureus,* mupirocin resistance has recently been found in *Streptococcus* species like *S. pseudintermedius* and S. *epidermidis S. pyogenes* (Lee, 2007). None of these species were found in the characterisation of the gut microbiome ofthe sheep in this study which suggests that there might be a microbial carrier of the *ileS* gene and mupirocin resistance that has not been identified and reported in literature or it is a free antibiotic resistance determinant within the gut not necessarily carried by any microbial species.

A class of synthetic antibiotics known as oxazolidinones is active against a variety of multidrug-resistant bacteria and is chemically characterized by the basic 2-oxazolidone nucleus. This drug is effective against MDR Gram-positive bacteria (GPB), namely vancomycin-resistant *enterococcus* (VRE), MRSA and *Mycobacterium tuberculosis* (Mtb) (Foti et al., 2021). Like mupirocin, this class of antibiotics was developed to combat the increasing level of resistance in gram positive bacteria like *S. aureus* (Pelton et al., 2000). Oxazolidinones work by inhibiting protein synthesis in bacteria(Diekema and Jones, 2001). Resistance to oxazolidinones is another cause for concernbecause it forms part of the new drugs that were developed to combat AMR. This suggest thatthe microbial population in the gut of Dohne Merino is fast evolving and becoming tolerant even to new drugs.

A broad-spectrum antibiotic called phenicol, whose full name is chloramphenicol, was discovered from Streptomyces venezuelae in 1947 (Roberts and Schwarz, 2016). Similar to the majority of antimicrobials mentioned in this research, chloramphenicol functions by preventing the creation of bacterial proteins (Yao and Moellering, 1999). Chlamydiae, Mycoplasmas, Rickettsiae, and Gram-positive and Gram-negative aerobic and anaerobic bacteria are all included in the substrate spectrum of chloramphenicol. (Schwarz et al., 2004). There is a variety of genes that confer resistance to phenicol,organized into 37 groups and have been identified in 70 bacterial genera (Roberts and Schwarz,2016). Some multidrug resistance genes that confer resistance through efflux pumps have beenreported to result in the export of phenicol as well, therefore yielding resistance to phenol (McMurry et al., 1994). One of such genes is the mef(A) gene (identified in this study). *Mef(A)*is one of the many macrolide efflux (mef) MFS-type transporter genes that confer macrolide resistance mostly in *Streptococcus* (as mentioned above) (Ambrose et al., 2005). The *Mef(A)* is the second MDR gene observed in this study which raises greater concern for MDR development.

According to Van Duijkeren et al. (2014), the fungus Pleurotus mutilus—now known as Clitopilus scyphoides—produces pleuromutilin, a naturally occurring antibiotic. But, tiamulin and valnemulin are the semi-synthetic derivatives of pleuromutilin used as veterinary medications, primarily in pigs. poultry and rabbits to treat various conditions like swine dysentery (*Brachyspira hyodysenteriae*), colitis (*Brachyspira pilosicoli*), ileitis (*Lawsonia intracellularis*) and enzootic pneumonia (*Mycoplasma hyopneumoniae*). Both tiamulin and valnemulin are also used as feedadditives (Sader et al., 2012), which can cause an increase in selection for pleuromutilin's resistance (Marshall and Levy, 2011). Besides the known pleuromutilin resistance genes (*vga* and *cfr*), reduced antibiotic susceptibility to the drug has been associated with the presenceof *lnu*(C) and *rplC* genes (Card et al., 2018). Of these two, the *lnu*(C) gene was part of the resistance genes found in this study(discussed above) and therefore it is the probable source of the pleuromutilin resistance observed.

The streptogramin antibiotics are naturally occurring compounds isolated from various members of the *Streptomyces* genus (Thal and Zervos, 1999). Interference with bacterial protein biosynthesis (also employed by streptogramin antimicrobials) is the most common mode of action in most antimicrobials (Johnston et al., 2002). Despite having been discovered in the 1950s, it was much later after their discovery that streptogramins have been approved for disease treatment followed by long use as feed additives. (Mukhtar and Wright, 2005). Streptogramin does not form part of the majorly used antimicrobials in South Africa and resistance to this group antimicrobials is rare (Eagar et al., 2012, Mthombeni et al., 2022).This therefore explains the low prevalence of streptogramin resistance in this study.

The mechanisms of resistance used by microbes to resist the drug depends on both the chemical structure of the antimicrobial and the ARG contained in the genome of that microbe. The general mechanisms of resistance for tetracycline include efflux pumps ribosomal protection, and enzymatic inactivation of tetracycline drugs (Grossman, 2016).

Resistance to nitroimidazole has been described as one of the most complex, resistant organisms have been found to employ several mechanisms to render this drug in ineffective (Van Der Wouden et al., 2000). These include impaired drug activation, high expression level of multidrug efflux pumps or DNA repair systems(Kalach et al., 2001). The current consensus is that nitroimidazole resistance is a complicated phenomenon that most likely involves multiple factors and as-yet-unidentified processes (Graves et al., 2020). Alauzet (2018) discovered that modifications to the overall patterns of gene expression persist even after the metronidazole selection pressure is removed, indicating that drug exposure results in significant and long-lasting alterations to gene expression. The establishment of resistant strains may be attributed to such persistent changes Hecht, 2006). This supports the conclusion made by Marshall and Levy (2011), that usage of antimicrobials induces high selection pressure for resistance.

Macrolides and lincosamides are chemically distinct but they have been found to share similar modes of action and resistance to them carried by a common gene, *mef*(A) (Seiple et al., 2016). These three mechanisms are how bacteria withstand the antibiotics macrolides and lincosamides: (1) by altering the target site through mutation or methylation, which stops the antibiotic from binding to its ribosome target, (2) by the antibiotic's efflux, and (3) by the medication's deactivation (Lai et al., 2022Most bacteria use enzyme-modifying enzymes, such as acetyl transferases, phosphotransferases, and nucleotidyltransferases as a mechanism of resistance to aminoglycosides. (Ramirez and Tolmasky, 2010).Resistance to oxazolidinones by achieved through a similar mechanism but instead of enzymemodification the microbes modify the ribosomal target site for this antimicrobial (Diekema andJones, 2001). Lasty resistance to chloramphenicol is due to an entirely different mechanism ofresistance, inactivation by chloramphenicol acetyltransferases (CATs) (Butaye et al., 2003, Schwarz et al., 2004).

The genes identified as core resistance genes in this study confer resistance to antimicrobials that have been found at to have a high prevalence of resistance in other studies (Castanon, 2007, Kırmusaoğlu, 2019). Overall, the observed AMR prevalence in this study is high compared to other studies that surveyed the AMR prevalence in sheep. We reasoned that this higher prevalence is as a result of using the metagenomics approach instead of the culture- based methods, additionally the participating farm doses the animals often which increases selection for resistance strains.

5.6 Conclusions

Metagenomics AMR screening is a potent technique of getting comprehensive picture of AMR prevalence in an environment without requiring a lot of resources and time to screen microbial species individually for antibiotic susceptibility. The findings of this study support literature reports that state that the usage of antimicrobials has a correlation with AMR resistance prevalence. Resistance to medicines that are commonly used South Africa was found to be higher than those that are rarely used (i.e macrolides and lincosamides). Microbials that are used as feed additives tend to have a have a higher prevalence of resistance as compared to those that are not even in areas where feed additives are not used, this can be attributed to the spread through the environment. Multidrug resistance genes increase the level of resistance even if the diversity of ARG present is low, meaning the diversity of ARG present will not always be an accurate presentation if the level of resistance. More studies that will focuses on ARG carrier species are needed, as some of the resistance phenotypes observed in this study could not be accounted for by the information available in literature which indicate novel data on ARG carrier species. Above all, this study reveals that the Dohne Merino sheep have an alarming antimicrobial resistance potential that could not only result in AMR but also MDR. The fact that the sheep farmers have not recognised signs of AMR in the Wauldby sheep is a sign of resilience in this breed considering the AMR findings obtained in this study.

6 GENERAL DISCUSSION

Ruminant livestock are of crucial importance to the global agriculture and economy, without livestock the economy of many countries would collapse. The importance of livestock is emphasised by their relevance to many of global challenges such as food security and AMR. The FAO recognises livestock to directly impact the attainment of 6 SDGs of : no poverty, zero hunger, responsible consumption and production, climate action, life on land and partnership for the goals. This high global relevance of livestock highlights the need for improved livestock farming and production strategies.

Challenges faced by livestock agricultural systems worldwide include inadequate pasture and high-quality feed, limited water resources, climate change, inadequate cattle breeding and management, illnesses, and parasites. (Eeswaran et al., 2022, Hursey, 1997). The weight of these challenges is much more pronounced in developing countries and small holderfarmers. South African farmers were greatly challenged by an increase in feed prices since 2020 which obliges farmers to rely more on pasture for feed. Pasture grazing leads to additionalchallenges of exposure to diseases, parasites, cross transfer of microbes between species and overall poor nutrition in less favourable environments such as the sourveld (Sihlobo, 2023). South Africa's wool exports suffered greatly due to the foot-and-mouth disease pandemic. Wool exports to China, which makes over 70% of South Africa's wool exports in terms of value was temporarily halted in the second quarter of 2022; as a result, the value of South Africa's wool exports fell by 21% (Sihlobo, 2023). This is one example of how challenges like disease outbreak in livestock has severe consequences on the farmers and the economy. Additionally, livestock farming has been shown to be linked with other global challenges such as AMR and global warming. In efforts to manage diseases and increase feed efficiency farmers use antimicrobials in livestock. In some cases, farmers have been found to overuse and misuse antimicrobials because of lack of knowledge, this exacerbates the AMR global challenge by increasing the selection pressure for AMR strains which leads to increase prevalence and spread of AMR genes and microbes. In terms of globalwarming some members of the gut microbiome in ruminants yield methane as an end-productof fermentation. Methane is a greenhouse gas that highly associated with global warming, meaning that ruminant livestock are contributors to the challenge of global warming.

To overcome these challenges, farming strategies for improved livestock need to be implemented. One of such strategies is the manipulation of the gut microbiome, especially in ruminants. The gut microbiome is a community of microbial organism that reside in the gut, these communities are composed of prokaryotic and eukaryotes cells , viruses, and archaea. The gut microbiome has been found to have an intricate relationship with their host and play very important roles in feed efficiency, digestion, host health, adaptation to environment and development of the immune system, the maintenance of intestinal homeostasis, mucosal and lymphoid structure development, and activation of the host immune cell repertoire (Jandhyala et al., 2015, O'Hara et al., 2020b). It is through the presence of the gut microbiome that ruminant livestock can digest complex plant fibre that other mammals cannot digest. Volatile fatty acids yielded by feed fermentation performed by the gut microbiome meets up to 70% ofthe energy needs of the host animals (O'Hara et al., 2020b). Bacteria and archaea found in the gut of ruminants carryout fermentation of feed (including the complex sugar polymers) in threestomach compartments before feed eventually flows into the last compartment which is similarto a non-ruminal stomach. The four gut compartments of ruminants are the rumen, reticulum, omasum and abomasum, feed flows from the rumen to the abomasum in that order after ingestion. Studies have revealed that a healthy gut microbiome is required for overall health and well-being of ruminant livestock (Arshad et al., 2021). A study that used meta-transcriptomics discovered that the Hu sheep (a native breed in China) rumen microbiome encoded a repertoire of novel cellulases (He et al., 2019). Roughage of inferior grade can be used and broken down for ruminant consumption by cellulases. According to another study, the amount of VFA in adult beef cattle and the gene expression level of ruminal epithelial cells were strongly connected with ruminal bacteria (Chen et al., 2011).

Fully understanding the gut microbiome is vital to informing new approaches to maximize ruminant production efficiency and tackle the challenges outlined above. Extensive research has been done on the gut microbiome of bigger ruminants such as cattle whilst small ruminants such as sheep remained understudied regardless of their importance to many small rural communities.The main objective of this study was to bridge the knowledge gap in gut microbial studies of small ruminants through the metagenomics approach analysis of the gut microbiome of DohneMerino sheep from the Eastern Cape province of South Africa.

