Effects of lactic acid bacteria as putative probiotics and host genetic profile on rumen microbial ecology of two South African goat breeds

By

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Gastro-intestinal and Biotechnology, Agricultural Research Council- Animal Production, Irene and the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa. The research was financially supported by ARC.

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 1: PLAGIARISM

I, Maake Takalani Whitney, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

_____

Signed: Maake, Takalani Whitney

Date: 10 January 2022

DECLARATION 2: PUBLICATIONS

My role in each paper and presentation is indicated. The * indicates corresponding author.

Chapter 3

1. Maake, T.W., Aiyegoro, O.A.* and Adeleke, M.A. 2019. Effect of probiotics on growth performance of South African goat breeds. 6th Annual PDP Agricultural Research Council-PDP Conference, held on 07 to 9 October 2019 Work was presented at ARC-Vegetable and Ornamental Plants, Roodeplaat, Pretoria. Presented by Maake T.W.
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Chapter 4

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2. Maake, T.W., Aiyegoro, O.A*. and Adeleke, M.A., 2021. Effects of *Lactobacillus rhamnosus* and *Enterococcus faecalis* Supplementation as Direct-Fed Microbials on Rumen Microbiota of Boer and Speckled Goat Breeds. Veterinary Sciences, 8(6), p.103. doi.org/10.3390/vetsci8060103.

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ABSTRACT

Over a decade ago, the use of antibiotics as feed additives has been banned in most European Union countries because of the following risks: development of antibiotic resistance in pathogenic microbiota, release of unmanageable antibiotics into the environment and antibiotic or chemical residues in animal products. Due to consumer's pressure and worries towards harmful effects of antibiotics as growth promoters, there was a need to think of alternatives to antibiotics. In recent years, probiotics have been preferred as a superior alternative to antibiotics because they no harmful attributes associated with antibiotics and also have the ability to stabilize the microbial diversity in the digestive tract, and promotes animal yield while preserving consumer's health. Probiotics have also been observed to improve the functions of rumen microflora, fermentation processes and improve digestion in ruminants. The study therefore aimed to evaluate the effect of supplementation of putative probiotics- *Lactobacillus rhamnosus* and *Enterococcus faecalis,* singly and in combination for two South African goat breeds.

The first objective of the study was to determine the effects of probiotics on feed intake and growth performance of Boer and Speckled goats. To achieve the first objective, a total of 18 Speckled and 18 Boer randomly selected goats were separated into five treatment groups according to gender and breed. The trial lasted for 30 days. The goats were fed with pellets. Also, fresh water and hay were provided *ad libitum*. The treatment groups were as follows: (T1) basal diet + *Lactobacillus rhamnosus* SCH; (T2) basal diet + *Enterococcus faecalis* 25a; T3 basal diet + probiotic *Lactobacillus rhamnosus* SCH and *Enterococcus faecalis* 25a; T4 (positive control) basal diet + antibiotic; and T5 (negative control) basal diet with no antibiotics and no probiotics. The animals were weighed before and after the trial to determine their growth performance. Ruminal contents were collected before and after trial to examine the changes in the ruminal pH. All the data collected were processed and analyzed using one-way analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS, version 9.4). The efficiency of oral administration of putative probiotics on growth performance of South African goats showed the best performance in weight gain, final body weight and feed conversion ratios. Gender and breed affected weight gain and body weight,

showing that male (18.4 kg) goats were heavier than females (15.3 kg) and that Boer goat had a faster percentage growth rate of 24% than Specked (18%). This indicates that Boer goats will reach the market weight faster; this is due to the effect of probiotics. Supplementation of probiotics had no effect on feed intake. The pH across all treatment groups decreased averagely from 7.01 to 6.18. The lowest pH of 6.18 was observed in treatment group 3 (combination of probiotics). The findings in this study suggest that probiotics may have beneficial effects in goats' nutrition by increasing weight gain and lowering pH.

The second aim of the study was to determine the effect of host genetic profile on rumen ecology and performance characteristics of two South African goat breeds. Gut microbiota compositions were determined by sequencing the V3-V4 region of the 16S rRNA gene from ruminal contents of 36 goats. A total of 1,260 operational taxonomic units were obtained and grouped in 19 Phyla and 97 Genera. *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Fibrobacters* were the most dominant Phyla in all the treatment groups, while *Prevotella* and *Anaerofustis* were the most abundant Genera. Archaeal genus *Vadin CA11,* decreased in Treatment 1, 2, 3 and 5, while an increase was observed only in treatment 5. The presence of this genus has potential to allow the microbiome to adapt quickly to environmental stress like diet changes. However, the abundance of this genus must be controlled because it can produce additional ammonium through methanogenesis. The presence of *Chlamydiae* was observed only in Treatment 5 showing that probiotics and antibiotics eliminate obligate pathogens. Our result indicates that probiotics promote microbial diversity.

The final objective was to examine the alterations triggered by the probiotics on the rumen microbial profiles of Boer and Speckled goats using the 50K SNP bead chip. Genome-wide association study was explored between genotype and the rumen microbiome composition. A total of 44 single-nucleotide polymorphisms dispersed across the goat genome were associated with the relative abundance of six microbial Genera: *BF311, Clostridium, Fibrobacter, Methanobrevibacter, Prevotella,* and *Ruminococcus*. A total of 47 candidate genes were identified within 1-Mb windows of the goat genome; *CPT1A, STC2, AGPAT3* and *ACSF3* genes were associated with fatty acid metabolism, while *GH, BMP, MSTN, GHR and STMN1* were associated with regulation of developmental growth. Our results suggest that 47 candidate genes may positively shape the microbiome and elucidate the association

between gastrointestinal (GI) microbiome and the host genome in two South African goat breeds used for this study.

Keywords: Boer goats, Speckled goats, lactic acid bacteria, rumen, GWAS, 50K single-nucleotide polymorphism

CHAPTER ONE

Introduction

1.1 Introduction

Over the years, antibiotics have been successfully used as feed additives to advance growth performance (Gustafson & Bowen, 1997). However, due to growing concerns of continuous use of antibiotic in animal feed resulting in antibiotic-resistant infections in humans and animals, the use of antibiotics as growth promoters have been banned in USA and European countries. Researchers were compelled to search for natural products which are safe and produce desirable effects (Retta, 2016).

Probiotics are microorganisms that are found in the gut, which are beneficial to health (Ozen & Dinleyici, 2015). The concept of probiotics evolved in 1907 when Metchnikoff first hypothesized that the long-life span of humans was caused by utilization of fermented milk products (Gogineni, 2013). From its inception, the probiotic concept developed from being a concept into proven solutions, providing improvement in animal production (Jørgensen *et al*., 2017).

In 2013, the International Scientific Association for Probiotics and Prebiotics (ISAPP) re-examined the concept of probiotics with specialties in gut microbiota, paediatric, family medicine, immunology, microbial genetics, food science and microbiology of probiotic bacteria (Hill *et al*., 2014). The ISAPP panel together with the Food and Agriculture Organization (FAO) and World Health Organization (WHO) had one objective of amending the probiotic definition to the current and anticipating applications (Hill *et al*., 2014).

When probiotics are orally administered in effective doses, they avert the colonization of pathogenic organisms as natural intestinal bacteria. Lactic acid bacteria (LAB) that are

common inhabitants of the human and animal intestines include species from the Genera *Bifidobacteria*, *Lactobacillus* and *Enterococcus* (Mohania *et al*., 2008).

Probiotics have been used in ruminants like sheep, goat, cattle, among others especially with respect to rumen fermentation and animal performance (Retta, 2016). South Africa contributes roughly 3 % of Africa`s commercial goats (Gwaze *et al*., 2009). As stated by the Department of Agriculture, Land Reform and Rural Development, goat market chain report (2019), South Africa contributes about 1% of the world goat meat production as approximately 63% are owned by smallholder sector (DAFF, 2019). Goat also known as "the poor man's cow", is raised for milk, and meat (Ncube, 2020). Many body parts of the goat are economically important, for example, pelts are used for rugs and robes, while skins are used for leather (Masika & Mafu, 2004).

Indigenous Veld goat (IVG) are multipurpose South African breeds that have not undergone any improvement in the past years (Mohlatlole *et al*., 2015). The biggest advantage of IVG is its resistance to diseases and its adaptability in unfavorable grazing circumstances (Gwaze *et al*., 2009). Specific varieties are generally named according to the geographical areas in which they are found, or the nations. However, this categorization does not contain thousands of indigenous goats which are located outside these specific locations across the country, all well adapted to their respective environments that have influenced their specific characteristics. The indigenous goats in South Africa vary in terms of coat type, horn, color, ear length and size (Pieters, 2007) .

For the supply of vitamins and proteins, ruminants depend on the symbiosis between the rumen microbes and the host (Castro-Carrera *et al*., 2014). Rumen microbes are involved in the digestion and fermentation of plant materials (Uyeno, 2015). The rumen microbial environment comprises a wide variety of strictly anaerobes like: ciliate protozoa, bacteria, archaea and fungi to complete digestion (Chaucheyras-Durand & Durand, 2010). Microbial feed additives can be used in ruminant feeds for the development of the adult rumen microflora, improve digestion, stabilize intestinal flora, improve well-being of the animal and improve meat and milk production (Retta, 2016). It is, therefore hypothesized that introducing probiotics to ruminants will be beneficial in manipulating the microbial ecosystem to maintain a balance in the gut microbes and thereby aimed at determining if Lactic Acid Bacteria (LAB) causes weight gain and affect ruminal pH of two South African

goat breeds for a duration of 30 days, followed by checking the effect of LAB on rumen microbiota using Illumina MiSeq platform targeting the V3-V4 region of the 16S rRNA gene. The study will also determine if there is a host genetic variation on the rumen microbiome composition of Boer and Speckled South African goat breeds.

1.2 Problem statement

The growing need for more and better animal products because of an increase in human population has led to researchers in the field of animal production to direct intensive effort to use biotechnological strategies to manipulate the microbial system (Prathap *et al*., 2021). The use of feed additives (such as monensin, antibiotics, buffers, tallow, probiotics or nitrogen compounds) to manipulate ruminal fermentation appeared to be the most researched approaches to modify rumen microbial diversity (Castillo-González *et al*., 2014). Antibiotics as growth promoters advance feed efficiency and growth development of the animal. However, because of growing concerns of antibiotic resistance gene transfer and the development of microbial resistance, the use of antibiotics as feed additives have been banned in most European countries (FAO, 2005). In addition, safety concerns have been raised regarding antibiotic residues in animal products. Therefore, these are new and motivation on developing natural products as feed additives to replace antibiotics (Khalid *et al*., 2011). Probiotics are naturally occurring microbes as that have desirable effects such as improvement in feed conversion efficiency, reduces mortality, improves growth performance and the overall well-being of the animal (Yirga, 2015). Lactic acid bacteria as probiotics have been previously used to increase acidity in the rumen in order to eliminate or reduce pathogenic microbes (Vieco-saiz *et al*., 2019).

The association between the host genetic profile and gut microbial composition has showed that rumen microbiome act as a polygenic trait in murine models (Paz *et al*., 2016). However, rumen microbial composition can fluctuate significantly even in well-established cohorts, since numerous factors other than host genetics modulate the microbiota (Parks *et al*., 2015). Factors which can affect the function and structure of gut microbiota in ruminants includes: diet, management, and dosages of antibiotics or probiotics (Hasan & Yang, 2019). Although the supplementation of probiotics in ruminants plays a vital role in maintaining healthy microbiome (Gaggìa et al., 2010). However, the interaction of probiotics with the whole microbial community currently remains unclear. A better understanding of how probiotics

contributes to rumen microbial community and the physiological function of its host is needed.

1.3 Hypothesis

This study hypothesizes that probiotics are efficient for the overall improvement of rumen microbial ecology.

1.4 Justification

Probiotics have been considered as alternatives to antibiotics because of safe microorganisms with the abilities to improve productivity and well-being of the animal (Retta, 2016). This was strongly driven by the increasing concern for the public health due to the long-term use of antibiotics and the fact that antibiotics have been completely banned as feed additives by the European Union (Papatsiros *et al*., 2013), because of risks of chemical residues in animal products, producing antibiotic resistance in pathogenic microbiota (Nagpal *et al*., 2012) and release of antibiotics into the environment (Martínez-Vaz *et al*., 2014).

As reported by different authors, probiotics have the potential to promote diverse gut microbiome (Grazul *et al*., 2016; Hemarajata & Versalovic, 2013; Musa *et al*., 2009; Uyeno, 2015), lower pH, increase the digestion, prevent invasion of enteric pathogens in the intestine (Kim *et al*., 2017), restore the gut microflora and nutrient absorption (Uyeno, 2015). Consequently, due to the above reasons, probiotics have the ability to improve the well-being of ruminants and productivity. Therefore, a better understanding on the effects of probiotics on digestibility, growth performance and nutrient intake of ruminants is needed.

1.5 Aims

The study aims at assessing the effects of LAB probiotics and host genetic profile on rumen ecology of two South African goat breeds.

1.6 Objectives

To achieve the aim, the following objectives have been set:

- To determine the effect of LAB probiotics on performance characteristics of the goat.
- To determine the effect of host genetic profile on rumen ecology and performance characteristics of two South African goat breeds
- To examine the alterations triggered by the probiotics on the rumen microbial profiles of the goat.

1.7 Outline of dissertation structure

Each chapter contains an abstract, introduction, materials and methods, results, discussion, and conclusion.

Chapter 2: Reviews the benefits of probiotics on the gastrointestinal microbial ecosystem of goats, and the current knowledge on the Boer and Speckled South African breeds.

Chapter 3: Evaluates the effect of putative probiotics- *Lactobacillus rhamnosus* and *Enterococcus faecalis,* singly and in combination on growth performance of Boer and Speckled South African goats.

Chapter 4: Focuses on the function of the host genome in shaping the gut microbiota composition.

Chapter 5: Focuses on identifying genomic regions that regulate gut microbial composition in Boer and Speckled South African goats using 16 rRNA sequencing and genome-wide association methods.

Chapter 6: Provides a critical discussion of the current research, conclusions and provides future direction of the study.

1.8 Ethical clearance

The procedures in this study were evaluated and approved by the Agricultural Research Council- Animal Production Institute Ethics Committee (APIEC17/23), prior to the commencement of the trial.

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CHAPTER TWO

Role of probiotics in gut microbiome: A review

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2.1 Abstract

Recent explorations of the rumen gut microbiota suggest that dietary manipulation plays a vital role in the development and function of the gastro-intestinal tract. Subsequently, higher body weight and feed efficiency in young ruminants. Feed additives such as probiotics, have the ability to restore the composition of the gut microbial communities, increase microbial diversity, reduces ruminal pH and produces short-chain fatty acids. Factors such as host genotype, physiological status and age, also plays a role in shaping the function and structure of gut microbiota. This review examines the impact of diet on rumen function and how probiotics plays a role in shaping the structure of the rumen. The knowledge about GIT has increased significantly due to the application of omics technologies, to determine microbial composition and functionality patterns in the rumen. These explorations can provide comprehensive insights in the interactions between rumen microbial activities and the host.

Keywords: Rumen, diet, probiotics, gut microbiota

2.2 Background

South Africa is one of the major goat-producing countries and contributes about 50% to the Southern African goat population (Dzomba *et al*., 2017; Mdladla *et al*., 2017). Southern African constitutes about 10% of dairy goats in sub-Saharan Africa (Kahi & Wasike, 2019). As stated by DAFF (2019), goats are found in all provinces of South Africa with KwaZulu-Natal, Eastern Cape and Limpopo contributing about 70% of all goats in South Africa. The commercial chevon industry, consist of the Boer, Kalahari Red and Savannah breeds (Visser & van Marle-Köster, 2018). South African goat husbandry is dominated by unimproved indigenous veld goat (IVG) ecotypes; the Nguni type (Mbuzi), Eastern Cape Xhosa lob-ear, Kunene-type Kaokoland, and Northern Cape Speckled.

The rumen is characterized by high microbial density, consisting of bacteria, fungi, archaea, and protozoa, which drive the underlying processes and functions under anaerobic environment (Malmuthuge & Guan, 2017; Wang *et al*., 2016). The rumen microbiome plays vital role in growth and development and in feed conversion of goats (Wang *et al.*, 2016) These microbes in the rumen help to degrade proteins, non-fiber carbohydrates and fibers into ammonia and volatile fatty acids. The metabolites produced are absorbed and digested to meet essential roles for growth, immunity and thermoregulation (Deusch *et al*., 2017).

Bacterial communities, the most abundant (Huang *et al*., 2021; Mackie *et al*., 2000), diverse (Deusch *et al*., 2017; Islam *et al*., 2019) and species that are metabolically active in the rumen interact synergistically to obtain energy for complete degradation of organic matter. The microbial diversity and host physiology can be manipulated by diet, even though the microbial community is mostly stable throughout the animals' life (Claus *et al*., 2011; Morgavi *et al*., 2013). Previous studies showed that dietary supplementation of probiotics had a positive effect in the balance of gastrointestinal microbiota in ruminants (Adjei-Fremah et al., 2018; Markowiak & Ślizewska, 2018; Uyeno, 2015). Probiotics are live microorganisms, which improves the host' gut microbiota (FAO, 2016; Uyeno, 2015). Probiotic strains include *Lactobacillus, Enterococcus, Bacillus* and *Bifidobacterium*, are considered beneficial to the host (Fijan, 2014). The *Lactobacilli* strains that have been previously used as probiotics in ruminants include *L. acidophilus, L. rhamnosus, L. casei*, and *L. helveticus*. These probiotic species have the ability to control and change the structure and function of the gastrointestinal tract (GIT), and can be used as individual species or multi-strain combination. The multi-strain probiotics have a broader effect on the growth performance of an animal because of synergy effect (Collado *et al*., 2007) . Although, the concept of probiotics have been used for many years, the precise method of action of microbial feed supplements for ruminants is still not well understood (Abd El-Tawab *et al*., 2016; Azzaz *et al*., 2016; Azad *et al*., 2018).

Probiotics have the ability to advance the gut microbiota by preventing the invasion of pathogens, lowers ruminal pH, and improve mucosal immunity. It is vital that the introduced microbes must not interfere with the microbial populations, which are already in to the environment. Additionally, putative probiotic strains have to meet certain requirements in order for them to survive, e.g., adhesion to intestinal mucosa and glycoproteins, bile and acid tolerance (Azzaz *et al*., 2016). Several researchers have reported the benefits of utilizing

probiotics as feed supplement including: increase in feed conversion ratio (FCR) and weight gain (Yirga, 2015; Adjei-fremah & Ekwemalor, 2018; Al-Shawi *et al*., 2020).

Several methods have been previously developed and successfully applied to study the ruminal microbiome, ranging from culture-dependent to high-throughput metagenomics approaches, including next generation sequencing (NGS) (Creevey *et al*., 2014; Felczykowska *et al*., 2012).

The development of NGS technology has decreased the use of Sanger sequencing. NGS sequencing technologies are high throughput, cost-effective and offer reasonable read lengths. Other technologies include: SOLiD (Sequencing by Oligonucleotide Ligation and Detection) 5500×l, Illumina HiSeq 2500, and PacBio RS II (Rhoads & Au, 2015). The Illumina HiSeq platforms have low error rate compared to Roche 454 and Torrent (Nevondo, 2016).

Furthermore, metagenomics studies have produced a lot of data about taxonomic diversity, genetic profiles, and interactions of the microbiota (Tanca et al., 2016).However, the research on gut microbiota is shifting from being a description of the taxonomic classification to a more broad functional potential of the microbial community (Tanca *et al*., 2016). The combination of different Omics approaches can overcome some barriers to analyze the microbiome in the rumen (Deusch *et al*., 2017). Despite the use of all these technologies to study rumen microbiome changes due to different diet supplementation, most studies are published mainly on cattle and sheep, as compared to goat (Cremonesi *et al*., 2018). This review explored the current research on the potential benefits of probiotics on the gastro-intestinal microbial ecosystems of ruminants and overall well-being of the animal.

2.3 Selection of probiotic strains

The selection of microbial strains to be used as probiotics is of great importance. The potential strains to be used should be characterized as normal microbiota of the target species. They must have the ability to colonize the gut and adhere to epithelial cells of the gut (Spivey *et al*., 2014). Furthermore, probiotic strains must not be absorbed or hydrolyzed before reaching the GIT. Probiotic strains have the ability to produce antimicrobial substances,

change the microbial population in the gut and controls the functioning of the ecosystem (Azad *et al*., 2018). Microbial species, usually bacteria (non-lactic acid and lactic acid bacteria), yeasts and fungi are considered as probiotics. Lactic acid bacteria are the most useful probiotics for farmers (Zhang *et al*., 2021).

2.4 Effect of probiotics on growth performance

A lot of studies on the effect of probiotic on goats have been done with the focus of supplementation with yeast culture in diets (Abd El-Ghani, 2004; Cai *et al*., 2021; Jinturkar *et al*., 2009) and few using *Lactobacillu*s (Apás *et al*., 2010; Utz *et al*., 2018). Kefir (mixture of yeast culture and bacteria) has also been used with an attempt to improve the performance of goat. However, no significant difference was found between the treatment groups and control, suggesting that new approaches or other probiotic strains were required for exploring efficacy on growth performance of goat kids (Ataþoðlu *et al*., 2011). Previous studies on the use of probiotics in ruminants have been reported on sheep and cows, as compared to goats (Makete, 2015). The impact of probiotics on growth performance of ruminants may vary, as some researchers reported an increased feed intake, feed conversion ratios (Abd El-Tawab *et al*., 2016; Arowolo & He, 2018; Izuddin *et al*., 2019) or daily weight gain in sheep ,cow and goat (Retta, 2016). Jinturkar *et al*., (2009) found that the supplementation of *Saccharomyces cerevisia* and *Lactobacillus acidophillus* separately and as multi-strain in feeds resulted in average daily weight gain (ADG) of 0.062 Kg as compared to controls in goats. Similarly, Salvedia and Supungco (2017) reported an increased in bodyweight on goat kids fed probiotic supplements as compared to controls (8.00 Kg for multi-strain, versus 3.50 kg control). Utz *et al*. (Utz *et al*., 2018) and Apas *et al*. (Apás *et al*., 2010) have reported that the application of probiotics in goats can remove mutagens and carcinogens.

2.5 Effect of probiotics on ruminal microbiome

A broader understanding of the ruminal microbiome is required for improvement of rumen function (Wang *et al*., 2016). Previously, investigations have been carried-out to study the composition and structure of ruminal microbiota using culture dependent methods (Islam *et al*., 2019) to 16S rRNA gene sequences (Guo *et al*., 2015). The ecology of microorganism has beneficial effect on growth performance and health of goats. However, instability of microbial ecosystem can occur during the weaning period, resulting in the shifts in the

gastrointestinal environment that can lead to diarrhea (Apás *et al*., 2010). Antibiotics have been used in the past for diarrheal treatment and other diseases in ruminants. However, the long-term use of antibiotics has led to antimicrobial resistance and antibiotic residues. It is for this reasons that the European Community is trying to prohibit the use of antibiotics in the livestock husbandry. Alternative strategies that improves gastrointestinal microbial communities, hence the well-being of the animal without using antibiotics as feed supplements, have been sought. Previous studies have shown that probiotics stabilize ruminal pH, increase volatile fatty acid (VFA) production, which result in an efficient functioning of the rumen Abd El-Tawab *et al* (2016). Some researchers have found a reduction in ruminal pH after supplementation with some *Lactobacillus* species (Lettat *et al*., 2012). In contrast, Abd El-Ghani (2004) has recorded an increase in ruminal pH. (Abd El-Tawab *et al*., 2016) have shown that supplementation of probiotics stabilized rumen pH leading to effective rumen functioning and also preventing the risk of sub-acute ruminal acidosis. Adjei-fremah *et al*., 2018; Kowalik *et al*., 2011 have reported a reduction in ruminal VFA in goats after the supplementation of probiotic in finishing diets. Whereas, some authors observed an increase in VFA production, which may have been attributed to the decrease in methane production (Abd El-Ghani, 2004; Medjekal *et al*., 2018). Nonetheless, other some researchers found no effect of probiotics on VFA concentrations in the rumen (Soren & Tripathi, 2013; Pragna *et al*., 2018).

2.6 Interactions between the host and microbial in the rumen

Associations between the host and microbiota are well defined in the GIT of ruminants and have been mainly studied to capitalize on growth performance of ruminant husbandry, despite having metabolic dysfunction like ruminal acidosis (Malmuthuge & Guan, 2017). Furthermore, major studies on links between the rumen microbiota and susceptibility to subacute acidosis have been reported more on cattle and sheep, and less on goats (Cremonesi *et al*., 2018). The presence or absence of microbial phylotypes affect microbial groups through interventions could be manipulated using dietary intervention with the aim of altering the host performance (Reigstad & Kashyap, 2015). Phylotypes refers to clusters which can be grouped according to similarities, equivalent to an evolutionary related group in line with classical Linnaean taxonomy or an operational taxonomic unit (OTU) defined as a cluster of similar sequences sharing ≥ 97% identity threshold (Mysara *et al*., 2017). Due to few microbial manipulation strategies in adult ruminants (Malmuthuge & Guan, 2017; Ulfina *et*

al., 2019), the demand in indulging in the interactions between the host and microbials in infantile ruminants, specifically goats has increased (Yáñez-Ruiz *et al*., 2015). Subsequently, there has been an increase in the interest in exploring the impact of rumen microbiota of goat kid (Han *et al*., 2015; Wang *et al*., 2018). The dietary manipulations in goat kids have been shown in the past to enhance the host phenotypes and microbial composition with the aim of producing lasting effects until adult life (Abecia *et al*., 2014). Therefore, these studies imply that microbial interventions during the early life of the animal can be more effective than the introducing changes in the later stages (Malmuthuge & Guan, 2017). The composition and the abundance of the ruminal microbiota vary depending on the host. This could mean that the microbial interventions will produce inconsistent results when applied to other ruminants. However, consistencies have been observed in goat kids by different authors (Abecia *et al*., 2014; Embarcadero-jiménez *et al*., 2014; Han *et al*., 2015; Wang *et al*., 2018; Yáñez-Ruiz *et al*., 2015). Therefore, there is an important need in understanding host-microbial interactions during the early life. Most of microbial intervention techniques have been studied from liquid phase microbiome, the epimural bacteria, which is the rumen-epithelial tissue associated microbes, maintains close interactions with the host. Some studies have reported that the epimural bacterial is more diverse than ruminal bacteria content found in the fluid phase. The changes that can occur in the rumen-epithelial tissue bacterial density are mainly related to gene expression of the host and ruminal acidosis. The epimural bacterial community plays an important role in the hydrolysis of urea, recycling of epithelial tissue and scavenging of oxygen (Liu *et al*., 2015). Thus, the insight on the functions of epimural microbiome should be taken into consideration in the future because it will provide a better understanding of the host-microbial interactions.

2.7 Diversity and richness of the rumen microbial ecosystem

The microbial structure and diversity of the rumen are influenced by different factors such as types of diet, sample collection methodology, DNA extraction method, 16s RNA gene target region, and sequencing (Latham, 2017). Research based on rumen microbiome previously depended on culture-based technologies on the isolation and characterization of the rumen microbiota (Chaucheyras-Durand & Ossa, 2014). The introduction of NGS technologies has opened the door to discover the diversity and complexity of the rumen microbiota (Behjati & Tarpey, 2013; Chaucheyras-Durand & Ossa, 2014). Rumen microbiota plays a vital role in shaping microbial pathways associated with metabolism (Rowland *et al*., 2018).

The diversity of the microbial community in the rumen using 16S rRNA metagenomics analysis is based on gene surveys on V1 to V3 regions. The first step for sequence processing is dataset cleaning and on average, about 20% is reduced in length. After sequence cleaning, fragments are assembled, followed by dataset specific clustering. Operational taxonomic units (OTUs) per dataset ranging from 520 to 2691 in length are assigned to taxonomic classification based on the 97 % sequence identity. When OTUs are clustered together they represent an estimation of community composition (Schmieder *et al*., 2010). A total of 20 bacterial Phyla are known to exist in the rumen, but the majority of them are rare (Creevey *et al*., 2014). Several studies on rumen microbiome of goat revealed that *Bacteriodetes, Proteobacteria* and *Firmicutes* are the most dominant Phyla (Kim *et al*., 2011; Wang *et al*., 2016; Wang *et al*., 2018; Lei *et al*., 2019). From the phylum *Bacteriodetes*, family *Prevotellaceae* and the class *Bacteroidia* are among the most abundant. Phyla such as *Acidobacteria, Chloroflexi*, *Spirochaetes*, and *Tenericutes* are also dominant in other ruminants like sheep and cattle (Park & Kim, 2020; Roggenbuck *et al*., 2014; Wang *et al*., 2018). The OTUs are considered diverse when they have a scaled phylogenetic distance of more than 0.25 (Creevey *et al*., 2014). Understanding diversity indices is vital because it has great effect in the function and health of an animal by providing the relatedness of species in a community (Willis, 2019). Alpha diversity metrics are determined from rarefied samples using Chao1 index and Shannon index to evaluate the diversity and community richness. Some researchers found that the diversity and abundance was higher in healthy adult Boer goats compared to kids goat and goats with diseases (Wang *et al*., 2018). It was also found that the communities revealed alpha diversity index increased in older goats (Lei *et al*., 2018). Beta diversity is also calculated from rarefied samples using principal coordinate analysis (PCoA), unweighted and weighted UniFrac (Campisciano *et al*., 2018).