Dohne Merino is a native S.A, dual purpose, high economic value sheep breed that is bred in Australia and Europe as well. The Dohne Merino was first developed to withstand harsh environmental conditions in the Eastern Cape before it started being reared in other places in South Africa and other countries as well. The breed has become very successful and continues to be a major contributor to the wool and meat industry in S.A. Sheep production is the most practised livestock operation in the Eastern Cape province and most farmers keep Dohne Merino sheep because their well adaption to the environment in the province. However, small sheep farmers in the Eastern Cape are still faced with challenges of increasing livestock production while achieving sustainable improvement (Ngqulana and Obi, 2019). The biggest obstacles faced by these farmers is disease control and low-quality feed. Dohne Merino in the Eastern Cape are pasture grazing and therefore are more exposed to pathogens in the field while also not getting enough nutrition from pasture feeding. As previously mentioned, the gut microbiome is highly advantageous to the host's overall health and well-being; nevertheless, certain pathogenic bacteria and AMR genes have been discovered to be part of the gut microbial environment. Therefore, it's critical to study the gut microbiome to identify harboured pathogens and AMR related genes and microbes, addition to attain better understanding of gut microbial’s roles and potentials in livestock improvement.

This study aims to characterise the gut microbial population of free-grazing Dohne Merino sheep from the Eastern Cape province with the aims of identifying microbial members, estimating microbial diversity, and uncovering the AMR potential in this challenges yet significant South African sheep breed. Shotgun metagenomics allows for uncultured and low abundance microbes to be detected in samples (Ranjan et al., 2016). Complex and diverse communities like the gut microbiota yield large-scale DNA sequence data that need to be analysed by computerized programming, therefore the combination of shotgun metagenomics and bioinformatics approach was most suitable for the current study.

In chapter 3 the composition and diversity of the microbial population of Dohne Merino sheep was assessed with focus on bacteria and archaea. A very diverse gut microbiome was discovered in the gut of Dohne Merino sheep with Bacteroides (53,9%) and Firmicutes (25,4%), dominating. These phyla are heavily involved in feed fermentation and digestion of complex polymers in the gut of ruminants. Many studies have also Bacteroides and Firmicutes to be the most abundant members of the gut microbial population in ruminants (Russell, 2002, Kamra, 2005, Li et al., 2012, Malmuthuge et al., 2015). The high abundance of complex feed fermenters was considered most beneficial for the Wauldbly Dohne Merino since the Wauldby farm is a sourveld with low nutrition, difficult to digest feed. Other present phyla include

Proteobacteria, Fibrobacteria, Methanobacteria and Thermoplasmata amongst others. Besides feed fermentation, immune defense is one of the most represented biological functions of the identified microbes in the gut of Dohne Merino(*Bacillus* and *Clostridia*). It was reasoned that these immune defense related microbes are as a result of the sheep's exposure to pathogens and parasites in the grazing field. Additionally, free roaming of Dohne Merino was also used to explain the presence of disease related bacteria such a Actinobacteria and Cyanobacteria. A comparative analysis of the different compartments found the reticulum to be the most diverse chamber and the abomasum as the least diverse. This contrasts with most literature where the rumen is recognised as the most diverse. It was suggested that since the environment has an influence the gut microbiome it was therefore acceptable that studies of different species in different environments would yield varying outcomes. Additionally, these findings were deemed reasonable because of the functions these compartments i.e. the abomasum harbours the least diversity of microbes because it is more acidic than the other compartments and receives feed last with less residue and microbes from the outside environment. Significant proportions of both the bacterial and archaeal populations could not be taxonomically classified by the tools used in this study because they remain uncultivated and therefore do not form part of databases known microbes identified through culture-based methods. This signifies the limitation of existing microbial databases for taxonomic classification and the power of metagenomics to unveil more accurate picture of diversity which would be impossible to obtain with other methods. However more studies are required to classify these unknown microbes and improve the existing databases. Most of the archaeal population could not be identified even at high levels of classification, this highlights the need for more archaeal identification and taxonomic classification studies.

Viruses are amongst the inhabitants of the gut of animals. Generally, viruses are difficulty to study compared to other microbes, this is because they cannot survive without a host and tend to mutate very easily. However, advances in technology have led to a better understanding of viruses. Chapter 4 utilised metagenomics sequencing and bioinformatic analysis to characterise the gut virome of Dohne Merino. A diverse population of viruses was discovered, with most viruses being phages. Five realms, 8 kingdoms and 13 phyla, 21 classes and 37 family/genus were found present. The phages belonged to realm Duplodnaviria which made up 77% if the virome. This result was expected because bacteria (which are hosts for phages) were found to be the most abundant members in chapter 3. Other realms present included Varidnaviria, Riboviria, Monodnaviria and Adnaviria. Most of the viruses' natural known hosts were bacteria and the most common nucleic acid structure amongst the viruses was dsDNA. Other nucleic acid structures found in this study that have been previously identified in viruses of the gut include -ssRNA, +ssRNA, ssDNA and dsRNA. As with bacteria, a low diversity and abundance of disease associated viruses was observed. This included the order Ortervirales which is linked to bovine leukemia immunodeficiency virus (BIV) and Jembrana disease virus (JEV), additionally the sheep were found to be carriers of Herpesviruses which is a causative agent for Malignant catarrhal fever (MCF) a deadly disease to cattle. Finding diseases related microbes in small abundances with no clinical signs in the animals was taken as a sign of breed resilience in Dohne Merino as this depicts their gut microbial's environment and immune system's ability to prevent over-growth of disease related microbes which would lead to illnesses. Yet the very presence of these disease-associated members is a warning of potential disease outbreaks sheep as well as other species such as cattle in the farm.

To deal with the constant challenges of disease, illness and feed shortages in livestock, farmers have become reliant on antimicrobials both as treatments to diseases and feed additives for growth promotion and improved feed efficiency. This has been found to be a major contributor to the challenge of AMR that we are facing globally. Antimicrobial Resistance (AMR) is the ability of microorganisms to withstand antibiotics drugs that should be deadly to them. AMR is not only a problem in livestock, but it is a threat to human health as well, as more microbes are becoming resistant to drugs that used to be effective against them. More than 1 million people died of AMR related deaths in South Africa in 2019. The WHO has named AMR a major global concern that requires surveillance and action. AMR surveillance involves the analysis of antimicrobial resistance genes (ARG) particular environments for an accurate measurement of AMR prevalence in a country and globally.

The gut environment of many animals has been found to be a reservoir of ARG, additionally resistance strains can transferred between animals and across species which acerbate the spread and selection for AMR strains. The objective of chapter 5 was to contribute the AMR global action plan through providing data that will expand the scope of AMR resistance in small ruminants. From the metagenomic analysis of the gut resistome of Dohne Merino, 12 ARGs (*nimJ*, *lnu*(C), *tet*(W), *tet*(O), *tet*(40), *tet*(Q), *tet*(44), *mef*(A), *ileS*, *aadE*, *aph(2")-If*, and *rpoB*) were identified. Two of these genes, the *mef*(A) and *aph(2")-If* gene are multi-drug resistance (MDR) genes conferring resistance to 7 and 4 antimicrobials respectively. Eight of 12 identified genes were found to be core resistance genes (*tet*(O,W,Q,40,44), *Inu*(c), *nimJ* and *mef*(A)), present in all the compartments of all the sampled animals. The identified ARGs translate resistance to 15 antimicrobials (Nitroimidazole, Tetracycline, Lincosamide, Amikacin; Gentamicin; Kanamycin; Tobramycin, Mupirocin, Streptomycin, Macrolide, Oxazolidinone, Phenicol, Pleuromutilin, Streptogramin and Rifamycin).With the prevalence of Nitroimidazole, Tetracycline, Lincosamide and Macrolide found in high abundances. These antimicrobial drugs are amongst the most commonly used drugs as treatment to diseases and feed additives. This finding supports literature reports that usage of specific antimicrobials correlates with the AMR prevalence of those drugs. The overall observed AMR is higher than what has been previously observed in other sheep studies. This is likely because the sheep in the current study are dosed often to deal with worms and heat-water diseases. The Wauldbly farm reports of no symptoms of AMR in the sheep despite the observed ARGs, this again proves breed resilience in Dohne Merino while giving warnings for potential future challenges with AMR in the farm.

In closing, this work has revealed great insights about the gut microbial population and environment of Dohne Merino sheep. A high microbial diversity and AMR prevalence has been uncovered. The microbial diversity findings closely align with work done in other studies.The microbial population analysis also revealed the need to improve microbial taxonomic classification databases especially for archaea and viruses. The AMR findings from the current study should be used to inform future decisions of antibiotic usage on sheep and livestock in general. Based on the outcomes of this study we recommend that further studies be conducted to make a concise connection between microbial species and ARGs in the gut of Dohne Merino.

7 CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusion

Dohne Merino sheep are generally known to be resilient to harsh conditions and diseases. The outcomes of this study support this consensus and reveals insights to factors that might be contributing to this breed's resilience. A diverse gut microbial population is most preferred in animals since it provides great stability and ability to withstand pathogens. Therefore, the high microbial diversity observed in the Dohne Merino sheep of this study can be considered a great contributor to the Dohne Merino's breed resilience. Additionally, exposure to pathogens in the grazing field was recognised as another potential contributor to resilience in Dohne Merino sheep. Disease linked bacteria and viruses were identified in the gut microbial population however, the animals showed no clinical symptoms. Furthermore, a high AMR prevalence with MDR genes was observed but the animals had no symptoms of AMR. Therefore, it is concluded that the studied Dohne Merino sheep showed resilience against viral and bacterial diseases as well AMR and MDR carrier pathogens.

7.2 Recommendations

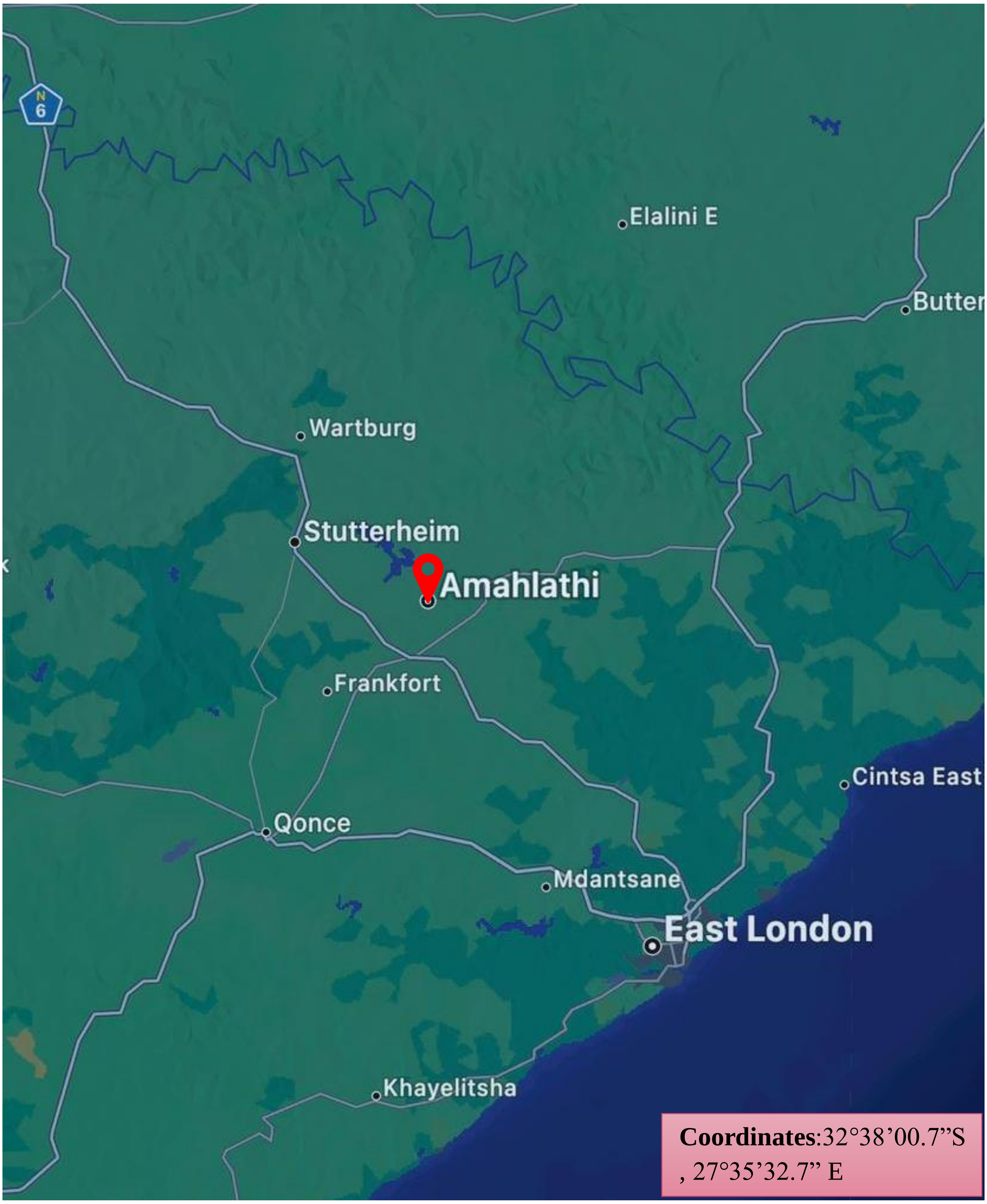
This study was able to provide an in-depth measure of the microbial diversity and a broad picture of the AMR prevalence in the gut of Dohne Merino sheep. Some observations made in the analysis were indicative of the fact that environmental factors play a role in the shaping of microbial diversity and AMR prevalence. We therefore recommend further studies that will explore the role of the environment in the gut microbial diversity and AMR, in order to gain insights on how "environmental" conditions can be used to favourably manipulate the gut microbiome. Full interpretation of this study's outcomes was significantly limited by various scientific gaps such as inadequate study of the last three stomach compartments, lack of sufficient knowledge in the role of viruses in the gut, limitations of taxonomic classification databases, and ARG carrier species. This lack of sufficient scientific knowledge makes it challenging to draw concise conclusions on some findings. Hence, it is recommended that more studies that will bridge these gaps be undertaken. This will aid future studies of the gut microbiome of sheep to better interpret their results and draw more concise conclusions. Though the metagenomics approach has much benefit in the measure of diversity in many complex ecosystems such as the gut, we recommend other techniques such as the culture-based method still be utilised. This will help maintain the balance between diversity measuring studies and taxonomic classification studies because, taxonomic classification studies remain crucial as they provide biological databases that are useful in metagenomic studies. The outcomes of this study also suggested a potential relationship between gut microbial diversity and AMR prevalence, we recommend that further studies be undertaken to investigate this relationship and its potential as an AMR control measure.