2.8 Conclusion

In ruminants, probiotics have proven to be a viable alternative to antibiotics, and their usage in farm animals continues to grow. The outcomes of probiotics may vary depending on the type of strain used. Factors such as dosage of probiotic, mode of administration, diet composition, number of viable microorganism present in each dose, and host animal determine the efficiency of probiotics. Recently multistrain probiotics, which comprises two

or more species, have gained a lot attention compared to monostrain probiotics because of positive synergistic effect. As a result, future research should be directed towards understanding the interaction between the host and combined microorganisms.

2.9 Recommendation

The aim of carrying-out research in rumen ecology of animal production field is to improve the rumen microbial fermentation in order to improve animal health and production. However, the knowledge regarding the interactions between the host and gut microbiota is still poorly understood in goats. The microbial OTUs are grouped based on similarities of 16S rRNA gene sequences which mostly analyses the presence or absence of certain OTUs and were based on known microbial taxonomy. Although previous studies have identified gut microbiome at functional and structural levels using metagenomics; the genome-wide associations (GWAS) were mainly based on existing knowledge of genetic variants to identify genotype-phenotype associations (Deelen *et al*., 2019). Therefore, there is no conclusion on whether the alterations in microbiome affect the host functions or the host physiological changes. Advanced technologies are required to identify metabolites of rumen. Future work should design metagenomics trials containing diet composition, management of animals, age, health status, host genetics, and volatile fatty acids (VFAs) production, in order to alter the rumen microbiota. These attempts can overcome the limitation practiced by previous researchers such as: no GWAS or any current technology can identify all the genetic components of complex traits (Tam *et al*., 2019). The attempts to overcome the limitations can unravel the impact of host genetics on rumen microbiome. The true impact of gut microbes in health can be achieved by moving beyond phylotyping using complimentary –mics' approaches to understand functional changes in the microbiota composition (Malmuthuge & Guan, 2017).

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CHAPTER THREE

Effect of lactic acid bacteria administered as feed supplement on weight gain and pH in South African goat breeds

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3.1 Abstract

Antibiotics as growth promoters are banned in most European countries, because of antibiotic resistance and residues in animal products, and the release of recalcitrant antibiotics into the environment. Probiotics are the preferred alternative, because of no harmful attributes when compared to antibiotics, and the additional advantage of the ability to stabilize the microbial communities in the gut. We investigated the effects of Lactic Acid Bacteria (LAB) on the growth performance of South African goats. Randomly selected 18 Speckled and 18 Boer goats were divided into five treatment groups according to sex and breed and were placed in an experimental trial for 30 days. Ruminal fluid was collected before and after the trial to monitor the changes in the pH. Data collected were analyzed using a one-way analysis of variance (ANOVA) (SAS, version 9.4). Results revealed that LAB supplementation had no effect on feed intake in all experimental groups. However, the average weight gain was higher in the LAB groups when compared to other groups. Sex and breed had effects on final body weight. Males (18.4 kg) goats are heavier than females (15.3 kg) averagely, irrespective of the breed, and the Boer goats are heavier than Speckled irrespective of gender. The pH decreased averagely from 7.01 to 6.18 across all treatments, confirming the modulatory effect of the administered lactic acid bacteria. The combination of probiotics (Treatment 3) showed the lowest pH value of 6.18, compared to the negative control with the pH value of 6.36. The findings in this study suggested that LAB as probiotics may induce beneficial effects in goats by enhancing weight gain, dropping the gut pH, thus maintaining an equilibrium of ruminal microbiota.

Keywords: Lactic acid bacteria; Boer goat; Speckled goat; pH; probiotic; weight gain

3.2 Introduction

The success of the goat husbandry is dependent upon healthy goats (Ekwemalor *et al*., 2017). Although goats have the ability to survive under harsh environment and utilize less favorable feeds, there is still a need to develop conventional feed for both smallholder and big commercial farmers (Sikosana *et al*., 2005.). Goats are an important source of meat (Wani *et al*., 2012), milk, skin, and fertilizer (Sikosana *et al*., 2005), and are easier to maintain than cattle, probably because of size, uncomplicated nutritional diets and resilience (Salvedia & Supungco, 2017). In the recent times, there is an increasing market demand for goat milk and cheese because of the extra nutritional values and uniqueness in tastes when compared to those of cattle (Adjei-fremah *et al*., 2018).

The South African goat meat industry is dominated by Boer and indigenous veld goat (IVG) breeds. The Boer goat is an improved breed that was developed for fast growth and finer meat production traits (Visser & van Marle-Köster, 2018). IVG are unimproved local varieties and are usually defined by the geographical area where they are found; most valued for their hardiness. The indigenous goats are marketed as having special characteristics of being resistant to tick-borne diseases (Mkwanazi *et al*., 2021).

A major problem in the goat industry is the production loss caused by gastrointestinal diseases (Ekwemalor *et al*., 2017). The occurrence of GIT parasites in goats is approximately 94% (Win *et al*., 2020). Coccidiosis is the most common gastrointestinal infection in ruminants, which caused by the parasites from the Genus *Eimeria* (Ratanapob *et al*., 2012). The common treatment for gastrointestinal parasites infection is the use of antibiotics to modify the gut microbiota. However, the long-term use of antibiotics has led to the worldwide concerns of development of antibiotic resistant microorganisms, which pose a threat to consumers' health and adding undesirable effect on the environment (Markowiak & Ślizewska, 2018). As a result, the use of antibiotics as feed additives was banned by the European Union since 2006 (Anadon, 2006). Therefore, all around the world, alternative natural substances including prebiotics, probiotics, medicinal plants among others, producing similar effect in animal nutrition has been sought as possible replacement to antibiotics (Markowiak & Ślizewska, 2018).

Probiotics are live microbes administered in adequate quantity as food supplements with beneficial characteristics on the host such as balancing the intestinal microbial (Bahari, 2017). Probiotics have positive effects on nutrient synthesis, growth performance and restoration of GIT microflora after the incidence of diarrhoea (Khalid *et al*., 2011). The commonly used probiotic bacterial species in ruminants include: *Lactobacillus, Enterococcus, Bifidobacterium, Lactococcus, Bacillus, Pediococcus, Leuconostos, Streptococcus* and *Saccharomyces species* (Yirga, 2015). The population of *Lactobacillus* and *Bifidobacterium* are low in ruminants, but studies have shown that supplementation of probiotics with these microorganisms increases growth (Adjei-Fremah *et al*., 2018). Recent studies have shown that the supplementation of probiotics as single or multi-strain feed in cattle, goat and sheep stabilizes the microbial communities in the digestive tract, improves growth performance, and the overall well-beings of the animal (Adjei-fremah & Ekwemalor, 2018.; Bidarkar *et al*., 2014).

The important purpose of livestock production is the provision of safe foods for human consumption taking into consideration the wellbeing of the animal. The food-borne pathogens such as *Salmonella* and *Campylobacter* can be transmitted through meat consumption to human (Gaggìa *et al*., 2010). In this context, probiotics can be used as feed additives to improve the health status of the animal, by producing a balanced microbiota within the gastrointestinal tract (GIT) with reduced risk of food-borne pathogen load. Probiotics are safe for animals as their mode of action is only restricted to the GIT and cannot be transferred to the food products like milk or meat (Yirga, 2015). Thus, the aim of this study was to evaluate the effects of two lactic acid bacteria as putative probiotic on growth performance of two South African goat breeds.

3.3 Materials and Methods

The procedures in this study were evaluated and approved by the Agricultural Research Council- Animal Production Institute Ethics Committee (APIEC17/23), prior to the commencement of the trial.

Lactic acid bacteria (LAB); *Lactobacillus rhamnosus* SCH (*Probiotic A*) and *Enterococcus faecalis* 25a1 (*Probiotic B*) as putative bacteria were isolated from the gastro-intestinal tract (GIT) of randomly selected eight indigenous South African Speckled goats. A combination of molecular sequencing (16S ribosomal RNA) and biochemical tests including; catalase test, gram staining, antibiotic resistance, acid tolerance and bile tolerance were used to characterize the probiotic properties of the isolated LAB. The putative probiotics were prepared in the Man, Rogosa and Shape (MRS) agar and stored in ultra-low freezer prior to use. The isolates were revived by inoculating in MRS broth (MRS; Oxoid, England), and then incubated under an obligate anaerobic condition at 37 °C for 48 hrs, and were administered to the goats singly and in combination.

The trial was conducted at the Small Stock Section of the Agricultural Research Council (Irene), Pretoria, South Africa. The Agricultural Research Council, Irene campus is located at 25° 55' South and 28° 12' East. All goats were treated in accordance with the established standards for the use of animals' ethical guidelines. The goats were vaccinated 15 days before the start of the trial to control diarrhea. A total of thirty-six goats including Boer and Speckled, randomly selected were used for this trial. The trial lasted for 30 days. The goats were assigned to 5 treatment groups (7 goats per treatment) according to breed and gender. Treatment 1- *Lactobacillus rhamnosus* SCH; Treatment 2- *Enterococcus faecalis* 25a1; Treatment 3- a combination of two probiotics (*Enterococcus faecalis* 25a1 and *Lactobacillus rhamnosus* SCH); Treatment 4 (positive control) – antibiotics and Treatment 5 (negative

control)-no antibiotics and no probiotics. The diet used was in the form of pellets to provide nutrient requirements regardless of the treatment as recorded in Table 3.1. Antibiotic lincospectin was added in the positive control group. The goats were supplied with sufficient hay and fresh water through self-feeders. The weekly administration of probiotics to goats was done orally using a dosing gun at the dosage of 5 ml of 2 X 10^9cfu/ML of fresh live culture per head. The oral feeding was done every week at 08:00 am for 4 weeks. The goats were weighed individually before and after the trial using a calibrated weighing scale. The total weight gain (TWG, kg) was estimated by obtaining the difference between the initial and final body weights. The growth performance indices were calculated as follows: Growth rate (%) = (final weight – initial weight) / (initial weight) * 100 (Hussein, 2014). Feed intake was calculated by subtracting the amount of feed left inside the feeding chambers from the total feed given weekly. Feed conversion ratio (FCR) was estimated by dividing feed intake by the total weight gain per trial pen.

Table 3. 1 Nutrient composition of the commercial diet

Nutrients[1]	**g (kg)**
Protein	150
Fat	25
Fibre	110
Calcium	8
Phosphorus	2
Urea	1
Chloride	9
Sodium	9
Magnesium	1
Potassium	6

Ruminal samples were collected before and after the trial, to examine pH using ororuminal collection method (Grünberg & Constable, 2009). Ruminal fluid was collected by placing a sterilized stomach tube through the mouth to the stomach of the goat. A volume of 40 mL of the collected rumen content was transferred to 50 ml tubes. Ruminal pH was measured using portable pH meter immediately after sampling. Ruminal fluid samples were kept on ice until they were stored at -80 ℃ for DNA extraction.

The data collected were subjected to one-way analysis of variance (ANOVA) using SAS (Statistical Analysis System), with treatments, sex, breed and time as classification factors. Dependent variables were weight gain, body weight and pH. Duncan's Multiple Range Test was adopted to determine the significance levels between them. The results were represented as the mean ± standard error (SE). The degree of statistical significance was set at $P<0.05$.

Microbial genomic DNA was extracted from collected ruminal fluid samples using PureLink Micobiome DNA Purification Kit (Thermofisher, South Africa) following the manufacturer's guidelines. Agarose gel electrophoresis was used to examine the quality of the DNA. Nano Drop (NaNoDrop 2000, United States) was used to measure the quantity of the DNA.

PCR amplifications were carried-out in a 25µl reaction mixture consisting of 2x KAPA Hi-Fi HotStat Ready Mix, 1µM 16s rRNA forward primer (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3') and 16s rRNA reverse primer (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3'), and 5ng/1µl template. The thermal cycler conditions were: 95°C for 3 min, 28 cycles (95°C for 30sec, 55°C for 30sec, 72°C for 30sec), 72°C for 5 min. PCR amplicons were visualized using 0.8% agarose gel electrophoresis. The 460 bp bands were excised from the

gels and purified using Nucleospin DNA/PCR cleanup kit following the manufacturer's guidelines. The excised amplicons were submitted for Illumina Miseq at the ARC-Biotechnology Platform.

A total of 10 500 581 paired-end reads generated using the Illumina MiSeq sequencer. Of the 10 500 581 raw reads, 9 858 186 paired-end reads remained from the data trimming process by the program: Trimmomatics. Using PANDAseq, a total of 7 789 215 reads were merged. These merged reads were denoised, and any chimeras within the samples were removed using Dada2. The OTU picking were done in Qiime2. A total of 12 175 representative taxa were identified and 1 607 770 reads were bacterial 16S rRNA sequences. The R studio was used to group OTUs according to taxonomic classification of treatment groups.

3.4 Results

The effects of treatments, breed, gender and time on growth performance of goats are presented in Table 3.2, and Table 3.3. The effect of probiotic supplementation on body weight, weight gain and pH values of growing goats are presented in Table 3.2. All the treatments improved in total weight gain. Significant difference on the final body weight was observed in treatment groups (P>0.05). The average daily gain was significantly higher in T3 (0.35 KG) and T4 (0.36 kg) as compared to the control (T5) (0.32 kg).

Table 3. 2 Growth performance and pH of goats influenced by probiotics treatment

Parameters	Treatment					P- value
	T1	T2	T3	T4	T5	
Initial body weight (kg)	16.2± 3.84 [a]	16.05±3.52 [a]	16.3±3.61 [a]	16.08± 3.53 [a]	16.0± 3.50 [a]	0.06
Final body weight (kg)	18.2± 3.73 [a]	17.4± 3.22 [a]	18.7±5.08 [a]	20.5± 5.54 [a]	16.87± 3.50 [a]	0.0035
Total weight gain (kg)	1.98± 1.10 [b]	1.32± 1.74 [b]	2.42± 1.25 [a]	4.42± 4.50 [a]	0.87± 0.58 [b]	0.0001
Percentage growth rate	12.2%	8.2%	14.8%	27.5%	5.7%	
Initial pH	6.99± 0.44	6.56± 0.42	7.12±0.41	7.5± 0.45	7.19± 0.43	0.57
Final pH	6.32± 0.41 [ab]	6.37±0.46 [a]	6.18±0.52 [b]	6.4±0.52 [b]	6.36±0.56 [a]	0.0001
Feed intake (kg/d)	0.8± 0.1	0.9±0.1	0.7±0.0	0.75±0.0	0.8±0.0	0.59
Average daily gain (kg/d)	0.07± 0.0 [a]	0.04± 0.0 [a]	0.08± 0.0 [a]	0.15± 0.0 [a]	0.03± 0.0 [a]	0.04
Feed conversion ratio	2.06± 0.8 [b]	2.21 ± 0.5 [a]	1.98 ± 0.4 [b]	2.0± 0.5 [b]	2.21± 0.7 [a]	0.001

[a-b]Mean values along a row with different superscripts are significantly different (P<0.05). T1= *Lactobacillus rhamnosus* SCH, T2= *Enterococcus faecalis* 25a1, T3= *Lactobacillus rhamnosus* SCH *and Enterococcus faecalis* 25a1, T4=Positive control, T5=Negative control.

Table 3.3 Effect of breed and sex on body weight, weight gain and pH of goats fed with different probiotics combinations

Parameters	Breed		Gender	
	Boer	Speckled	Male	Female
Initial Body weight (kg)	14.4 ± 3.93 [a]	14.2 ± 5.08 [a]	15.8 ± 2.63 [a]	14.7 ± 1.21 [b]
Final Body weight	17.8±5.18 [a]	16.7± 3.20 [b]	18.4± 4.46 [a]	16.3± 3.61 [b]
Average weight gain (kg)	3.40± 3.80 [a]	2.50± 1.60 [b]	2.42± 1.25 [b]	4.42± 4.50 [a]
Percentage growth rate (%)	24%	18%	17%	11%
Initial pH	7.12 ± 0.42 [a]	7.12 ± 0.42 [a]	7.12±0.41 [b]	7.5± 0.45 [a]
Final pH	6.80± 0.53 [a]	6.34±0.55 [b]	6.77± 0.57 [a]	6.65± 0.52 [b]
Feed intake (kg/d)	0.84± 0.0 [a]	0.87±0.0 [a]	0.89±0.0 [a]	0.83±0.0 [b]
Average daily gain (kg/d)	0.12± 0.0 [a]	0.06± 0.0 [b]	0.09± 0.0 [a]	0.086± 0.0 [a]
Feed conversion ratio	2.06± 0.0 [b]	2.43 ± 0.1 [a]	2.48 ± 0.0 [a]	1.95± 0.5 [b]

[a-b]Mean values across a row with different superscripts are significantly different (P<0.05).

Table 3.3 shows the effect of probiotics on the type of breed, gender and time in the entire goat population. Significant difference was observed in the final body weight and pH between the Specked and Boer goats. A significant difference was observed between the two breeds on weight gain with the respective weights of 3.40 kg for Boer and 2.50 kg for Speckled. A faster growth rate was observed on Boer goats (24 %) as compared to Speckled (18%). Significant difference was observed in final body weights on gender type as male goats (18.4 kg) were heavier than females in body weight (16.3 kg). Time had significant effect in body weight as the goats weighed 15. 7 kg at day 0 and 18.3 kg at day 30 across all

treatments. This might have been affected by lowering of ruminal pH from 7.01 to 6.33. There was no interactive effect between treatments, sex and breed.

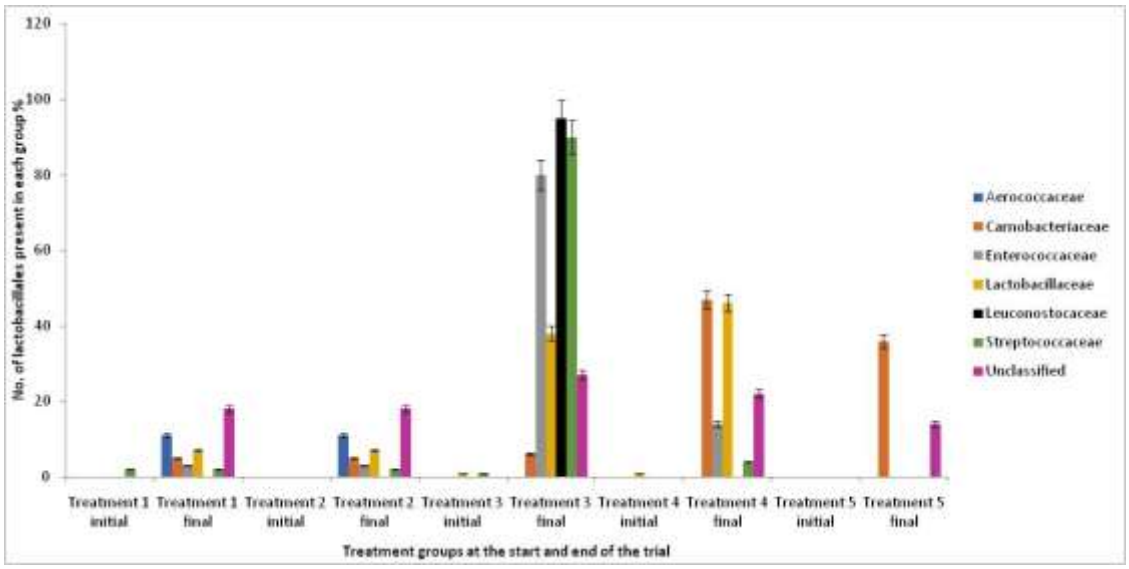

Figure 3.1 Effect of different treatments on the abundance of families from Lactobacillaes, present in the rumen of goat kids from Day 1 to Day 30

Figure 3.1 shows the different families of lactobacillales present in the rumen of the goat initially or day 1 and after the trial in all the treatment groups. T3 and T4 show many *Enterococcus* and *Lactobacillus* compared to the negative control group (T5). The *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae* and unclassified families were observed in all the treatments groups at the end of the trial. All six families (*Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Streptococcacea. and Lactobacillaceae*) were observed after LAB supplementation in T3.

3.5 Discussion

In animal feeds, probiotics are incorporated as feed additives to stabilize microbial communities in the GIT (Ekwemalor *et al*., 2017). The efficacy of probiotics is dependent upon genetics, type of animal, age, stress, and well-being of the animal (Yirga, 2015). In the present study, the weight gained in different treatment groups was observed in Table 3.2: antibiotic group (T4) gained about 56 % weight, T1 (26 %), T2 (24 %), combination of two probiotics (T4) (44 %) and the control group (T5) (16 %). These results are in conformity with the findings by Salvedia & Supungco, (2017) who observed significant increase of total weight gain of 3.5 Kg of goats fed *Lactobacillus plantarum* and *Saccharomyces cerevisiae* compared to the negative control group. Jinturkar *et al*. (2009) also observed that goats fed *Lactobacillus* had higher total weight gain as compared to the control group. A heavier weight gain observed from the LAB treated groups cannot be assumed as the excess nutrients intake from the pellets as each experimental goat was given same amount of feed. However, the increase can be linked to the nutrient digestion caused by the interaction of probiotics with the microbial flora. Lactic acid bacteria are known to improve nutrients digestibility (Mudgal & Baghel, 2010) and improved health of the animal (Bahari, 2017). The group supplemented with *Lactobacillus rhamnosus* SCH showed heavier weight gain than the two control groups. The group supplemented with *Enterococcus faecalis* 25a1 only (T2) also showed better growth performance than the negative control group. As reported by different authors, the use of *Lactobacillus species* and *E. faecalis* stains as probiotic have beneficial effects on weaned piglets by reducing certain pathogenic *E. coli* strain and diarrhea (Starke *et al*., 2015; Vahjen & Simon, 2015). The European Food Safety Authority (EFSA), Food and Drug Administration (FDA), feed industries and the European Commission recognizes *E. faecalis* and other Lactic acid bacteria as feed supplement and is safe to use (Rychen *et al*., 2018).

The average weight gain was statistically significant between antibiotic (T4) and the combination of two probiotics (T3) groups. This shows that the multi-strain probiotics and antibiotics groups have same effect on promoting growth of SA goats. The use of probiotics as feed additives have been observed to improve microbial ecology, feed conversion ratio, and nutrient intake resulting

in improved weight gain in animals (Khalid *et al*., 2011; Oyetayo & Oyetayo, 2005; Salvedia & Supungco, 2017). Supplementation of probiotics improved feed efficiency, nutrient synthesis resulting in better weight gain. Singh *et al*. (2019) reported that the use of multi-strain probiotic or combination of probiotics improved the overall mean weight gain. This was observed in Table 3.2, which showed heavier weight gained from goats fed with a combination of probiotics as compared to goats fed with *Lactobacillus rhamnosus* SCH only and *Enterococcus faecalis* 25a1 only. The LAB treatment group performed better in all parameters used, than the control groups. These results suggest that lactic acid bacteria can be used in finishing diets. Bahari (2017) has indicated that the supplementation of probiotics improves the microbial ecosystem and hence improves the growth of ruminants. The average weight gain in LAB treatment groups can be improved by increasing dosage. Studies have shown that higher dosage (Gaggìa *et al*., 2010; Sazawal *et al*., 2006) and using more than three strains of probiotics (Salvedia & Supungco, 2017) can be used to achieve higher weight gain. *Lactobacillales* belong to the phylum of the *Firmicutes*, and has 6 families. The positive performance of multi-strain probiotics showed that a combination *Lactobacillus rhamnosus* and *Enterococococcus faecalis* were suitable potential probiotic strains as an inhabitant of the host organism. They have shown the ability to adhere, colonize the epithelial cells of the gut and exert positive effects on the host. Microbial density and diversity of *Lactobacillae* family increased in T3, confirming the theory that maximal populations are observed in areas where the pH range is close to moderate (AL-Shawi *et al*., 2020). The abundance of *Lactobacillus* emphasizes that gut microbiota plays vital in growth performance and overall animal' health. A high level of unassigned family was observed within all treatment groups. Shen *et al*. (2017) also found high number of unclassified OTUs in all the treatment groups. The introduction of LAB in the rumen also assisted with the change in ruminal bacterial community composition, as more bacteria from the family of *Lactobacillales* were observed in T3 and T4. LAB species constitute the order *Lactobacillales* (Salvetti *et al*., 2012). Studies have shown that rumen microbiome can be altered by several factors including geographical location, season, health, age, stress, feed additives and diet (Lee *et al*., 2019). *Lactobacillus* species plays a vital role in stabilizing ruminal pH. When the pH decreases below 5.6 it increase the pH level (Lettat *et al*., 2012).

Boer goats are known to be bigger in body weight, rapid growth rate and high-quality carcass traits compared to other goat breeds *(*Gwaze *et al*., 2009; Mohlatlole *et al*., 2015). The growth rate is normally affected by weaning methods and adaptation (Lu, 2001). In this study, the total weight gain of Boer goats was 20 % more than Speckled goats. As observed in Table 3, the percentage growth rate of Boer goats was 24% and 18% for Speckled goats. Speckled goat is one of the indigenous goat breeds which can survive in harsh environments and it is for this reason why they are known to be lighter in body weight and slow in weight gain. Although the growth performance of indigenous goat breeds of South Africa is generally poor, partially as a result of parasite challenge and low nutrition. It can be improved by the availability of forage (Gwaze *et al*., 2009; Dzomba *et al*., 2017). Overall results showed that at the beginning of the trial (day 1), all the goats were lighter (15.7kg) in body weight as compared to the post-trial (day 30) (18, 3kg). The body weight has increased by 24 %. The differences in body weight might have been affected by lower pH and stabilization of microbial flora (Singh *et al*., 2019). The decrease in ruminal pH level was observed through time, from 7.01 to 6.18 (Table 3). A decrease in ruminal pH was earlier recorded (Abd El-Ghani, 2004; Abd El-Tawab *et al*., 2016). Studies have shown that *Lactobacillus* strains decrease ruminal pH by producing lactic acid and decreasing volatile fatty acids (VFA) absorption in the rumen (Aschenbach *et al*., 2011; Goto *et al*., 2016; Russell & Wilson, 1996). Based on these results, bacterial probiotics have lowered the ruminal pH. A moderate pH leads to nutrient absorption, resulting in improved weight gain. High ruminal pH inhibits population of lactacte ulizers, which could lead to ruminants being susceptible to severe ruminal acidosis (Nocek *et al*., 2002). Generally, male goats are usually heavier than females (Masika *et al*., 1998). The majority of cases have observed that male animals are born heavier than females with sexual dimorphism in size and body weight maintained through post-weaning into adulthood.

3.6 Conclusion

The efficiency of oral administration of putative probiotics on growth performance of South African goats showed best performance in weight gain, final body weight and feed conversion ratios. Boer goats gained higher weight than Speckled goats, showing that Boer goats will reach the marketed weight faster. These results suggest that the combination of probiotics or using one probiotic improved the ecology of the ruminal microbiome and decreases the ruminal pH.

Probiotics have beneficial effect on growth performances of animals and are suitable to be used as growth promoters. Most importantly, current evidence suggests that the use of probiotic cultures improve growth performance in goats and can be a possible replacement to antibiotics. The limitation of the study includes the difficulty in identifying the type of bacterial strain that will be effective for growth performance. Therefore, there is a need to broaden the research beyond the use of identified strains used as probiotics into tailored characteristics to produce variety of products. Genomics studies can be applied to determine the interaction of probiotics with the microbiota, which can result in better growth performance.

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CHAPTER FOUR

Effects of *Lactobacillus rhamnosus* and *Enterococcus faecalis* supplementation as direct fed microbials on rumen microbiota of Boer and Speckled goats breeds

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4.1 Abstract

The effects on rumen microbial communities of direct-fed probiotics, *Lactobacillus rhamnosus* and *Enterococcus faecalis*, singly and in combination as feed supplements to Boer and Speckled goats were studied using the Illumina Miseq platform targeting the V3-V4 region of the 16S rRNA microbial genes from sampled rumen fluid. Thirty-six goats of both the Boer and Speckled were divided into five experimental groups: (T1) = diet + *Lactobacillus rhamnosus*; (T2) = diet + *Enterococcus faecalis*; (T3) = diet + *Lactobacillus rhamnosus* + *Enterococcus faecalis*; (T4, positive control) = diet + antibiotic and (T5, negative control) = diet without antibiotics and without probiotics. Our results revealed that *Bacteroidetes*, *Firmicutes*, *Euryarchaeota*, *Proteobacteria*, and *TM7* dominate the bacterial communities. In our observations, *Lactobacillus rhamnosus* and *Enterococcus faecalis* supplements reduced the archaeal population of *Methanomassiliicoccus* in the T1, T2 and T3 groups, and caused an increase in the T4 group. *Chlamydiae* were present only in the T5 group, suggesting that probiotic and antibiotic inhibit the growth of pathogens in the rumen. We inferred, based on our results, that *Lactobacillus rhamnosus* and *Enterococcus faecalis* favour the survival of beneficial microbial communities in the goats' rumen. This may lead to an overall improved feed efficiency and growth rate.

Keywords: 16S rRNA; Illumina sequencing; Boer goat; Speckled goat; Probiotic; Lactic acid bacteria; Rumen

4.1 Introduction

Goats are raised for meat, milk, cheese, skin, hair, and plays important roles in religious and cultural ceremonies in South Africa. South Africa has a successful goat industry comprising of commercial and indigenous goat breeds (Visser and van Marle-Köster, 2018). The Boer, Savannah and Kalahari, are commercially developed breeds that have turned out to be important worldwide (Mohlatlole *et al*., 2015). South African goats are recognized for their rapid growth and good meat carcass traits (Pophiwa *et al*., 2017). Goat as a ruminant can breakdown plants material through fermentation in the rumen using anaerobic microbiota such as bacteria, fungi, and protozoa, which convert feeds into energy (Li *et al*., 2018). In goats, and in other ruminants, the rumen microbial diversity and the host physiology can be manipulated by diet, even though the microbial community is mostly stable throughout the animals' life (Langda *et al*., 2020). Successfully, antibiotics are in use to enhance beneficial gut microbial diversity. However, the long-lasting exploit of antibiotics has led to worldwide concerns of antibiotic resistant microorganism, which poses threat to humans health and the environment (Markowiak & Ślizewska, 2018). As a result, the use of antibiotic as feed additives was placed on a ban in most European Union countries since 2006 (FAO, 2005). Therefore, alternatives including probiotics are possible replacement for antibiotics (Markowiak & Ślizewska, 2018). The use of probiotics have been previously shown to improve nutrient digestibility, prevent pathogen invasion in the gut, improve balance in the GIT microbiota and overall well-being of ruminants (Bahari, 2017).

In recent years, metagenomics analysis provides more details about taxonomic diversity and interactions of the gut microbiomes. More details about microbiomes can provide insights on rumen microbial communities and possible applications in animal husbandry. Studies have shown that ruminants can adapt to new diets and contribute to the well-being of the animal (Luton *et al*., 2002). As microbiome communities are of great importance in the breakdown and absorption of nutrients, it is important to determine the effect of direct fed microbes on the rumen microbiota (Henderson *et al*., 2015). Therefore, in this study, we explored the microbial diversity and composition in the rumen of Boer and Speckled goats, under the same feeding regimen, supplemented with *Lactobacillus rhamnosus* and *Enterococcus faecalis*, as putative probiotics.

4.2 Materials and Methods

4. 2.1 Animals, treatments, and sampling

All animal experimental procedures were performed under protocols approved by the Agricultural Research Council- Animal Production Institute Ethics Committee (APIEC17/23), before the commencement of the trial. The trials were done at GI Microbiology and Biotechnology unit, and the Small Stocks Unit in Irene, of Agricultural Research Council-Animal Production Institute, Gauteng Province. The Agricultural Research Council, Irene campus is located at 25° 55' South and 28° 12' East.

Fresh faecal samples from indigenous veld goats (IVG) were used to isolate lactic acid bacteria (LAB). IVGs are known to resist parasites and can survive in unfavourable grazing land (Visser and van Marle-Köster, 2018). 16S ribosomal RNA sequencing and biochemical tests such as gram staining, antibiotic resistance, bile and acid tolerance were used to identify and characterize the isolated LAB. The potential probiotic bacteria were prepared on De Man Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, Hampshire, England) anaerobically, and preserved in 25% glycerol in the ultra-low freezer. The two putative probiotics were revived by inoculation in MRS broth. For suspension, MRS broth was inoculated with, 1% (*v*/*v*) culture and incubated anaerobically at 37 °C overnight prior to administering.

Goats were treated in accordance with the established standards for the use of animals' ethical guidelines. The goats were vaccinated (CDT Vaccine) against *Clostridium tetani* (Tetanus) and *Clostridium perfringens* type C and D (overeating disease), 15 days before the start of the trials to control diarrhoea. A total of thirty-six goats, average age 25 weeks old, including Boer and Speckled, randomly selected were used for this trial. The trial lasted for 30 days after initial 30 days of adaptation. The body weights of goats in the beginning of the trial animals were: Boer males (15.8 ± 2.6 kg), Boer females (14.7 ± 1.2 kg), Speckled males (14.4 ± 3.9 kg), and Speckled females (14.2 ± 5.1 kg). The goats were separated per treatment according to breed (treatment 1= 4 Boer and 4 Speckled, treatment 2= 3 Boer and 3 Speckled, treatment 3= 3 Boer and 3 Speckled, treatment 4= 4 Boer and 4 Speckled, treatment 5= 4 Boer and 4 Speckled) and sex (treatment 1= 4 males and 4 females, treatment 2= 2 males and 4 females, treatment 3= 3

males and 3 females, treatment 4= 4 males and 4 female, treatment 5= 4 males and 4 females) into the trial shelters. The ratio of female or males within breed was as follows: 10 males + 8 females for Boer goats and 8 males + 10 females for Speckled goats. The five experimental treatments were as follows: (T1) = diet + *Lactobacillus rhamnosus*; (T2) = diet + *Enterococcus faecalis*; (T3) = diet + *Lactobacillus rhamnosus* + *Enterococcus faecalis*; (T4) [positive control] = diet + antibiotic; and (T5) [negative control] = diet without antibiotics and without probiotics. The diet used was in the form of pellets to provide nutrient requirements regardless of the treatment as recorded in Table 1. Antibiotic lincospectin was added to the diet in the positive control (T4) group. Freshwater and hay were provided *ad libitum* for all the goats. The weekly administration of probiotics to goats was done orally using a dosing gun at the dosage of 5 ml of 2×10^9cfu/ML of fresh live culture per head (Ataþoðlu *et al*., 2010), repeated every week at 08:00 am for 4 weeks. The goats were weighed individually before and after the trial using a calibrated weighing scale.

Ruminal samples were collected from all the goats at the beginning of the trial and on the last day of the trial using ororuminal collection method (Grünberg and Constable, 2009). About 100 mL of ruminal fluid samples were collected before and after the trial at 07:30 am before feeding on day 1 and day 30 of the trial, by inserting a sterilized tube to the stomach through the mouth of the goat. A volume of 40 mL kept on ice of the collected rumen content was transferred to 50 ml centrifuge tubes and centrifuged at a speed of 10000 × g for 15 minutes. The collected supernatants were transferred into other clean and sterile tubes, and stored immediately at −80°C until DNA extraction. The pH of the ruminal fluid; measured using a pH meter immediately after the collection, and results recorded as shown in Table 2, adopted from our previously published article (Maake *et al*., 2021).

4.2.2 DNA Extraction, PCR Amplification and MiSeq Sequencing

Total genomic DNA was extracted from ruminal fluid samples using PureLink Microbiome DNA Purification Kit (Thermofisher, South Africa) according to the manufacturer's guidelines. The quantity of the DNA; assessed using Qubit 4 Fluorimeter (Invitrogen, South Africa). The extracted DNA samples were used as templates for amplifying V3-V4 region using the following primers, which have overhang adapter (Klindworth *et al*., 2013): 16SAplicon PCR Reverse

Primer=5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCT AATCC3', and 16S Amplicon PCR Forward Primer = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3'. PCR reaction was carried out as follows: 2.5 µl microbial genomic DNA (5ng/ µl), 5 µl of amplicon reverse primer (1 µM), 5 µl of amplicon forward primer (1 µM), 12.5 µl of 2XKAPA Hi-Fi Hot Start Ready Mix (KAPA Biosystems, South Africa), with the following conditions on the thermal cycler: initial denaturation at 95°C for 3 min, 25 cycles (95°C for 30 seconds, 55°C for 30 seconds 72°C for 30 seconds) and a final extension at 72°C for 5 min. Amplicons were visualized using agarose gel electrophoresis. Band size of 550 bp was excised from the gel and purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) according to the manufacturer's guidelines.

Illumina MiSeq library preparation and sequencing was carried out at the ARC- Biotechnology Platform, South Africa, and raw data generated submitted to the NCBI database (PRJNA579264).

4.2.3 Data Analysis

The raw data that was generated from Illumina sequencer MiSeq was trimmed using Trimmomatics version 0.36, whereby universal adapter sequences and low quality sequences were removed. PANDAseq was used to merge the trimmed reverse and forward reads. The merged sequences were imported to Qiime2 version 2018.8 for analysis. Using DADA2, the imported reads were denoised and trimmed. Representative reads were picked using Green gene database and Operational taxonomic units (OTU) table was generated. OTUs at a relative abundance $\geq 0.05\%$ of the total reads in at least one sample were retained. The R Studio (Version 3.5.3) with phyloseq package was used to carry out further analysis on taxonomic classification and diversity. Alpha diversity of samples was calculated using three indices: Shannon index, Simpson index and Chao1 index. For multivariate analysis, Non-metric Multidimensional

Scaling Plots (NMDS) was calculated based on Bray-Curtis Dissimilarity distances (Anderson, 2014).

4.3 Results

4.3.1 OTU clustering and taxonomic annotation of the goat rumen microbiome

To better understand the OTU information and their taxonomic annotation, tags and OTU were calculated. The taxonomic classification between Boer and Speckled was compared, showing dominant Phyla and Genera for both the sampling period (at the beginning of the trial and end of the trial) (Fig 4.1a). Microbial abundance at species level was also evaluated by comparing the abundance between day 1 and day 30 of the trial (Fig 4.1b). An increase of Order *Lactobacillaes* in Day 30 was observed as compared to Day 1 of the trial.

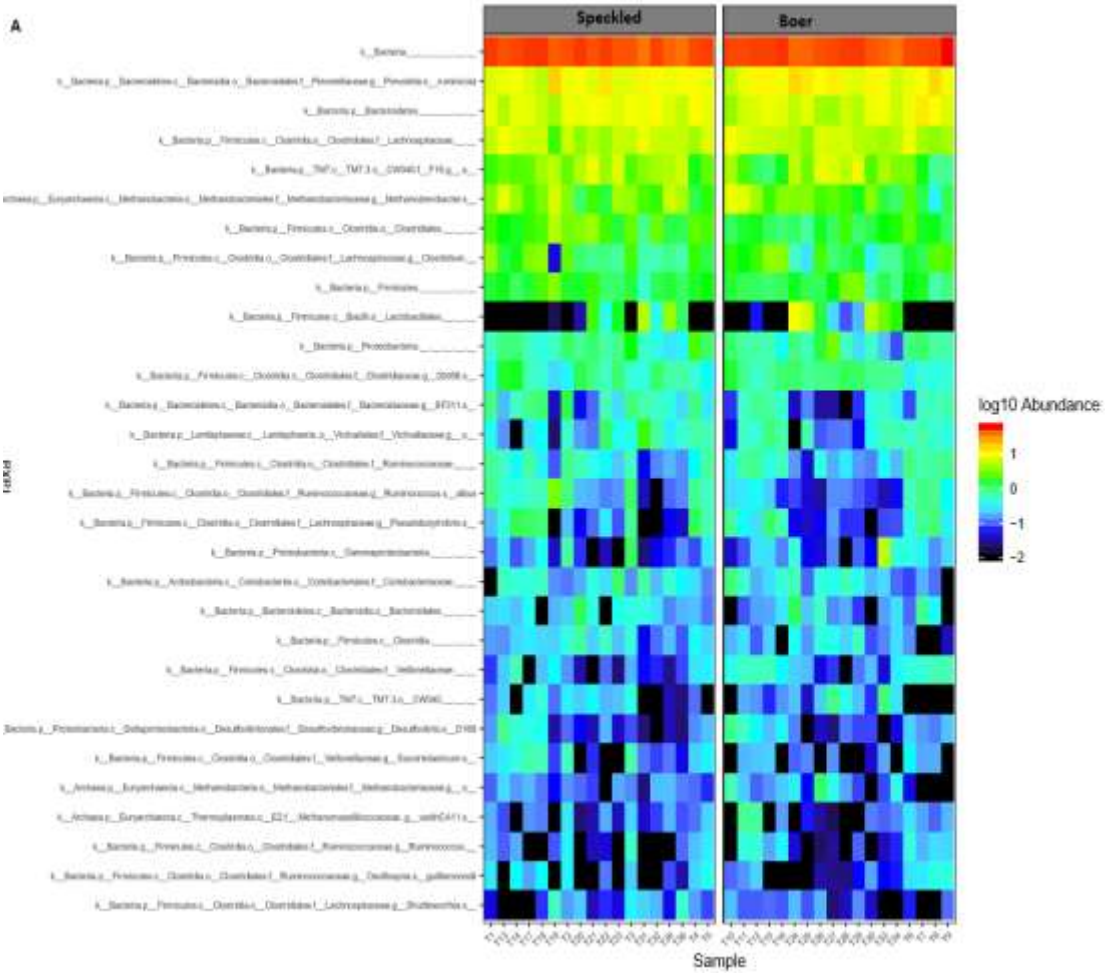


Figure 4.1 ***cont.***

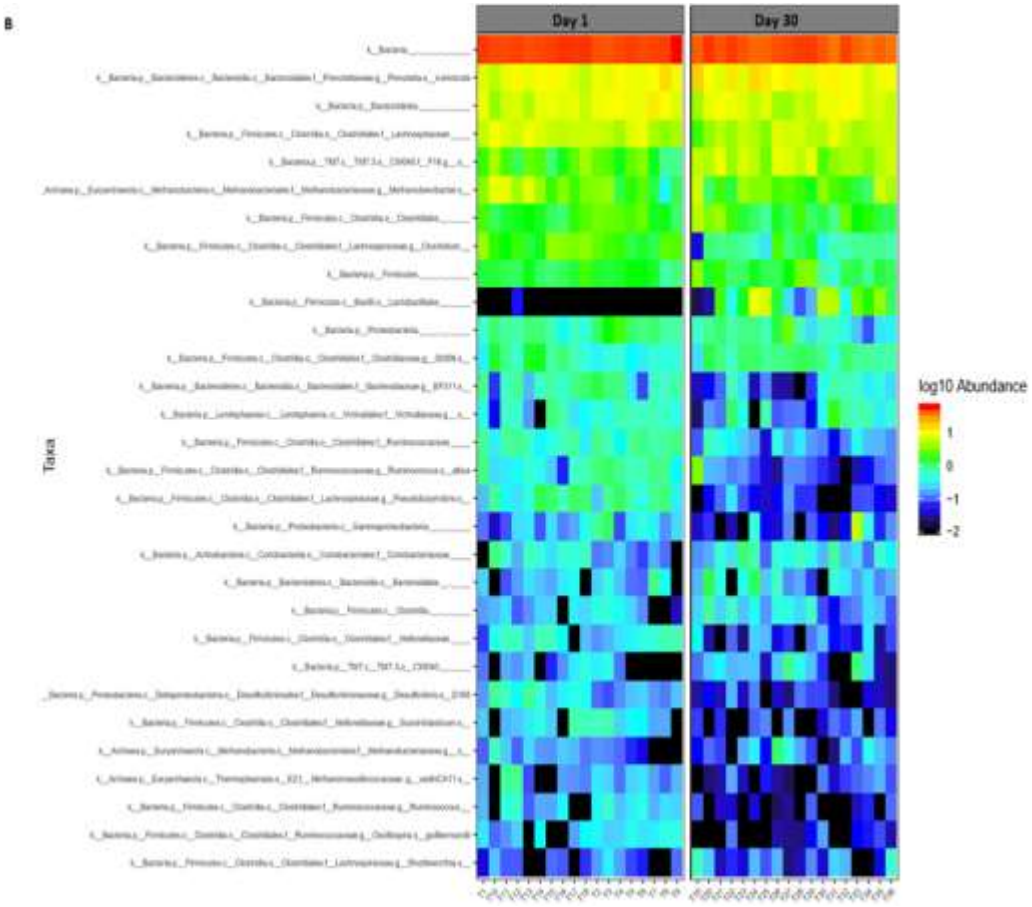


Figure 4.1 Relative abundance of rumen microbial communities between Boer and Speckled goats (A) between Day 1 and Day 30 of the trial (B). T1-T18 are samples before the treatments were administered and T19-T36 are samples collected at the end of the trial. Relative values for each genus depicted by color intensity according to the legend provided on the scale of −2 to 1.

Using the OTUs, a Venn diagram (Fig 4.2a.) was created to show the number of OTUs shared between the goat breeds. The number of mutual OTU in rumen samples between two breeds was 3251, representing 59% of shared OTU, while Speckled goats had 23% and Boer goats 17% (Fig 4.2a). The most frequently abundant 36 OTUs were observed among all the treatments (Fig 4.2b). The distribution patterns showed that core OTUs may perform same basic functions among the five treatment groups.

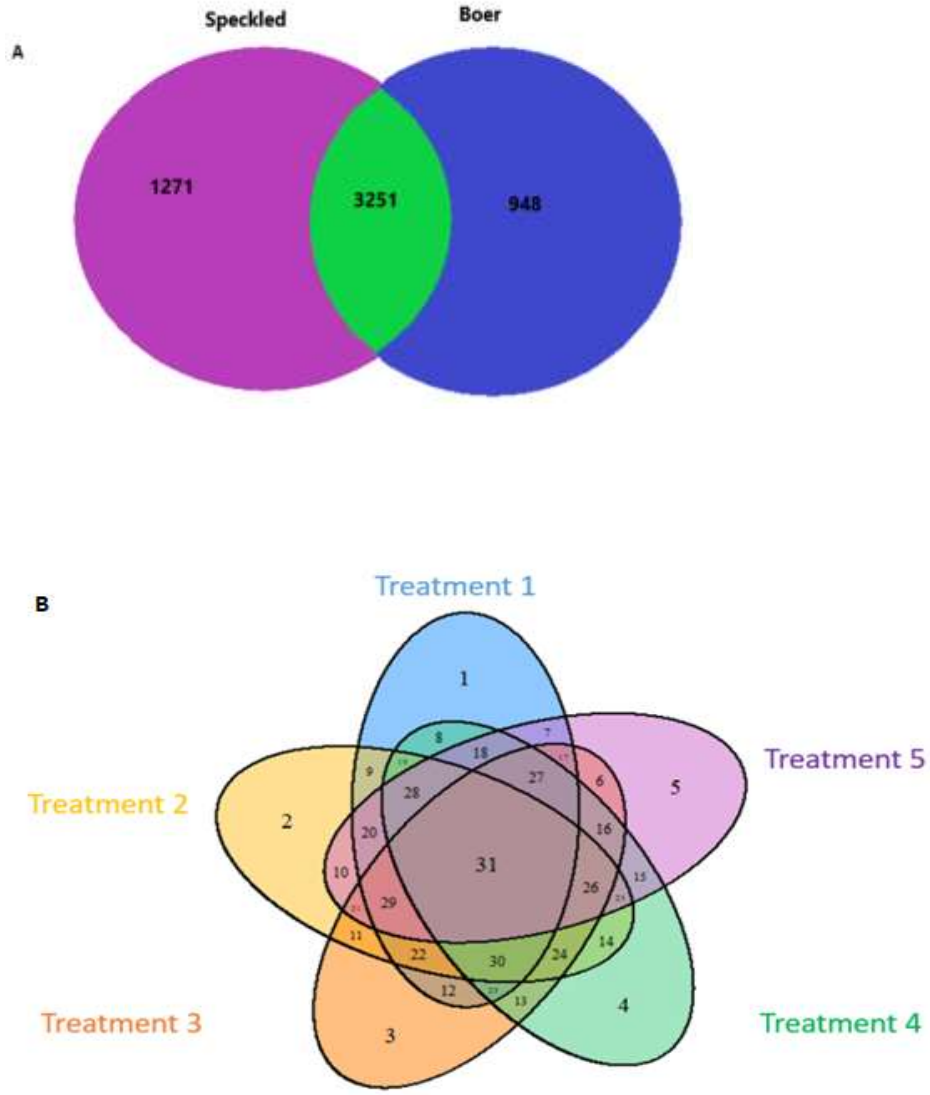
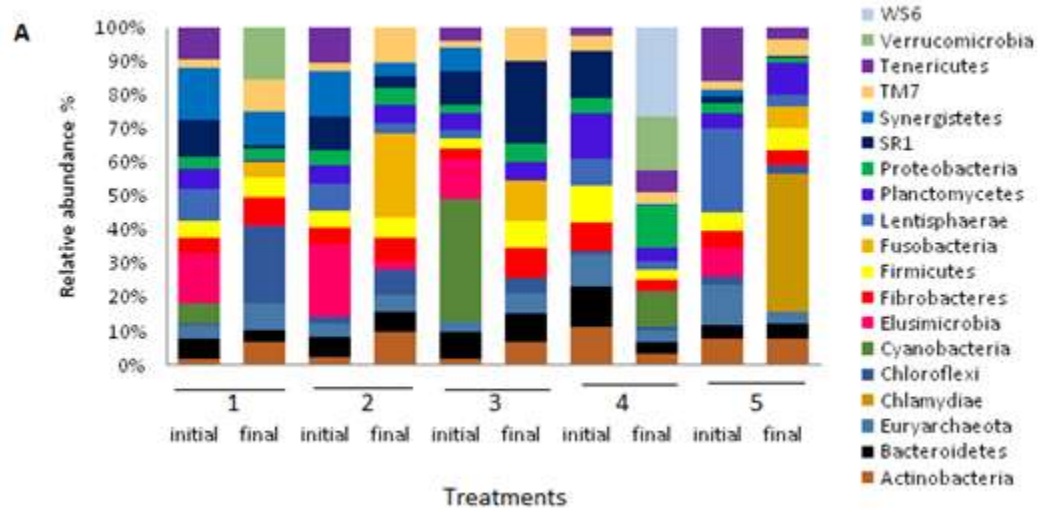


Figure 4.2 Venn diagrams of number of operational taxonomic units of bacteria at day 30 of the trial between 2 goat breeds (A) Speckled (purple) and Boer (blue); and 5 treatment groups (B): treatment 1 (blue), treatment 2 (yellow), treatment 3 (orange), treatment 4 (green), treatment 5 (purple). The numbers in the diagrams represent how many OTUs

were unique in the five treatment groups or shared (similar) between sections as their areas overlaps.

4.3.2 Bacterial and archaeal composition

The taxonomic classification resulted in naming of 19 Phyla, 28 Classes, 39 Orders, 72 Families, and 97 Genera across bacteria and archaeal domains.



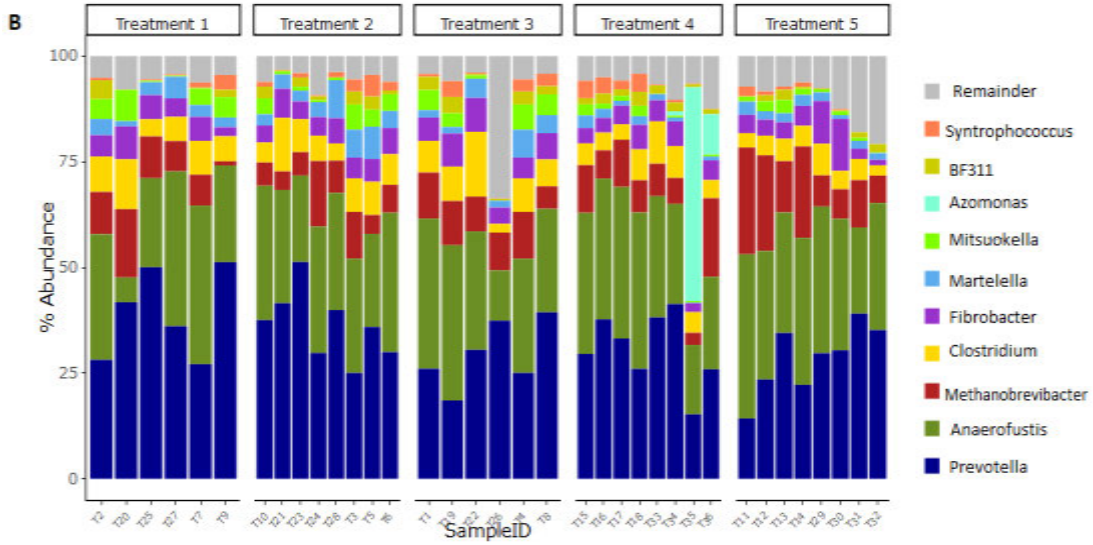


Figure 4.3 *cont.*

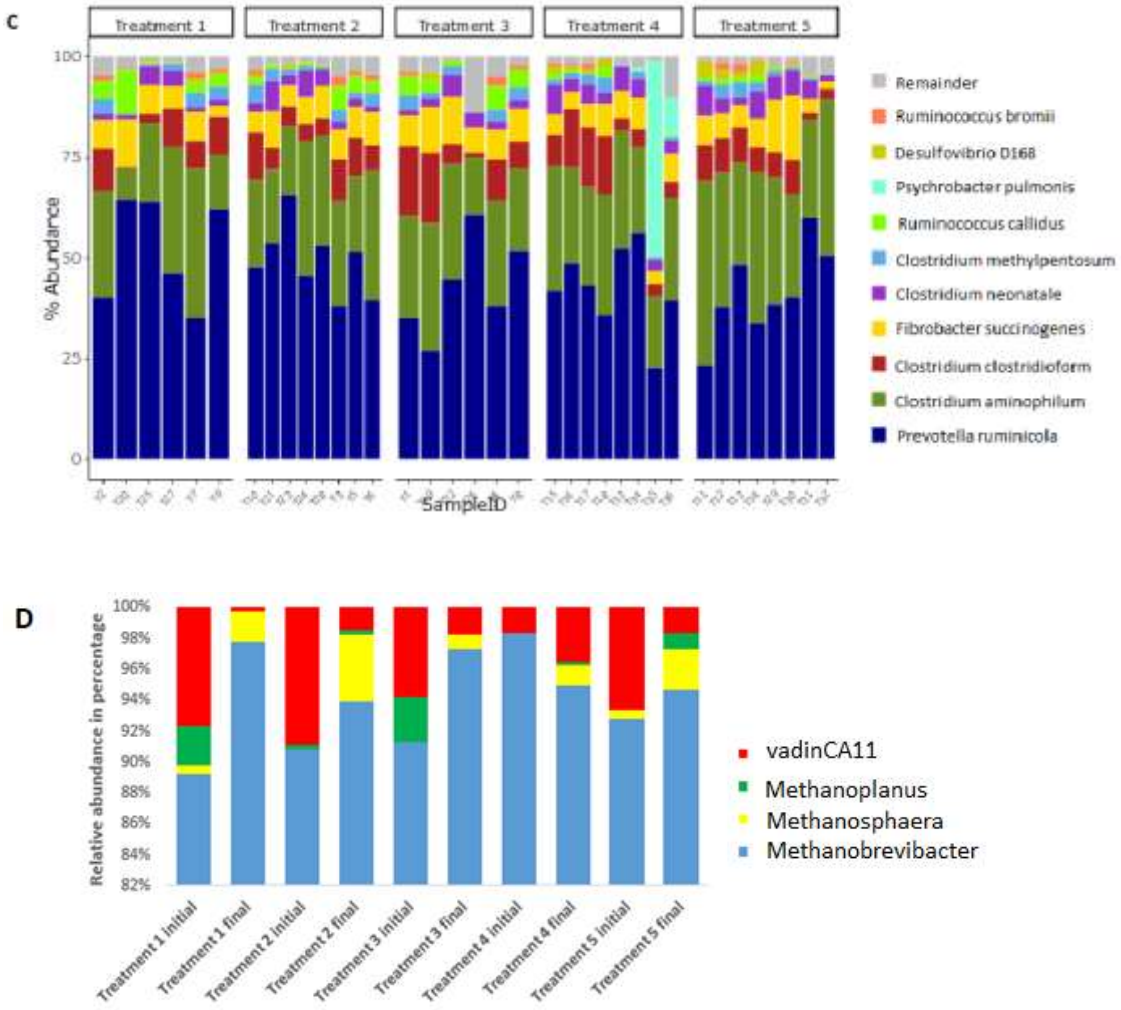


Figure 4. 3 Relative abundance of microbial communities across the five treatment groups of the trial at A) Phylum, B) Genus, C) Species levels. The abundance at Genus level was also shown D). "Remainder" includes all Phyla or Genera with less than 1% relative abundance. T1-T18 indicates samples collected at the beginning of the trial and T19-T36 were collected at the end of the trial. Each bar represents the average relative abundance of each bacterial or archaeal taxon within a group.

The predominant Phyla across all treatment groups were: *Firmicutes*, *Bacteroidetes*, *TM7*, *Fibrobacteres*, *Actinobacteria*, *Proteobacteria* and *Euryarchaeota*. The abundance of

Cyanobacteria, *Verrucomicrobia* and *Tenericutes* were more enriched in the rumen microbiota of Treatment 4 group (Fig. 4.3a)

At the Genus level, the most predominant Genera are *Prevotella*, *Anaerofustis*, *Clostridium*, *Fibrobacter*, and *Martelella* (Fig. 4.3b). The most predominant bacterial species includes *Prevotella ruminicola*, *Clostridium aminophilum*, and *Fibrobacter succinogenes* (Fig. 4.3c). *Ruminococcus callidus*, *Ruminococcus bromii* and *Clostridium spp* were observed in high abundance in all treatment groups. Across all treatment groups, the archaeal community was dominated by Methanobrevibacter, followed by *VadinCA11*, *Methanoplanus* and *Methanosphaera*. The genus *VadinCA11* decreased in all the treatment group except on treatment group 4 (Fig. 4.3d).