8 APPENDIX

Table A1 Bacterial species identified in the gut of Dohne Merino sheep

- *Absiella sp, 9CBEGH2*
- *Alloprevotella sp, E39*
- *Anaerobutyricum hallii*
- *Bacteroides caecimuris*
- *Bacteroides cellulosilyticus*
- *Bacteroides coprosuis*
- *Bacteroides fragilis*
- *Bacteroides heparinolyticus*
- *Bacteroides thetaiotaomicron*
- *Bacteroides uniformis*
- *Bacteroides xylanolyticus*
- *Bacteroides zoogleformamans*
- *Bibersteinia trehalosi*
- *Bifidobacterium animalis*
- *Bifidobacterium bifidum*
- *Bifidobacterium choerinum*
- *Bifidobacterium longum*
- *Bifidobacterium pseudolongum*
- *Blautia producta*
- *Blautia sp. SC05B48*
- *Burkholderia gladioli*
- *Butyrivibrio fibrisolvens*
- *Butyrivibrio hungatei*
- *Butyrivibrio proteoclasticus*
- *Chryseobacterium camelliae*
- *Cloacibacillus porcorum*
- *Clostridiales bacterium CCNA10*
- *Clostridioides difficile*
- *Clostridium botulinum*
- *Clostridium sp, SY8519*
- *Clostridium tetani*
- *Cutibacterium acnes*
- *Dehalococcoides mccartyi*
- *Denitrobacterium detoxificans*
- *Enterococcus cecorum*
- *Escherichia coli*
- *Ethanoligenens harbinense*
- *Eubacterium cellulosolvens*
- *Faecalibacterium prausnitzii*
- *Fibrobacter succinogenes*
- *Fusobacterium necrophorum*
- *Intestinibaculum porci*
- *Kosakonia cowanii*
- *Lachnoclostridium sp. YL32*
- *Lachnospira eligens*
- *Lachnospiraceae bacterium KM106-2*
- *Leifsonia xyli*
- *Longibaculum sp. KGMB06250*
- *Mannheimia haemolytica*
- *Microbacterium lemovicicum*
- *Microbacterium testaceum*
- *Muribaculaceae bacterium DSM 108610*
- *Nocardiopsis alba*
- *Olsenella umbonate*
- *Pandoraea pnomenusa*
- *Pantoea agglomerans*
- *Pantoea ananatis*
- *Pantoea vagans*
- *Paracoccus sp. Arc7-R13*
- *Pedobacter sp. KBS0701*
- *Petrimonas mucosa*
- *Prevotella dentalis*
- *Prevottela denticola*
- *Prevotella fusca*

- *Propionibacterium australiense*
- *Pseudobutyrivibrio xylanivorans*
- *Pseudomonas extremoustralis*
- *Pseudomonas oryzihabitans*
- *Pseudomonas palleroniana*
- *Pseudomonas protegens*
- *Pseudomonas sp. 02C 26*
- *Roseburia hominis*
- *Roseburia intestinalis*
- *Ruminococcus albus*
- *Ruminococcus bicirculans*
- *Ruminococcus champanellensis*
- *Ruminococcus sp, JE7A12*
- *Streptococcus sp, HSISB1*
- *Succinivibrio dextrinosolvens*
- *Tannerella forsythia*
- *Treponema phagedenis*
- *Treponema succinifaciens*
- *Prevotella intermedia*
- *Prevotella jejuni*
- *Prevotella melaninogenica*
- *Prevotella ruminicola*
- *Prevotella scopos*
- *Prevotella sp, oral taxon 299*
- *Ruthenibacterium lactatiformans*
- *Selenomonas ruminantium*
- *Shewanella polaris*
- *Slackia heliotrinireducens*
- *Stenotrophomonas rhizophila*
- *Stenotrophomonas sp. 364*
- *Stenotrophomonas sp. LM091*
- *Streptococcus equinus*
- *Streptococcus gallolyticus*
- *Streptococcus infantarius*
- *Streptococcus sp, CNU 77-61*
- *Streptococcus sp, CNU G3*



Coordinates:32°38’00.7”S , 27°35’32.7” E

Figure A1 GPS coordinates of Amahlathi Local municipality in the Eastern Cape Province in South Africa. The Amahlathi local municipality is where the Wauldby farm for Dohne Merino sheep is located.

9 REFERENCES

AARESTRUP, F. M., JENSEN, V. F., EMBORG, H.-D., JACOBSEN, E. & WEGENER, H. C. J. A. J. O. V. R. 2010. Changes in the use of antimicrobials and the effects on productivity of swine farms in Denmark. 71**,** 726-733.

ACAR KIRIT, H., BOLLBACK, J. P. & LAGATOR, M. 2022. The role of the environment in horizontal gene transfer. *Molecular Biology and Evolution,* 39**,** msac220.

ADAM, P. S., BORREL, G., BROCHIER-ARMANET, C. & GRIBALDO, S. J. T. I. J. 2017. The growing tree of Archaea: new perspectives on their diversity, evolution and ecology. 11**,** 2407-2425.

AL-DOBAIB, S. & MOUSA, H. 2009. Benefits and risks of growth promoters in animal production. *J Food Agric Environ,* 7**,** 202-8.

AL-MASAUDI, S., EL KAOUTARI, A., DRULA, E., AL-MEHDAR, H., REDWAN, E. M., LOMBARD, V. & HENRISSAT, B. J. F. I. M. 2017. A metagenomics investigation of carbohydrate-active enzymes along the gastrointestinal tract of Saudi sheep. 8**,** 666.

ALAUZET, C., LOZNIEWSKI, A. & MARCHANDIN, H. 2019. Metronidazole resistance and nim genes in anaerobes: A review. *Anaerobe,* 55**,** 40-53.

ALCOCK, B. P., HUYNH, W., CHALIL, R., SMITH, K. W., RAPHENYA, A. R., WLODARSKI, M. A., EDALATMAND, A., PETKAU, A., SYED, S. A. & TSANG, K. K. 2023. CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic acids research,* 51**,** D690-D699.

ALEXANDER, T. & PLAIZIER, J. J. A. F. 2016. From the Editors: The importance of microbiota in ruminant production. 6**,** 4-7.

ALIAN, F., RAHIMI, E., SHAKERIAN, A., MOMTAZ, H., RIAHI, M. & MOMENI, M. J. G. V. 2012. Antimicrobial resistance of Staphylococcus aureus isolated from bovine, sheep and goat raw milk. 8**,** 111-114.

AMBROSE, K. D., NISBET, R. & STEPHENS, D. S. 2005. Macrolide efflux in Streptococcus pneumoniae is mediated by a dual efflux pump (mel and mef) and is erythromycin inducible. *Antimicrobial agents and chemotherapy,* 49**,** 4203-4209.

ANDERSON, C. L., SULLIVAN, M. B. & FERNANDO, S. C. 2017. Dietary energy drives the dynamic response of bovine rumen viral communities. *Microbiome,* 5**,** 1-19.

ANGELIDAKI, I., KARAKASHEV, D., BATSTONE, D., PLUGGE, C. & STAMS, A. 2011. Methods in methane metabolism. Part A: methanogenesis. *Methods in enzymology,* 494.

ARSHAD, M. A., HASSAN, F.-U., REHMAN, M. S., HUWS, S. A., CHENG, Y. & DIN, A. U. J. A. N. 2021. Gut microbiome colonization and development in neonatal ruminants: Strategies, prospects, and opportunities. 7**,** 883-895.

BÄCKHED, F., LEY, R. E., SONNENBURG, J. L., PETERSON, D. A. & GORDON, J. I. J. S. 2005. Host-bacterial mutualism in the human intestine. 307**,** 1915-1920.

BAHRNDORFF, S., ALEMU, T., ALEMNEH, T. & LUND NIELSEN, J. J. I. J. O. G. 2016. The microbiome of animals: implications for conservation biology. 2016.

BALDWIN, N. & WHITTON, B. J. J. O. A. P. 1992. Cyanobacteria and eukaryotic algae in sports turf and amenity grasslands: a review. 4**,** 39-47.

BANKEVICH, A., NURK, S., ANTIPOV, D., GUREVICH, A. A., DVORKIN, M., KULIKOV, A. S., LESIN, V. M., NIKOLENKO, S. I., PHAM, S. & PRJIBELSKI, A. D. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology,* 19**,** 455-477.

BARBA, M., CZOSNEK, H. & HADIDI, A. J. V. 2014. Historical perspective, development and applications of next-generation sequencing in plant virology. 6**,** 106-136.

BARTLEY, S. 2022. *AMRmageddon: the Material Risks of AMR to Animal Health and the Livestock Sector* [Online]. [Accessed 11 Nov 2023].

BAUER, M., HEROLD, D., BRITTON, R., STOCK, R., KLOPFENSTEIN, T. & YATES, D. 1995. Efficacy of laidlomycin propionate to reduce ruminal acidosis in cattle. *Journal of animal science,* 73**,** 3445-3454.

BECKER, R., MARSHALL, S. & ARNOLD, P. D. J. J. O. D. S. 1963. Anatomy, development, and functions of the bovine omasum. 46**,** 835-839.

BECKER, R. B., WILCOX, C., SIMPSON, C., GILMORE, L. & FECHHEIMER, N. 1964. Genetic aspects of actinomycosis and actinobacillosis in cattle.

BELANCHE, A., PATRA, A. K., MORGAVI, D. P., SUEN, G., NEWBOLD, C. J. & YÁÑEZ-RUIZ, D. R. J. F. I. M. 2020. Gut Microbiome Modulation in Ruminants: Enhancing Advantages and Minimizing Drawbacks. 11.

BERGMAN, E. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological reviews,* 70**,** 567-590.

BERNARD, K., SHUTTLEWORTH, L., MUNRO, C., FORBES-FAULKNER, J., PITT, D., NORTON, J. & THOMAS, A. 2002. Propionibacterium australiense sp. nov. derived from granulomatous bovine lesions. *Anaerobe,* 8**,** 41-47.