4.3.3 Comparison of bacterial diversity

Alpha diversity was used as a measure of diversity within rumen microbiota. Alpha diversity of gut microbiota was shown to be influenced by breed, sex and treatment. Three indices were determined (Shannon, Simpson, and Chao1) (Fig. 4.4). All three indices showed an increment on the final day in treatment groups (1 to 4). Treatment 5 only showed an increment in Chao1 index. The differences were consistent in Shannon index and Chao1 index across the five treatment groups at sub-sample depth point. Rumen microorganism present in Treatment 4 group (H = 2.4) had a higher Shannon index than that of Treatment group 1 (H = 2.0), 2 (H = 2.05), 3 (H = 1.90) and 5 (H = 2.21). The values of 3 indexes were significantly higher in Treatment group 4 as compared to other treatments, indicating that the alpha diversity of rumen Microbiome was higher in treatment group 4. Significant difference in alpha diversity was observed between Boer and Speckled goats. Speckled goats had higher Shannon and Simpson indices (Fig. 4.5).

Samples were found to be dispersed according to treatment groups. Same pattern was observed across all NMDS among breed and sex are presented in Fig 4.6.

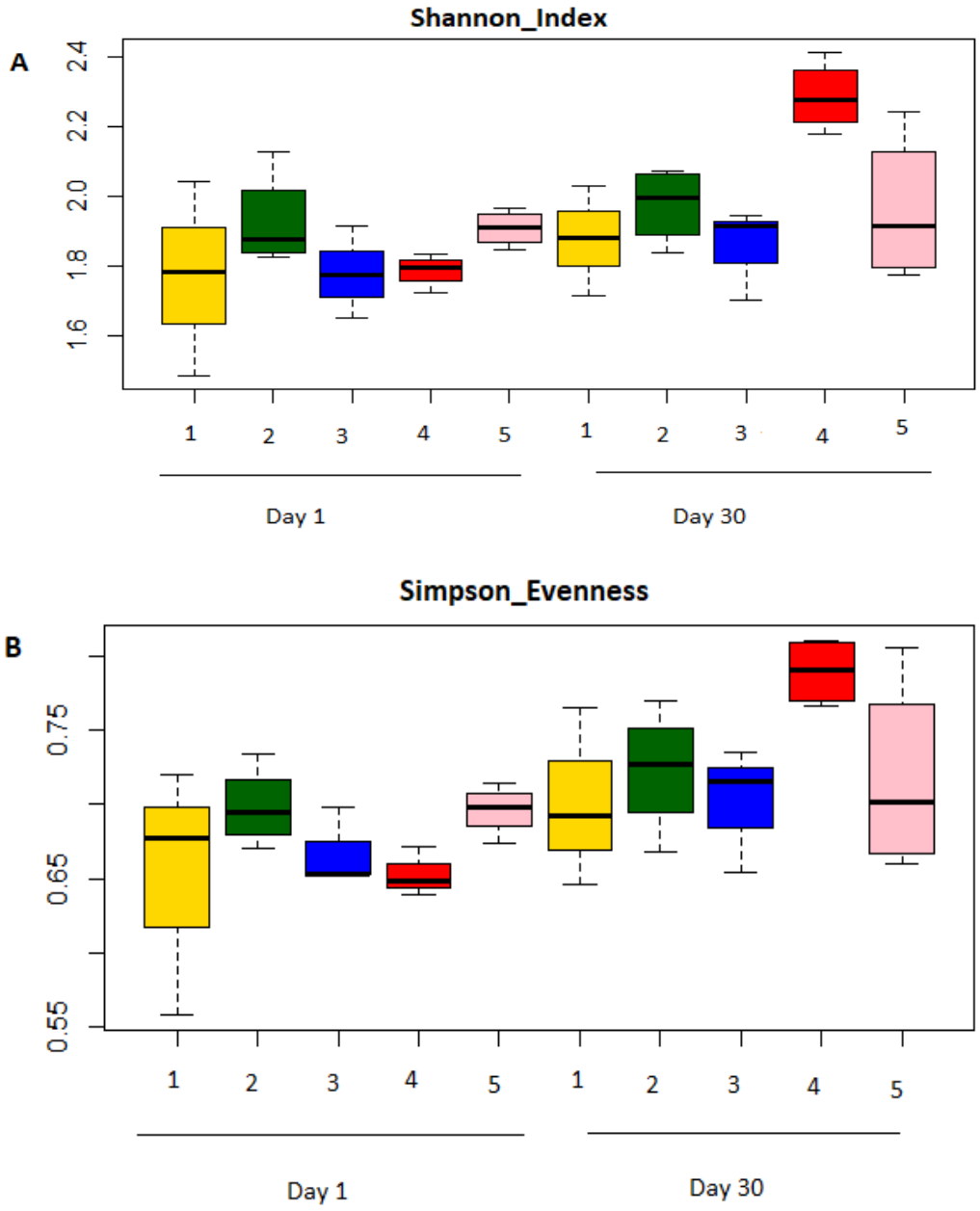


Figure 4.4 ***cont.***

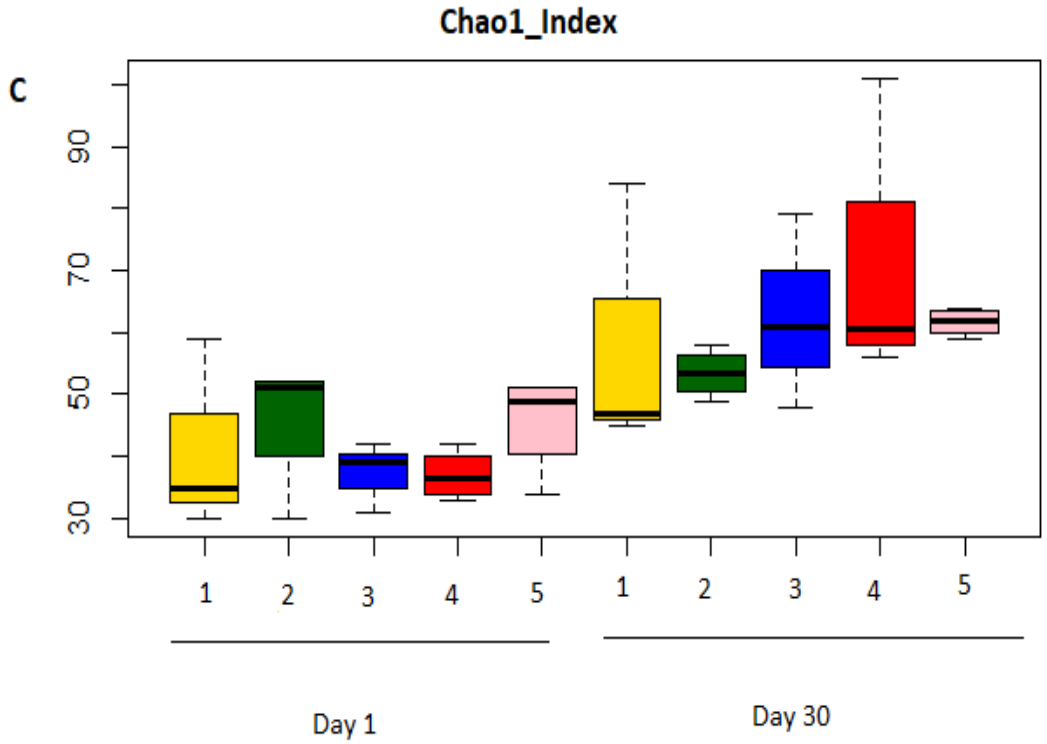


Figure 4.4 Comparison of alpha diversity metrics of communities between Day 1 and Day 30 by Shannon index (A), Simpson evenness (B) and Chao1 (C), respectively. Yellow (treatment 1), green (treatment 2), blue (treatment 3), red (treatment 4) and pink (treatment 5). Treatments groups are as follows: 1. Diet with *Enterococcus faecalis* (yellow); 2. Diet with *Lactobacillus rhamnosus* (green); 3. Diet with combination of *Lactobacillus rhamnosus* and *Enterococcus faecalis* (blue); 4. Diet with antibiotics, no probiotics (positive control; red) 5. Diet without antibiotics, no probiotics (negative control; pink). The top and bottom boundaries indicate the 75thand 25th quartile values, respectively. The horizontal lines within each box represent median values.

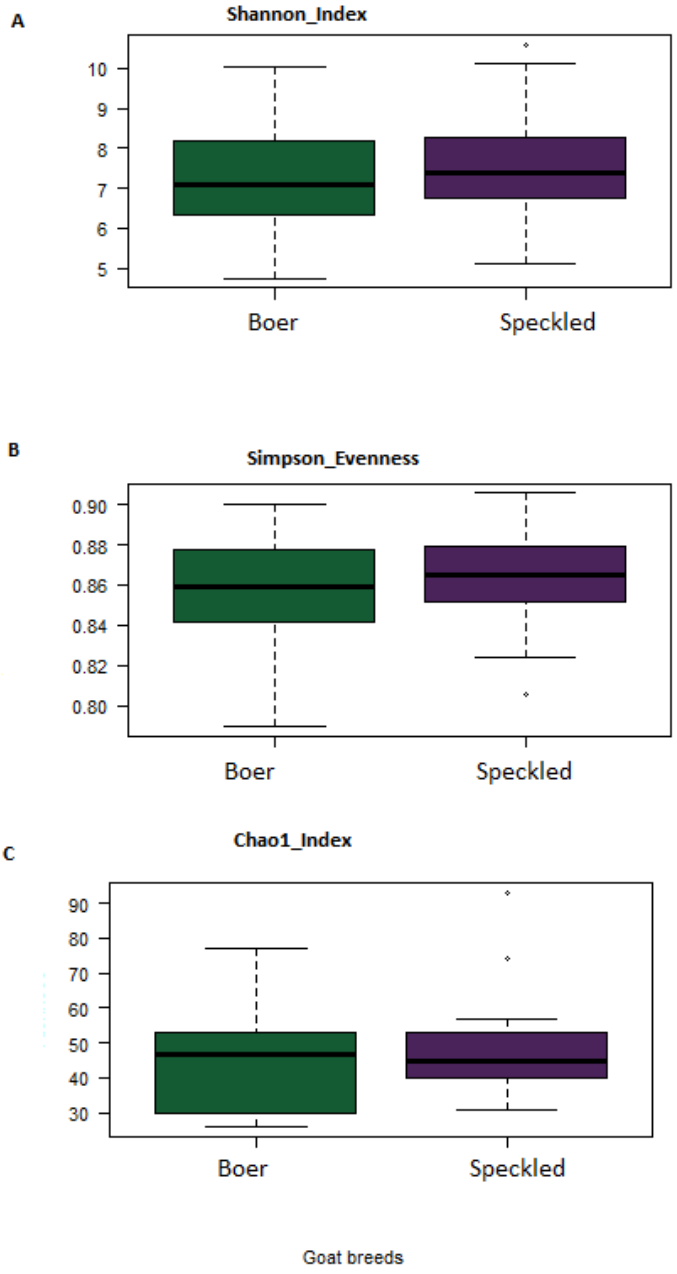
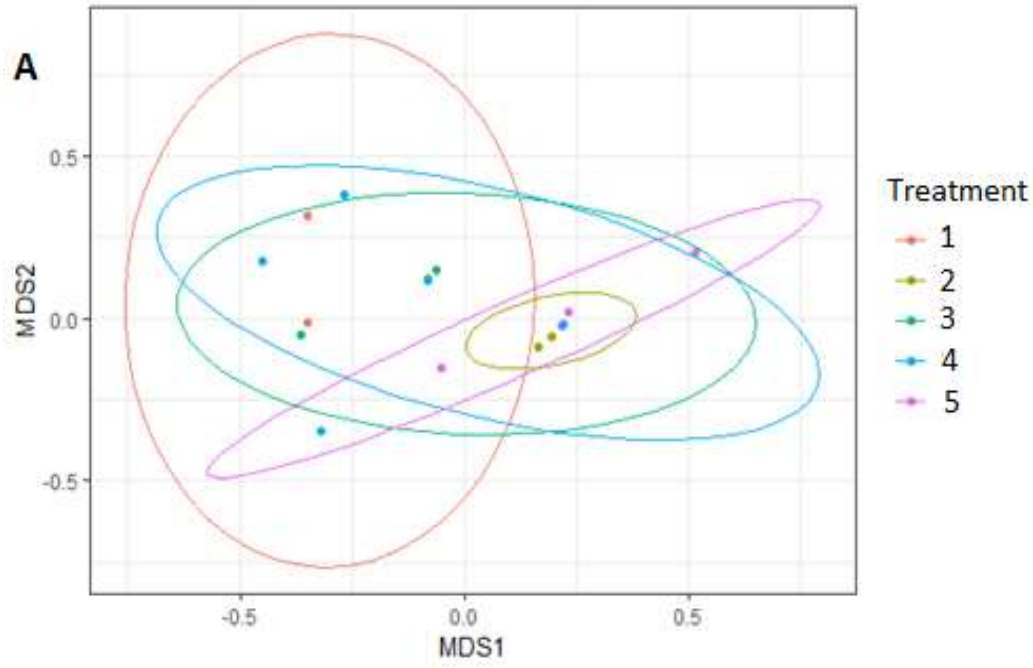


Figure 4.5 Alpha diversities within Boer and Speckled goats on Day 30 of the trial. Blue (Boer), and purple (Speckled). Three indices were measured; Shannon index (A) Simpson evenness (B) and Chao1 (C), respectively. The top and bottom boundaries indicate the $75^{th}$and 25_{th} quartile values, respectively. The horizontal lines within each box represent median values.



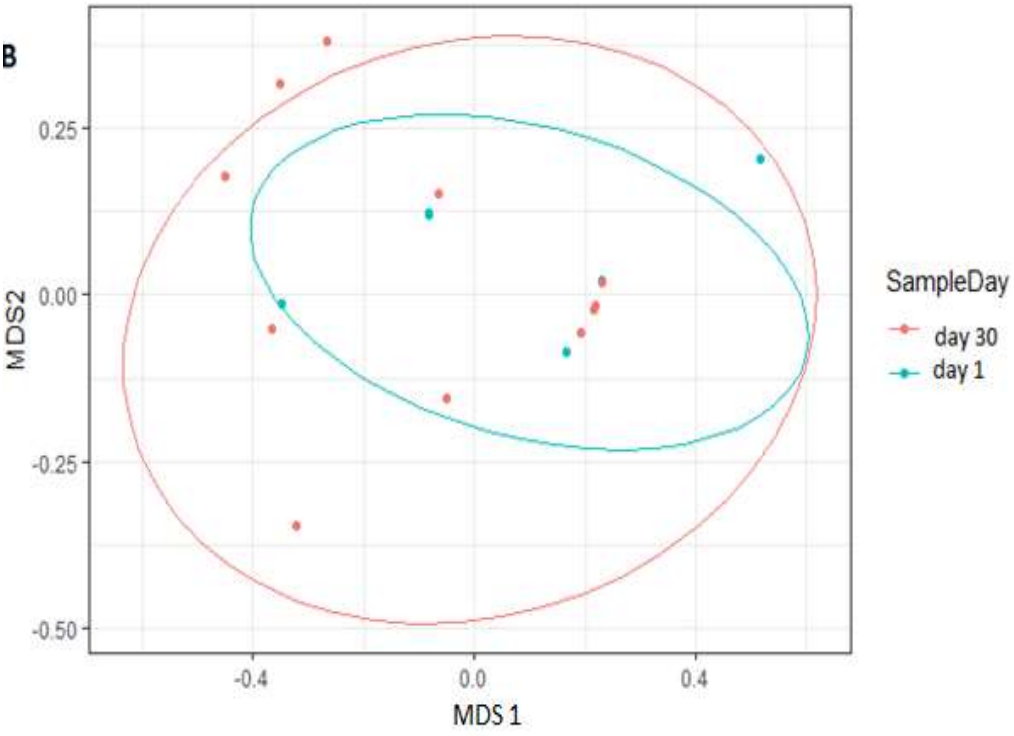


Figure 4.6 ***cont.***

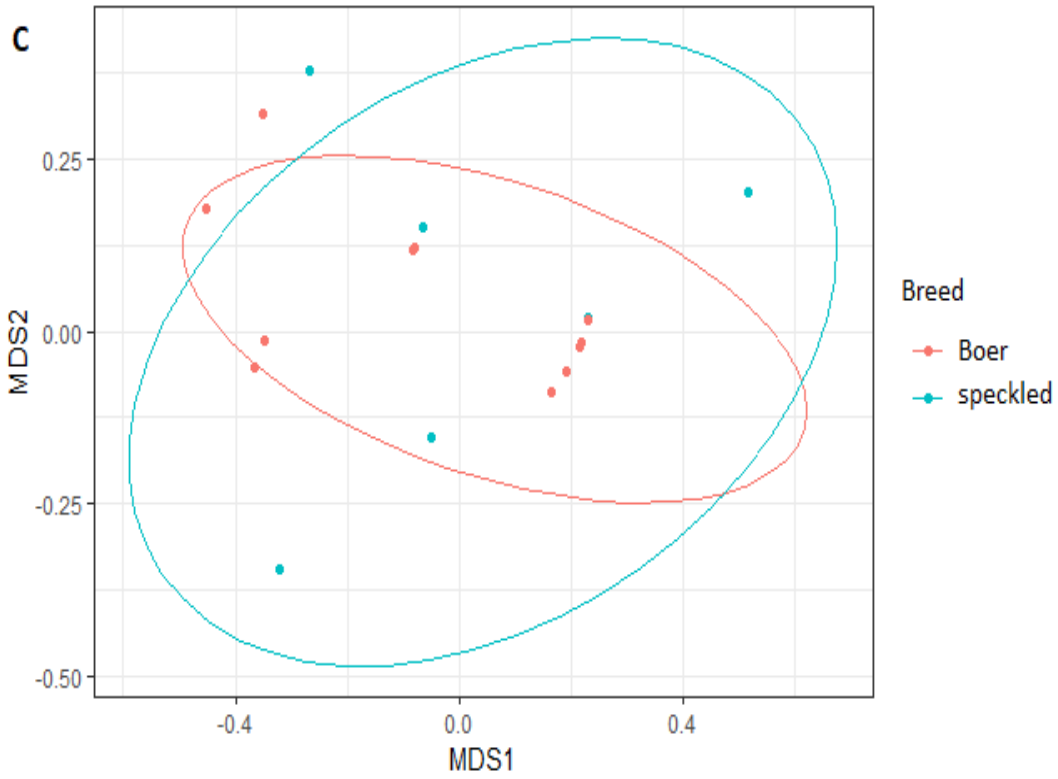


Figure 4.6 ***cont.***

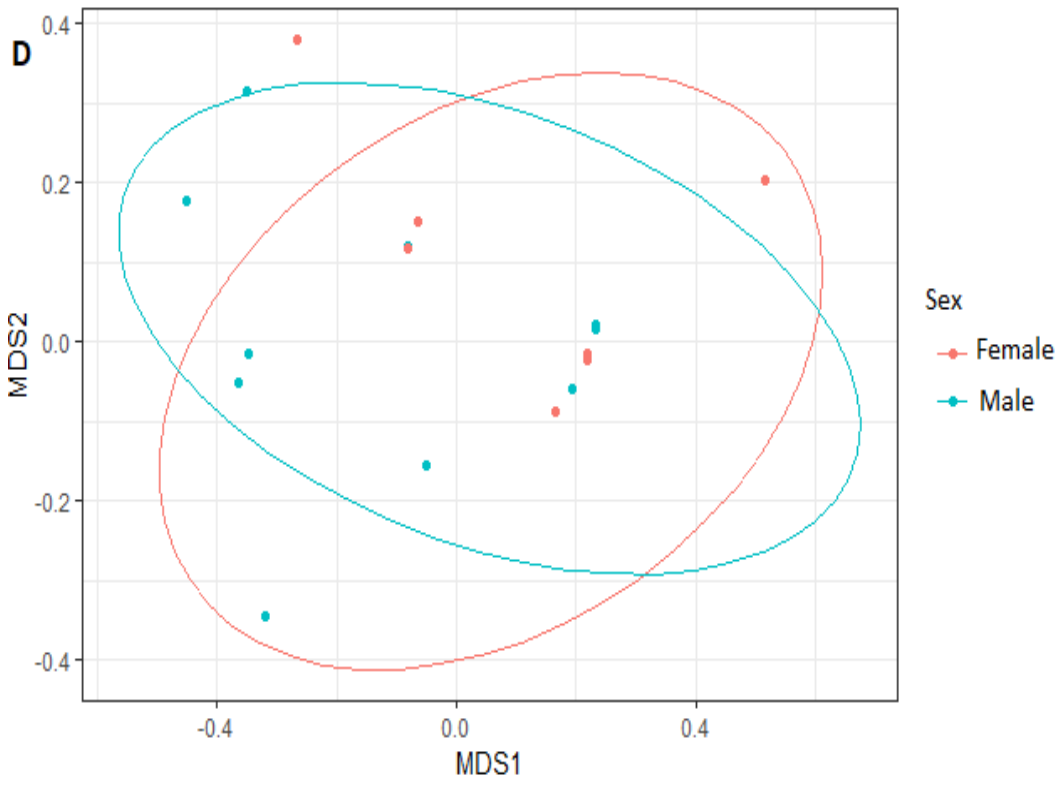


Figure 4.6 Non-metric Multidimensional Scaling Plots (NMDS) based on Bray-Curtis Dissimilarity distances in rumen content of goats treated with *Lactobacillus rhamnosus* (Treatment 1), *Enterococcus faecalis* (Treatment 2), combination of *Lactobacillus rhamnosus* and *Enterococcus faecalis* (Treatment 3), antibiotic (Treatment 4), and negative control (Treatment 5). Each point represents a sample and the colours represent: treatments (A), sample day (B), breed (C), sex (D).

Analysis of similarity (ANOSIM) showed that there were many similarities in microbial composition in the rumen across treatments 1, 2, 4 and 5 (Fig. 4.7a). ANOSIM also showed that no differences were observed in the rumen microbial structure between Boer and Speckled goats (Fig. 4.7b).

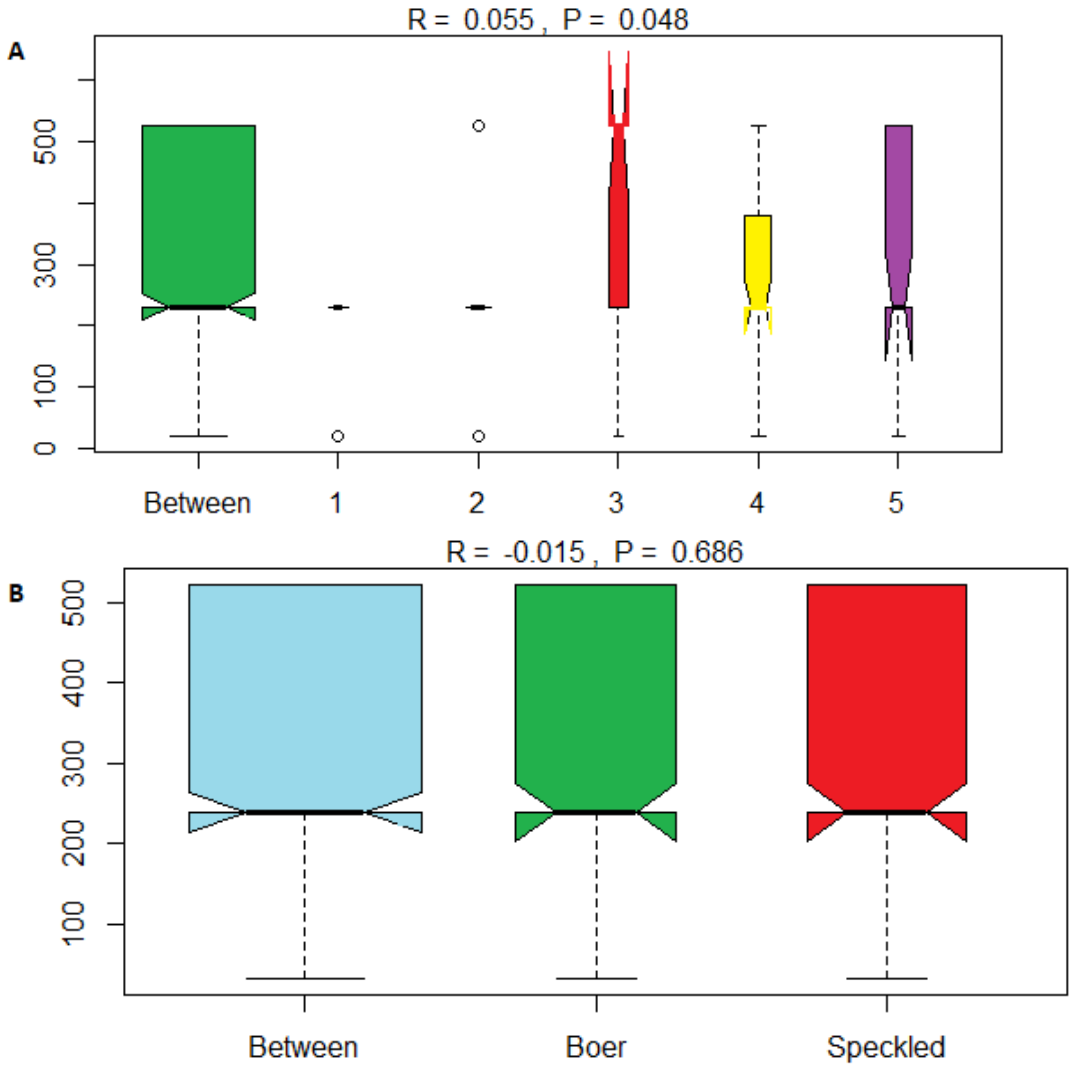


Figure 4.7 Adonis plots showing similarities between treatments. Analysis of similarities (ANOSIM) of the differences in structure of bacterial community in rumen of goats between treatments (A) 1 = (Treatment 1); 2 = (Treatment 2); 3 = (Treatment 3, red); 4 = (Treatment 4, orange); 5 = (Treatment 5, purple). The difference in structure of bacterial community between Boer and Speckled goats were also shown (B). Y axis shows the ranks of dissimilarity. The ends of the whiskers symbolize the minimum and maximum of all the data within the group. "Between" signifies the variation between the five treatment groups, the nearer the R-value is to 1, the greater the dissimilarity between the breeds and treatment. Negative R-value symbolizes greater similarity between the breeds.

Table 4.1 Nutrient composition of the commercial diet fed to goats (g/kg dry matter)

Nutrients[1]	g (kg)
Protein	150
Fat	25
Fibre	110
Calcium	8
Phosphorus	2
Urea	1
Chloride	9
Sodium	9
Magnesium	1
Potassium	6

Table 4.2 Effect of probiotic treatments on ruminal pH of Boer and Speckled goats (Adopted from Maake *et al.*,2021 (Maake *et al.*, 2021)**)**

Parameter	T1	T2	T3	T4	T5	Boer	Speckled	P-value
Initial pH	6.99±0.44	6.56±0.42	7.12±0.41	7.5±0.45	7.19±0.43	7.12±0.42	7.12±0.42	0.57
Final pH	6.32±0.41	6.37±0.46	6.18±0.52	6.4±0.52	6.36±0.56	6.80±0.53	6.34±0.55	0.0001

4.4 Discussion

In recent years, the association between the rumen microbial community and the host has revealed to have a significant part in the host's well-being (Jiao *et al.*, 2015; Lei *et al.*, 2019). Several studies have shown that lactic acid bacteria (LAB) have favourable effects on host (Khafipour *et al.*, 2016; Khalid *et al.*, 2011; Thomas *et al.*, 2017). In the present study, we evaluated microbial abundance and diversity of five treatment groups of Boer and Speckled goat

breeds. The rumen microbiota was altered by the supplementation of antibiotics and probiotics. A decrease in the ruminal pH was also observed in all the treatment groups. Ruminal pH plays a significant role in preserving the internal balance in the rumen environment; therefore, it is important to keep a moderately stable pH for ruminal fermentation. Franzolin & Dehority (2010) also observed a decrease in pH of Angus x Hereford steers from average of 6.27 to 5.81 because of diet to maintain the stability of the gut microbiota. The surety that a decrease in pH value has decrease, was observed by a decrease of Phylum Tenericutes in the treatment groups (Guo *et al*., 2014), as the survive at pH 6.8-7.2 (Tyagi *et al*., 2021).

In both breeds, a microbial diversity of 19 bacteria Phyla (5470 OTUs), was observed. The rarefaction curve was constructed to show that the sequencing depth of the sample was sufficient (Supplementary Fig.1; Appendix 1). When compared to other studies, this study illustrated the high abundance of bacteria in the rumen of goat's breeds at various taxonomic levels (Langda *et al*., 2020).

Irrespective of dietary treatment groups, *Bacteroidetes, Firmicutes* and *Proteobacteria* were found to be the dominant phyla across all the treatments, with a high abundance of *Ruminococcaceae*, *Prevotellaceae*, *Lachnospiraceae* and low abundance of *Veillonellaceae* and *Bacteriodaceae*. These result were in harmony with previous work by Wang *et al*.(2016), who identified microbial using Next-generation sequencing technique for goats. Furthermore, Cremonesi *et al*. (2018) also found similar results. In this study, *Prevotellaceae* was identified as the dominant bacterial family in all the treatment groups in day 1 and day 30. *Bacteroidaceae and Prevotellaceae* are known to be the main families that plays a vital role in the degradation of the feed in goats (Lei *et al*., 2019). Archaea, which accounts for about 4 % of the ruminal microbes (Paul *et al*., 2015), *Methanobrevibacter* was the main genus, supporting previous studies on rumen of goats (Cremonesi *et al*., 2018; Min *et al*., 2019), sheep (Min *et al*., 2019), and cattle (Sirohi *et al*., 2010). *Methanobrevibacter* is a genus from *Methobacteriaceae,* obligate anaerobes that give rise to methane as a major catabolic product (Cersosimo *et al*., 2015; Iqbal *et al*., 2018).

The observed increment of some of microbial abundance in the rumen from Day 1 through Day 30 may emphasise the increased microbes which breakdown carbohydrates and fibre ingested by the animal. The presence of species (*Ruminococcus callidus*, *Ruminococcus bromii* and *Clostridium spp*.) which promote degradation of cellulose into soluble carbohydrates (Ratti *et al*., 2014). *Ruminococcus bromii* comprises of anaerobic Gram-positive cocci with a fermentative metabolism which serve as substrates for growth (Chassard *et al*., 2012). Correspondingly, *Bacteroidales* was found in more abundance in all treatment groups and the levels remained constant throughout the study. An increase of *Lactobacillaes* after the trial indicates that *Lactobacillus* contributes to the growth of the animal (Hussein *et al*., 2021). Shabana *et al*. (2021) also recorded an increase in *Lactobacillus* as the age of the goat increases.

The increase in abundance of the Genera *Lachnospiraceae* and *Bacillus* in the negative control group (Treatment 5) in the gut may be due to undisturbed ecosystem. The genus *Bacillus* is a genus characterized by high proteolytic activity (Ratti *et al*., 2014). Whereas, members of the family *Lachnospiraceae* are associated with butyrate production through carbohydrate digestion (Bi *et al*., 2018; Matthews *et al*., 2019). Lachnospiraceae remained constant throughout the study. The genus *vadinCA11* from the order *Methanomassiliicocca,* which was initially high in all the treatment groups, decreased in all treatments expect Treatment 4 (the antibiotic group) (Fig. 4.3D). The presence of this genus has potential to allow the microbiome to adapt quickly to environmental stress like diet changes. However, the abundance of this genus must be controlled because it can produce additional ammonium through methanogenesis (Matthews *et al.*, 2019). Therefore it is of great importance to include feed which decreases methane production (Sirohi *et al*., 2010), without affecting fermentation and fibre degradation (Martinez-Fernandez *et al*., 2016). The presence of fibrolytic bacteria, *Fibrobacter succinogenes*, a fibre-degrading bacteria was also observed (Fig. 4.3C). Fibrolytic bacteria is responsible for fibre digestion in the rumen (Loor *et al*., 2016). Previous studies have also shown that *Fibrobacter succinogenes* and at least two *Ruminococcus species* functions together to maximize the utilization of fiber components such as cellulose and hemicellulose by ruminants (Koike & Kobayashi, 2009). The presence of *Ruminococcus callidus* and *Ruminococcus bromii* were also observed to assist in fibre degradation.

Alpha diversities within treatments revealed that the microbial diversity was altered with an increase in the richness and overall diversity of the bacterial species observed with Treatment 4 (positive control) showing higher diversity than other treatment groups, followed by Treatment 3 (combination of *Lactobacillus rhamnosus* and *Enterococcus faecalis*). Breed variation also affected diversity, as Speckled goats had higher Shannon index and Simpson index values than Boer goats. These analyses demonstrate that the host genotype plays an important role in maintaining the rumen microbial structure and functions. The results are in agreement with other studies that investigated rumen microbial diversity in cows (Thomas *et al*., 2017), sheep (Couch *et al*., 2020; Mani *et al*., 2021) and goat (Han *et al*., 2015; Langda *et al*., 2020). Jia *et al*.2018 reported that no significant difference observed in microbial richness among the lambs (Dorper x Thin-tailed Han) when fed *Bacillus licheniformis* and *Saccharomyces cerevisiae* as alternatives to monensin.

Beta diversity showed no significant differences between the Boer and Speckled goat breeds and also between treatments 1, 2, 4 and 5, suggesting that there was no distinct diversity in the rumen microbiota of the treatment group. This result was also supported by the Adonis plot (Fig 4.7). The R value was 0.055. The closer the R-value is to 1, would mean that the greater the difference between the treatments. Noel *et al*. (2019) also observed that diet had no significant effect on dissimilarities between microbial communities.

4.5 Conclusion

Our study observed that the administration of lactic acid bacteria as putative probiotics showed beneficial effects on the rumen microbial structure and abundance. Although there were some variations in microbial communities between treatments, similar rumen Phyla (*Bacteriodetes, Actinobacteria, Firmicutes, Tenericutes* and *Fibrobacter*) were abundant in all the treatment groups. The observed rich and diverse microbiome could be the effects of the direct fed microbials to maintain the balance of gut microbiota, hence, the well-being of the animal. In addition, it is noted that the breed variation has an effect on microbial diversity of the rumen. We inferred, based on our results, that *Lactobacillus rhamnosus* and *Enterococcus faecalis* improved microbial composition, which shows that probiotic supplementation can help maintain the

balance of gut microbiota and enhance ruminal bacterial composition and structure in the goats' rumen.

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CHAPTER FIVE

Host genetic variation on the rumen microbiome composition of Boer and Speckled South African goat breeds

Submitted to Scientific Report

5.1 Abstract

The study assessed the host genetic factor effects on the rumen microbiome composition in two South African goat breeds. Microbial DNA was extracted from 36 ruminal fluids of Boer and Speckled goats. A total of 1,261 OTUs acquired from Illumina MiSeq were normalized according to their relative abundance, resulting in 97 Genera. The goats were genotyped using Illumina Goat 50K SNP BeadChip. Subsequently, a genome-wide association study was explored between genotype and the rumen microbiome composition. A total number of 44 single-nucleotide polymorphisms dispersed across the goat genome were associated with the relative abundance of six microbial Genera: *BF311, Clostridium, Fibrobacter, Methanobrevibacter, Prevotella,* and *Ruminococcus.* A total of 47 candidate genes were identified within 1-Mb windows of the goat genome; *CPT1A, STC2, AGPAT3* and *ACSF3* genes were associated with fatty acid metabolism, while *GH, BMP, MSTN, GHR and STMN1* were associated with regulation of developmental growth. *SOCS2* gene was also identified, which is associated with receptor signaling pathway via STAT. Immune system signaling pathway, growth hormone signaling and ghrelin-mediated regulation of food intake and energy homeostasis were identified as significant pathways. Our results suggest that 47 candidate genes may positively shape the microbiome and elucidate the association between gastrointestinal (GI) microbiome and the host genome in South African goat breeds.

Keywords: Rumen microbiome, Boer goats, Speckled goats, GWAS, 50K single-nucleotide polymorphism, Genetic variation

5.2 Introduction

The physiology of the gut is modulated by dietary factors (feed composition and intake), environment and host genetics (Abbas *et al*., 2020). The research interest on the effect of the host genome on the microbiome has been increasing in recent years (Crespo-Piazuelo *et al*., 2019; Firkins *et al*., 2008; Malmuthuge *et al*., 2015). The gut microbiome has a vital role in maintaining homeostasis, inhibiting pathogens, nutrient digestion and animal health (Elghandour et al., 2020). The ability to improve the host's health is largely dependent on the knowledge of rumen microbiology (Zhu *et al*., 2016). Therefore, there is much interest in determining whether host genetics controls the microbiome composition (Zubiria *et al*., 2018).Studies have also shown that the gut microbiome is regulated by a relationship linking host genetic variation in SNPs and relative abundance of gut microbiota, hence genome-wide association studies (GWAS)(Onzima *et al.*, 2018; Rahmatalla *et al*., 2018).

GWAS in livestock production has been previously used to find candidate genes associated with quantitative traits that are economically important (Scholtens *et al*., 2020). Previous studies have shown that a synergistic effect exists with the host to perform several functions in immunity, homeostasis and metabolism(Awany *et al.*, 2019). Studies have also shown that host genetic variation shapes microbial composition, providing a starting point towards understanding the complex interaction between animals and microbiome (Zubiria *et al*., 2018). GWAS can be used in a breeding program to select individual animals with microbiome composition for a particular breeding goal (Mkize *et al*., 2021). The objective of the study was to identify genomic regions that regulate gut microbial composition in Boer and Speckled South African goats using 16 rRNA sequencing and genome-wide association methods.

5.3 Materials and Methods

5.3.1 Experimental animals

The study was conducted according to a protocol approved by the Agricultural Research Council- Animal Production Ethics Committee (APIEC17/23) to protect the animal's welfare,

before the commencement of the trial. All methods were carried out in accordance with relevant guidelines and regulations. We confirmed that all authors complied with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments guidelines). A total of 36 South African goats (18 Boer and 18 Speckled) were used in this study. The goats were fed a commercially available pelletized feed, freshwater and hay (*ad libitum*). Details on the feed formulation are provided in Supplemental Table S1. With the aid of EDTA coated vacutainer tubes and needles, 10 ml of blood was collected from the jugular vein, while ruminal fluids were obtained using stomach tubes before and after the trial. The samples were preserved at -20 ℃ until DNA extraction.

5.3.2 Microbial DNA extraction and 16S rRNA sequencing

Microbial DNA was extracted from ruminal fluid samples using PureLink Microbiome DNA Purification Kit (Thermofisher, South Africa) following the manufacturer's guidelines. The concentration and purity of the DNA were assessed using agarose gel electrophoresis and Nanodrop spectrophotometer (ND1000; Thermo Fisher Scientific, Wilmington, DE, USA), respectively. 16S metagenomic sequencing library preparation guideline (Illumina, San Diego, USA) was used for amplification of V3–V4 hypervariable region of 16S rRNA gene using extracted DNA as templates. Illumina Miseq sequencing was carried-out at ARC-Biotech Platform. Raw reads generated from Illumina Miseq were submitted to National Center of Biotechnology Information (NCBI) database, Sequence Read Archive (SRA) (Accession number: PRJNA579264).

5.3.3 Data Analysis

The raw data that was generated from Illumina sequencer MiSeq was trimmed using Trimmomatics version 0.36, whereby universal adapter sequences and low quality sequences were removed. PANDAseq was used to merge the trimmed reverse and forward reads. The merged sequences were imported to Qiime2 version 2018.8 for analysis. Using DADA2, the imported reads were denoised and trimmed. Representative reads were picked using Green gene database and Operational taxonomic units (OTU) table was generated. OTUs at a relative abundance $\geq 0.05\%$ of the total reads in at least one sample were retained. The R Studio (Version

3.5.3) with phyloseq package was used to to group OTU according to the taxonomic classification using *tax_glom* from phyloseq package (http://www.r-project.org/).

5.3.4 Host DNA extraction and SNP genotyping

DNA was extracted from whole blood samples using PureLink DNA Purification Kit (Thermofisher, South Africa) following the manufacturer's guidelines. The genomic DNA was quantified using Qubit 3.0 Fluorometer (Life Technologies). Genotyping was carried-out at ARC Biotechnology platform. A total of 36 goats were genotyped using the Illumina goat SNP50 bead chip (Tosser-Klopp *et al*., 2014). SNP genotypes were obtained using GenomeStudio software to generate input files. Quality control (QC) was done using PLINK1.9 software (Purcell *et al*., 2007). SNP quality parameters included variations from Hardy-Weinberg Equilibrium > 0.001, missing genotypes > 0.01minor allele frequency < 0.01, and individuals that were unsuccessful for genotyping > 0.1. After QC, a set of 47 853 SNPs out of 53347 SNPs was available for genome-wide association (GWAS) analysis.

5.3.5 GWAS analysis

GWAS was performed between 47 853 SNPs and microbiota composition at Genus level. Samples were normalized based on relative abundance. GWAS was performed on 6 Genera. The chosen genera were present in more than 90% of the samples and comprised more than 0.5% of the total annotated reads. PLINK and false discovery rate (FDR) method using *p.adjust* function built-in on R (Benjamini, 1995),were used to perform GWAS analysis.

5.3.6 Gene annotation and prediction

To identify candidate genes, 1Mb region within significant SNPs was defined, and a record of candidate genes along this region was created using Ensembl Genome Browser (www.ensembl.org). NCBI database was used to identify the functions related to the genes. Central sub-network connection was created using genemania (www.genemania.org), while signaling pathways were produced using BioPlanet 2019 and KEGG 2021 from Enrichr (www.maayanlab.cloud/Enrichr.org).

5.4 Results and Discussion

Microbial composition within goat rumen: Taxonomic classification of normalized OTUs resulted in the identification of 19 Phyla and 97 Genera across Bacteria and Archaea (Figure 5.1).

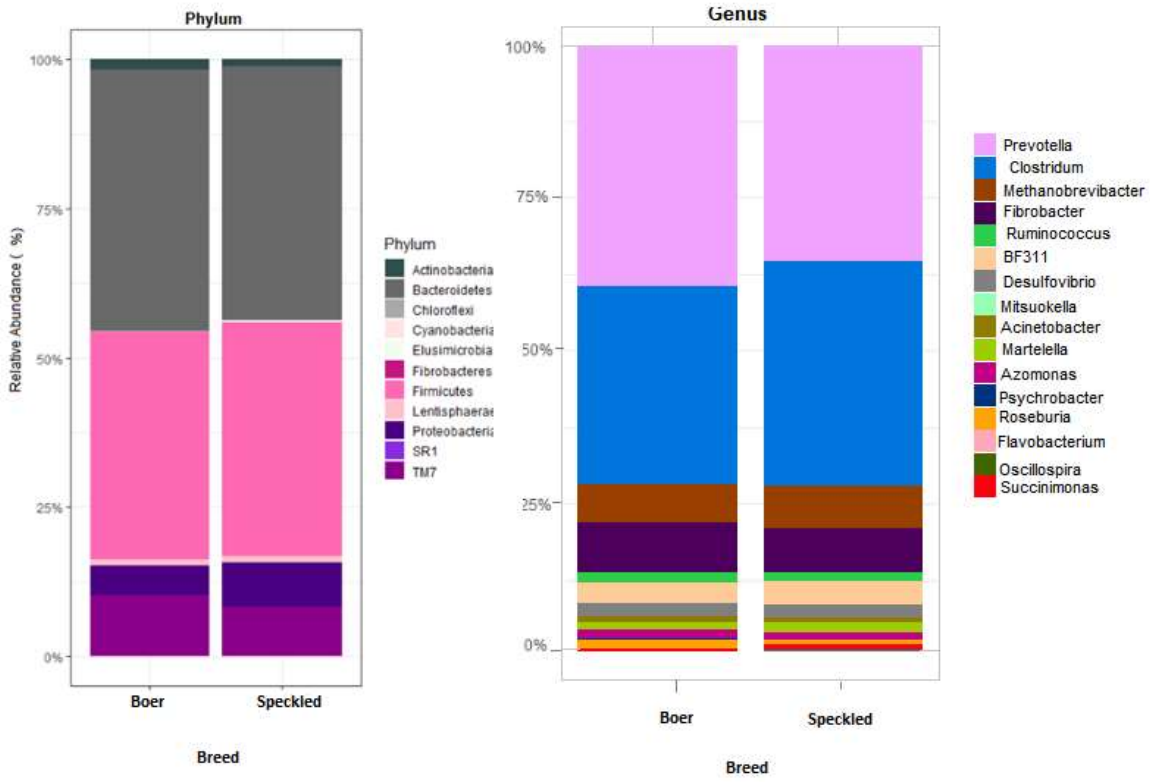

Figure 5.1 Bar plots of OTUs grouped by Phyla and Genera for Boer and Speckled goats.

Candidate Genes and SNPs : GWAS was performed using 47853 SNPs and the relative abundance of six microbial genera; *BF311, Clostridium, Fibrobacter, Methanobrevibacter, Prevotella,* and *Ruminococcus*. The Genera *Clostridium, Fibro*bacter, and *Ruminococcus* are from the phylum Firmicutes, while *Prevotella* and *BF311* are from the *Bacteroidetes*; *Methanobrevibacter* belongs to Euryarchaeota Phylum from the Archaea domain. The allotment of SNPs was different among the chromosomes, with chromosomes 6 having the highest number of SNPs in both Boer (0.686) and Speckled (0.669), while the least number of SNPs was observed in chromosome 18 (0.658) for Boer and chromosome 29 (0.6312) for Speckled (Figure 5.2). Significant association signals (FDR < 0.1) were found in all six Genera. Most associations were observed from *Prevotella* and *Clostridium* Phyla. A total of 44 significant SNPs dispersed

across 29 chromosomes of the *Capra hircus* (goat) genome (Table 5.1, Figure 5.3). A total of 47 candidate genes were found in the regions linked with relative abundance of six Genera. The list of significant genes was recorded in Table 5.1.

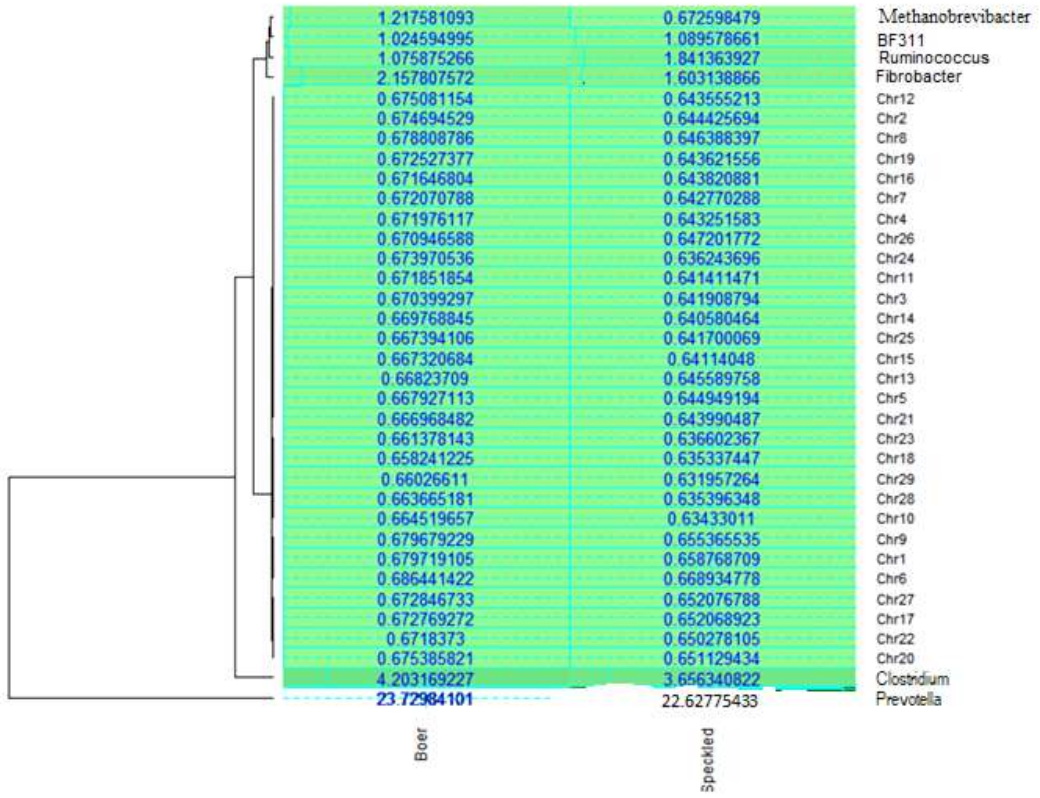

Figure 5.2 Heatmap showing abundance of SNPs across all 29 chromosomes. Associations of six Genera: *BF311, Clostridium, Fibrobacter, Methanobrevibacter, Prevotella,* and *Ruminococcus* with chromosomes were shown.

BF311: The relative abundance of *BF311*, a genus from Bacteroidetes, was significantly associated with polymorphism in five chromosomes: Chr4, 28, 10, 25 and 23. *CACNA2D1* is located in Chr *4*. *CACNA2D1* encodes protein in calcium channel complex *and* have been previously mapped in ruminants for meat QTL (qualitative trait loci) (Hou *et al.*, 2010). *AGT* (Angiotensinogen), located in Chr 28, is a protein-coding gene associated with the conversion of angiotensin 1 to angiotensin 2 and also with cardiac conduction (Lu *et al.*, 2016). *PTGER2* (prostaglandin E receptor 2), located in Chr 10 was also observed in the *BF311* genus. *TRIM56* (located in Chr 25) and *IRF4/5* (located in Chr 23) prevents viral replication. *PTGER2*,

CACNA2D1 and *AGT* have been previously observed to function together in hypoxia-mediated cellular responses and also in the renin secretion pathway on indigenous goats (Zonaed *et al*., 2020).

Clostridium: Significant genes associated with *Clostridium* were *PDK2*, *PDK1*, *SLC2A4PROX1, ENPEP, EPAS1* and *IGF1*. Most of these genes play a vital role in high altitude adaptation (Childebayeva *et al*., 2021). *PDK2*, *PDK1*, *SLC2A4* genes regulate lipid metabolic process (Jeong *et al*., 2012). *SOCS2* was found to be associated with cellular response to peptide hormone stimulus, receptor signaling pathway via STAT, and response to growth hormone (Dehkhoda *et al*., 2018). *ENPEP* and *EPAS1* regulate temperature homeostasis, while *NOX4* and *IDE* were responsible for peptidyl-tyrosine modification. *IGF1* activate protein kinase activity and calcium ion transport. *PROX1* was associated with growth developmental growth and regulation of protein serine/threonine kinase activity. MAPK4 regulates interleukin, which is important for maintaining gut homeostasis (Crespo-Piazuelo *et al*., 2019).

Fibrobacter: *RYR1* gene was associated with growth development, calcium ion transport, cardiac conduction, and regulation of blood circulation (Amburgey *et al*., 2013). *ADCY4* and *ROCK1* regulate insulin secretion (Chun *et al*., 2012) and were associated with adrenergic signaling in several pathways (Hartmann *et al*., 2015). *ACNA1C* was associated with the oxytocin signaling pathway (Zonaed *et al*., 2020).

Methanobrevibacter : The relative abundance of *Methanobrevibacter*, a genus from Archaea domain, was significantly associated with polymorphism in five chromosomes: chr19, 29, 18, 14 and 15. *CPT1A and RDH16*genes are associated with fatty acid metabolism (Skotte *et al*., 2017). *SREBF1* and *APOL3* are regulators of lipid homeostasis (Wu & Näär, 2019). *MYADM* promote an increase in body weight in sheep (Gonzalez *et al*., 2013).

Prevotella: *Prevotella* was one of the dominant Genera in the rumen microbiota (Figure 5.1). Seven genes were associated with *Prevotella* in five chromosomic regions of the goat genome. Chromosome 12 has two significant genes: *ABCC4* and *PRAME* associated with metabolic pathways, signaling on the central neural system and involved in the immune system (Nalini *et*

al., 2013).Three protein-coding genes (*TRIM5*, *LDM2* and *CFH*) were observed, which play an essential role in preventing viral replication and is associated with cytokine signaling in the immune system (Ganser-Pornillos & Pornillos, 2019). *TBX15* and *DGCR8* genes are located in Chr 3, and are responsible for increasing the body size of domesticated goats (Wang *et al*., 2016). *TBX15* is vital for skeletal development (Singh *et al*., 2005; Wang *et al*., 2016), while *DGCR8* is vital for bone development. Other genes were ; *ACSF3*,*STC2*, and *AGPAT3*, all previously associated with fatty acids or cell metabolism (Crespo-Piazuelo *et al*., 2019).

Ruminococcus: Seven candidate genes (*HMGXB3, SLC26A2, BMP*, *GH*, *GHR, MSTN* and *LEP*) were found to be associated with *Ruminococcus*. Most of the genes associated with *Ruminococcus* were related to growth hormones. *GH, BMP*, GHR, and *MSTN* are responsible for regulation of developmental growth (Abbas *et al*., 2020). *GHR* is responsible for activating protein kinase activity and cellular response towards stimuli to growth hormone (Dias *et al*., 2017). *GHR* gene was previously observed to be involved with milk production in goats (Zonaed et al., 2020). *HMGXB3* and SLC26A2 were associated with meat traits of goat (Zonaed *et al*., 2020). *LEP* gene is responsible for positive regulation of receptor signaling pathway via JAK/STAT (Morris *et al*., 2018).

Table 5.1 Significant genes associated with six Genera across 29 chromosomes of the goat genome

SNP Name	Chromosome	Genus	Gene	Related function (www.ensembl.org)
snp16362-scaffold1725-339300	4	*BF311*	*CACNA2D1*	transport calcium, cardiac conduction
snp19552-scaffold1970-1035575	28	*BF311*	*AGT*	activation of protein kinase activity, cardiac conduction
snp49379-scaffold703-7045041	10	*BF311*	*PTGER2*	
snp38374-scaffold485-2194757	25	*BF311*	*TRIM56*	Restricts viral diarrhea by inducing interferon-stimulated genes
snp39675-scaffold505-368570	23	*BF311*	*IRF4/5*	Restricts virus replication
snp38375-scaffold486-16164	5	*Clostridium*	*PDK2*	regulation of lipid metabolic process
snp38376-scaffold486-54217	5	*Clostridium*	*SOCS2*	receptor signaling pathway via STAT, related to growth of the body
snp54512-scaffold831-77802	11	*Clostridium*	*NOXA1*	

snp4229-scaffold1131-46760	6	*Clostridium*	*ENPEP*	regulate systemic arterial blood pressure
snp56549-scaffold893-1070513	11	*Clostridium*	*EPAS1*	temperature homeostasis
snp38088-scaffold4745-30811	1	*Clostridium*	*IFNGR2*	
snp54892-scaffold84-631244	24	*Clostridium*	*MAPK4*	
snp37171-scaffold452-595809	19	*Clostridium*	*SLC2A4*	Associated with regulation of metabolic processes
snp47376-scaffold666-1300349	5	*Clostridium*	*IGF1*	activation of protein kinase activity, animal organ maturation, bone development
snp30498-scaffold3363-112737	26	*Clostridium*	*IDE*	Plays an essential role in the breakdown of insulin
snp54961-scaffold840-1219604	29	*Clostridium*	*NOX4*	peptidyl-tyrosine modification

snp22123-scaffold2185-126993	15	*Clostridium*	*P2RX3*	calcium ion transport
snp58604-scaffold955-89907	16	*Clostridium*	*PROX1*	regulate protein kinase activity
snp38086-scaffold474-2541566	2	*Clostridium*	*PDK1*	regulate lipid metabolism
snp33158-scaffold388-1250275	10	*Clostridium*	*PHF6*	
snp49379-scaffold703-7045041	10	*Fibrobacter*	*ADCY4, ROCK1*	regulate insulin secretion
snp55096-scaffold846-261083	18	*Fibrobacter*	*RYR1*	calcium ion transport
snp34473-scaffold405-1787350	5	*Fibrobacter*	*ACNA1C*	Involved in the oxytocin signaling pathway
snp14625-scaffold1589-3086304	18	*Methanobrevibacter*	*RDH16*	

snp1389-scaffold1038-1590541	19	*Methanobrevibacter*	*SREBF1*	Critical regulator of lipid homeostasis
snp21640-scaffold2119-285990	29	*Methanobrevibacter*	*CPT1A*	Involved in fatty acid metabolism
snp14624-scaffold1589-3028746	18	*Methanobrevibacter*	*MYADM*	Associated with body weight gain
snp13382-scaffold1516	14	*Methanobrevibacter*	*APOL3*	Associated with lipid transport
snp35599-scaffold429-588455	15	*Methanobrevibacter*	*STIM1*	Involved in body weight gain
snp39124-scaffold498-1821142	12	*Prevotella*	*ABCC4, PRAME*	Involved with the immune system
snp39398-scaffold500-1458426	16	*Prevotella*	*CFH*	Protein coding
snp32205-scaffold365-11004	15	*Prevotella*	*TRIM5*	Prevent viral replication
snp30844-	6	*Prevotella*	*LDB2*	Protein coding

scaffold340-2083066				
snp12394-scaffold1471-55356	3	*Prevotella*	*TBX15,DGCR8*	Responsible for body size
snp30573-scaffold339-685946	7	*Ruminococcus*	*HMGXB3*	Associated with meat traits
snp30573-scaffold339-685946	7	*Ruminococcus*	*SLC26A2*	Associated with meat traits
snp37170-scaffold452-563029	19	*Ruminococcus*	*GH*	Related to the growth and development
snp3792-scaffold112-5567638	16	*Ruminococcus*	*BMP*	regulation of developmental growth
snp40392-scaffold515-4009362	2	*Ruminococcus*	*MSTN*	regulate developmental growth
snp30983-scaffold342-929432	20	*Ruminococcus*	*GHR*	activation of protein kinase activity, related to the growth and development

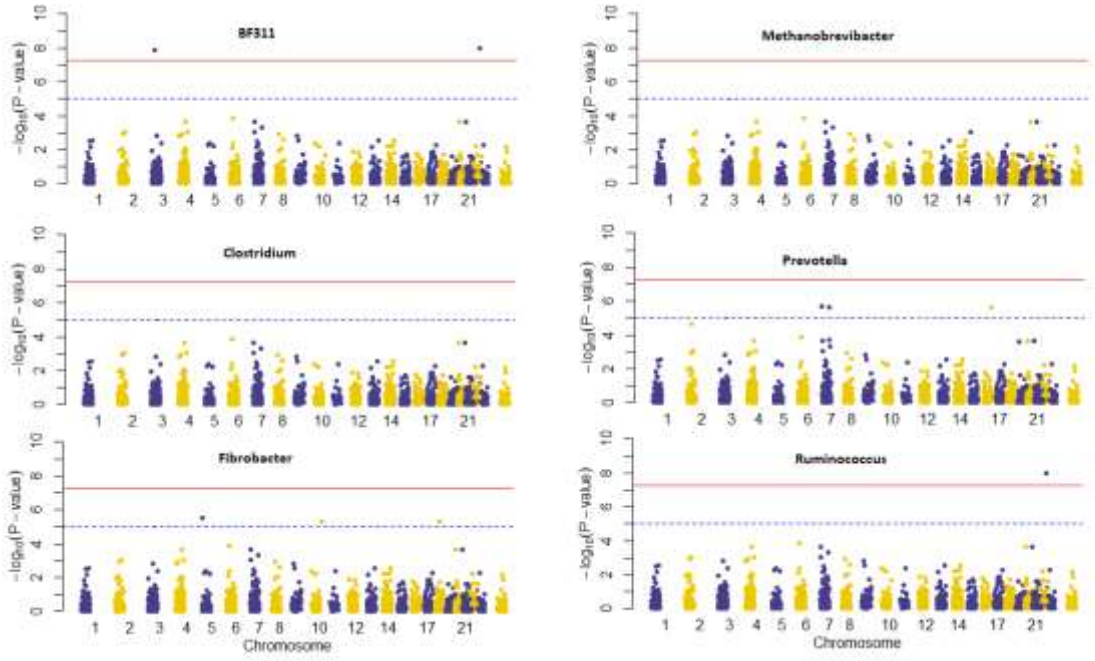

Figure 5.3 Manhattan plot showing SNPs associated with most abundant rumen microbiota (*BF311, Clostridium, Fibrobacter, Methanobrevibacter, Prevotella* and *Ruminococcus*) across the 29 goat chromosomes.