BETTENCOURT, E. M. V., TILMAN, M., HENRIQUES, P. D. D. S., NARCISO, V. & CARVALHO, M. L. D. S. 2013. The economic and sociocultural role of livestock in the wellbeing of rural communities of Timor-Leste.

BINDA, C., LOPETUSO, L. R., RIZZATTI, G., GIBIINO, G., CENNAMO, V., GASBARRINI, A. J. D. & DISEASE, L. 2018. Actinobacteria: a relevant minority for the maintenance of gut homeostasis. 50**,** 421-428.

BOLGER, A. M., LOHSE, M. & USADEL, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics,* 30**,** 2114-2120.

BROOK, I. 2008. Microbiology and management of abdominal infections. *Digestive diseases and sciences,* 53**,** 2585-2591.

BRUNSON, J. L., KHORETONENKO, M. V. & STOKES, K. Y. 2016. Herpesviruses. *Vascular Responses to Pathogens***,** 123-136.

BRYANT, M. P. J. B. R. 1959. Bacterial species of the rumen. 23**,** 125-153.

BURGESS, M. 2016. *Wauldby Dohne Merinos: on the veld for 68 years!* [Online]. Farmer's Weekly. Available: https://www.farmersweekly.co.za/animals/wauldby-dohne-merinos-on-the-veld-for-68-years/ [Accessed 13 Nov 2023].

BUTAYE, P., CLOECKAERT, A. & SCHWARZ, S. 2003. Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. *International journal of antimicrobial agents,* 22**,** 205-210.

CALDWELL, S. J., HUANG, Y. & BERGHUIS, A. M. 2016. Antibiotic binding drives catalytic activation of aminoglycoside kinase APH (2 ″)-Ia. *Structure,* 24**,** 935-945.

CAMARGO, A. P., ROUX, S., SCHULZ, F., BABINSKI, M., XU, Y., HU, B., CHAIN, P. S., NAYFACH, S. & KYRPIDES, N. C. 2023. You can move, but you can't hide: identification of mobile genetic elements with geNomad. *bioRxiv***,** 2023.03. 05.531206.

CANANI, R. B., DI COSTANZO, M., LEONE, L., PEDATA, M., MELI, R. & CALIGNANO, A. J. W. J. O. G. W. 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. 17**,** 1519.

CARD, R. M., STUBBERFIELD, E., ROGERS, J., NUNEZ-GARCIA, J., ELLIS, R. J., ABUOUN, M., STRUGNELL, B., TEALE, C., WILLIAMSON, S. & ANJUM, M. F. 2018. Identification of a new antimicrobial resistance gene provides fresh insights into pleuromutilin resistance in Brachyspira hyodysenteriae, aetiological agent of swine dysentery. *Frontiers in microbiology,* 9**,** 1183.

CASTANON, J. J. P. S. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. 86**,** 2466-2471.

CASTILLO-GONZÁLEZ, A., BURROLA-BARRAZA, M., DOMÍNGUEZ-VIVEROS, J. & CHÁVEZ-MARTÍNEZ, A. J. A. D. M. V. 2014. Rumen microorganisms and fermentation. 46**,** 349-361.

CHANG, J., YAO, X., ZUO, C., QI, Y., CHEN, D. & MA, W. J. B. V. R. 2020. The gut bacterial diversity of sheep associated with different breeds in Qinghai province. 16**,** 1-9.

CHAUMEIL, P.-A., MUSSIG, A. J., HUGENHOLTZ, P. & PARKS, D. H. 2020. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database. Oxford University Press.

CHEN, S., LUO, S. & YAN, C. J. A. 2021. Gut Microbiota Implications for Health and Welfare in Farm Animals: A Review. 12**,** 93.

CHEN, Y.-B., LAN, D.-L., TANG, C., YANG, X.-N. & LI, J. 2015. Effect of DNA extraction methods on the apparent structure of yak rumen microbial communities as revealed by 16S rDNA sequencing. *Pol J Microbiol,* 64**,** 29-36.

CHEN, Y., PENNER, G. B., LI, M., OBA, M. & GUAN, L. L. 2011. Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. *Applied and environmental microbiology,* 77**,** 5770-5781.

CHOLEWIŃSKA, P., CZYŻ, K., NOWAKOWSKI, P. & WYROSTEK, A. 2020. The microbiome of the digestive system of ruminants–a review. *Animal health research reviews,* 21**,** 3-14

CHOLEWIŃSKA, P., GÓRNIAK, W. & WOJNAROWSKI, K. 2021. Impact of selected environmental factors on microbiome of the digestive tract of ruminants. *BMC veterinary research,* 17**,** 1-10.

CHRISTOPHERSON, M. R., DAWSON, J. A., STEVENSON, D. M., CUNNINGHAM, A. C., BRAMHACHARYA, S., WEIMER, P. J., KENDZIORSKI, C. & SUEN, G. J. B. G. 2014. Unique aspects of fiber degradation by the ruminal ethanologen Ruminococcus albus 7 revealed by physiological and transcriptomic analysis. 15**,** 1-13.

CIVITAREALE, C., FIORI, M., BALLERINI, A. & BRAMBILLA, G. 2004. Identification and quantification method of spiramycin and tylosin in feedingstuffs with HPLC–UV/DAD at 1 ppm level. *Journal of pharmaceutical and biomedical analysis,* 36**,** 317-325.

CLEMMONS, B. A., VOY, B. H. & MYER, P. R. 2019. Altering the gut microbiome of cattle: considerations of host-microbiome interactions for persistent microbiome manipulation. *Microbial ecology,* 77**,** 523-536.

CLIFFORD, K., DESAI, D., DA COSTA, C. P., MEYER, H., KLOHE, K., WINKLER, A. S., RAHMAN, T., ISLAM, T. & ZAMAN, M. H. 2018. Antimicrobial resistance in livestock and poor quality veterinary medicines. *Bulletin of the World Health Organization,* 96**,** 662.

COMALADA, M., BAILON, E., DE HARO, O., LARA-VILLOSLADA, F., XAUS, J., ZARZUELO, A., GALVEZ, J. J. J. O. C. R. & ONCOLOGY, C. 2006. The effects of short-chain fatty acids on colon epithelial proliferation and survival depend on the cellular phenotype. 132**,** 487-497.

COMMON, G., DEY, A. & PAUL, S. S. J. I. J. A. N. 2014. Gastrointestinal nematodiasis in ruminants: Nutritional consequences and ameliorations. 31**,** 101-109.

CREEVEY, C. J., KELLY, W. J., HENDERSON, G. & LEAHY, S. C. 2014. Determining the culturability of the rumen bacterial microbiome. *Microbial biotechnology,* 7**,** 467-479.

DADASHI, M., HAJIKHANI, B., DARBAN-SAROKHALIL, D., VAN BELKUM, A. & GOUDARZI, M. 2020. Mupirocin resistance in Staphylococcus aureus: A systematic review and meta-analysis. *Journal of global antimicrobial resistance,* 20**,** 238-247.

DAVIES, R., WALES, A. J. C. R. I. F. S. & SAFETY, F. 2019. Antimicrobial resistance on farms: a review including biosecurity and the potential role of disinfectants in resistance selection. 18**,** 753-774.

DE KLERK, H. & HEYDENRYCH, H. 1990. BLUP analysis of genetic trends in the Dohne Merino. *Proceedings of the Proceedings of the 4th World Congress on Genetics applied to Livestock Production, Edinburgh***,** 23-27.

DEL PRADO, A., LYNCH, J., LIU, S., RIDOUTT, B., PARDO, G. & MITLOEHNER, F. 2023. Animal board invited review: Opportunities and challenges in using GWP* to report the impact of ruminant livestock on global temperature change. *animal***,** 100790.

DIEKEMA, D. J. & JONES, R. N. 2001. Oxazolidinone antibiotics. *The Lancet,* 358**,** 1975-1982.

DISTEL, R., VILLALBA, J. & LABORDE, H. J. J. O. A. S. 1994. Effects of early experience on voluntary intake of low-quality roughage by sheep. 72**,** 1191-1195.

DLAMINI, N. M., VISSER, C., SNYMAN, M. A., SOMA, P. & MUCHADEYI, F. C. 2019. Genomic evaluation of resistance to Haemonchus contortus in a South African Dohne Merino flock. *Small Ruminant Research,* 175**,** 117-125.

DOH-SA 2018. Surveillance for antimicrobial resistance and consumption of antibiotics in South Africa. *In:* NATIONAL DEPARTMENT OF HEALTH, S. A. (ed.). 2018: National Department of Health Pretoria, South Africa.

DOYLE, E. K., PRESTON, J. W., MCGREGOR, B. A. & HYND, P. I. 2021. The science behind the wool industry. The importance and value of wool production from sheep. *Animal Frontiers,* 11**,** 15-23

DYSON, H. J. 2023. Vital for Viruses: Intrinsically Disordered Proteins. *Journal of Molecular Biology,* 435**,** 167860.

DZOMBA, E., VAN DER NEST, M., MTHEMBU, J., SOMA, P., SNYMAN, M., CHIMONYO, M. & MUCHADEYI, F. 2023. Selection signature analysis and genome-wide divergence of South African Merino breeds from their founders. *Frontiers in Genetics,* 13**,** 932272.

EAGAR, H., SWAN, G. & VAN VUUREN, M. 2012. A survey of antimicrobial usage in animals in South Africa with specific reference to food animals. *Journal of the South African Veterinary Association,* 83**,** 1-8.

EESWARAN, R., NEJADHASHEMI, A. P., FAYE, A., MIN, D., PRASAD, P. V. & CIAMPITTI, I. A. 2022. Current and future challenges and opportunities for livestock farming in West Africa: Perspectives from the case of Senegal. *Agronomy,* 12**,** 1818.

ESCOBAR-ZEPEDA, A., VERA-PONCE DE LEÓN, A. & SANCHEZ-FLORES, A. J. F. I. G. 2015. The road to metagenomics: from microbiology to DNA sequencing technologies and bioinformatics. 6**,** 348.

EVSEEV, P., GUTNIK, D., SHNEIDER, M. & MIROSHNIKOV, K. 2023. Use of an Integrated Approach Involving AlphaFold Predictions for the Evolutionary Taxonomy of Duplodnaviria Viruses. *Biomolecules,* 13**,** 110.

FAO. n.d. *Synthesis—Livestock and the Sustainable Development Goals: Global Agenda for Sustainable Livestock* [Online]. Available: ww.livestockdialogue.org/fileadmin/templates/res_livestock/docs/2016/Panama/FAO-AGAL_synthesis_Panama_Livestock_and_SDGs.pdf [Accessed 15 September 2023].

FERNANDO, B. R. 2012. *Metagenomic analysis of microbial communities in the bovine rumen,* Oklahoma State University.

FIRKINS, J. L. & YU, Z. 2015. RUMINANT NUTRITION SYMPOSIUM: How to use data on the rumen microbiome to improve our understanding of ruminant nutrition1,2. *Journal of Animal Science,* 93**,** 1450-1470.

FITZGERALD, S. D., COOLEY, T. M. J. J. O. Z. & MEDICINE, W. 2007. Clinical Challenge. 38**,** 604-606.

FLETCHER, S. 2015. Understanding the contribution of environmental factors in the spread of antimicrobial resistance. *Environmental health and preventive medicine,* 20**,** 243-252.FLIEGEROVÁ, K. K., KERSTIN & KIRK, PAUL & VOIGT, KERSTIN. 2015. Rumen Fungi. *Rumen Microbiology: From Evolution to Revolution***,** 15.

FOTI, C., PIPERNO, A., SCALA, A. & GIUFFRÈ, O. 2021. Oxazolidinone antibiotics: chemical, biological and analytical aspects. *Molecules,* 26**,** 4280.

FRIEDERSDORFF, J. C., KINGSTON-SMITH, A. H., PACHEBAT, J. A., COOKSON, A. R., ROOKE, D. & CREEVEY, C. J. 2020. The isolation and genome sequencing of five novel bacteriophages from the rumen active against Butyrivibrio fibrisolvens. *Frontiers in microbiology,* 11**,** 1588.

FUHRMEISTER, A. S. & JONES, R. N. The importance of antimicrobial resistance monitoring worldwide and the origins of SENTRY antimicrobial surveillance program. Open forum infectious diseases, 2019. Oxford University Press US, S1-S4.