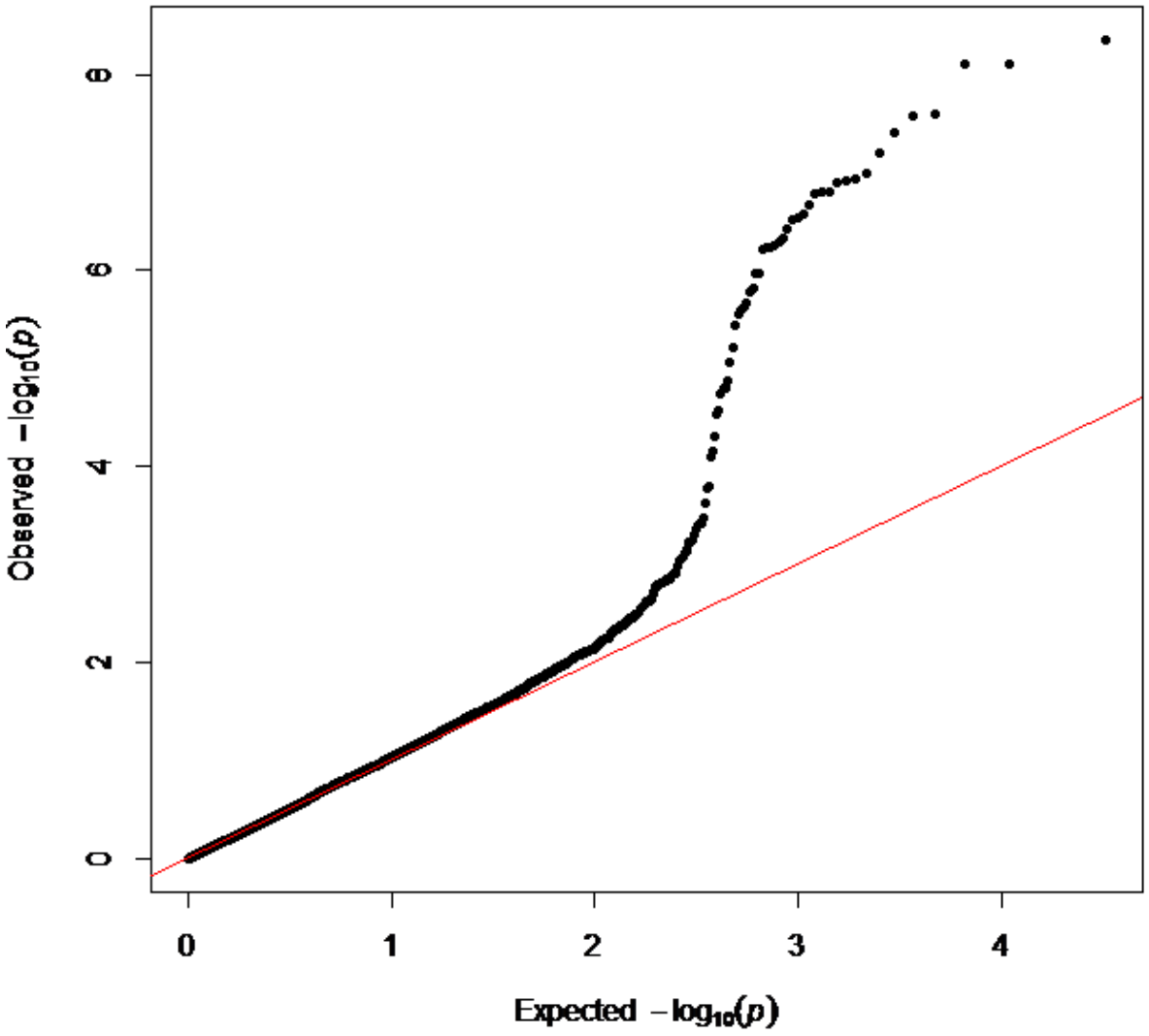

Figure 5.4 Q-Q plot measuring deviation from the projected distribution using p values. Straight line (red) line signifies the estimated distribution.

Quantile-quantile (Q-Q) plot for genome-wide association with the relative abundance of microbiota, demonstrated deviation from the estimated distribution signifying population stratification (Figure 5.4).

Gene networks involved in biological and molecular functions were identified by Genemania. Growth development was the most significant gene network with GH having the most interaction (Figure 5.5). No gene co-expression observed on *SGTA*, while no physical interaction observed in *MAPK4*, *P2RX3*, *CPT1A*

and *IFNGR2*. The physical interaction was observed with genes related to growth development: *IGF-1*, *POU1F1*, *MSTN* and *BMP*.

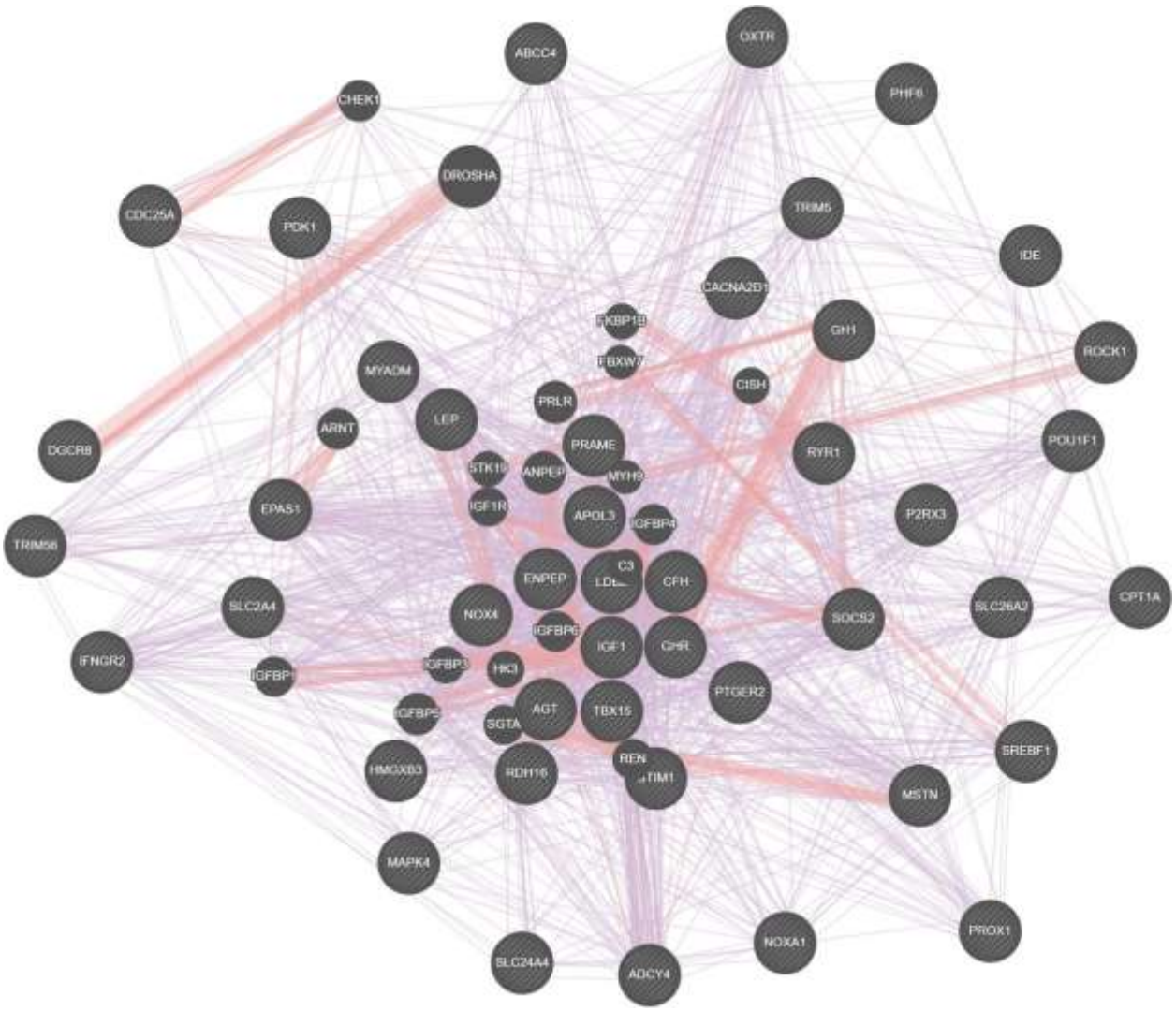

Figure 5.5 Interaction network of significant genes that control the goat microbiome. Red lines indicate physical interactions, while purple lines represent gene co-expression.

The significant pathways have been depicted in Table 5.3 based on –log10 (p-value) (Supplementary Table 1). Significant pathways identified were immune system signaling by interferon, growth hormone signaling and insulin signaling pathways.

Table 5.2 *P*-values of top 10 KEGG pathways for GWAS data: each pathway with a *P*-value <0.005. *Q*-value represents adjusted *P*-value. Pathways were predicted using KEGG 2021 and BioPlanet 2021 (www.enrichr.org).

Pathways	p-value	q-value
Ghrelin-mediated regulation of food intake and energy homeostasis	4.14E-15	1.58E-12
Insulin-like growth factor (IGF) activity	1.02E-11	1.94E-09
Growth hormone receptor signaling	4.69E-09	5.96E-07
Adipogenesis	1.00E-08	9.54E-07
AMPK signaling pathway	1.26E-08	9.63E-07
Myometrial relaxation and contraction pathways	3.33E-08	2.11E-06
Renin-angiotensin system	2.20E-07	1.20E-05
Immune system signaling by interferons, prolactin, and interleukins	3.03E-07	1.44E-05
Insulin signaling pathway	2.77E-06	9.59E-05

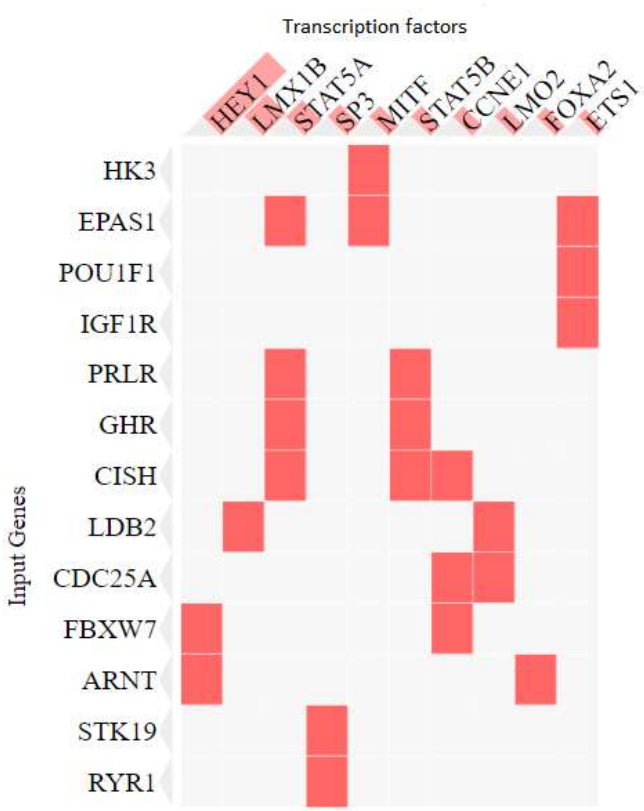

Figure 5.6 Correlation between transcription factors and associated genes.

Regulatory analysis using the Transcription Factor PPIs algorithm showed STAT5A as the most significant transcription factor (TF), with the adjusted P- value of 0.02. STAT5A control four genes: *EPAS1*, *PRLR*, *GHR* and *CIISH* (Figure 5.6). Two TFs, STAT5A and STAT5B, are known to be activator of transcription (Farashi & Kryza, 2020).

5.5 Conclusion

This study identified an association linking the goat genome and the relative abundance of six bacterial Genera (*BF311*, *Clostridium*, *Fibrobacter*, *Methanobrevibacter*, *Prevotella*, and *Ruminococcus*). The candidate genes observed function in immune defense mechanisms, growth development and metabolic processes. Most associations were observed from *Prevotella* and *Clostridium* Phyla. Our results indicated the significance of host genetic variation in the shaping of the microbiome composition in Boer and Speckled South African goats.

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CHAPTER SIX

General Discussion, Conclusion and Recommendation

6.1 General Discussion

The study set out to investigate the effects of lactic acid bacteria (LAB) as putative probiotics on growth performance, rumen microbial communities and host genetic factor on the rumen microbiome composition in Boer and Speckled South African goat breeds. This was carried out in an effort to enhance weight gain resulting from a beneficial modulation of intestinal microflora and subsequently a GWAS was conducted in search for significant SNPs that regulate the rumen gut microbiota.

The first analysis looked at the use of probiotics as growth promoters in an attempt to substitute probiotics as alternatives to antibiotics. *Lactobacillus rhamnosus* and *Enterococcus faecalis* were used as direct-fed probiotics singly and in combination to both Boer and Speckled goats as summarized in Chapter 3. The use of LAB as feed supplements was a success as average weight gain was higher in LAB groups (Treatment 1, 2 and 3) as compared to the negative control group (Treatment 5= diet without antibiotics and without probiotics). Total weight gain of T1 = 1.95kg, T2 = 1.32kg, T3 = 2.42kg, T4= 4.4kg and T5 = 0.87kg were recorded and feed intake was calculated. Results showed that LAB supplementation had no significant effect on feed intake. The pH decreased averagely from 7.01 to 6.18 in all the treatment groups, confirming modulation effect of the intestinal digestion in goats caused by lactic acid bacteria. The pH of T3 (combination of probiotics) decreased to the lowest of 6.18, confirming the synergistic adhesion effects on animal performance. Although monostrain probiotics were beneficial, combining different strains has achieved better performance (Lambo *et al*., 2021).

The use of probiotics as feed supplements has been previously successfully used in sheep (Alejandro *et al*., 2014),and other goat breeds (Ataþoðlu *et al*., 2011; Vosooghi-poostindoz *et al*., 2014; Kumar *et al*., 2016; Salvedia & Supungco, 2017; Srivastava *et al*., 2017) to promote growth. By balancing VFA in the rumen, the use of probiotic has prevented ruminal acidosis in some goats (Kumar *et al*., 2016). The use of multi-strains produced desirable weight gain as compared to when LAB strains were used singly. This result conforms to previous finding on goat kids whereby *Lactobacillus plantarum* BS was used in combination with *S. cerevisiae* 2030 (Jinturkar *et al*., 2009; Salvedia & Supungco, 2017). The current study also compared LAB supplementation between two breeds, whereby Boer goats (3.4kg) were heavier than

Speckled goats (2.5kg). Pophiwa *et al*., 2017 also observed that South African Boer goats develop faster than Indigenous veld goats (Kalahari, Speckled, etc). Boer goats are usually selected for rapid growth development, while Speckled are considered for selection programs because they have the best genetic potential (Dzomba *et al*., 2017; Ncube, 2020).Although an African goat breed have previously explored growth enhancement with probiotics, Saanen goats (Ataþoðlu *et al*., 2011), there was no comparison between breeds. The male goats recorded a growth rate of 17%, while female goats were 11%. The faster growth rate was supported by Masika *et al*. (1994) , who observed that male goats were heavier than female goats. However, no probiotic studies have been recorded. Studies focusing on supplementation of probiotic have been observed (Srivastava *et al*., 2017). The success of LAB shows that probiotics can enhance growth performance and that it is usable in different breeds of interest.

Chapter 4 focused on the effects of *Lactobacillus rhamnosus* and *Enterococcus faecalis* singly, and in combination on rumen microbial communities. Irrespective of treatments provided, rumen microbial compositions of goats were dominated by *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. 16S rRNA analysis was also supported by findings from Li *et al*. (2019), who observed that the rumen of goats was dominated by *Euryarchaeota*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes*. Microbial analysis revealed that *Lactobacillus rhamnosus* and *Enterococcus faecalis* increased microbial diversities as compared to other treatments without LAB. The proportion of *Lactobacillus* is usually low in kids and increases through growth stages in goats (Li *et al*., 2019), Order *Lactobacillales* increased in Day 30. *Lactobacillus* assists in the degradation of carbohydrates and glycans, and makes a foremost contribution to the host (Scheiman *et al*., 2019). The presence of *Chlamydiae* was observed in treatment group 5, without probiotics and antibiotics, showing that probiotics and antibiotics eliminate pathogens. Ability to produce bacteriocin is an important trait in probiotic selection because bacteriocin eliminates pathogens within the gut through bacteriocidal activity (Raabis & Li, 2018). The genus *VadinCA11* decreased in all treatment groups except in treatment group with antibiotic (T4). Other studies also observed the decrease of *VadinCA11* with the aid of dietary additives to reduce methane emissions (Bowen *et al*., 2020). The microbial richness was measured by Shannon index and Chao1 showed an increase as the number of days progressed in all the treatment groups, which was consistent with previous reports (Li *et al*., 2019). Breed variation was observed to play a significant role in alpha diversities, as Speckled goats had higher Shannon and Simpson indices. However, no dissimilarities were observed between the breeds and treatments in the beta diversities, which could mean that there was no distinct diversity in the gut microbiome of goats.

With all the advancements in goat genomics to unlock the genetic potential that affects goat production in South Africa, limited information still exist to determine the association of the goat with their microbiome.

Illumina Goat SNP50K Beadchip and Illumina Miseq were used to study genetic potential of South African goat breeds as summarized in Chapter 5. Genome- wide association was performed to assess the effects of genetic factors on the rumen microbiota of two SA goat breeds. A total of 44 significant SNPs identified using GWAS and most of them are known to be economically important. Candidate genes such as *CPT1A*, *STC2*, *AGPAT3* and *ACSF3* were associated with fatty acid metabolism. These genes may positively shape the microbiome. Results are in agreement with other studies whereby genes like *STC2*, *AGPAT3* and *CPTIA* regulate metabolism, physiological pathways, and phenotype expression (Zonaed *et al*., 2020). While, genes like *GH, BMP, MSTN, GHR and STMN1* were associated with growth development. With growth quality being the most important economic trait in goat husbandry, other researchers also found *GH1* and *GHR* to be the most significant genes associated with Boer and indigenous South African goat breeds (Ncube, 2020). Snp30983-scaffold342-929432 associated with *Ruminococcus* on chromosome 20 had the highest *p*-value and was in close proximity with *GHR* gene. Snp19552-scaffold1970-1035575 at Chr 28 for *BF311* in position 32731923, followed by Snp16362-scaffold1725-339300 in Chr 4 in position 10253048.

6.2 Conclusion

The efficiency of probiotic cultures showed best performance in weight gain and feed conversion ratios in Boer and Speckled South African goats. The use of *Lactobacillus rhamnosus* and *Enterococcus faecalis* as single and in combination decreased ruminal pH, suggesting that they can improve the ecology of ruminal microbiome. The study also observed the association between the goat genome and relative abundance of six Genera: *BF311, Clostridium, Fibrobacter, Methanobrevibacter, Prevotella,* and *Ruminococcus*. Candidate genes associated with growth development, immune response and fatty acid metabolism were identified. The current evidence suggests that the use of probiotics promotes growth performance and could improve goat husbandry. Therefore, probiotics are possible replacement to antibiotics.

6.3 Recommendations

Based on this study, we recommend the following:

- The use of *Lactobacillus rhamnosus* and *Enterococcus faecalis* in combination as putative probiotics to achieve higher weight gain, effective nutrient digestion and maintain ruminal pH.
- Synergism of probiotics can be used to improve microbial composition and structure, in order to maintain the balance of gut microbiota in indigenous goats.
- Breed variation plays a vital role in regulating the rumen microbial community structure and achieve higher microbial diversity. Speckled goats can be used to achieve higher alpha diversity measured by

Shannon and Chao1 indexes. Breed variation can be further investigated to improve fitness traits in goat production in South Africa.

- With all the progress in goat genomics to unlock genetic potential that affects goat production in South Africa, limited information still exists to establish the relationship between the goat genotype with its microbiome.

6.4 References

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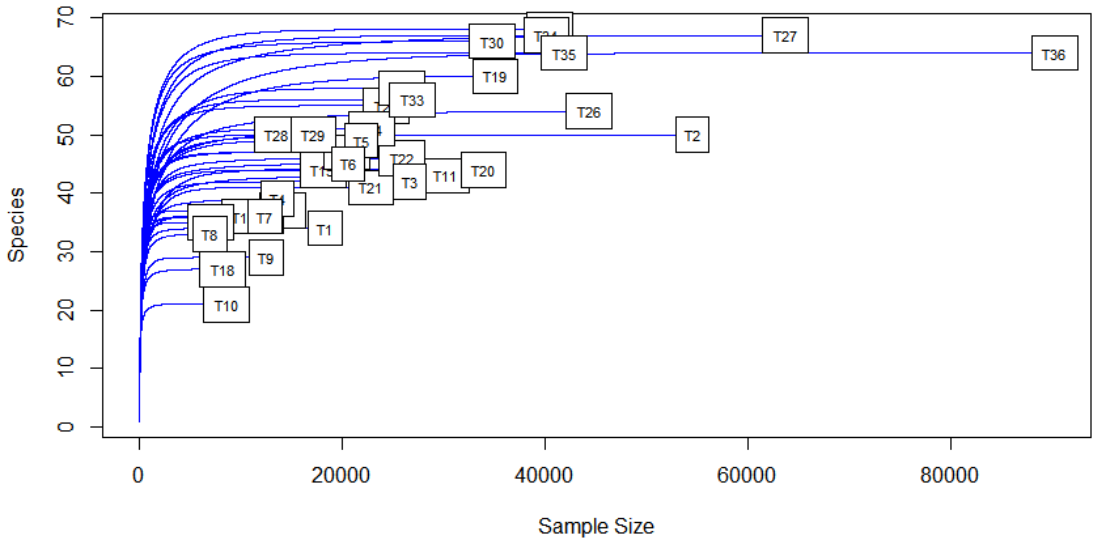
List of Appendices



Appendix 1 (Supplementary Figure 1) Rarefaction curves for rumen microbial communities for each sample, showing species accumulation in the goats. T1-T18 indicates samples collected at the beginning of the trial (day 1) and T19-T36 were collected at the end of the trial (day 30).

Appendix 2 (Supplementary Table 1) P-values of top KEGG pathways for GWAS data: each pathway with a P-value <0.005. Q-value represents adjusted P-value. Pathways were predicted using KEGG 2021 and BioPlanet 2021 (www.enrichr.org).

Pathways	p-value	q-value
Ghrelin-mediated regulation of food intake and energy homeostasis	4.14E-15	1.58E-12
Insulin-like growth factor (IGF) activity regulation by insulin-like growth factor binding proteins (IGFBPs)	1.02E-11	1.94E-09
Growth hormone receptor signaling	4.69E-09	5.96E-07
Adipogenesis	1.00E-08	9.54E-07
Diabetes pathways	1.26E-08	9.63E-07
Myometrial relaxation and contraction pathways	3.33E-08	2.11E-06

Renin-angiotensin system	2.20E-07	1.20E-05
MicroRNAs in cardiomyocyte hypertrophy	3.03E-07	1.44E-05
IGF-1 receptor and longevity	3.56E-07	1.51E-05
Fibroblast growth factor 1	1.35E-06	5.15E-05
Insulin signaling pathway	2.77E-06	9.59E-05
Jak-STAT signaling pathway	3.34E-06	0.000106
Prolactin receptor signaling pathway	1.38E-05	0.000405
Ghrelin biosynthesis, secretion, and deacylation	1.70E-05	0.000462
Neuroactive ligand-receptor interaction	2.55E-05	0.000649
Endochondral ossification	5.92E-05	0.00141
AMPK signaling	6.66E-05	0.001447
IGF1 receptor signaling through beta-arrestin	6.83E-05	0.001447
Progesterone-mediated oocyte maturation	0.000166311	0.003335
BDNF signaling pathway	0.000185536	0.003449
Integrated cancer pathway	0.00019011	0.003449
Immune system signaling by interferons, interleukins, prolactin, and growth hormones	0.000270881	0.004504
Disease	0.000279216	0.004504
Nuclear signaling by ErbB4	0.000283727	0.004504
Gene expression regulation by hypoxia-inducible factor	0.000357673	0.005451
Cdc25 and Chk1 regulatory pathway in response to DNA damage	0.000446169	0.006538
Reversal of insulin resistance by leptin	0.000544193	0.007486
Type 2 diabetes mellitus	0.000550176	0.007486
Signaling events mediated by PTP1B	0.000617533	0.008113
Angiotensin-converting enzyme 2 regulation of heart function	0.000768587	0.009761
Complement cascade regulation	0.000894836	0.010998
Leptin signaling pathway	0.000985265	0.011731
ATR activation in response to replication stress	0.001030376	0.011896
Rb tumor suppressor/checkpoint signaling in response to DNA damage	0.001175147	0.012971
Immune system	0.001191601	0.012971
HIF-1 transcriptional activity in hypoxia	0.001238565	0.013108
Adipocytokine signaling pathway	0.001293678	0.013321
ACE inhibitor pathway	0.00132909	0.013326
Long-term depression	0.001468213	0.014343
FSH regulation of apoptosis	0.001533583	0.014607
Cytokine-cytokine receptor interaction	0.001585493	0.014733
Prolactin activation of MAPK signaling	0.00179067	0.016244
Skeletal muscle hypertrophy is regulated via AKT/mTOR pathway	0.001845372	0.016351
Transcriptional regulation of white adipocyte differentiation	0.001931047	0.016721
IGF1 signaling pathway	0.002035424	0.017233
TGF-beta regulation of extracellular matrix	0.002134534	0.01768
AKT signaling pathway	0.002234359	0.018113
Signaling events mediated by PRL	0.002442121	0.019384

Calcium signaling pathway	0.002543004	0.019474
Transmembrane transport of small molecules	0.00255564	0.019474
Regulatory RNA pathways	0.002883897	0.02113
Cellular response to hypoxia	0.002883897	0.02113
Interleukin-9 regulation of target genes	0.003117799	0.022074
Signaling by ERBB4	0.003303522	0.022074
RORA activates circadian expression	0.003360302	0.022074
BAD phosphorylation regulation	0.003360302	0.022074
Cell cycle: G2/M checkpoint	0.003360302	0.022074
eIF4E and p70 S6 kinase regulation	0.003360302	0.022074
Skeletal myogenesis control by HDAC and calcium/calmodulin-dependent kinase (CaMK)	0.003870889	0.02458
Sema4D in semaphorin signaling	0.003870889	0.02458
Senescence and autophagy	0.003939083	0.024603
Dilated cardiomyopathy	0.004051725	0.024899
Ovarian infertility genes	0.004138862	0.02503
HIF-2-alpha transcription factor network	0.005294007	0.031031
EPO receptor signaling	0.005294007	0.031031
Lipid metabolism regulation by peroxisome proliferator-activated receptor alpha (PPAR-alpha)	0.005558033	0.031918
Oocyte meiosis	0.005696722	0.031918
mTOR signaling pathway	0.005696722	0.031918
p53 activity regulation	0.00642132	0.03495
Prolactin regulation of apoptosis	0.00642132	0.03495
Trefoil factor initiation of mucosal healing	0.006579762	0.034957
Focal adhesion	0.006606062	0.034957
Hexose transport	0.007992716	0.041715
G2/M checkpoints	0.008365436	0.043071
SLC-mediated transmembrane transport	0.008541547	0.043391
HNF3B pathway	0.009133891	0.04579
Delta Np63 pathway	0.009932691	0.049087
p53 signaling pathway	0.010049321	0.049087
ATM pathway	0.010343343	0.04926
HES/HEY pathway	0.010343343	0.04926
Developmental biology	0.011030261	0.051883
Integrated breast cancer pathway	0.012785693	0.059407
RAGE pathway	0.015836832	0.071267
Cyclin B2-mediated events	0.015899373	0.071267
Chk1/Chk2(Cds1)-mediated inactivation of cyclin B-Cdk1 complex	0.015899373	0.071267
Circadian rhythm	0.01685052	0.074652
Oncostatin M	0.017557783	0.076891
Ubiquitin-mediated degradation of phosphorylated Cdc25A	0.017891216	0.077167
Fatty acid, triacylglycerol, and ketone body metabolism	0.018025932	0.077167
Glioma	0.018421575	0.077985
Semaphorin interactions	0.018958545	0.078041
Activation of C3 and C5	0.019049269	0.078041
Vasopressin-like receptors	0.019049269	0.078041

Pathways in cancer	0.020278423	0.082192
NFAT involvement in hypertrophy of the heart	0.020608664	0.08231
MicroRNA regulation of DNA damage response	0.02117162	0.08231
Renal cell carcinoma	0.02117162	0.08231
Complement and coagulation cascades	0.02117162	0.08231
Melanoma	0.021740958	0.083359
SARS coronavirus protease	0.02218924	0.083359
SLC26 family multifunctional anion exchangers	0.02218924	0.083359
Leishmaniasis	0.022316633	0.083359
Integrins in angiogenesis	0.023486813	0.086661
Peptide G-protein coupled receptors	0.023655401	0.086661
Glycolysis and gluconeogenesis	0.02408123	0.08738
Complement cascade	0.025288492	0.088501
Mitochondrial pathway of apoptosis: caspases	0.025288492	0.088501
SREBP control of lipid biosynthesis	0.025319318	0.088501
Import of palmitoyl-CoA into the mitochondrial matrix	0.025319318	0.088501
Apoptosis regulation	0.02590125	0.089049
G alpha (i) signaling events	0.025943489	0.089049
G2/M DNA damage checkpoint	0.028439533	0.094221
Prostanoid ligand receptors	0.028439533	0.094221
Sodium/calcium exchangers	0.028439533	0.094221
Elevation of cytosolic calcium levels	0.028439533	0.094221
Response to elevated platelet cytosolic calcium	0.029054566	0.095429
Activation of Src by protein tyrosine phosphatase alpha	0.031549916	0.101869
E2F-enabled inhibition of pre-replication complex formation	0.031549916	0.101869
HIF-1 degradation in normoxia	0.032353156	0.103584
Prostate cancer	0.033029842	0.10487
Alternative complement pathway	0.034650497	0.106466
Sonic Hedgehog (SHH) receptor PTCH1 regulation of cell cycle	0.034650497	0.106466
E2F1 destruction pathway	0.034650497	0.106466
Erythropoietin-mediated neuroprotection through NF-kB	0.034650497	0.106466
Actin cytoskeleton regulation	0.035844505	0.109254
Transport of inorganic cations/anions and amino acids/oligopeptides	0.037205248	0.111469
Pyruvate dehydrogenase (PDH) complex regulation	0.037741306	0.111469
Opening of calcium channels triggered by depolarization of the presynaptic terminal	0.037741306	0.111469
Facilitative sodium-independent glucose transporters	0.037741306	0.111469
Interferon-gamma signaling pathway	0.038639993	0.11238
TSH regulation of gene expression	0.038639993	0.11238
Adenylate cyclase inhibitory pathway	0.040822374	0.116968
Integrin-mediated cell adhesion	0.040831266	0.116968
GPCR ligand binding	0.04225856	0.119454
Apoptosis	0.04250809	0.119454
Signal transduction	0.043787648	0.119454
Chagas disease	0.043824397	0.119454

Interferon gamma signaling regulation	0.043893731	0.119454
Interleukin-6 regulation of target genes	0.043893731	0.119454
Lectin-induced complement pathway	0.043893731	0.119454
G alpha i pathway	0.04689689	0.123379
Erythrocyte differentiation pathway	0.046955406	0.123379
Validated nuclear estrogen receptor beta network	0.046955406	0.123379
Eicosanoid ligand-binding G-protein coupled receptors	0.046955406	0.123379
Cyclin A/B1-associated events during G2/M transition	0.046955406	0.123379

Appendix 3 GLM outputs

The SAS System

The GLM Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	5	12345
Breed	2	1 2
Gender	2	1 2
Time	2	1 2

Number of Observations Read	64
Number of Observations Used	64

The GLM Procedure

Dependent Variable: Bodyweight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	361.992528	51.713218	3.50	0.0035
Error	56	826.937472	14.766741		
Corrected Total	63	1188.930000			

R-Square	Coeff Var	Root MSE	Bodyweight Mean
0.304469	22.65439	3.842752	16.96250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	42.1914583	10.5478646	0.71	0.5857
Breed	1	40.6406250	40.6406250	2.75	0.1027
Gender	1	170.4798200	170.4798200	11.54	0.0013
Time	1	108.6806250	108.6806250	7.36	0.0088

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	48.3867287	12.0966822	0.82	0.5184
Breed	1	51.6523422	51.6523422	3.50	0.0667
Gender	1	170.4798200	170.4798200	11.54	0.0013
Time	1	108.6806250	108.6806250	7.36	0.0088

Dependent Variable: Weight gain

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	234.0356876	33.4336697	5.65	<.0001
Error	56	331.2818124	5.9157467		
Corrected Total	63	565.3175000			

R-Square	Coeff Var	Root MSE	Weight gain Mean
0.413990	93.32300	2.432231	2.606250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	194.1833333	48.5458333	8.21	<.0001
Breed	1	39.6900000	39.6900000	6.71	0.0122
Gender	1	0.1623543	0.1623543	0.03	0.8690
Time	1	0.0000000	0.0000000	0.00	1.0000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	194.2842604	48.5710651	8.21	<.0001
Breed	1	39.2122833	39.2122833	6.63	0.0127
Gender	1	0.1623543	0.1623543	0.03	0.8690
Time	1	0.0000000	0.0000000	0.00	1.0000

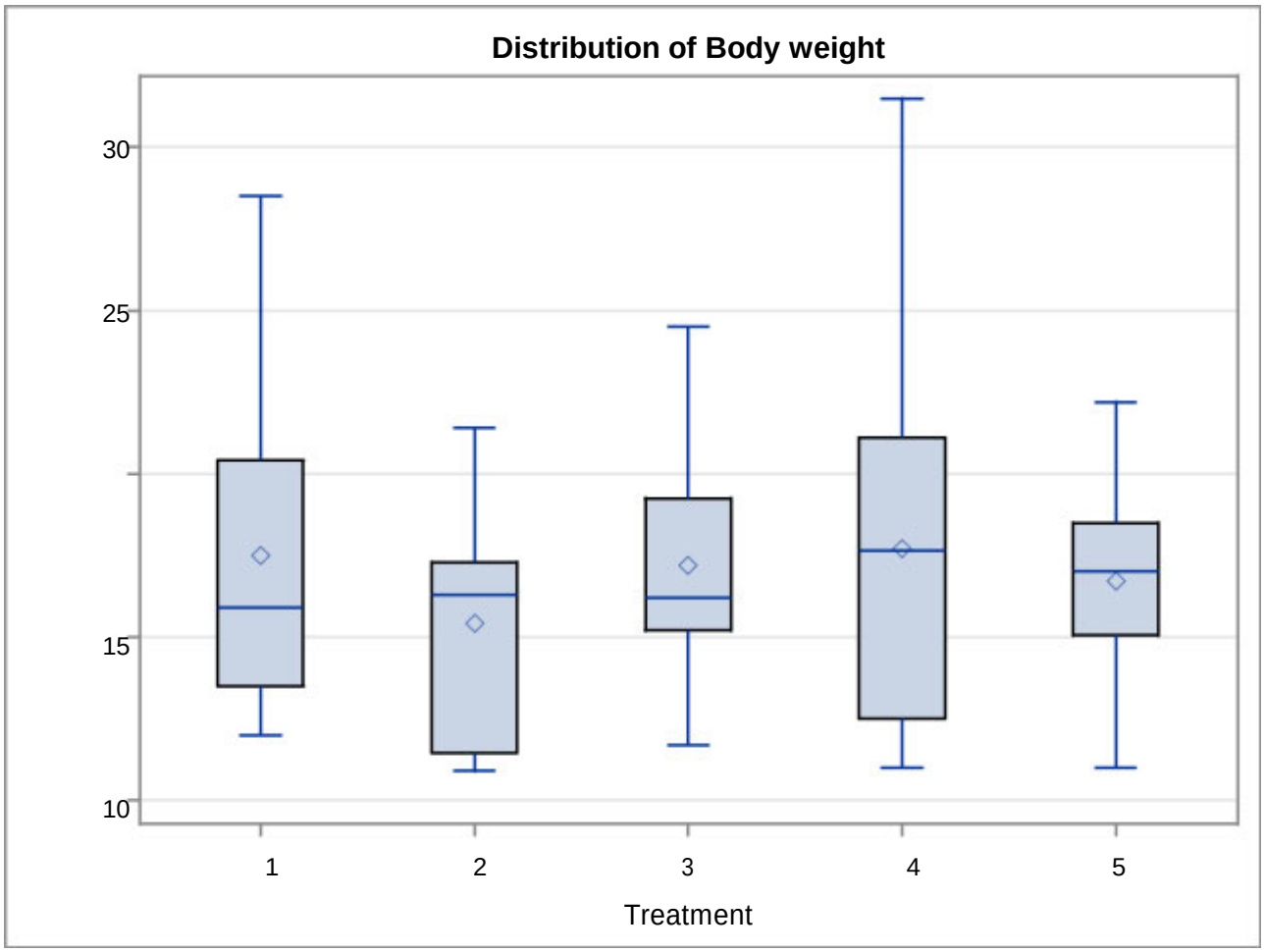
Dependent Variable:pH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	12.13194131	1.73313447	14.44	<.0001
Error	56	6.71983369	0.11999703		
Corrected Total	63	18.85177500			

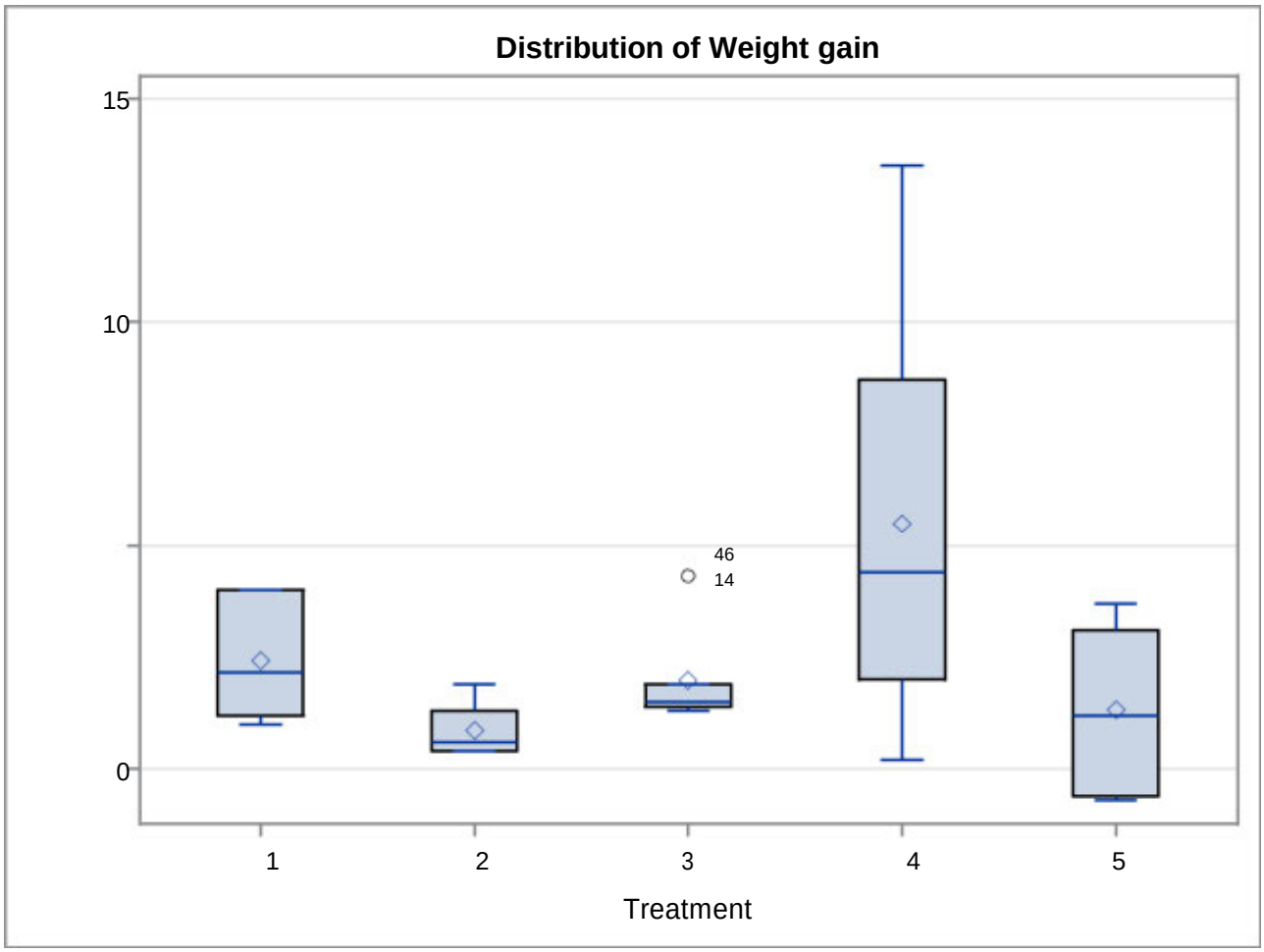
R-Square	Coeff Var	Root MSE	Ph Mean
0.643544	5.159168	0.346406	6.714375

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	1.75052500	0.43763125	3.65	0.0104
Breed	1	0.41602500	0.41602500	3.47	0.0679
Gender	1	0.46358506	0.46358506	3.86	0.0543
Time	1	9.50180625	9.50180625	79.18	<.0001

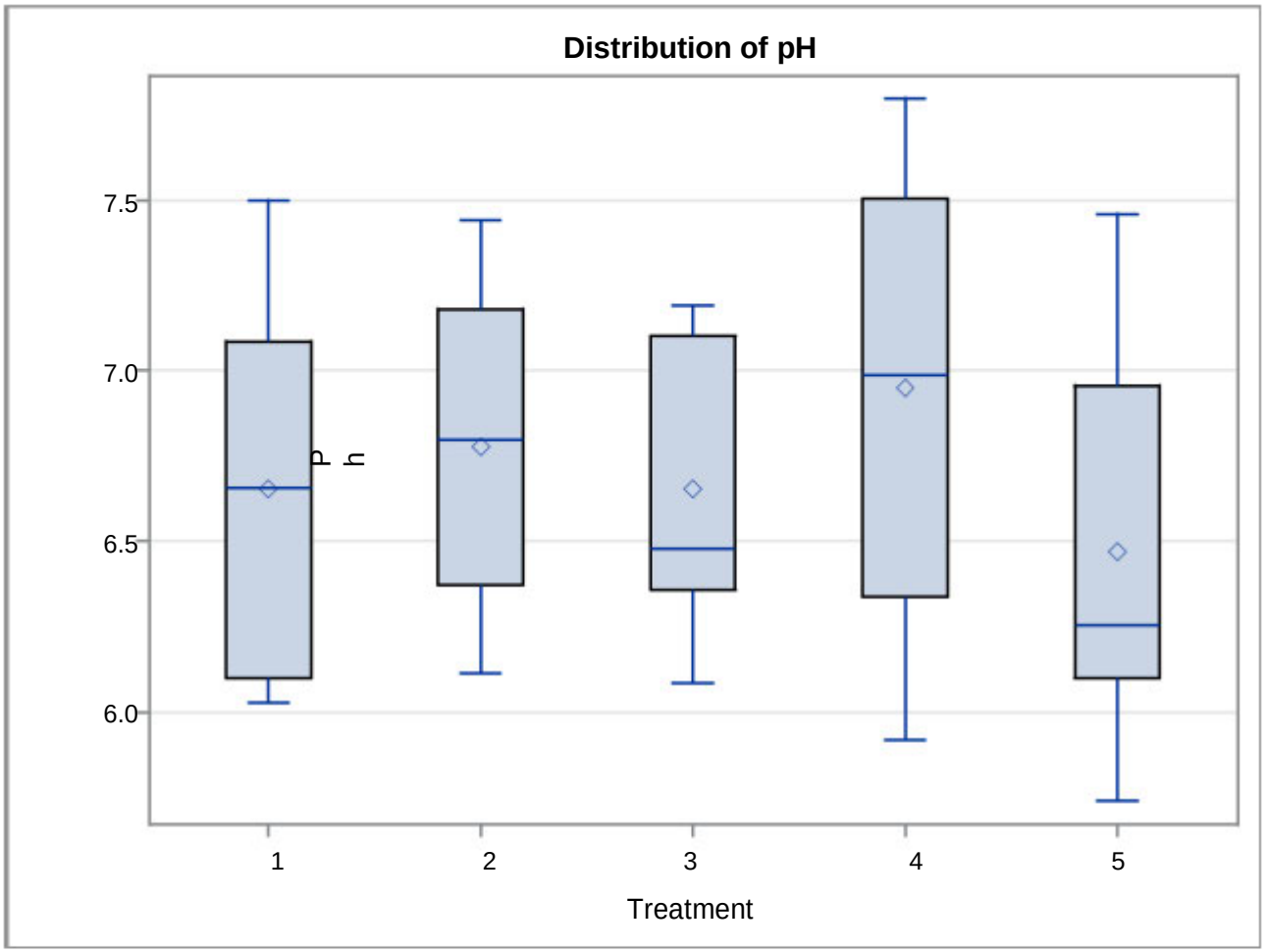
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	1.91130336	0.47782584	3.98	0.0065
Breed	1	0.47158009	0.47158009	3.93	0.0523
Gender	1	0.46358506	0.46358506	3.86	0.0543
Time	1	9.50180625	9.50180625	79.18	<.0001


Distribution of Body weight
30
25
15
10
1
2
3
4
5
Treatment

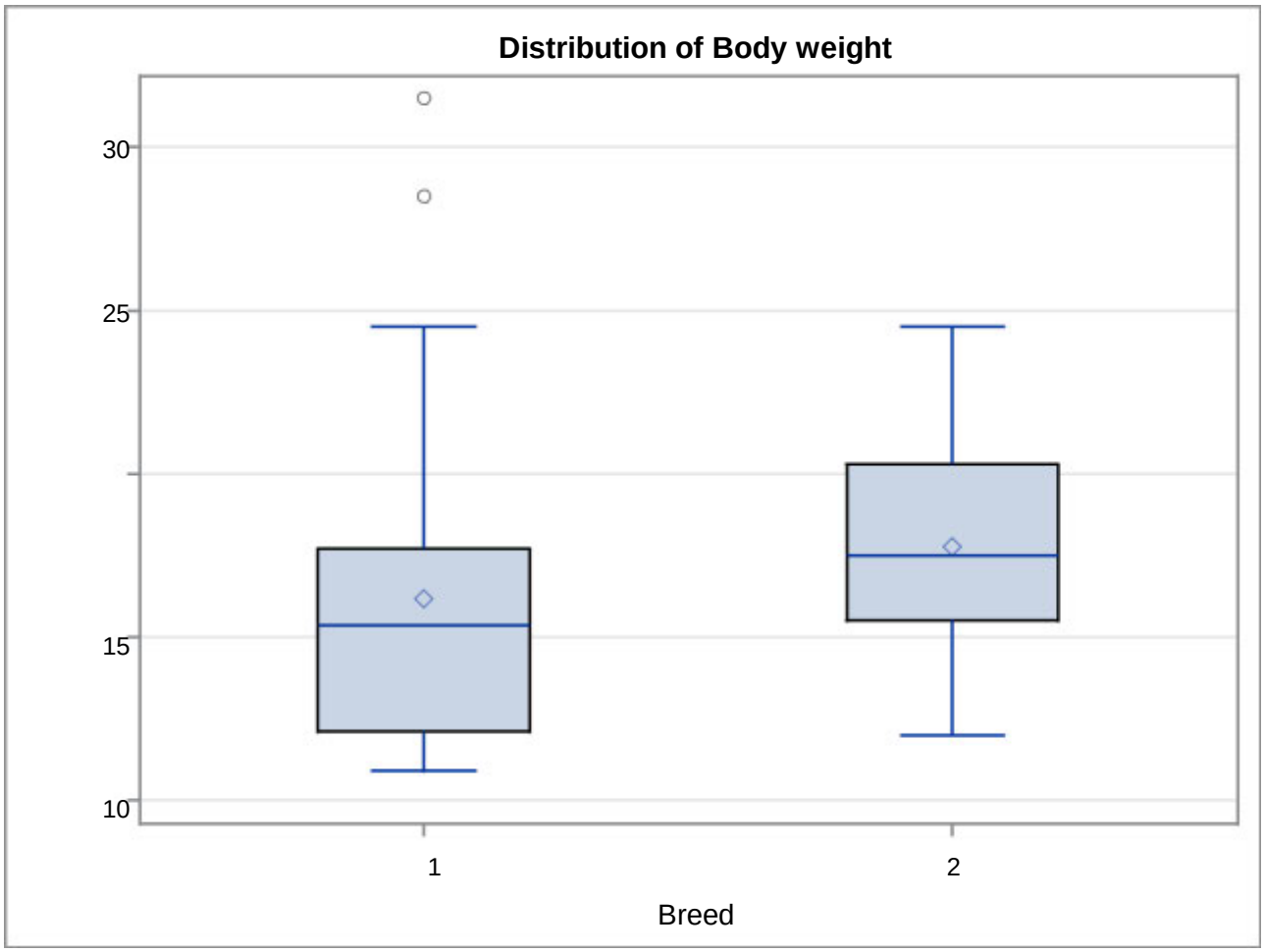
The GLM Procedure

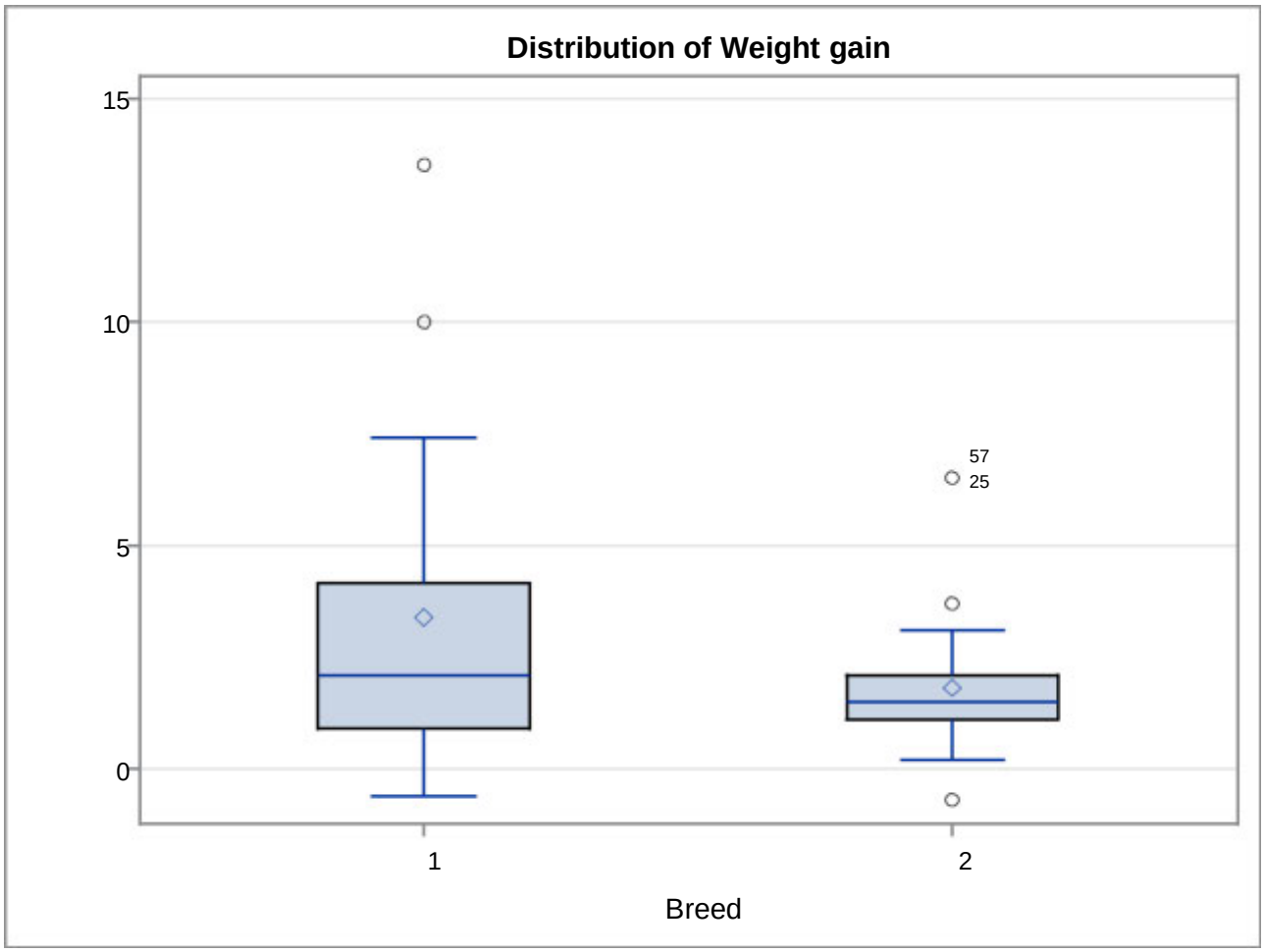


The GLM Procedure

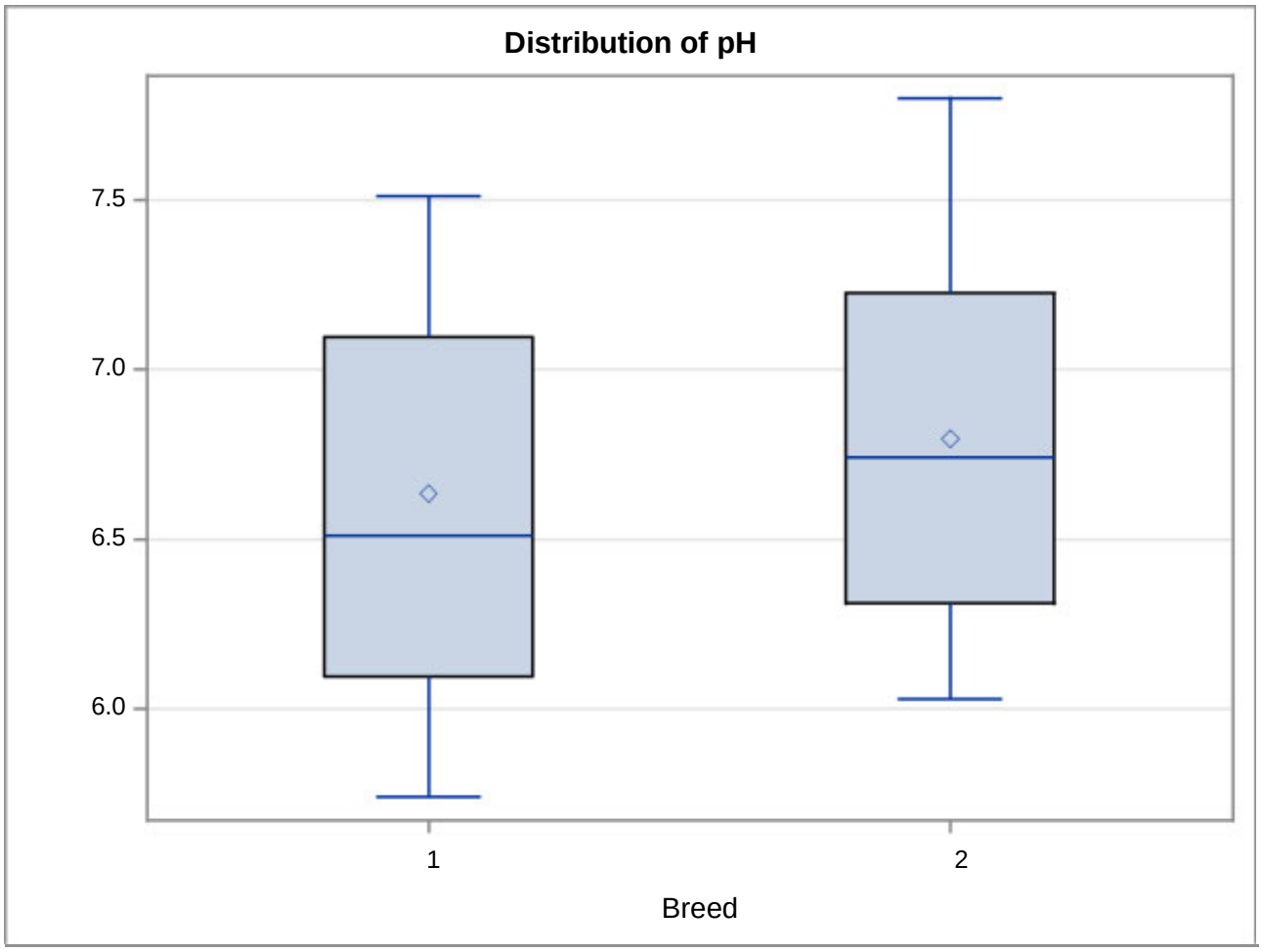


Level of Treatment	N	Body weight		Weight gain		pH	
		Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1	12	17.5083333	5.08052580	2.41666667	1.25685996	6.65166667	0.52339509
2	12	15.4333333	3.49917739	0.86666667	0.57735027	6.77666667	0.46388348
3	12	17.1916667	3.73690643	1.98333333	1.09944890	6.65250000	0.41149671
4	16	17.7187500	5.54198746	5.48750000	4.49842565	6.94750000	0.65613515
5	12	16.7083333	3.21656461	1.31666667	1.73877351	6.46583333	0.55599965