GAÏA, M., MENG, L., PELLETIER, E., FORTERRE, P., VANNI, C., FERNANDEZ-GUERRA, A., JAILLON, O., WINCKER, P., OGATA, H. & KRUPOVIC, M. 2023. Mirusviruses link herpesviruses to giant viruses. *Nature***,** 1-7.

GILBERT, R. A., KELLY, W. J., ALTERMANN, E., LEAHY, S. C., MINCHIN, C., OUWERKERK, D. & KLIEVE, A. V. 2017. Toward understanding phage: host interactions in the rumen; complete genome sequences of lytic phages infecting rumen bacteria. *Frontiers in microbiology,* 8**,** 2340.

GILBERT, R. A. & KLIEVE, A. V. 2015. Ruminal viruses (bacteriophages, archaeaphages). *Rumen microbiology: From evolution to revolution.* Springer.

GILBERT, R. A., TOWNSEND, E. M., CREW, K. S., HITCH, T. C., FRIEDERSDORFF, J. C., CREEVEY, C. J., POPE, P. B., OUWERKERK, D. & JAMESON, E. 2020a. Rumen virus populations: technological advances enhancing current understanding. *Frontiers in microbiology,* 11**,** 450.

GIRI, N. 2021. Bacteriophage structure, classification, assembly and phage therapy. *Biosciences Biotechnology Research Asia,* 18**,** 239-250.

GODBEER, S. M., GOLD, R. M. & LAWHON, S. D. 2014. Prevalence of mupirocin resistance in Staphylococcus pseudintermedius. *Journal of clinical microbiology,* 52**,** 1250-1252.

GODBER, O. F. & WALL, R. 2014. Livestock and food security: vulnerability to population growth and climate change. *Global change biology,* 20**,** 3092-3102.

GONZÁLEZ-MÉNDEZ, A. S., TÓRTORA PÉREZ, J. L., ROJAS-ANAYA, E. & RAMÍREZ ÁLVAREZ, H. 2023. Study of the Genetic Expression of Antiretroviral Restriction Factors and Acute Phase Proteins in Cattle Infected with Bovine Leukemia Virus. *Pathogens,* 12**,** 529.

GONZÁLEZ HERNÁNDEZ, M. A., CANFORA, E. E., JOCKEN, J. W. & BLAAK, E. E. J. N. 2019. The short-chain fatty acid acetate in body weight control and insulin sensitivity. 11**,** 1943.

GRANADOS-CHINCHILLA, F. & RODRÍGUEZ, C. 2017. Tetracyclines in food and feedingstuffs: from regulation to analytical methods, bacterial resistance, and environmental and health implications. *Journal of Analytical Methods in Chemistry,* 2017.

GRAVES, K. J., NOVAK, J., SECOR, W. E., KISSINGER, P. J., SCHWEBKE, J. R. & MUZNY, C. A. 2020. A systematic review of the literature on mechanisms of 5-nitroimidazole resistance in Trichomonas vaginalis. *Parasitology,* 147**,** 1383-1391.

GROSSMAN, T. H. 2016. Tetracycline antibiotics and resistance. *Cold Spring Harbor perspectives in medicine,* 6**,** a025387.

GUILLOTEAU, P., MARTIN, L., EECKHAUT, V., DUCATELLE, R., ZABIELSKI, R. & VAN IMMERSEEL, F. J. N. R. R. 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. 23**,** 366-384.

GULYAEVA, A., GARMAEVA, S., KURILSHIKOV, A., VICH VILA, A., RIKSEN, N. P., NETEA, M. G., WEERSMA, R. K., FU, J. & ZHERNAKOVA, A. 2022. Diversity and ecology of Caudoviricetes phages with genome terminal repeats in fecal metagenomes from four Dutch cohorts. *Viruses,* 14**,** 2305.

GUO, H., ZHOU, G., TIAN, G., LIU, Y., DONG, N., LI, L., ZHANG, S., CHAI, H., CHEN, Y. & YANG, Y. J. A. 2021. Changes in Rumen Microbiota Affect Metabolites, Immune Responses and Antioxidant Enzyme Activities of Sheep under Cold Stimulation. 11**,** 712.

GUO, W., LI, Y., WANG, L., WANG, J., XU, Q., YAN, T. & XUE, B. 2015. Evaluation of composition and individual variability of rumen microbiota in yaks by 16S rRNA high-throughput sequencing technology. *Anaerobe,* 34**,** 74-79.

HA, A. D., MONIRUZZAMAN, M. & AYLWARD, F. O. 2023. Assessing the biogeography of marine giant viruses in four oceanic transects. *ISME communications,* 3**,** 43.

HAIDER, N., ROTHMAN-OSTROW, P., OSMAN, A. Y., ARRUDA, L. B., MACFARLANE-BERRY, L., ELTON, L., THOMASON, M. J., YEBOAH-MANU, D., ANSUMANA, R. & KAPATA, N. 2020. COVID-19—zoonosis or emerging infectious disease? *Frontiers in Public Health,* 8**,** 763.

HANNING, I. & DIAZ-SANCHEZ, S. J. M. 2015. The functionality of the gastrointestinal microbiome in non-human animals. 3, 1-11.

HAQ, I. U., CHAUDHRY, W. N., AKHTAR, M. N., ANDLEEB, S. & QADRI, I. 2012. Bacteriophages and their implications on future biotechnology: a review. *Virology journal,* 9, 1-8.

HARDY, B. J. A. B. 2002. The issue of antibiotic use in the livestock industry: what have we learned? 13, 129-147.

HARVEY, E. & HOLMES, E. C. 2022. Diversity and evolution of the animal virome. *Nature Reviews Microbiology,* 20, 321-334.

HE, B., JIN, S., CAO, J., MI, L. & WANG, J. 2019. Metatranscriptomics of the Hu sheep rumen microbiome reveals novel cellulases. *Biotechnology for biofuels,* 12, 1-15.

HECHT, D. W. 2006. Anaerobes: antibiotic resistance, clinical significance, and the role of susceptibility testing. *Anaerobe,* 12, 115-121.

HENDERSON, G., COX, F., GANESH, S., JONKER, A., YOUNG, W. & JANSSEN, P. H. J. S. R. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. 5, 1-15.

HENDERSON, G., COX, F., GANESH, S., JONKER, A. & YOUNG, W. J. P. O. 2012. Rumen microbial community composition varies with diet and host, but a. 7, e38571.

HENNESSEY, M., WHATFORD, L., PAYNE-GIFFORD, S., JOHNSON, K. F., VAN WINDEN, S., BARLING, D. & HÄSLER, B. J. P. V. M. 2020. Antimicrobial & antiparasitic use and resistance in British sheep and cattle: a systematic review. 105174.

HEPWORTH, K. 2006. Managing internal parasitism in sheep and goats.

HERNÁEZ, J. R., CUCCHI, M. E. C., CRAVERO, S., MARTINEZ, M. C., GONZALEZ, S., PUEBLA, A., DOPAZO, J., FARBER, M., PANIEGO, N. & RIVAROLA, M. J. M. G. 2018. The first complete genomic structure of Butyrivibrio fibrisolvens and its chromid. 4.

HESS, M., PAUL, S. S., PUNIYA, A. K., VAN DER GIEZEN, M., SHAW, C., EDWARDS, J. E. & FLIEGEROVÁ, K. J. F. I. M. 2020. Anaerobic fungi: past, present, and future. 11.

HODGINS, D. C., CONLON, J. A. & SHEWEN, P. E. 2002. Respiratory viruses and bacteria in cattle. *Polymicrobial diseases*, 213-229.

HOLMAN, D. B. & GZYL, K. E. J. F. M. E. 2019. A meta-analysis of the bovine gastrointestinal tract microbiota. 95, fiz072.

HOOGENRAAD, N., HIRD, F., HOLMES, I. & MILLIS, N. F. 1967. Bacteriophages in rumen contents of sheep. *Journal of General Virology,* 1, 575-576.

HOOK, S. E., WRIGHT, A.-D. G. & MCBRIDE, B. W. J. A. 2010. Methanogens: methane producers of the rumen and mitigation strategies. 2010.

HU, W., PAN, J., WANG, B., GUO, J., LI, M. & XU, M. J. E. M. 2021. Metagenomic insights into the metabolism and evolution of a new Thermoplasmata order (Candidatus Gimiplasmatales). 23, 3695-3709.

HURSEY, B. 1997. Towards the twenty-first century-the challenges facing livestock production. *World Animal Review.*

HUWS, S. A., CREEVEY, C. J., OYAMA, L. B., MIZRAHI, I., DENMAN, S. E., POPOVA, M., MUÑOZ-TAMAYO, R., FORANO, E., WATERS, S. M. & HESS, M. J. F. I. M. 2018b. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. 9, 2161.

IHME. 2023. *The burden of antimicrobial resistance (AMR) in South Africa* [Online]. [Accessed 29/11/2023 2023].

IRANZO, J., KRUPOVIC, M. & KOONIN, E. 2016. The double-stranded DNA virosphere as a modular hierarchical network of gene sharing. mBio 7: e00978-16.

IVARSSON, M., SCHNÜRER, A., BENGTSON, S. & NEUBECK, A. J. F. I. M. 2016. Anaerobic fungi: a potential source of biological H2 in the oceanic crust. 7, 674.

JANDHYALA, S. M., TALUKDAR, R., SUBRAMANYAM, C., VUYYURU, H., SASIKALA, M. & REDDY, D. N. 2015. Role of the normal gut microbiota. *World journal of gastroenterology: WJG,* 21, 8787.

JANSSEN, P. H., KIRS, M. J. A. & MICROBIOLOGY, E. 2008. Structure of the archaeal community of the rumen. 74, 3619-3625.

JOHNSTON, N. J., MUKHTAR, T. A. & WRIGHT, G. D. 2002. Streptogramin antibiotics: mode of action and resistance. *Current drug targets,* 3, 335-344.

JOTWANI, R. & GUPTA, U. 1991. Virulence factors in Bacteroides fragilis group. *The Indian Journal of Medical Research,* 93, 232-235.

KALACH, N., BERGERET, M., BENHAMOU, P. H., DUPONT, C. & RAYMOND, J. 2001. High levels of resistance to metronidazole and clarithromycin in Helicobacter pylori strains in children. *Journal of clinical microbiology,* 39**,** 394-397.

KAMRA, D. N. J. C. S. 2005. Rumen microbial ecosystem. 124-135.

KAZIMIERCZAK, K. A., SCOTT, K. P., KELLY, D. & AMINOV, R. I. 2009. Tetracycline resistome of the organic pig gut. *Applied and environmental microbiology,* 75**,** 1717-1722.

KHALIL, A., BATOOL, A. & ARIF, S. J. R. 2022. Healthy Cattle Microbiome and Dysbiosis in Diseased Phenotypes. 2**,** 134-156.

KILGOUR, R., WATERHOUSE, T., DWYER, C. & IVANOV, I. 2008. Farming systems for sheep production and their effect on welfare. *The welfare of sheep***,** 213-265.

KIM, J. Y., WHON, T. W., LIM, M. Y., KIM, Y. B., KIM, N., KWON, M.-S., KIM, J., LEE, S. H., CHOI, H.-J. & NAM, I.-H. J. M. 2020. The human gut archaeome: identification of diverse haloarchaea in Korean subjects. 8**,** 1-17.

KıRMUSAOĞLU, S. 2019. *Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods,*BoD–Books on Demand.

KLIEVE, A. V., GREGG, K. & BAUCHOP, T. 1991. Isolation and characterization of lytic phages from Bacterioides ruminicola ss brevis. *Current Microbiology,* 23**,** 183-187.

KOONIN, E. V., KRUPOVIC, M. & YUTIN, N. 2015. Evolution of double-stranded DNA viruses of eukaryotes: from bacteriophages to transposons to giant viruses. *Annals of the New York Academy of Sciences,* 1341**,** 10-24.

KOTSINYAN, A., SAHAKYAN, H. & ZAKARYAN, H. 2022. Identification of the Branching Order within the Kingdom Bamfordvirae. *bioRxiv***,** 2022.05. 25.493380.

KOTZÉ, J. 1951. The development of a muttonwoolled sheep for the sour-grassveld area. *Farming in South Africa,* 28**,** 110-113.

KRUPOVIC, M., KUHN, J. H., WANG, F., BAQUERO, D. P., DOLJA, V. V., EGELMAN, E. H., PRANGISHVILI, D. & KOONIN, E. V. 2021. Adnaviria: a new realm for archaeal filamentous viruses with linear A-form double-stranded DNA genomes. *Journal of Virology,* 95**,** 10.1128/jvi. 00673-21.

KWOK, K. T., NIEUWENHUIJSE, D. F., PHAN, M. V. & KOOPMANS, M. P. 2020. Virus metagenomics in farm animals: a systematic review. *Viruses,* 12**,** 107.

LA REAU, A. J. & SUEN, G. J. J. O. M. 2018. The Ruminococci: key symbionts of the gut ecosystem. 56**,** 199-208.

LAI, C. K., NG, R. W., LEUNG, S. S., HUI, M. & IP, M. 2022. Overcoming the rising incidence and evolving mechanisms of antibiotic resistance by novel drug delivery approaches–an overview. *Advanced Drug Delivery Reviews,* 181**,** 114078.

LAPORTE-URIBE, J. & GIBBS, S. Differential passage of rumen bacterial populations to the abomasum in sheep. Proceedings of the New Zealand Society of Animal Production, 2009. New Zealand Society of Animal Production, 242-243.

LARSSON, D. J. & FLACH, C.-F. 2022. Antibiotic resistance in the environment. *Nature Reviews Microbiology,* 20**,** 257-269.

LARTIGUE, A., BURLAT, B., COUTARD, B., CHASPOUL, F., CLAVERIE, J.-M. & ABERGEL, C. 2015. The megavirus chilensis Cu, Zn-superoxide dismutase: the first viral structure of a typical cellular copper chaperone-independent hyperstable dimeric enzyme. *Journal of Virology,* 89**,** 824-832.

LECLERCQ, R. 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clinical infectious diseases,* 34**,** 482-492.

LEE, V. 2007. Anti-gram positive agents of natural product origins.

LEFKOWITZ, E. J., DEMPSEY, D. M., HENDRICKSON, R. C., ORTON, R. J., SIDDELL, S. G. & SMITH, D. B. 2018. Virus taxonomy: the database of the International Committee on Taxonomy of Viruses (ICTV). *Nucleic acids research,* 46**,** D708-D717.

LETTAT, A., NOZIÈRE, P., SILBERBERG, M., MORGAVI, D. P., BERGER, C. & MARTIN, C. J. B. M. 2012. Rumen microbial and fermentation characteristics are affected differently by bacterial probiotic supplementation during induced lactic and subacute acidosis in sheep. 12**,** 1-12.

LEVY, B. & JAMI, E. J. F. I. M. 2018. Exploring the prokaryotic community associated with the rumen ciliate protozoa population. 9**,** 2526.

LEVY, S. 2014. Reduced antibiotic use in livestock: how Denmark tackled resistance. NLM-Export.

LI, H., TAUS, N. S., LEWIS, G. S., KIM, O., TRAUL, D. L. & CRAWFORD, T. B. 2004. Shedding of ovine herpesvirus 2 in sheep nasal secretions: the predominant mode for transmission. *Journal of clinical microbiology,* 42**,** 5558-5564.

LI, J., LI, Y., ZHOU, Y., WANG, C., WU, B. & WAN, J. J. B. R. I. 2018. Actinomyces and alimentary tract diseases: a review of its biological functions and pathology. 2018.

LI, J., SI, H., DU, H., GUO, H., DAI, H., XU, S. & WAN, J. J. B. M. 2021. Comparison of gut microbiota structure and Actinobacteria abundances in healthy young adults and elderly subjects: a pilot study. 21**,** 1-10

LI, R. W., CONNOR, E. E., LI, C., BALDWIN, V., RANSOM L & SPARKS, M. E. J. E. M. 2012. Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. 14**,** 129-139.

LIU, K., ZHANG, Y., XU, Q., ZHENG, N., ZHAO, S., HUANG, G. & WANG, J. J. A. N. 2020. Ruminal microbiota-host interaction and its effect on nutrient metabolism.

LIU, Y., ZHOU, Z., PAN, J., BAKER, B. J., GU, J.-D. & LI, M. J. T. I. J. 2018. Comparative genomic inference suggests mixotrophic lifestyle for Thorarchaeota. 12**,** 1021-1031.

LOBO, R. R. & FACIOLA, A. P. 2021. Ruminal phages–a review. *Frontiers in microbiology,* 12**,** 763416.

LOPETUSO, L. R., SCALDAFERRI, F., PETITO, V. & GASBARRINI, A. J. G. P. 2013. Commensal Clostridia: leading players in the maintenance of gut homeostasis. 5**,** 1-8.

LOZUPONE, C. A., STOMBAUGH, J. I., GORDON, J. I., JANSSON, J. K. & KNIGHT, R. 2012. Diversity, stability and resilience of the human gut microbiota. *Nature,* 489**,** 220-230.

MACLEOD, F., KINDLER, G. S., WONG, H. L., CHEN, R. & BURNS, B. P. J. A. M. 2019. Asgard archaea: diversity, function, and evolutionary implications in a range of microbiomes. 5**,** 48.

MAFUNA, T., SOMA, P., TSOTETSI-KHAMBULE, A., HEFER, C., MUCHADEYI, F., THEKISOE, O. & PIERNEEF, R. 2021. Bacterial profiling of Haemonchus contortus gut microbiome infecting Dohne Merino sheep in South Africa. *Scientific reports,* 11**,** 5905.

MAHASHI, M., MGWALI, N. & OBI, A. 2019. Assessing socio-economic factors influencing wool production in kolomana villages of Eastern Cape, South Africa. *South African Journal of Agricultural Extension,* 47**,** 59-74.

MALIK, P., TRIVEDI, S., KOLTE, A., SEJIAN, V., BHATTA, R. & RAHMAN, H. 2022. Diversity of rumen microbiota using metagenome sequencing and methane yield in Indian sheep fed on straw and concentrate diet. *Saudi Journal of Biological Sciences,* 29**,** 103345.

MALMUTHUGE, N., GRIEBEL, P. J. & GUAN, L. L. J. F. I. V. S. 2015. The gut microbiome and its potential role in the development and function of newborn calf gastrointestinal tract. 2**,** 36.

MALMUTHUGE, N. & GUAN, L. L. J. A. F. 2016. Gut microbiome and omics: a new definition to ruminant production and health. 6**,** 8-12.

MANSON, J. M., RAUCH, M., GILMORE, M. S. J. G. M. & SYSTEM, R. O. T. I. 2008. The commensal microbiology of the gastrointestinal tract. 15-28.

MÄNTYNEN, S., LAANTO, E., OKSANEN, H. M., PORANEN, M. M. & DÍAZ-MUÑOZ, S. L. 2021. Black box of phage–bacterium interactions: Exploring alternative phage infection strategies. *Open biology,* 11**,** 210188.

MARKIEWICZ, L., DRAZKOWSKA, K. & SIKORSKI, P. J. 2021. Tricks and threats of RNA viruses–towards understanding the fate of viral RNA. *RNA biology,* 18**,** 669-687.

MARSHALL, B. M. & LEVY, S. B. J. C. M. R. 2011. Food animals and antimicrobials: impacts on human health. 24**,** 718-733.

MARTHA, R., ANDREW, D., ANDREY, V. & SHAUN, H. 2011. Phages in nature. *Bacteriophage,* 1**,** 31-45.

MARX, V. J. N. 2013. The genome jigsaw. 501**,** 263-268.

MBATHA, C. 2021. Livestock production and marketing for small emerging farmers in South Africa and Kenya: comparative lessons. *South African Journal of Agricultural Extension,* 49**,** 141-161.

MCCRACKEN, V. J. & LORENZ, R. G. J. C. M. 2001. The gastrointestinal ecosystem: A precarious alliance among epithelium, immunity and microbiota: Microreview. 3**,** 1-11.

MCGORUM, B. C., PIRIE, R. S., GLENDINNING, L., MCLACHLAN, G., METCALF, J. S., BANACK, S. A., COX, P. A. & CODD, G. A. J. V. R. 2015. Grazing livestock are exposed to terrestrial cyanobacteria. 46**,** 1-10.

MCKINNEY, C. W., LOFTIN, K. A., MEYER, M. T., DAVIS, J. G. & PRUDEN, A. 2010. Tet and sul antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. *Environmental science & technology,* 44**,** 6102-6109.

MCLOUGHLIN, S., SPILLANE, C., CLAFFEY, N., SMITH, P. E., O'ROURKE, T., DISKIN, M. G. & WATERS, S. M. J. F. I. M. 2020. Rumen microbiome composition is altered in sheep divergent in feed efficiency. 11**,** 1981.

MCMURRY, L. M., GEORGE, A. & LEVY, S. 1994. Active efflux of chloramphenicol in susceptible Escherichia coli strains and in multiple-antibiotic-resistant (Mar) mutants. *Antimicrobial agents and chemotherapy,* 38**,** 542-546.

MEDJEKAL, S. & GHADBANE, M. 2021. Sheep digestive physiology and constituents of feeds. *Sheep Farming-An Approach to Feed, Growth and Health.* IntechOpen.

MEINEKE, B., HEIMGÄRTNER, J., LAFRANCHI, L. & ELSÄSSER, S. J. J. A. C. B. 2018. Methanomethylophilus alvus Mx1201 provides basis for mutual orthogonal pyrrolysyl tRNA/aminoacyl-tRNA synthetase pairs in mammalian cells. 13**,** 3087-3096.

MENZEL, P., NG, K. L. & KROGH, A. 2016. Fast and sensitive taxonomic classification for metagenomics with Kaiju. *Nature communications,* 7**,** 11257.

MIHARA, T., NISHIMURA, Y., SHIMIZU, Y., NISHIYAMA, H., YOSHIKAWA, G., UEHARA, H., HINGAMP, P., GOTO, S. & OGATA, H. 2016. Linking virus genomes with host taxonomy. *Viruses,* 8**,** 66.

MILLER, T. L. & JENESEL, S. E. J. J. O. B. 1979. Enzymology of butyrate formation by Butyrivibrio fibrisolvens. 138**,** 99-104.

MORGAVI, D. P., RATHAHAO-PARIS, E., POPOVA, M., BOCCARD, J., NIELSEN, K. F. & BOUDRA, H. 2015. Rumen microbial communities influence metabolic phenotypes in lambs. *Frontiers in Microbiology,* 6**,** 1060.

MTHOMBENI, T. C., BURGER, J. R., LUBBE, M. S. & JULYAN, M. 2022. Antibiotic consumption in the public sector of the Limpopo province, South Africa, 2014–2018. *Southern African Journal of Infectious Diseases,* 37.

MUKHTAR, T. A. & WRIGHT, G. D. 2005. Streptogramins, oxazolidinones, and other inhibitors of bacterial protein synthesis. *Chemical reviews,* 105**,** 529-542.

MUPFUNYA, C. R., QEKWANA, D. N. & NAIDOO, V. 2021a. Antimicrobial use practices and resistance in indicator bacteria in communal cattle in the Mnisi community, Mpumalanga, South Africa. *Veterinary medicine and science,* 7**,** 112-121.

MUPFUNYA, C. R., QEKWANA, D. N., NAIDOO, V. J. V. M. & SCIENCE 2021b. Antimicrobial use practices and resistance in indicator bacteria in communal cattle in the Mnisi community, Mpumalanga, South Africa. 7**,** 112-121.

MURCIA, P., DONACHIE, W. & PALMARINI, M. 2009. Viral pathogens of domestic animals and their impact on biology, medicine and agriculture. *Encyclopedia of Microbiology***,** 805.

NAMONYO, S., WAGACHA, M., MAINA, S., WAMBUA, L. & AGABA, M. 2018. A metagenomic study of the rumen virome in domestic caprids. *Archives of virology,* 163**,** 3415-3419.

NAQVI, S. & SEJIAN, V. J. A. J. O. A. S. 2011. Global climate change: role of livestock. 3**,** 19-25.

NAYFACH, S., CAMARGO, A. P., SCHULZ, F., ELOE-FADROSH, E., ROUX, S. & KYRPIDES, N. C. 2021. CheckV assesses the quality and completeness of metagenome-assembled viral genomes. *Nature biotechnology,* 39**,** 578-585.

NEWBOLD, C. J., DE LA FUENTE, G., BELANCHE, A., RAMOS-MORALES, E. & MCEWAN, N. R. J. F. I. M. 2015. The role of ciliate protozoa in the rumen. 6**,** 1313.

NGQULANA, A. & OBI, A. 2019. The impact of extension intensities on income of sheep producers in the Eastern Cape Province of South Africa. *South African Journal of Agricultural Extension,* 47**,** 54-60.

NICHOLSON, J. K., HOLMES, E. & WILSON, I. D. 2005. Gut microorganisms, mammalian metabolism and personalized health care. *Nature Reviews Microbiology,* 3**,** 431-438.

NOGRADO, K., UNNO, T., HUR, H.-G. & LEE, J.-H. 2021. Tetracycline-resistant bacteria and ribosomal protection protein genes in soils from selected agricultural fields and livestock farms. *Applied Biological Chemistry,* 64**,** 1-9.

ODUNIYI, O. S., RUBHARA, T. T. & ANTWI, M. A. 2020. Sustainability of livestock farming in South Africa. Outlook on production constraints, climate-related events, and upshot on adaptive capacity. *Sustainability,* 12**,** 2582.

O'HARA, E., NEVES, A. L., SONG, Y. & GUAN, L. L. J. A. R. O. A. B. 2020a. The role of the gut microbiome in cattle production and health: driver or passenger? 8**,** 199-220.

O'HARA, E., NEVES, A. L. A., SONG, Y. & GUAN, L. L. 2020b. The Role of the Gut Microbiome in Cattle Production and Health: Driver or Passenger? *Annual Review of Animal Biosciences,* 8**,** 199-220.

OIKONOMOU, G., TEIXEIRA, A. G. V., FODITSCH, C., BICALHO, M. L., MACHADO, V. S. & BICALHO, R. C. J. P. O. 2013. Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of Faecalibacterium species with health and growth. 8**,** e63157.

OLTJEN, J. & BECKETT, J. 1996. Role of ruminant livestock in sustainable agricultural systems. *Journal of Animal science,* 74**,** 1406-1409.

ONDOV, B. D., BERGMAN, N. H. & PHILLIPPY, A. M. 2011. Interactive metagenomic visualization in a Web browser. *BMC bioinformatics,* 12**,** 1-10.

OWENS, F. N. & HANSON, C. F. J. J. O. D. S. 1992. External and internal markers for appraising site and extent of digestion in ruminants. 75**,** 2605-2617.

ÖZTÜRK, H. & GURSEL, G. 2021. Rumen physiology: microorganisms, fermentation and manipulation. *Ankara Üniversitesi Veteriner Fakültesi Dergisi,* 68**,** 423-434.

PALESE, P. 1998. RNA virus vectors: where are we and where do we need to go? *Proceedings of the National Academy of Sciences,* 95**,** 12750-12752.

PARISH, J. A., RIVERA, J. D. & BOLAND, H. T. 2009. Understanding the ruminant animal digestive system.

PARMAR, N. R., JAKHESARA, S. J., MOHAPATRA, A. & JOSHI, C. G. 2016. Rumen virome: an assessment of viral communities and their functions in the rumen of an Indian buffalo. *Current Science***,** 919-925.

PARTE, A. C., CARBASSE, J. S., MEIER-KOLTHOFF, J. P., REIMER, L. C., GÖKER, M. J. I. J. O. S. &

MICROBIOLOGY, E. 2020. List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. 70, 5607.

PATYRA, E., PRZENIOSŁO-SIWCZYŃSKA, M., GRELIK, A. & KWIATEK, K. 2019. Use of tetracyclines in feeds: causes and consequences. *Medycyna Weterynaryjna,* 75, 280-286.

PAYET, J. P. & SUTTLE, C. A. 2013. To kill or not to kill: the balance between lytic and lysogenic viral infection is driven by trophic status. *Limnology and Oceanography,* 58, 465-474.

PAYNTER, M., EWERT, D. & CHALUPA, W. 1969. Some morphological types of bacteriophages in bovine rumen contents. *Applied microbiology,* 18, 942-943.

PELTON, S., FIGUEIRA, M., ALBUT, R. & STALKER, D. 2000. Efficacy of linezolid in experimental otitis media. *Antimicrobial agents and chemotherapy,* 44, 654-657.

PERKINS, D., HOGUE, J. S., FAIRCHOK, M., BRAUN, L. & VISCOUNT, H. B. 2008. Mupirocin resistance screening of methicillin-resistant Staphylococcus aureus isolates at Madigan Army Medical Center. *Military medicine,* 173, 604-608.

POLTRONIERI, P., SUN, B. & MALLARDO, M. 2015. RNA viruses: RNA roles in pathogenesis, coreplication and viral load. *Current genomics,* 16, 327-335.

PRECUP, G. & VODNAR, D.-C. J. B. J. O. N. 2019. Gut Prevotella as a possible biomarker of diet and its eubiotic versus dysbiotic roles: a comprehensive literature review. 122, 131-140.

PRYDE, S. E., DUNCAN, S. H., HOLD, G. L., STEWART, C. S. & FLINT, H. J. J. F. M. L. 2002. The microbiology of butyrate formation in the human colon. 217, 133-139.

PULINA, G., FRANCESCONI, A. H. D., STEFANON, B., SEVI, A., CALAMARI, L., LACETERA, N., DELL'ORTO, V., PILLA, F., AJMONE MARSAN, P. & MELE, M. 2017. Sustainable ruminant production to help feed the planet. *Italian Journal of Animal Science,* 16, 140-171.

RAHMAN, M. T., SOBUR, M. A., ISLAM, M. S., IEVY, S., HOSSAIN, M. J., EL ZOWALATY, M. E., RAHMAN, A. T. & ASHOUR, H. M. 2020. Zoonotic diseases: etiology, impact, and control. *Microorganisms,* 8, 1405.

RAMIREZ, M. S. & TOLMASKY, M. E. 2010. Aminoglycoside modifying enzymes. *Drug resistance updates,* 13, 151-171.

RANJAN, R., RANI, A., METWALLY, A., MCGEE, H. S., PERKINS, D. L. J. B. & COMMUNICATIONS, B. R. 2016. Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon sequencing. 469, 967-977.

REARDON, S. 2023. Antibiotic use in farming set to soar despite drug-resistance fears. *Nature,* 614, 397-397.

RINNINELLA, E., RAOUL, P., CINTONI, M., FRANCESCHI, F., MIGGIANO, G. A. D., GASBARRINI, A. & MELE, M. C. J. M. 2019. What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. 7, 14.

ROBERTS, M. C. & SCHWARZ, S. 2016. Tetracycline and phenicol resistance genes and mechanisms: importance for agriculture, the environment, and humans. *Journal of environmental quality,* 45, 576-592.

ROEHE, R., DEWHURST, R. J., DUTHIE, C.-A., ROOKE, J. A., MCKAIN, N., ROSS, D. W., HYSLOP, J. J., WATERHOUSE, A., FREEMAN, T. C. & WATSON, M. J. P. G. 2016. Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. 12, e1005846.

ROUND, J. L. & MAZMANIAN, S. K. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nature reviews immunology,* 9, 313-323.

RUSSELL, J. B. 2002. *Rumen microbiology and its role in ruminant nutrition,* Department of Microbiology, Cornell University.

RUST, J. & RUST, T. J. S. A. J. O. A. S. 2013. Climate change and livestock production: A review with emphasis on Africa. 43, 255-267.

RYU, W.-S. 2016. *Molecular virology of human pathogenic viruses,* Academic Press.

SADER, H. S., PAUKNER, S., IVEZIC-SCHOENFELD, Z., BIEDENBACH, D. J., SCHMITZ, F. J. & JONES, R. N. 2012. Antimicrobial activity of the novel pleuromutilin antibiotic BC-3781 against organisms responsible for community-acquired respiratory tract infections (CARTIs). *Journal of antimicrobial chemotherapy,* 67, 1170-1175.

SAMUELSON, J. 1999. Why metronidazole is active against both bacteria and parasites. *Antimicrobial agents and chemotherapy,* 43, 1533-1541.

SANJUÁN, R., ILLINGWORTH, C. J., GEOGHEGAN, J. L., IRANZO, J., ZWART, M. P., CIOTA, A. T., MORATORIO, G., GAGO-ZACHERT, S., DUFFY, S. & VIJAYKRISHNA, D. 2021. Five challenges in the field of viral diversity and evolution. Frontiers Media SA.

SCHOEMAN, S., CLOETE, S. & OLIVIER, J. J. L. S. 2010. Returns on investment in sheep and goat breeding in South Africa. 130, 70-82.

SCHUIJT, T. J., VAN DER POLL, T., DE VOS, W. M. & WIERSINGA, W. J. J. T. I. M. 2013. The intestinal microbiota and host immune interactions in the critically ill. 21**,** 221-229.

SCHWARZ, S., KEHRENBERG, C., DOUBLET, B. & CLOECKAERT, A. 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS microbiology reviews,* 28**,** 519-542.

SEEMANN, T. 2016. ABRicate: mass screening of contigs for antiobiotic resistance genes. *San Francisco, CA: GitHub.*

SEIPLE, I. B., ZHANG, Z., JAKUBEC, P., LANGLOIS-MERCIER, A., WRIGHT, P. M., HOG, D. T., YABU, K., ALLU, S. R., FUKUZAKI, T. & CARLSEN, P. N. 2016. A platform for the discovery of new macrolide antibiotics. *Nature,* 533**,** 338-345.

SEITZ, K. W., LAZAR, C. S., HINRICHS, K.-U., TESKE, A. P. & BAKER, B. J. J. T. I. J. 2016. Genomic reconstruction of a novel, deeply branched sediment archaeal phylum with pathways for acetogenesis and sulfur reduction. 10**,** 1696-1705.

SERWECIŃSKA, L. 2020. Antimicrobials and antibiotic-resistant bacteria: a risk to the environment and to public health. *Water,* 12**,** 3313.

SHARMA, D. K., UPADHAYA, P. & BHARDWAJ, S. 2020. Intelligent Cloud Computing and Bioinformatics Data Analysis. *Applications of Cloud Computing.* Chapman and Hall/CRC.

SIHLOBO, W. 2023. Challenges facing the SA livestock industry *AgriBusiness Research*.

SMITH, C. J., ROCHA, E. R. & PASTER, B. J. J. P. 2006. The medically important Bacteroides spp. in health and disease. 7**,** 381-427.

SOLDEN, L. M., NAAS, A. E., ROUX, S., DALY, R. A., COLLINS, W. B., NICORA, C. D., PURVINE, S. O., HOYT, D. W., SCHÜCKEL, J. & JØRGENSEN, B. 2018. Interspecies cross-feeding orchestrates carbon degradation in the rumen ecosystem. *Nature microbiology,* 3**,** 1274-1284.

SONG, P., QIN, W., HUANG, Y., WANG, L., CAI, Z. & ZHANG, T. 2020. Grazing management influences gut microbial diversity of livestock in the same area. *Sustainability,* 12**,** 4160.

SOUNDARARAJAN, M., MARINCOLA, G., LIONG, O., MARCINIAK, T., WENCKER, F. D., HOFMANN, F., SCHOLLENBRUCH, H., KOBUSCH, I., LINNEMANN, S. & WOLF, S. A. 2022. Farming practice influences antimicrobial resistance burden of non-aureus staphylococci in pig husbandries. *Microorganisms,* 11**,** 31.

SPANG, A., CACERES, E. F. & ETTEMA, T. J. J. S. 2017. Genomic exploration of the diversity, ecology, and evolution of the archaeal domain of life. 357**,** eaaf3883.

STEELE, M. A., PENNER, G. B. & CHAUCHEYRAS-DURAND, F. J. J. O. D. S. 2016. Development and physiology of the rumen and the lower gut: Targets for improving gut health. 99**,** 4955-4966.

STERN, A., TE YEH, M., ZINGER, T., SMITH, M., WRIGHT, C., LING, G., NIELSEN, R., MACADAM, A. & ANDINO, R. 2017. The evolutionary pathway to virulence of an RNA virus. *Cell,* 169**,** 35-46. e19.

STOJANOV, S., BERLEC, A. & ŠTRUKELJ, B. J. M. 2020. The influence of probiotics on the firmicutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. 8**,** 1715.

STRANGE, J. E., LEEKITCHAROENPHON, P., MØLLER, F. D. & AARESTRUP, F. M. 2021. Metagenomics analysis of bacteriophages and antimicrobial resistance from global urban sewage. *Scientific Reports,* 11**,** 1600.

SUGIHARTONO, T. & MIFTAHUSSURUR, M. 2022. Antimicrobial resistance profile by metagenomic and Metatranscriptomic approach in clinical practice: opportunity and challenge. *Antibiotics,* 11**,** 654.

SUTTON, T. D. & HILL, C. 2019. Gut bacteriophage: current understanding and challenges. *Frontiers in Endocrinology,* 10**,** 784.

SWANSON, K. C. 2019. Small intestinal anatomy, physiology, and digestion in ruminants.

SWEET, M. J. & BULLING, M. T. 2017. On the importance of the microbiome and pathobiome in coral health and disease. *Frontiers in Marine Science,* 4**,** 9.

TANCA, A., FRAUMENE, C., MANGHINA, V., PALOMBA, A., ABBONDIO, M., DELIGIOS, M., PAGNOZZI, D., ADDIS, M. F. & UZZAU, S. 2017. Diversity and functions of the sheep faecal microbiota: a multi-omic characterization. *Microbial biotechnology,* 10**,** 541-554.

THAKER, M., SPANOGIANNOPOULOS, P. & WRIGHT, G. D. 2010. The tetracycline resistome. *Cellular and Molecular Life Sciences,* 67**,** 419-431.

THAL, L. A. & ZERVOS, M. J. 1999. Occurrence and epidemiology of resistance to virginiamycin and streptogramins. *Journal of Antimicrobial Chemotherapy,* 43**,** 171-176.

THAMAGA-CHITJA, J. M. & MOROJELE, P. J. J. O. H. E. 2014. The context of smallholder farming in South Africa: Towards a livelihood asset building framework. 45**,** 147-155.

THOMAS, F., HEHEMANN, J.-H., REBUFFET, E., CZJZEK, M. & MICHEL, G. J. F. I. M. 2011. Environmental and gut bacteroidetes: the food connection. 2**,** 93.

TOKARZ-DEPTUŁA, B., NIEDŹWIEDZKA-RYSTWEJ, P., CZUPRYŃSKA, P. & DEPTUŁA, W. 2019. Protozoal giant viruses: agents potentially infectious to humans and animals. *Virus genes,* 55**,** 574-591.

TOMLEY, F. M. & SHIRLEY, M. W. 2009. Livestock infectious diseases and zoonoses. The Royal Society.

TREDWAY, L. P., STOWELL, L. J. & GELERNTER, W. D. J. G. C. M. P. T. R. I., SAN DIEGO 2006. Yellow spot and the potential role of cyanobacteria as turfgrass pathogens. 83-86.

TRENT, A. M. J. V. C. O. N. A. F. A. P. 1990. Surgery of the bovine abomasum. 6**,** 399-448.

TRETTER, L., PATOCS, A. & CHINOPOULOS, C. J. B. E. B. A.-B. 2016. Succinate, an intermediate in metabolism, signal transduction, ROS, hypoxia, and tumorigenesis. 1857**,** 1086-1101.

TROUTT, H. F. & AMSTUTZ, H. J. T. B. P. 1972. Physiological aspects of the omasum and abomasum. 2-7.

TURNER, D., SHKOPOROV, A. N., LOOD, C., MILLARD, A. D., DUTILH, B. E., ALFENAS-ZERBINI, P., VAN ZYL, L. J., AZIZ, R. K., OKSANEN, H. M. & PORANEN, M. M. 2023. Abolishment of morphology-based taxa and change to binomial species names: 2022 taxonomy update of the ICTV bacterial viruses subcommittee. *Archives of Virology,* 168**,** 74.

VAKULENKO, S. B. & MOBASHERY, S. 2003. Versatility of aminoglycosides and prospects for their future. *Clinical microbiology reviews,* 16**,** 430-450.

VAN DEN BOGERT, B., BOEKHORST, J., PIROVANO, W. & MAY, A. 2019. On the role of bioinformatics and data science in industrial microbiome applications. *Frontiers in Genetics,* 10**,** 466224.

VAN DER MERWE, D., BRAND, T. & HOFFMAN, L. 2020. Wool production in Dohne Merino, Dormer, Merino and South African Mutton Merino lambs. *South African Journal of Animal Science,* 50.

VAN DER WOUDEN, E., THIJS, J., VAN ZWET, A. & KLEIBEUKER, J. 2000. Review article: nitroimidazole resistance in. *Helicobacter pylori***,** 7-14.

VAN DUIJKEREN, E., GREKO, C., PRINGLE, M., BAPTISTE, K. E., CATRY, B., JUKES, H., MORENO, M. A., POMBA, M. C. M. F., PYÖRÄLÄ, S. & RANTALA, M. 2014. Pleuromutilins: use in food-producing animals in the European Union, development of resistance and impact on human and animal health. *Journal of Antimicrobial Chemotherapy,* 69**,** 2022-2031.

VAN, T. T. H., YIDANA, Z., SMOOKER, P. M. & COLOE, P. J. J. J. O. G. A. R. 2020. Antibiotic use in food animals worldwide, with a focus on Africa: Pluses and minuses. 20**,** 170-177.

WAGHORN, G. & REID, C. 1983. Rumen motility in sheep and cattle given different diets. *New Zealand journal of agricultural research,* 26**,** 289-295.

WALKER, P. J., SIDDELL, S. G., LEFKOWITZ, E. J., MUSHEGIAN, A. R., ADRIAENSSENS, E. M., ALFENAS-ZERBINI, P., DAVISON, A. J., DEMPSEY, D. M., DUTILH, B. E. & GARCÍA, M. L. 2021. Changes to virus taxonomy and to the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2021). *Archives of Virology,* 166**,** 2633-2648.

WANAPAT, M. J. A. A. J. O. A. S. 2000. Rumen manipulation to increase the efficient use of local feed resources and productivity of ruminants in the tropics. 13**,** 59-67.

WANG, S. P., RUBIO, L. A., DUNCAN, S. H., DONACHIE, G. E., HOLTROP, G., LO, G., FARQUHARSON, F. M., WAGNER, J., PARKHILL, J. & LOUIS, P. J. M. 2020. Pivotal roles for pH, lactate, and lactate-utilizing bacteria in the stability of a human colonic microbial ecosystem. 5**,** e00645-20.

WANG, Z., FU, H., ZHOU, Y., YAN, M., CHEN, D., YANG, M., XIAO, S., CHEN, C. & HUANG, L. J. M. B. 2021. Identification of the gut microbiota biomarkers associated with heat cycle and failure to enter oestrus in gilts. 14**,** 1316-1330.

WARGO, J. A. J. S. 2020. Modulating gut microbes. 369**,** 1302-1303.

WEXLER, H. M. J. C. M. R. 2007. Bacteroides: the good, the bad, and the nitty-gritty. 20**,** 593-621.

WHO. 2021. *Antimicrobial resistance* [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance [Accessed 15/09/2023 2023].

WILLIAMS, A., COLEMAN, G., HOBSON, P. & STEWART, C. J. T. R. P. 1997. The rumen microbial ecosystem. 73-139.

WILLIAMS, C. L., THOMAS, B. J., MCEWAN, N. R., REES STEVENS, P., CREEVEY, C. J. & HUWS, S. A. J. F. I. M. 2020. Rumen protozoa play a significant role in fungal predation and plant carbohydrate breakdown. 11**,** 720.

WILLIAMS, S. J. J. O. P. & BACTERIOLOGY 1965. Observations on the flora of the alimentary tract of animals and factors affecting its composition. 89**,** 95-122.

WISE, R., HART, T., CARS, O., STREULENS, M., HELMUTH, R., HUOVINEN, P. & SPRENGER, M. 1998. Antimicrobial resistance. British Medical Journal Publishing Group.

WOLIN, M., MILLER, T. & STEWART, C. 1997. Microbe-microbe interactions. *The rumen microbial ecosystem.* Springer.

WOO, A. C., GAIA, M., GUGLIELMINI, J., DA CUNHA, V. & FORTERRE, P. 2021. Phylogeny of the Varidnaviria morphogenesis module: congruence and incongruence with the tree of life and viral taxonomy. *Frontiers in Microbiology,* 12**,** 704052.

WRIGHT, A.-D. G. & PIMM, C. J. J. O. M. M. 2003. Improved strategy for presumptive identification of methanogens using 16S riboprinting. 55**,** 337-349.

WRIGHT, A., KENNEDY, P., O'NEILL, C., TOOVEY, A., POPOVSKI, S., REA, S., PIMM, C. & KLEIN, L. J. V. 2004. Reducing methane emissions in sheep by immunization against rumen methanogens. 22**,** 3976-3985.

WUBAH, D. A. J. B. O. F. I. & METHODS, M. 2004. Anaerobic zoosporic fungi associated with animals. 501-510

XIE, X., YANG, C., GUAN, L. L., WANG, J., XUE, M. & LIU, J. X. J. F. I. M. 2018. Persistence of cellulolytic bacteria fibrobacter and treponema after short-term corn stover-based dietary intervention reveals the potential to improve rumen fibrolytic function. 9**,** 1363.

XUE, D., CHEN, H., LUO, X., GUAN, J., HE, Y. & ZHAO, X. 2018a. Microbial diversity in the rumen, reticulum, omasum, and abomasum of yak on a rapid fattening regime in an agro-pastoral transition zone.*Journal of Microbiology,* 56**,** 734-743.

XUE, D., CHEN, H., LUO, X., GUAN, J., HE, Y. & ZHAO, X. J. J. O. M. 2018b. Microbial diversity in the rumen, reticulum, omasum, and abomasum of yak on a rapid fattening regime in an agro-pastoral transition zone. 56**,** 734-743.

YÁÑEZ-RUIZ, D. R., ABECIA, L. & NEWBOLD, C. J. J. F. I. M. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. 6**,** 1133.

YAO, H., LIU, D., WANG, Y., ZHANG, Q. & SHEN, Z. 2017. High prevalence and predominance of the aph (2 ″)-If gene conferring aminoglycoside resistance in Campylobacter. *Antimicrobial agents and chemotherapy,* 61**,** e00112-17.

YAO, J. & MOELLERING, R. 1999. Antibacterial Agents en Manual of Clinical Microbiology. *Patrick Murray y col.*

YAO, J. D. & MOELLERING JR, R. C. 2011. Antibacterial agents. *Manual of clinical microbiology***,** 1041-1081.

ZÁRATE, S., TABOADA, B., YOCUPICIO-MONROY, M. & ARIAS, C. F. 2017a. Human virome. *Archives of medical research,* 48**,** 701-716.

ZÁRATE, S., TABOADA, B., YOCUPICIO-MONROY, M. & ARIAS, C. F. J. A. O. M. R. 2017b. Human virome. 48**,** 701-716.

ZENG, Y., ZENG, D., NI, X., ZHANG, Y., ZHU, H. & TANG, Y. J. C. J. O. A. N. 2015. A comparison study on bacteria diversity of rumen, reticulum, omasum and abomasum from sheep using PCR-DGGE analysis. 27**,** 298-304.

ZHANG, H., SHAO, M., HUANG, H., WANG, S., MA, L., WANG, H., HU, L., WEI, K. & ZHU, R. J. F. I. M. 2018a. The dynamic distribution of small-tail han sheep microbiota across different intestinal segments. 9**,** 32.

ZHANG, Y.-Z., SHI, M. & HOLMES, E. C. 2018b. Using metagenomics to characterize an expanding virosphere. *Cell,* 172**,** 1168-1172.

ZHANG, Y., ZHANG, X., LI, F., LI, C., LI, G., ZHANG, D., SONG, Q., LI, X., ZHAO, Y. & WANG, W. J. A. 2021. Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. 15**,** 100161.

ZHU, Z., DIFFORD, G. F., NOEL, S. J., LASSEN, J., LØVENDAHL, P. & HØJBERG, O. 2021. Stability assessment of the rumen bacterial and archaeal communities in dairy cows within a single lactation and its association with host phenotype. *Frontiers in Microbiology,* 12**,** 636223.

ZUPPI, M., HENDRICKSON, H. L., O'SULLIVAN, J. M. & VATANEN, T. 2022. Phages in the gut ecosystem. *Frontiers in Cellular and Infection Microbiology,* 11**,** 822562.