Distribution of Body weight
30
25
15
10
1
2
Breed

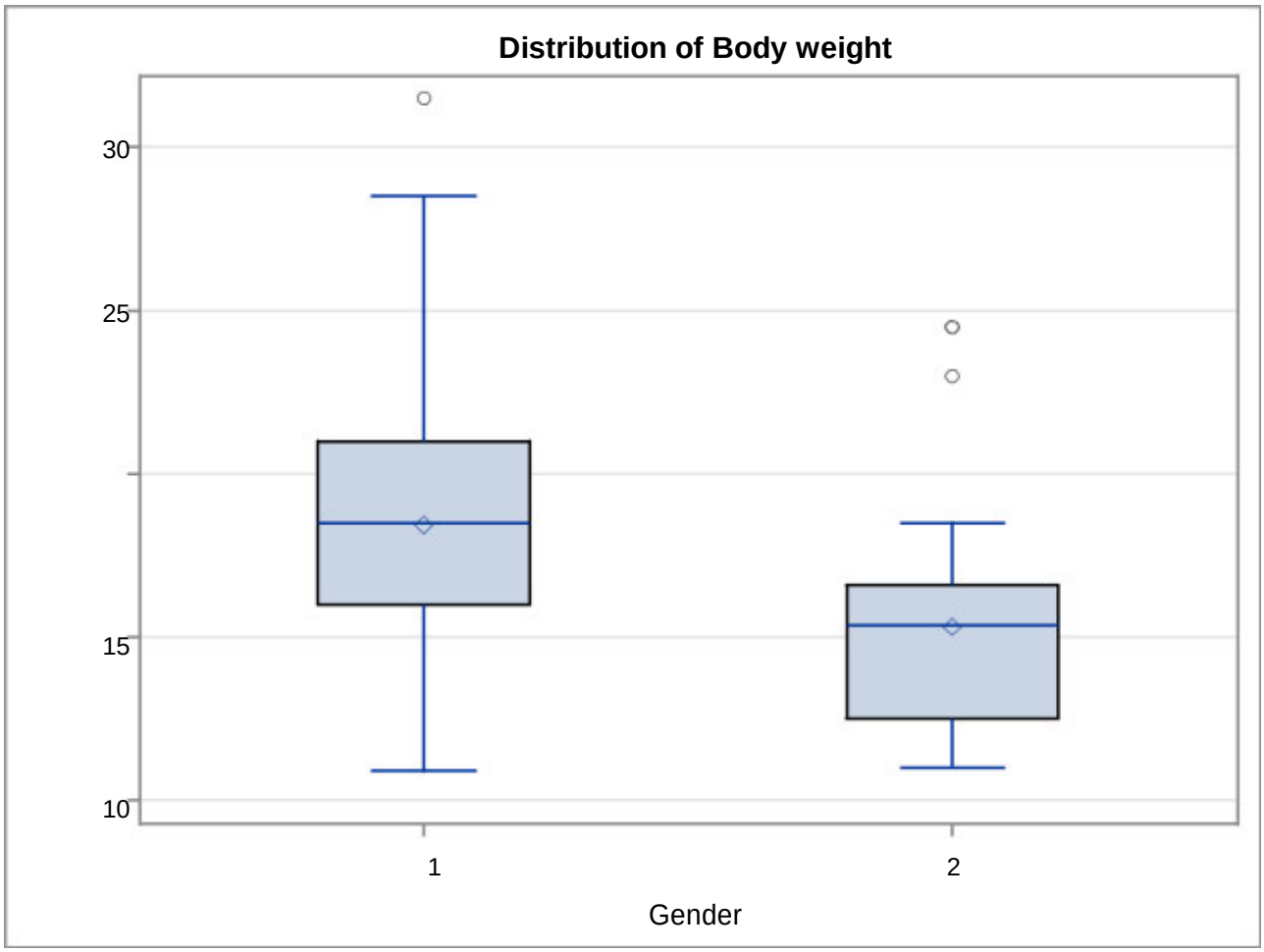

Distribution of Weight gain
15
10
5
0
57
25
1
2
Breed

The GLM Procedure

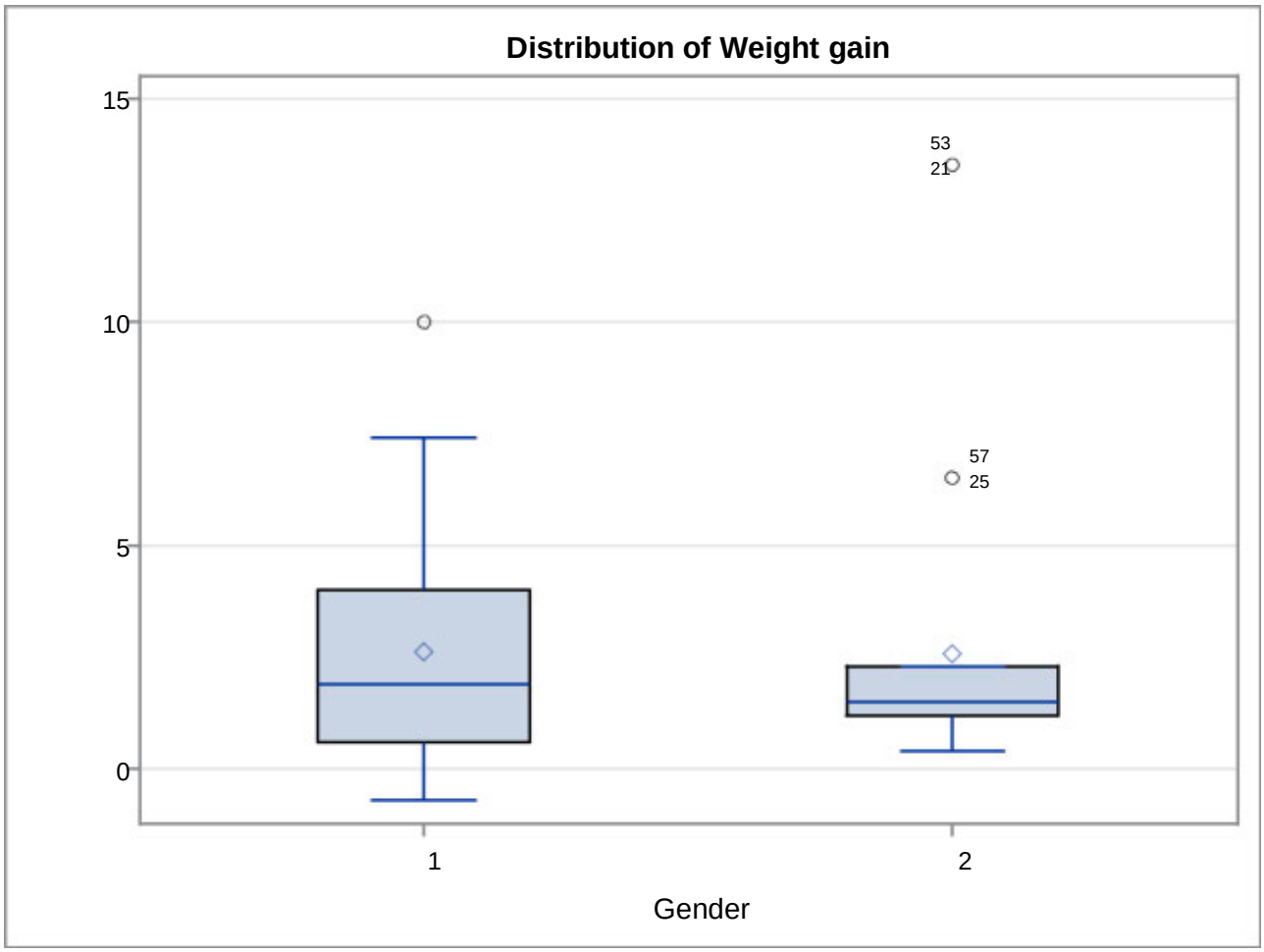


Level of Breed	N	Body weight		Weight gain		pH	
		Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1	32	16.1656250	5.17747039	3.39375000	3.79549287	6.63375000	0.53792762
2	32	17.7593750	3.19928010	1.81875000	1.59685932	6.79500000	0.55257170

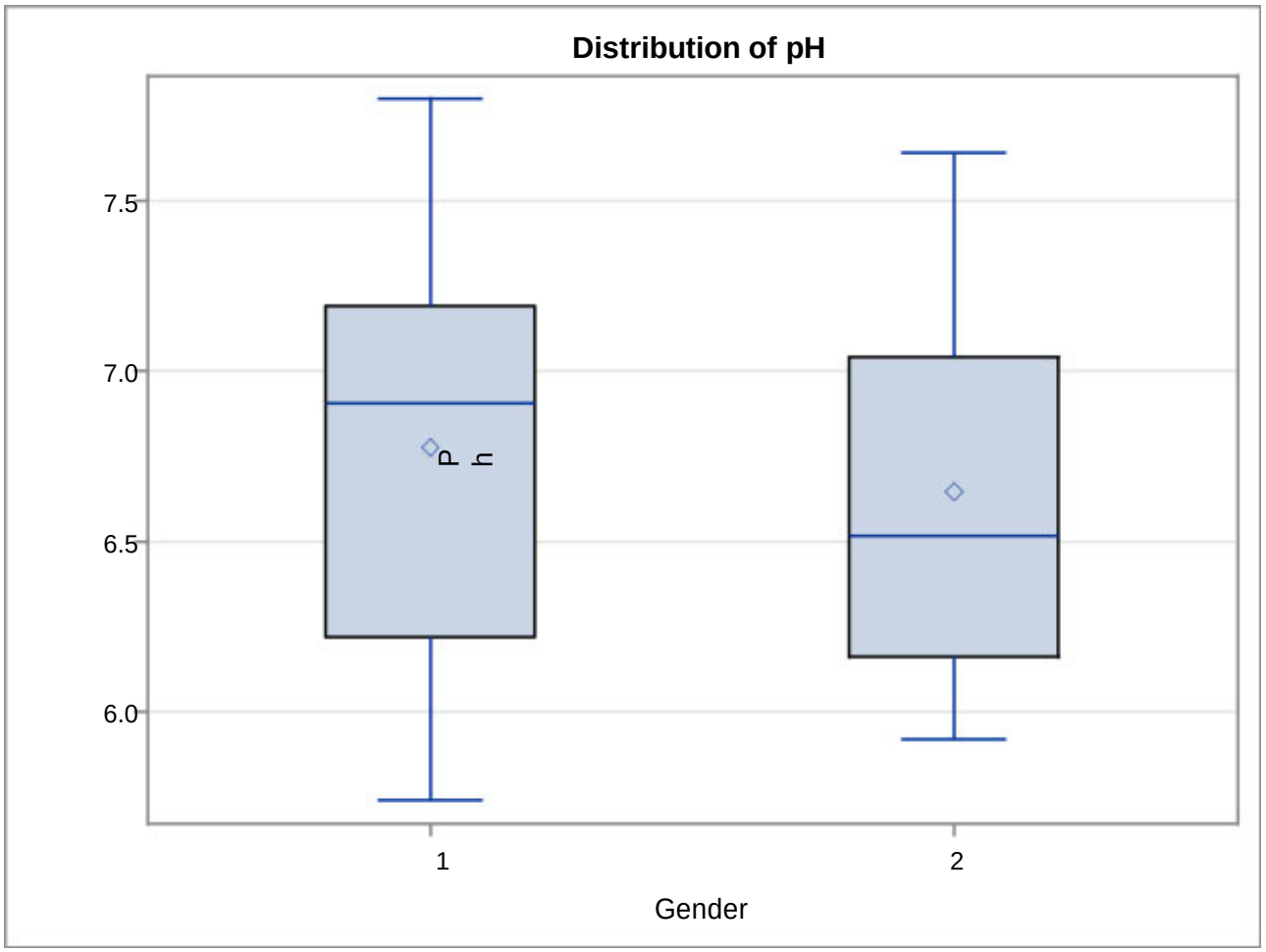
The GLM Procedure



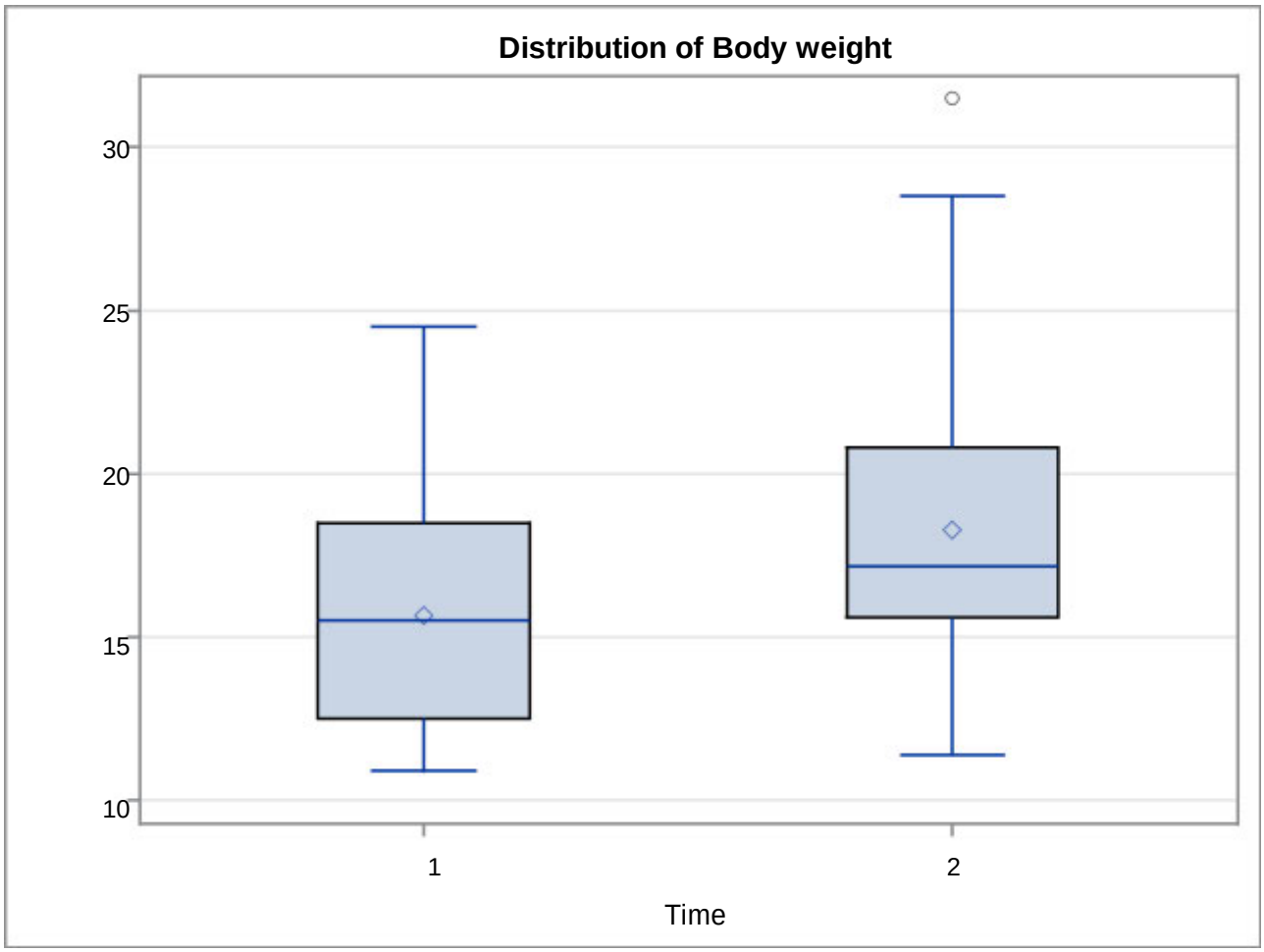
The GLM Procedure

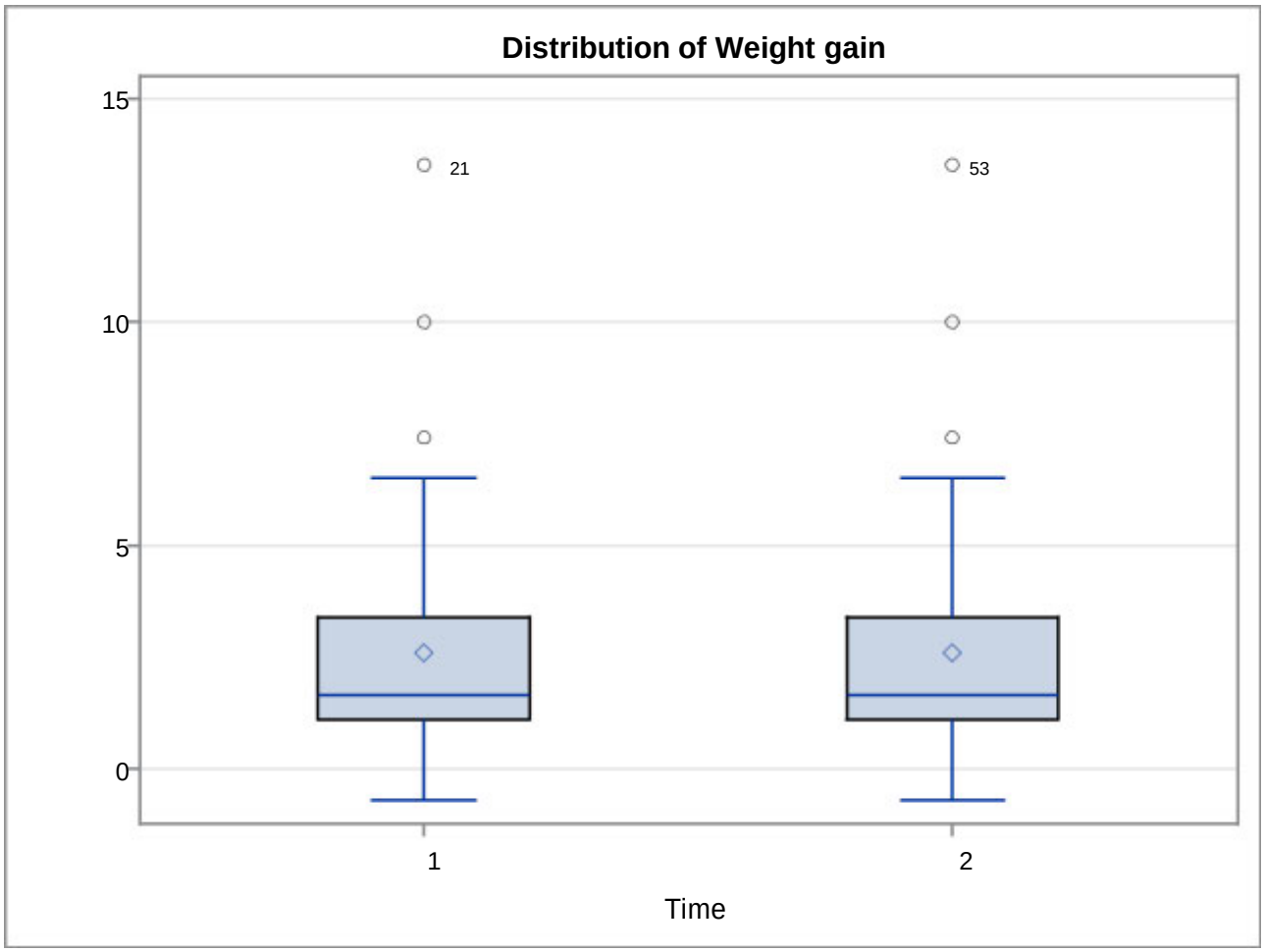


The GLM Procedure

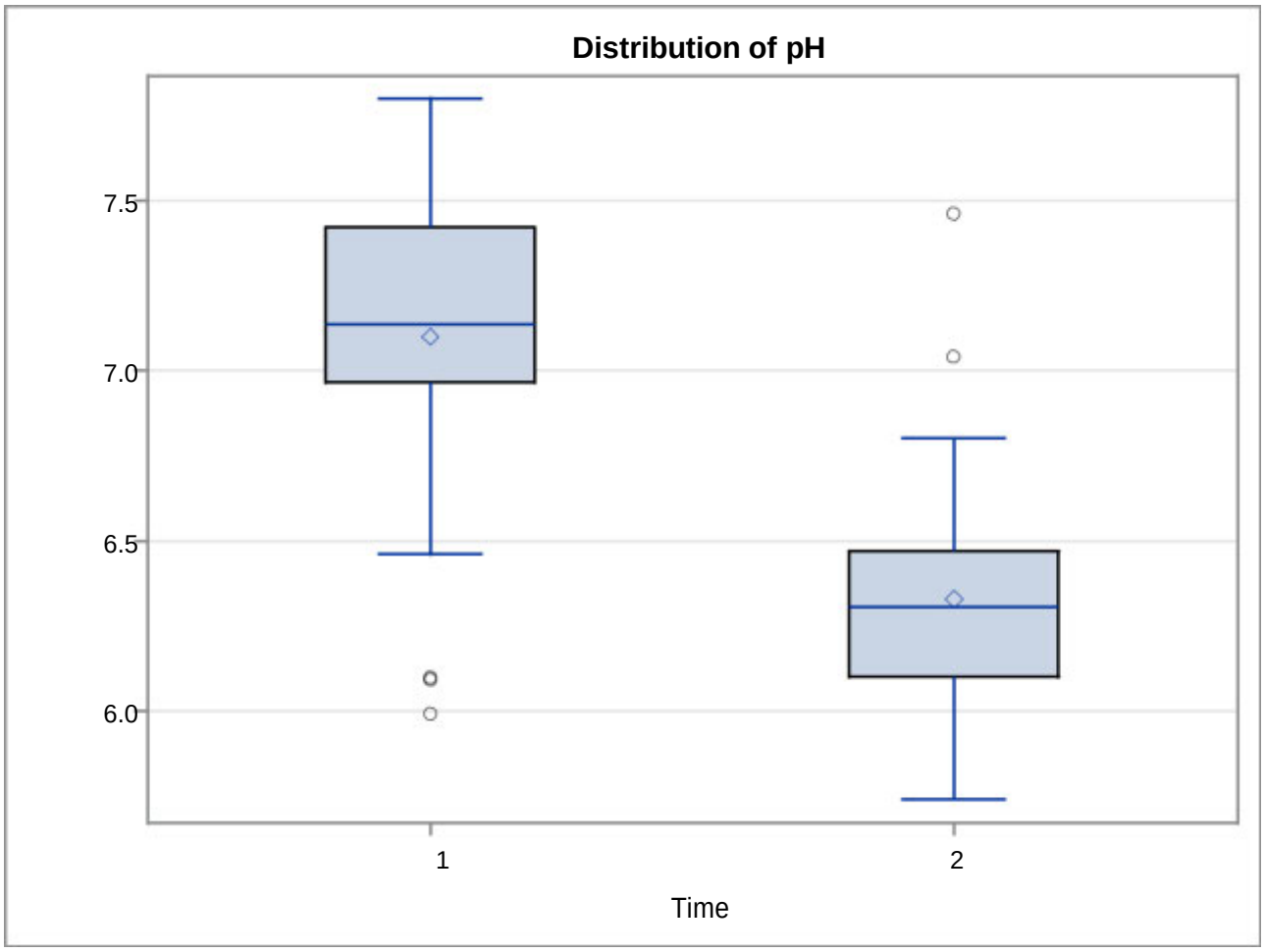


Level of Gender	N	Body weight		Weight gain		pH	
		Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1	34	18.4176471	4.46375780	2.62352941	2.77489381	6.77411765	0.57353300
2	30	15.3133333	3.60944483	2.58666667	3.27579770	6.64666667	0.51654912


Distribution of Body weight
30
25
20
15
10
1
2
Time

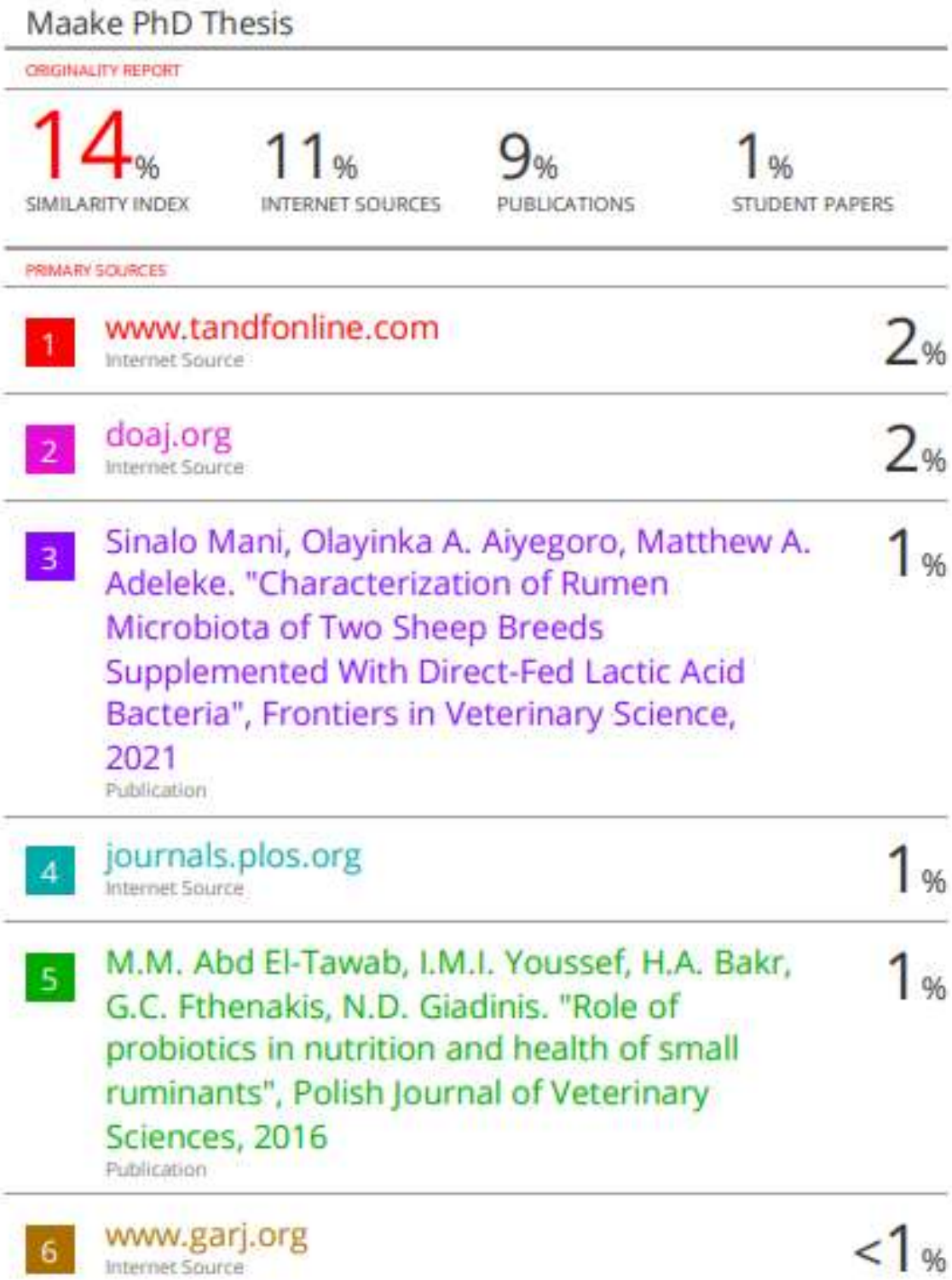

Distribution of Weight gain
15
10
5
0
21
53
1
2
Time

The GLM Procedure



Level of Time	N	Body weight		Weight gain		pH	
		Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1	32	15.6593750	3.75807598	2.60625000	3.01960663	7.09968750	0.43835090
2	32	18.2656250	4.55232017	2.60625000	3.01960663	6.32906250	0.33084798

Appendix 4: Turnitin Report



Maake PhD Thesis

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Appendix 5: Ethical Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 June 2018

Ms Takalani Whitney Maake (217082173)
School of Life Sciences
Westville Campus

Dear Ms Maake,

Protocol reference number: AREC/052/018D
Project title: Effect of lactic acid bacteria (LAB) fed as putative probiotics and genetic characterization of rumen microbiome in South African goats

Full Approval – Research Application

With regards to your application received on 08 June 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 11 June 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee