DIVERSITY OF THE GUT MICROBIOME OF CHICKEN FED WITH BLACK SOLDIER FLY LARVAE -BASED FEEDS

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Diversity of the Gut Microbiome of Chicken Fed With Black Soldier Fly Larvae -Based Feeds

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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This thesis has been submitted for examinations with our approval has the university supervisors

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DEDICATION

I dedicate this work to my parents Mr. Moses Ndotono and Mrs. Gladys Wanjuhi for their immense support throughout my study, and my siblings, Charity Ndotono and Grace Ndotono, for always cheering me on and supporting me the best they can. Thank you all so much for the love and kindness you have shown me, the financial and moral support, and for always encouraging me to scale to greater heights. God bless you all so much and I love you all.

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ABBREVIATIONS AND ACRONYMS

AMR	Antimicrobial Resistance
AMPs	Antimicrobial Peptides
ARGs	Antibiotic Resistance Genes
BSF	Black Soldier Fly
BSFL	Black Soldier Fly Larvae
CARD	Comprehensive Antibiotic Resistance Database
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
FM	Fishmeal
GIT	Gastrointestinal tract
KEBS	Kenya Bureau of Standards
LAB	Lactic Acid Bacteria
OTU	Operational Taxonomic Unit
ONT	Oxford Nanopore Technologies
PCR	Polymerase Chain Reaction
PCoA	Principal Coordinate Analysis
rDNA	Ribosomal DNA
SCFAs	Short Chain Fatty Acids

ABSTRACT

Industrial rearing of insects, especially the black soldier fly, is gaining momentum in recent years because of the increase in food and feed insecurity, high prices of animal feeds and animal proteins, and population growth. This in turn has led to increased global demand for alternative sources of protein apart from traditional livestock products. This study focused on evaluating the gut microbial community dynamics of both the layer and broiler chickens that have been fed on BSF larvae-based diet. The bacterial communities were characterized using high throughput Oxford nanopore sequencing of the full-length bacterial 16S rRNA gene and downstream analysis was done using the QIIME2 pipeline and R software. The layer pullets were allotted 5 dietary treatments that were formulated as follows: control diet (T1): 100% FM + 0% BSFL, T2: 25% BSFL + 75% FM; T3: 50% BSFL + 50% FM; T4: 75% BSFL + 25% FM, and T5: 100% BSFL + 0% FM and the broiler chicken were allotted four dietary treatments, T1 (25% DI + 75% BSFL), T2 (50% DI + 50% BSFL), T3 (75% DI + 25% BSFL) and T4 (100% fishmeal + 0% DI + BSFL). From the findings, it was observed that the predominant phyla in the gut of both the layers and broilers were Firmicutes (90%), Proteobacteria (7%), and Bacteroidetes (2%). At the genus level, the abundant bacteria identified in layer pullets were the *Lactobacillus* (93%), *Enterococcus* (2%), *Bacteroides* (2%), and *Blautia* (2%) among others. In broilers, the predominant bacteria were *Enterococcus* (70%), *Lactobacillus* (25%), *and Ruminococcus* (3%). A significant increase in the abundance of the beneficial lactic acid-producing bacteria was observed in diets that had BSFL inclusion, especially the *Lactobacillus*, and *Enterococcus*. The BSFL-based feeds supported almost similar microbial communities as the conventional fishmeal with changes observed in microbial abundance and this supports the replacement of fishmeal with insect-based feeds without a negative impact on the GIT of chicken. Our findings unravel complex gut microbial shifts in chickens fed BSFL-based feeds and therefore underpins the potential roles of beneficial bacteria identified as promising prebiotics and probiotics in reshaping the gut microbiota to maintain good gut health and improve the overall health status of the birds.

CHAPTER ONE

INTRODUCTION

1.1 Background

Industrial rearing of insects has been on the rise over the years because of an increase in food and feed insecurity, high prices of animal proteins, population growth, and the global increase in demand for proteins. Food wastage and population growth have contributed largely to food insecurity issues (FAO, 2011b). Furthermore, insects have been used traditionally as food and feed especially in African countries. Moreover, there are about 1,900 species of edible insects that have been identified across the world (Huis et al., 2013).

Poultry has the potential to bridge the gap between the demand and supply of protein because of its high feed conversion rates (Shepon et al., 2016). In the tropics, a large number of smallholder farmers contribute significantly to food security by rearing poultry for domestic and local markets (Frempong et al., 2019). Chicken meat contributes to the daily dietary needs of humans as it contains protein, minerals, vitamins (niacin, riboflavin, and vitamin B6), and other nutrients (Schiavone et al., 2019).

The poultry sector is one of the fast-growing industries and Kenya's demand and consumption of chicken meat is expected to hit 165,000 metric tons by 2030, up from 55,000 metric tons in 2000 (FAO, 2011a). This projected increase in demand is based on rapid urbanization, middle-class growth, and ever-increasing global health views promoting poultry products as a better protein source than red meat (Meijer-Willems et al., 2018).

The accessibility of safe, affordable feed in terms of energy, protein, mineral, and vitamin sources is among the major constraints faced by chicken farmers in Kenya hampering this industry from attaining its full production potential (Mutisya et al., 2020). Protein is an essential key ingredient and one of the most expensive components required in poultry feed. The most common and widely preferred conventional sources of protein in animal feed include soybean, fishmeal, cotton or sunflower seed cakes, and other plant proteins (Sun et al., 2013).

However, soybean meal and fishmeal are available in low quantities due to land unavailability for production, global cost fluctuation, human consumption of soybean and fishmeal, and other constraints making them much more expensive for farmers to afford (Onsongo et al., 2018). As such, it is important to identify other more affordable, scalable, and sustainable high-quality protein sources to achieve favorable economic returns from animal feed utilization.

The black soldier fly (Diptera: stratiomyidae), *Hermetia illucens*, is a common free-living insect commonly reared for industrial purposes because of its capacity for bioremediation of organic wastes. They are popular as essential decomposers due to their ability to convert organic waste substrates into biomass containing high-quality protein (Müller et al., 2017). Since they feed on a variety of organic wastes, they could probably acquire microorganisms from these substrates (beneficial or not) which may be transferred to animals that are fed on them.(De Smet et al., 2018).

Previous studies have demonstrated that the intestinal microbiome of poultry is significantly influenced by the dietary ingredients; the nutrient levels of fat, protein, and carbohydrates; the inclusion of feed additives; and dietary supplementation using feed enzymes (Torok et al., 2011). The chicken diet is seen as the greatest determinant of the gut microbiota and any diet change including feed components and feed additives will certainly affect the composition and diversity of the gut microbiome which in turn may lead to adverse effects on productivity, feed efficiency, and overall health (Kohl, 2012). The chicken gut microbiota harbours a complex microbial community that plays vital roles in nutrient utilization and adsorption, growth and development, production of short chain fatty acids and overall health status (Oakley et al., 2014). The microbial diversity is stretched across the entire gut with the main sites being the crop and the ceca (Gabriel et al., 2007).

There is inadequate knowledge on the potential of black soldier fly larvae meal to transfer pathogenic and beneficial microbes present in the rearing substrates through feed and their impact on the gut health of poultry. Therefore, this study focused primarily on the gut microbial community and their interactions with chicken when fed on insect-based feeds through metagenomics. This information is crucial and

would provide insight on how to improve the practical adoption of safe insect-based products.

The study's working hypothesis was that the analysis of microbiota could lead to the identification of species with unique characteristics that can be isolated and exploited for more research. As such, questions arise on the overall impact of the microbial community more so, the gut microbiota of animals that feed on BSF in this case poultry. Other than playing an important role in its host by providing proteins, the BSF larvae inclusion in the chicken diet could have an improved effect on the chicken gut and overall health (Coretti et al., 2017).

1.2 Statement of the problem

The poultry farming sub-sector, which makes up a bigger portion of the livestock sector, is crucial to the Kenyan economy by being a source of food, income, raw materials, and employment to many (Magothe et al., 2012a). As the human population continues to expand, the demand for poultry products is expected to increase to about 164.6 thousand metric tonnes by the year 2030 in Kenya (Omondi, 2022). Food insecurity is becoming a major concern in developing countries and food and feed production hence must increase significantly to satisfy the increasing demands of the growing world's population (FAO, 2015). Despite poultry being an attractive enterprise, their production is constrained by expensive and poor-quality food (Wambua et al., 2022). Currently, many people have adopted the practice of industrial rearing of insects as a sustainable alternative to try and curb the issue of food insecurity with the most popular insect being *Hermetia illucens*. BSF has been widely promoted as an alternative replacement to conventional protein sources such as soybean and fishmeal, in poultry diets (S. H. Khan, 2018). Unfortunately, extensive research has been devoted to the nutritional value of the BSF and optimization of rearing conditions (Shaphan Y. Chia et al., 2020; Shaphan Yong Chia et al., 2018), thus overlooking the transfer of the pathogenic species and contaminants to animals fed on BSF larvae feeds that are found in the substrates the BSF are reared on. There is still much to be discovered about the impact of insect-based feeds especially on the gut microbiota of poultry. According to Boccazzi et al, (2017), insects for feed are usually processed with their intestinal contents that can harbor various transmissible microorganisms and

the microbiota can also be found on the exoskeleton which can come about during farming and processing. Hence, the safety of the insect diet is a crucial aspect to ensure the safe practical adoption of insect-based feeds as a protein source for animals. While some studies have shown the short-term benefits of insect-based feeds on gut health (A. Józefiak et al., 2019; Marono et al., 2017), the long-term effects are not well understood. Research is needed to evaluate the potential risks of long-term feeding of insects on gut health, including the impact on the development of antibiotic resistance and the potential for dysbiosis. Moreover, further research is needed to determine how insect-based feeds affect the gut microbiota and the mechanisms of action behind any observed effects. The present study aimed at identifying and characterizing the gut microbial dynamics of layer and broiler chickens that have been fed with black soldier fly larvae meal as a dietary protein source, to better understand the impact of this alternative protein source on the gut microbiota of poultry.

1.3 Justification of the study

There is increasing interest in using insect-based feeds, such as black soldier fly larvae meal, as a sustainable and cost-effective alternative protein source in poultry diets. However, the impact of such feeds on the gut microbiota of poultry is not well understood. The gut microbiota plays a critical role in nutrient metabolism, immune function, and overall health of the host, and changes in gut microbiota composition have been linked to various health issues in poultry, including poor performance and susceptibility to diseases.

Therefore, investigating the impact of black soldier fly larvae meal on the gut microbiota of chickens is important for understanding the potential benefits and risks associated with this alternative protein source. Identifying and comparing bacterial communities in chickens fed with black soldier fly larvae meal and those fed with traditional protein sources can provide valuable insights into the potential effects of this alternative feed on the gut microbiota. This information can inform the development of optimized insect-based feed formulations and ultimately contribute to the development of more sustainable and healthy poultry production systems. It is also hoped that insect-based feeds will provide research opportunities to study microbiota-host interactions and help to identify novel genes that can be exploited in

biotechnology applications. Industrial rearing BSF will also increase employment opportunities and increase cash income for smallholder farmers in Kenya.

1.4 Hypothesis

The microbial community of chickens fed with BSF larvae-based feeds plays a key role in the gut health and overall health status of the chicken.

1.5 Objectives

1.5.1 General objective

To identify and compare bacterial diversities in the gut of layer and broiler chicken fed on black soldier fly larvae - based feeds in Kenya.

1.5.2 Specific objectives

1. To identify, characterize and compare bacterial community dynamics from the layer chicken gut microbiome fed on BSF-based diets at different inclusion levels.
2. To identify and characterize microbial communities from the broiler chicken gut fed BSF-Desmodium-based diets at different inclusion levels.
3. To identify the potential beneficial or pathogenic bacterial communities from host chicken gut fed with insect- based feeds.
4. To identify possible antibiotic resistance genes in layer chicken samples using AMR-WIMP workflow on the EPI2ME software.

CHAPTER TWO

LITERATURE REVIEW

2.1 Background information

Entomophagy is a Greek term that describes the practice of eating insects either by humans or animals. Insects have remained a popular food for centuries with entomophagy being practiced in various regions of the world including Central and Southern America, Africa, Asia, New Zealand and other areas. About 1900 species of insects have been identified as food for both humans and animals (Huis et al., 2013).

Insects have proved to be a good source of food and feed and over the years and insects for food and feed have gained momentum due to various issues arising such as increase in food and feed insecurity, rising costs of animal feeds and animal proteins, and drastic population growth (FAO, 2015). Increased population growth will lead to an increased demand for animal proteins and other alternative solutions to feed sources need to be implemented (Fasolato et al., 2018). Not only is the pressure on assuring food security increased, but so is the need to reduce excessive production of organic waste (Parfitt et al., 2010). Insects in general have been playing an important role in pollination during plant reproduction and they also improve the soil fertility through bioconversion (Scudder, 2007). They are also important to humans such as bees that produce honey and silkworms that produce silk (Scudder, 2007). The most consumed insects identified globally include: caterpillars e.g. mealworms, grasshoppers and crickets, termites, black soldier flies and others (Schlüter et al., 2017). While many of the reported species have the potential to be used as food and feed, others are preferred for feed production rather than for human consumption such as the black soldier flies (Van et al., 2013).

2.2 Life cycle and morphology of *Hermetia illucens*

Black soldier fly, *H. illucens*, is found in the phylum Arthropoda within the order Diptera. Diptera is a group of insect flies that have a pair of functional wings and a pair of vestigial hind wings. It is also found in the Stratiomyidae family alongside other species such as *stratiomys*, *odontomyia*, *oxycera* etc. They have similar developmental stages.

BSF life cycle takes about 45 days to complete metamorphism. The females lay about 500 eggs in cracks and crevices near or in decaying matter such as manure or organic substrates (Diclaro & Kaufman, 2012) which then hatch into larvae after about 4 days and appear pale yellow or creamy white in colour. The larvae pass through six instars taking about 18 days to completely develop (**Figure 2.1**). Fully developed larvae are 27mm in length, about 6 mm in width and have a dull whitish appearance in colour with a small projecting head (Lanka, 2018). During the larval stages, the larvae are insatiable feeders until they pupate where they stop feeding (Y.S. Wang & Shelomi, 2017). Pupation takes about 14 days and the exoskeleton darkens in colour. The adults live for about 5-8 days feeding on fat reserves stored during larvae stages (Barros et al., 2018).

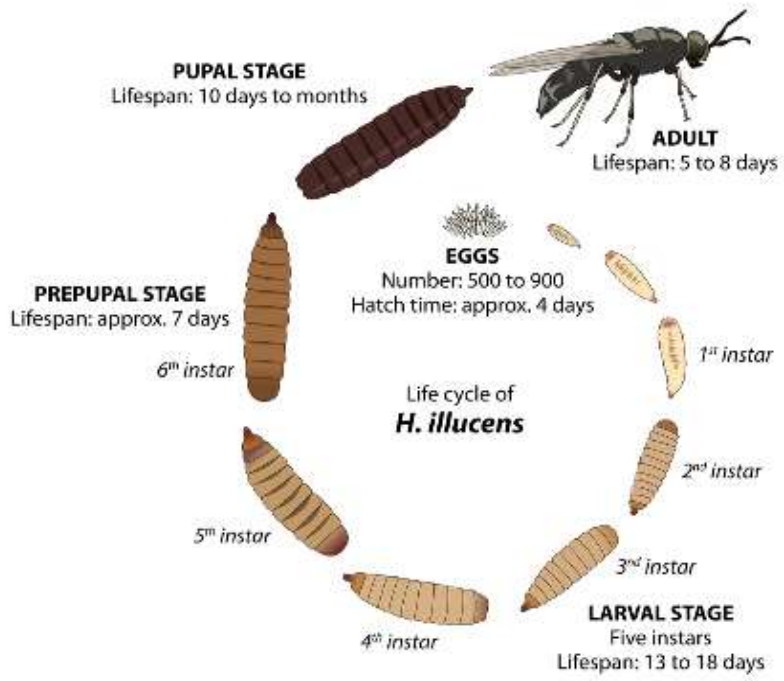


Figure 2.1: Developmental stages of the life cycle of *Hermetia illucens* (De Smet et al., 2018).

2.3 Economic relevance of black soldier fly

The use of BSF can help address two global problems: the vast accumulation of organic waste and food insecurity arising from unsustainable food production

(Badenhorst, 2017). The BSF larvae has a wide range of feeding diet from manure, rotten fruits, kitchen waste and a variety of other organic wastes (Jeon et al., 2011). They have powerful chewing mouth parts that help them to digest the organic compounds in the organic substrates before the compounds have time to decompose and this eliminates the odour from the organic substrates thus reducing pollution (Mertenat et al., 2019) and for these reason, they have been termed as effective manure decomposers. They have also helped in manure management that has reduced environmental damage that can result due to accumulations of manure (Lalander et al., 2013).

As the BSF larvae digest the organic compounds, they convert the organic waste nutrients into 42% crude protein, chitin and 35% fat feed stuff (Müller et al., 2017; Liu et al., 2017*;* Oliveira et al., 2016)). This is known as bioconversion and the high-quality crude protein can be exploited to produce improved protein animal feeds that can be used as an alternative source of protein for animals making BSF to be a potential replacement of conventional animal feeds such as soybean and fishmeal (Onsongo et al., 2018) . The fat from the larvae can also be extracted and exploited to produce biodiesel (Li et al., 2015). The chitin produced can be derived as it used in several industries such as the cosmetic industry, textile industries, pharmaceuticals (Caligiani et al., 2018).

The BSF larvae also have other useful compounds such as calcium, phosphorous and nitrogen free extract (Gligorescu et al., 2018*)*. The high calcium content could halt effects of metabolic bone diseases. The residues of these organic substrates after bioconversion can also be used as rich fertilizers (Mazza et al., 2018).

The BSF larvae's feeding habit discourages the development of pest flies such as house flies and blow flies. As they churn the manure and compost heaps, they make it more liquid and less suitable for oviposition and development of pest fly's larvae thus reducing them. They also emit substances that repel these 'dirty' flies (Ahrens et al., 2016) .

BSF naturally thrive in high densities and can be reared in small spaces thus they can be easily managed. They have a high rate of production and they grow very rapidly (in two weeks of hatching they can grow to 15000 times their size) (Nakamura et al.,

2016). Furthermore, BSF larvae are self- harvesting. When they are ready to metamorphosize, they leave their feeding area in search of dark places to complete their metamorphism (Y.S. Wang & Shelomi, 2017). This makes them to be less laborious when being reared as the farmers don't have to move them.

BSF have been known to reduce *Escherichia coli* in dairy manure and they could also provide potential chemical precursors to producing biodiesel (Li et al., 2015*;* Sanford et al., 2008). In addition to this, they minimize food wastage where the food wastes are recycled, and bio converted. This will help immensely in promoting food loss reduction and improve food security (FAO, 2011b).

Since the BSF larvae are being used as feed supplements to animal feeds (Onsongo et al., 2018), they can be easily and economically transported unlike the unprofitable manure and these can reduce the need to import concentrates that are added to the animal feeds making it a more cost effective feed supplement (Müller et al., 2017).

A study conducted by (Cai et al., 2018) has demonstrated that BSF larvae can provide environmentally friendly manure treatment dependent on their capacity to adequately and rapidly degrade tetracycline. This showed that nearly 97% of tetracycline was degraded within 12 days in a BSF larvae treatment system. Past research has also shown that utilization of BSF larvae can smother the spread of three pharmaceuticals (carbamazepine, roxithromycin and trimethoprim) and two pesticides (azoxystrobin and propiconazole) into the environment with no bioaccumulation distinguished in the BSF larvae (Ahrens et al., 2016). This promising ability is advantageous, particularly to lessen anti-microbial deposits in the environment. However, BSF's feeding habit can make them pick up a variety of microorganisms while feeding from the organic wastes making their exoskeleton and intestinal gut to provide peculiar environments for microbial community which can harbour specialized bacteria that can be transmitted to animals fed on BSF (De Smet et al., 2018).

2.4 Chicken production and challenges in Kenya

Chicken production in Kenya is a significant aspect of the country's agricultural sector. It provides a source of income and nutrition for small-scale farmers and rural

households. The demand for chicken meat and eggs has been increasing in the country, driven by population growth, urbanization, and changing dietary preferences (Wambua et al., 2022).

However, the production of chicken in Kenya faces several challenges, one of which is feeding. The cost of feed is high in Kenya, and this makes it difficult for small-scale farmers to afford to feed their chickens adequately. The high cost of feed is due to factors such as the high cost of raw materials, the cost of transportation, and import tariffs on some feed ingredients. There is also limited availability of feed ingredients in Kenya, which means that some farmers may have to import feed or use alternative sources of feed. This can be expensive and may not always be a viable option (Magothe et al., 2012b).

Poor quality feed is also another constraint. The quality of feed available in Kenya is often poor, which can impact the growth and health of the chickens. Poor quality feed can also lead to low egg production and poor meat quality (Wambua et al., 2022). On the other hand, many small-scale farmers lack the technical knowledge needed to produce balanced feed rations for their chickens. This can result in malnourished chickens and poor production. Moreover, diseases and pests can have a significant impact on chicken production in Kenya, leading to high mortality rates and decreased production. Some diseases, such as Newcastle disease and avian influenza, can be prevented through vaccination, but this can be expensive for small-scale farmers (Evans et al., 2021).

In conclusion, feeding is a major challenge facing chicken production in Kenya and to overcome these challenges, there is a need for investment in research and development for alternative feed sources, improved access to feed ingredients, and training for small-scale farmers on feed production and management to improve poultry production in Kenya.

2.5 Gut microbiota of chicken

The gut, as an organ, is lined by a continuous layer of epithelial cells which maintain the structural and functional integrity of the gut. In humans and even animals, the

gastrointestinal tract is known to harbour a vast and intricate ecological niche in which various domains of life which include bacteria, fungi, archaea, protozoa and viruses survive (Huang et al., 2016). The microbiome has been found to implicate the digestion and formation of polymers, maintaining gut peristalsis, maintaining the intestinal mucosa integrity, stimulation of the immune system and colonization resistance against pathogens (Kogut & Arsenault, 2016). Similarly, the gut microbiota composition of chicken harbours a complex microbial community that plays vital roles in nutrient utilization and adsorption, growth and development, production of short chain fatty acids and overall health status(Yeoman et al., 2012).

The domestic chicken forms the basis of the protein industry and the interactions between the gut microflora and the chicken plays a key role in host physiological development, health and nutrition, and food safety (Oakley et al., 2014). The microbial diversity is stretched across the entire gut with the main sites being the crop, ceca and small intestines (Gabriel et al., 2007). The gizzard and proventiculus have lower pH levels which reduce the number of bacteria populations (Apajalahti & Vienola, 2016) thus they are not included in the main sites that are studied. The chicken ceca is the most studied organ for microbial analysis as it harbors the highest number of microbial cell densities that are important sites for water regulation, carbohydrates fermentation, recycling urea and formation of short chain fatty acids with the most dominant species being the *Clostridiaceae* while the crop and the small intestines are dominated by facultative anaerobes *lactobacillus*, *enterococci* and *coliforms* (Fravalo et al., 2015).

The chicken gut microflora is affected by many factors including diet, age of the chickens, location and rearing environment, animal strain and sex (Clavijo & Flórez, 2018). Rinttilä, T., Apajalahti (2013) demonstrated that chicks at day seven are dominated by three genera of the order Clostridiales (*flavonifractor, pseudoflavonifractor* and *lachnospiracea*) while at day 21 and day 42 the genus *faecalibacterium and Roseburia* predominates respectively.

The gut microbes have been shown to play an important role in feed absorption and digestion drawing an association between the gut microbiota and feed utilization efficiency (Chen et al., *2018)*. The diet of poultry has the greatest impact on the

intestinal microbiome as the dietary components serve as substrates for the growth of gut bacteria (Oakley et al., 2014). The microbiota may be influenced by the dietary ingredients, nutrient levels of fat, protein and carbohydrates, inclusion of feed additives and dietary supplementation using feed enzymes (Torok et al., 2011). The microbiota may also be affected by the form of cereal included in the diet including whole, milled or pellets (Gabriel et al., 2007).

In a study conducted by Hammons et al, (2010), it was demonstrated that even the smallest variations in the dietary cereal grain composition affected the intestinal bacteria at strain level. The observations illustrated that corn-soybean ratios favoured *Lactobacillus agilis* strain R5 while wheat middling favoured *Lactobacillus agilis* strain R1.

Insect- based diet has a high potential as an alternative source of protein for poultry and few studies are available in literature on the effects of the BSF larvae meal on the chicken intestinal microbiome (D. Józefiak et al., 2016). A study by Pretorius, (2011) showed that augmentation of BSF larvae meal in broiler diet did not have any negative effect on weight gain, feed intake and feed efficiency. In addition to this, Leiber et al, (2015) also reported that replacement of layer diet with BSF larvae meal did not affect egg production and feed conversion efficiency.

Nevertheless, the source of the dietary protein may also affect the gut microbiome in poultry. Plant-based proteins such as *Desmodium intortum*, which is widely used by smallholder farmers as a fodder crop, have also been suggested to be a valuable feed additive for poultry feed (Teguia & Beynen, 2005). These plants have high-quality protein, can be grown between and under crops, and fixes nitrogen, therefore increasing crop yields and reducing the need for nitrogen fertilizers (Martens et al., 2012). In Africa at large, *Desmodium* is being used as an intercrop in a novel cropping system model which is known as push-pull technology, which was developed by the International Centre of Insect Physiology and Ecology (icipe) for integrated management of pests such as fall armyworm on cereal crops and has been adopted by over 200,000 smallholder farmers (Z. R. Khan et al., 2018). The cinnamon plant, from the genus *Cinnamomum*, is another plant that has been recently approved as poultry

feed additive because it produces bioactive compounds that improve immunity, metabolism, growth performance, and overall poultry health (Ali et al., 2021).

Sun et al, (2013) reported that use of fermented cottonseed meal as a protein source increased the *Lactobacilli* populations in broiler chicken unlike soybean meal which is the most used protein source. Furthermore, the use of green tea powder as a feed additive towards chicken health affects the bacterial diversity by promoting the prevalence *of Proteobacteria* while use of mulberry leaf powder enhances prevalence of *Bacteriodetes* (Chen et al., 2019).

Rodríguez, (2012) reported that various feed additives in the poultry diet can influence the gut microbiome, reduce enteric pathogens and demonstrated that dietary enzymes such as xylanase and β- glucanase normally increase the intestinal lactic acid bacteria and decreases pathogenic bacteria such as *E. coli*. A study conducted by Tangomo et al, (2020) tested the efficacy and safety of *Dacryodes edulis* plant which has prebiotic potential as a feed additive in chicken diets and found that this plant significantly reduced the colonies forming unit of *Escherichia coli* and *Salmonella* sp. in the gut of the chickens.

The importance of the gut microbiota cannot be understated. Chicken gut commensal bacteria usually provide protection to the mucosal membrane by modulating the immune response but different rearing conditions and environment may bring about pathogenic bacteria which can cause morphological changes in the gut. This has been demonstrated by Shang et al, (2018) who reported that chickens infected with *Clostridium perfringens* have a significant reduced length of the intestinal villi. In this regard, control of pathogens from the chicken farms is of great importance because they may cause gastrointestinal tract infections that may be brought about by bacteria such as *Salmonella* and *Campylobacter* from consumption of various products and for this reason, it's important to analyse the feeds fed to chicken(Gabriel et al., 2007).

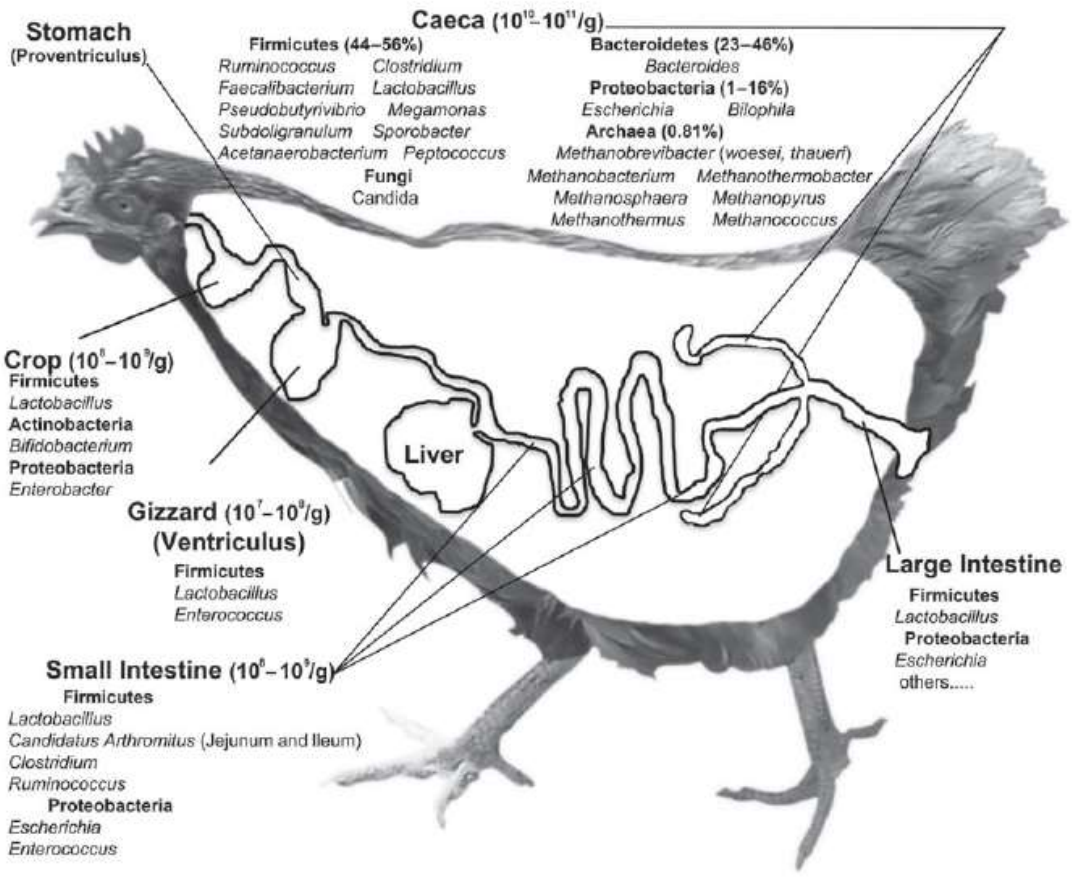


Figure 2.2: Bacterial diversity as shown from different sites of the chicken gut (Yeoman et al., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethical statement

All experiments, mainly feeding and later extraction of guts for analysis, were conducted with strict adherence to the approved experimental guidelines and procedures by the Institutional Animal Care and Use Committee (IACUC) at the Kenya Agricultural and Livestock Research Organization (KALRO) -Veterinary Science Research Institute (VSRI); approval Code No.: KALRO - VSRI/IACUC019/30082019.The chickens were handled carefully to ensure minimum distress

3.2 Chicken rearing and diet formulations

3.2.1 Layers

The chickens were reared at the Poultry Research Unit in KALRO located in Naivasha, Nakuru County, Kenya (Lat 0.6835 ° S, longitude 36.4012 ° E) as described by Sumbule et al, (2021). In summary, a total of 250 one-day-old ISA BROWN female chicks were sourced from Kenchic Limited, Nairobi. During the first two weeks of acclimatization, all the 1-day old birds (chicks) were kept together in a brooder, which was a round deep litter floor covered with a 7.6 cm-thick layer of wood shavings bedding. The area was fitted with 250 Watts infra-red bulbs to provide heating during the brooding period. For a period of 14 days, the young birds were provided the control (100% FM inclusion ratio) diet and water *ad libitum* for 14 days. However, birds that showed signs of deformity or weakness (25 chicks) were carefully removed.

After 14 days, they were kept in different floor pens (1 m × 1 m) each with five chicks. The chicks were assigned randomly to one of the five feeding regimes using a completely randomized design throughout the entire feeding phase. Each experimental set-up was replicated nine times. The birds were given access to both feed and clean water *ad libitum* daily. The conditions inside the rearing facility were kept at 30 ± 1 ◦C with a relative humidity of 70 ± 2%. Within the first 4 weeks, 24 h of lighting was used to stimulate feed and water intake among the chicks. This was later followed by a gradual decrease of hours of lighting to adapt to natural conditions with a dark: light

cycle of 12 h:12 h by the end of the chick stage. Further lighting conditions were maintained with a dark: light cycle of 12:12 h throughout the grower phase. The vaccination program of the birds followed the agreed guidelines for the prevention of any disease-causing agents. Vitamins were administered in water each time a new batch of feed was introduced and after vaccination. Sawdust was used as bedding in the pens and changed every 3 weeks to avoid ammonia build-up and bacterial infection. The birds were reared for 20 weeks.

All ingredients required for the formulation of the various diet types for starter diets (Table 3.1) and grower diets (Table 3.2) were sourced from a local miller (Josiche General Traders Ltd in Nakuru, Kenya). The BSF larvae were obtained from the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. A standard diet of commercialized conventional fishmeal (FM) served as the control diet [Treatment 1 (T1): 100 %FM + 0 %BSFL], while the other 4 diets were composed of (T2): 25 % BSFL + 75 % FM; T3: 50 % BSFL + 50 % FM; T4: 75 % BSFL + 25 % FM, and T5: 100 % BSFL + 0 % FM. All diets for the entire experimental period were formulated at once as described by Sumbule et al, (2021) following standard protocols to meet the nutrient requirements of the birds. The hens were reared under appropriate conditions following protocols and guidelines as instructed by the Federation of Animal Science Societies (FASS 2010) until the hens were ready for sample collection.

Comprehensive analytical estimates of the nutrient quality and the proximate analysis of the formulated diets were also undertaken to ensure the crude protein levels for each treatment diet were within the acceptable values for the birds before the commencement of the experiments as described by Sumbule et al, (2021).

Table 3.1: Ingredients composition of the formulated diets fed to layer chicks for eight (8) weeks

Ingredients (%)	T1 (control)	T2	T3	T4	T5
Maize germ	60.0	60.0	60.0	60.0	60.0
Soybean meal	21.0	21.0	21.0	21.0	21.0
Fishmeal	10.0	7.5	5.0	2.5	0.0
BSFL	0.0	2.5	5.0	7.5	10.0
Vegetable oil	2.0	2.0	2.0	2.0	2.0
Limestone	5.0	5.0	5.0	5.0	5.0
Dicalcium phosphate	1.5	1.5	1.5	1.5	1.5
Iodized salt (NaCl)	0.3	0.3	0.3	0.3	0.3
Layer premix [a]	0.2	0.2	0.2	0.2	0.2

Super layer premix contents per 2.5 kg: Vit. (Vitamin) A: 8,000,000 IU/kg, Vit. D3:2,000,000 IU/kg, Vit. E: 3000 mg, Vit. K3: 2000 mg, Vit B2: 3500 mg, Pantothenic Acid: 6600 mg, Niacin:20,000 mg, Folic Acid: 550 mg, Vit. B12: 6 mg, Choline chloride: 200,000 mg, Lysine: 350 mg, Methionine:120 mg, Manganese: 63,000 mg, Iron: 23,000 mg, zinc: 63,000 mg, Copper: 14,000 mg, Cobalt: 1000 mg, Iodine:2000 mg, Selenium: 100 mg and BHT: 120,000 mg. Abbreviation: BSFL-black soldier fly larvae. FM- fishmeal Diet 1—0% BSFL, Diet 2—25% BSFL and 75% FM, Diet 3—50% BSFL and 50% FM, Diet 4–75% BSFL and 25% FM and Diet 5—100% BSFL

Table 3.2: Ingredients composition of the formulated diets fed to grower layer chicken for 12 weeks

Ingredients (%)	T1 (control)	T2	T3	T4	T5
Maize germ	50.0	50.0	50.0	50.0	50.0
Pollard (wheat)	19.0	19.0	19.0	19.0	19.0
Soybean meal	13.0	13.0	13.0	13.0	13.0
Fishmeal	10.0	7.5	5.0	2.5	0.0
BSFL	0.0	2.5	5.5	7.5	10.0
Limestone	5.0	5.0	5.0	5.0	5.0
Dicalcium phosphate	2.0	2.0	2.0	2.0	2.0
Iodized salt (NaCl)	0.3	0.3	0.3	0.3	0.3
Layer premix	0.2	0.2	0.2	0.2	0.2

Super layer premix contents per 2.5 kg:Vit. (Vitamin)A: 8,000,000IU/kg, Vit. D3:2,000,000 IU/kg, Vit. E: 3000 mg, Vit. K3: 2000 mg, Vit B2: 3500 mg, Pantothenic Acid: 6600 mg, Niacin:20,000 mg, Folic Acid: 550 mg, Vit. B12: 6 mg, Choline chloride: 200,000 mg, Lysine: 350 mg, Methionine:120 mg, Manganese: 63,000 mg, Iron: 23,000 mg, zinc: 63,000 mg, Copper: 14,000 mg, Cobalt: 1000 mg, Iodine:2000 mg, Selenium: 100 mg and BHT: 120,000 mg. Abbreviation: BSFL-black soldier fly larvae. FM- fishmeal Diet 1—0% BSFL, Diet 2—25% BSFL and 75% FM, Diet 3—50% BSFL and 50% FM, Diet 4–75% BSFL and 25% FM and Diet 5—100% BSFL

3.2.2 Broilers

The study was mainly based and conducted at *icipe*, Duduville, Kasarani sub-county, Nairobi. The rearing was done as described by Mutisya et al, (2020). In summary, the BSFL colony was initiated at the *icipe's* Animal Rearing and Containment Unit (ARCU) under controlled conditions of temperature 28 ± 1º C and 70 ± 2% relative humidity. The 5th instar larvae were harvested from the colony and cleaned by washing them in hot water at 84º C for 10 min. The clean BSFL were oven-dried at 120º C using a hot air circulating drying oven (Henan Forchen Machinery, Henan, China). The dried BSFL were ground using a Munch hammer mill model M6FFC – 230 (Wuppertal, Germany) into a powdered larval meal which was used in the formulation of the various test diets.

Greenleaf desmodium was harvested from one of the *icipe's* push-pull plots located at *icipe*, Duduville, and the harvested biomass was dried under a shade and later ground into powder using a milling machine as previously described.

At the beginning of the experiment, 120 one-day-old broiler chicks (Cobb500) were sourced from Kenchic Ltd. hatchery in Thika, Kenya, and reared for 42 d. The chicks were placed in a common brooder room of 5 × 6 feet for the first 7 d and temperatures were maintained between 33°C and 35°C using light heating bulbs of 250 W suspended at 45 cm height over the brooder to provide heat. All the chicks were fed on starter mash (conventional feed) purchased from Unga Feeds Ltd. (Nairobi, Kenya). The chicks were fed at a rate of at least 125 g per day per chick. The chicks were randomly distributed in 12 pens each measuring 1.5 m × 1.8 m × 1.5 m in a poultry house. Sexing was done to ensure that each pen contained 10 chicks (5 females and 5 males). Each treatment was replicated 3 times in a completely randomized design. The whole experiment comprised 120 chickens, with 30 chickens in each treatment, including the control.

After the 7 d brooding period, the chicks were weighed individually to obtain their initial weight before being introduced to starter diets of the BSFL-*Desmodium* formulations. The chickens were fed daily and feeding was *ad libitum.* Finisher diets containing BSFL and *Desmodium* were provided to the chicks after 21 d. Clean drinking water was provided *ad libitum* until the end of the rearing period when the chickens were isolated for slaughtering. The chickens were maintained at 28 ± 1°C, RH of 65 ± 5%, and a photoperiod of 12L:12D. All the chickens were vaccinated against Newcastle disease on the 14th day.

The test diets were formulated according to the recommendations of the Kenya Bureau of Standards (KEBS) as guided by the NRC (1994) specifications for broiler starter and finisher feeds as described by Mutisya et al, (2020). The diets were prepared by replacing fishmeal on the control diet with a mixture of BSFL and *Desmodium intortum* as shown in Table 3.3. All the ingredients required for both the starter diets (Table 3.4) and finisher diets (Table 3.5) were sourced from Unga Limited, Kenya. Comprehensive analytical estimates of the nutrient quality and the proximate analysis

of the formulated diets were also undertaken to ensure the crude protein levels for each treatment diet were within the acceptable values for the birds before the commencement of the experiments as described by Mutisya et al, (2020).

Table 3.3: Summary of broiler diet formulations into four treatments using combinations of BSFL (Black Soldier Fly larvae) and Desmodium intortum in place of the conventional fishmeal diet.

Treatment group, T	Diet Formulation
T1 (Treatment 1)	25% *Desmodium intortum* +75% BSFL
T2 (Treatment 2)	50% *D. intortum* + 50% BSFL
T3 (Treatment 3)	75% *D. intortum* + 25% BSFL
T4 (Control)	100% Commercial fishmeal

Table 3.4: Feed composition for Broiler starter (Diets (g/kg) as fed) of Experimental Diets.

Ingredients (%)	Control	T1	T2	T3
Maize germ	528.8	527.0	540.0	550.3
Wheat pollard	104.0	108.0	97.9	201.6
Corn oil	24.6	16.3	11.4	4.0
Fish meal	16.3	0.0	0.0	0.0
Desmodium intortum	0.0	82.7	165.3	247.0
BSFL	0.0	247.9	165.3	82.7
Limestone	10.0	10.0	10.0	10.0
Salt	3.6	3.6	3.6	3.6
Di -calcium phosphate	0.5	1.0	3.3	3.2
Broiler premix[1]	2.5	2.5	2.5	2.5
Mycotoxin binder	1.0	1.0	1.0	1.0

1Broiler premix (provided per kg of diet) = Vitamin A (I.U) 6250000,Vitamin D3 (I.U) 1000000,Vitamin E (I.U) 15000,Vitamin K3 (Mg) 1000,Vitamin B1 (Mg) 500,Vitamin B2 (Mg) 2500, Vitamin B6 (Mg) 2500, Vitamin B12 (Mg) 10, Pantothenic acid (Mg) 600, Nicotinic acid (Mg) 15000, Folic acid (Mg) 500, Biotin (Mg) 35, Choline chloride (Mg) 150000, Iron (Mg) 20000, Copper(Mg) 2500, Zinc (Mg) 25000, Manganese (Mg) 15000, Iodine (Mg) 600, Cobalt (Mg) 400, BHT (Anti-oxidant, Mg) 125000 T1=25% *D. intortum*+75%BSFL, T2=50% *D. intortum*+50% BSFL and T3=75% *D. intortum*+25% BSFL and Control= Commercial feed.

Tab**le 3.5: Feed composition for Broiler starter finisher diets (Diets (g/kg) as fed) of Experimental Diets.**

Ingredients (%)	Control	T1	T2	T3
Maize germ	550.0	526.0	534.0	576.5
Wheat pollard	201.6	200.5	198.2	165.5
Corn oil	27.2	22.1	15.2	5.4
Fish meal	191	0.0	0.0	0.0
Desmodium intortum	0.0	55.0	110.0	165.0
BSFL	0.0	165.0	110.0	55.0
Limestone	22.6	22.6	22.6	22.6
Salt	3.6	3.6	3.6	3.6
Di -calcium phosphate	0.5	1.7	2.9	3.3
Broiler premix[1]	2.5	2.5	2.5	2.5
Mycotoxin binder	1.0	1.0	1.0	1.0

1Broiler premix (provided per kg of diet) = Vitamin A (I.U) 6250000,Vitamin D3 (I.U) 1000000,Vitamin E (I.U) 15000,Vitamin K3 (Mg) 1000,Vitamin B1 (Mg) 500,Vitamin B2 (Mg) 2500, Vitamin B6 (Mg) 2500, Vitamin B12 (Mg) 10, Pantothenic acid (Mg) 600, Nicotinic acid (Mg) 15000, Folic acid (Mg) 500, Biotin (Mg) 35, Choline chloride (Mg) 150000, Iron (Mg) 20000, Copper(Mg) 2500, Zinc (Mg) 25000, Manganese (Mg) 15000, Iodine (Mg) 600, Cobalt (Mg) 400, BHT (Anti-oxidant, Mg) 125000 T1=25% *D. intortum*+75%BSFL, T2=50% *D. intortum*+50% BSFL and T3=75% *D. intortum*+25% BSFL and Control= Commercial feed.

3.3 Sample collection

A subset of 75 layer chickens was selected randomly with 15 chickens per treatment being used for sample collection while for broilers 5 chickens per treatment were selected. The chickens were euthanized and sacrificed professionally and the whole gut was harvested from each chicken and samples excised from the 8 major sections of the gut including the esophagus, crop, proventriculus, gizzard, duodenum, small intestine, large intestine, and ceca sections. The carcasses were disposed of using safe methods. The samples were then placed in clean Eppendorf tubes and stored at 4 ºC on ice for transportation to the Arthropod Pathology Unit at *icipe*, Kenya for further analysis.

3.4 Genomic DNA extraction and 16S rRNA amplification

Gut contents were excised from the inner epithelial tissues and used for genomic DNA extraction using Isolate II Genomic Extraction kit (Bioline, London, UK) following the manufacturer's instructions. The concentration and quality of DNA was determined using a nanodrop 2000/2000c spectrophotometer (Thermo Fischer Scientific, Wilmington, USA) and those with a good quality range (1.8 - 2.0), based on A_{260nm}/A_{280nm} selected for metagenomics analyses. Sequencing of the full length ~1500 bp bacterial 16S rRNA gene was performed using Oxford Nanopore Technologies (ONT) Minion device using R9.4.1 flow cells and the libraries were prepared with pooled DNA for multiplexing using the 16S barcoding kit SQK - 16S024. Library preparation with PCR step was conducted using the following components: 10 pmol μL^{-1} of each 16S barcode, 10 ng μL^{-1} of DNA template, 0.625 U μL^{-1} *MyTaq* DNA polymerase (Bioline), and 5 X *MyTaq* reaction buffer (5 mM dNTPs, 15 mM $MgCl_2$, stabilizer and enhancer) (Bioline). The reactions were set up in a total reaction volume of 50 µL and run in a Master cycler Nexus gradient thermal cycler (Eppendorf, Germany), under the following conditions: initial denaturation for 2 min at 95 ˚C, followed by 35 cycles of denaturation for 30 s at 95 ˚C, annealing for 40 s at 55 ˚C, extension for 1 min at 72 ˚C, and a final extension step of 10 min at 72 ˚C. The libraries were then purified using a Bioline kit following the manufacturer's instructions and pooled together before loading into the flow cells for Minion sequencing.

3.5 Sequencing and data analysis

The sequencing runs were done for 4 hrs with live base calling performed using the Albacore tool (v2.3.4) on the Minknow (v 20.10.3) software on the ONT cloud. The reads that passed sequencing generated FASTQ files which were uploaded to EPI2ME software v 2020.11.19 (https://epi2me.nanoporetech.com) for qualitative, real-time species identification from metagenomic samples. The FASTQ - 16S workflow (v 2020.04.06) on EPI2ME was used to characterize the reads and assign taxonomy to the genus level using NCBI as the reference database and relative cumulative abundance plots were generated. A minimum abundance threshold of 0.1 % was used to select the most abundant taxa and those that were below this threshold were collapsed into others. However, EPI2ME is a limited tool since it does not give the

diversity parameters such as alpha and beta diversity. Thus, QIIM2 –v 2020.8 (Bolyen et al., 2019) pipeline was used for further processing to generate the reads needed for bacterial diversity statistics. On the QIIME2 pipeline, the adapters were trimmed using the trimmomatic tool (v 0.39) and the reads were demultiplexed using the ONT porechop tool (v0.2.4). The demultiplexed reads were checked for chimeric sequences using the VSEARCH- Qiime2 tool and the chimeric sequences found were filtered out using the UCHIIME tool. The reads were then aligned to MAFFT and taxonomy was assigned using SILVA 132 database as the reference database. OTUs were generated from the reads using a 97 % similarity threshold. The reads were then rarefied to even sampling depths and both alpha and beta diversity were calculated. Alpha diversity was calculated using the Shannon and Chao1 index in the phyloseq package in R (McMurdie & Holmes, 2013). Beta diversity was calculated using the Bray Curtis and unweighted unifrac distance methods. PERMANOVA was used to compare statistical significance differences between the microbial communities across the different treatments using the Adonis package in R. Antimicrobial resistance (AMR) was evaluated using the WIMP - ARMA workflow on the EPI2ME ONT platform which referenced the reads against the Comprehensive Antibiotic Resistance Database (CARD) to generate reads conferring to antibiotic resistance genes.

Figure 3.1: A pictorial representation of the Nanopore Minion sequencer topology (https://nanoporetech.com/).

CHAPTER FOUR

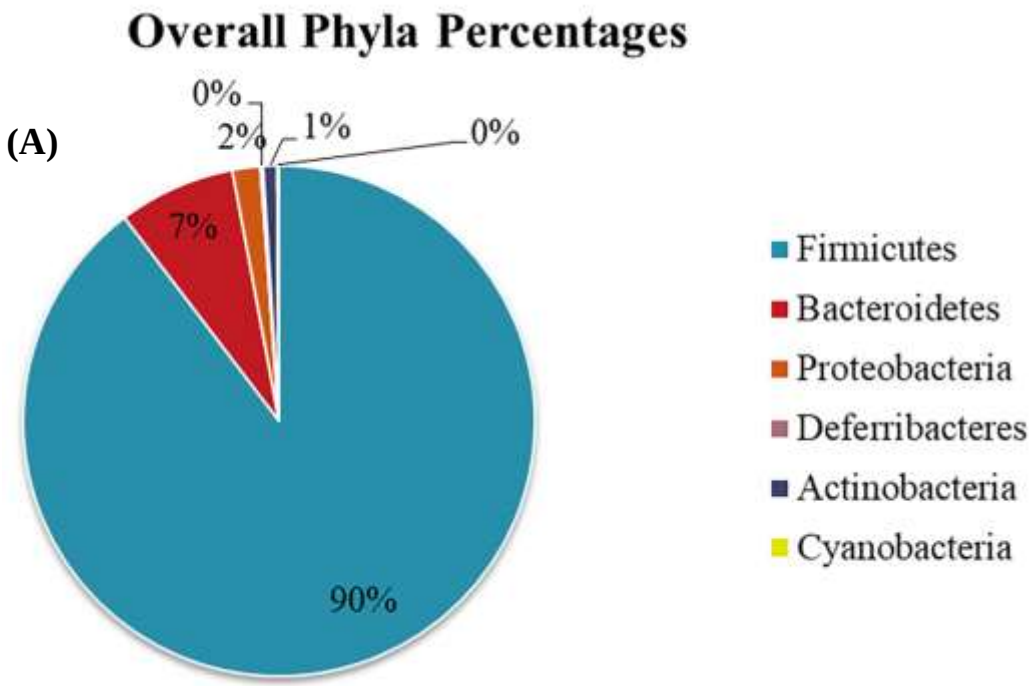
RESULTS

4.1 LAYERS

4.1.1 Bacterial abundance composition

About 400,064 reads were generated from which 338,087 were classified. Out of these reads, T1 which represented the control group had 40,769 reads, whereas the T2 group (25 % BSF) had 38,255 reads, T3 (50 % BSF) produced 13,806 reads, T4 (75 % BSF) had 5,884 reads, and T5 (100 % BSF) had 258,356 reads. The most abundant phyla observed across all the treatment groups included Firmicutes (90 %) followed by Bacteroidetes (7 %) and lesser portions of Proteobacteria (2 %) and Actinobacteria (1 %) (**Figure 4.1 A, B, C**).

(A)



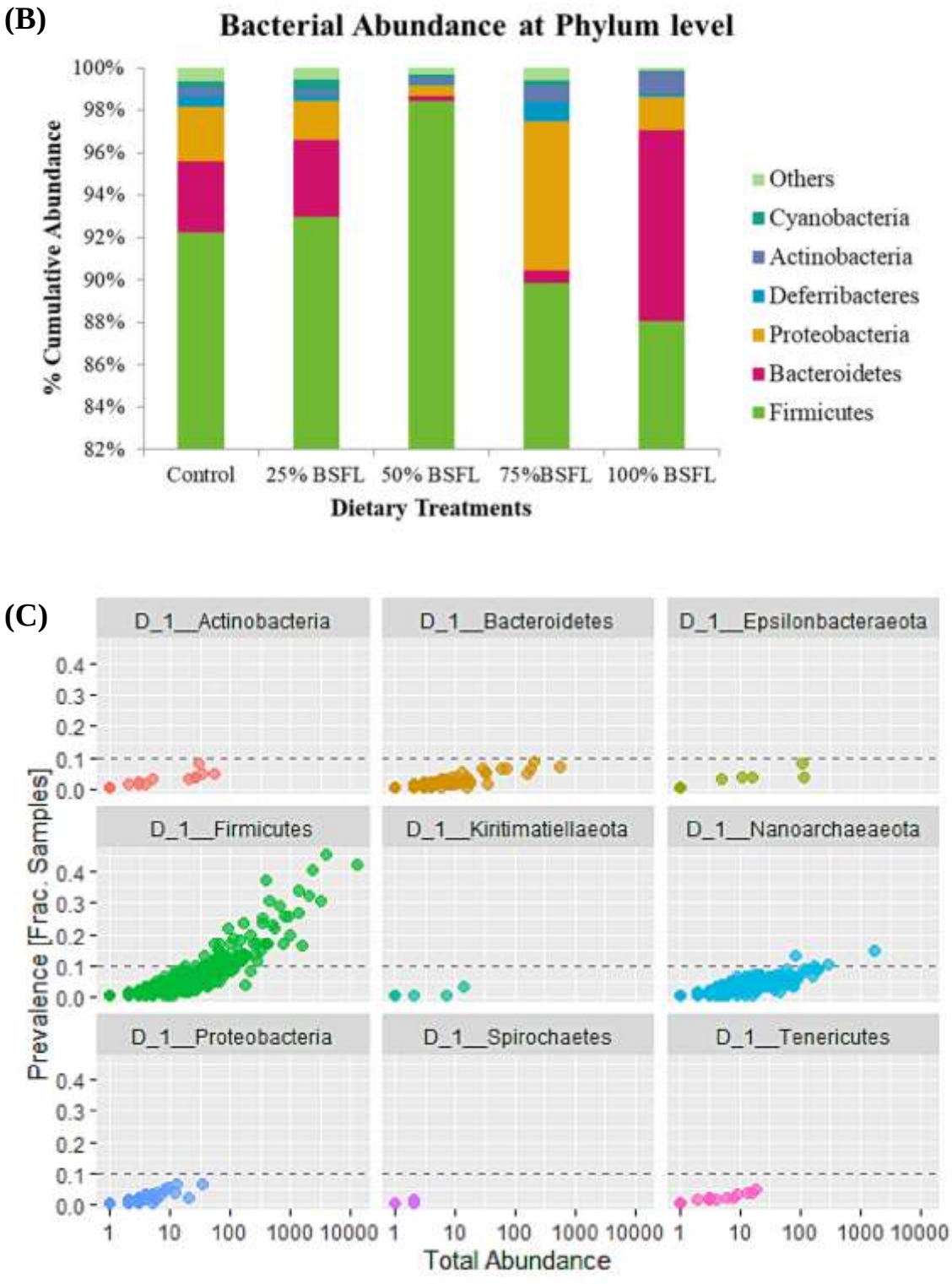


Figure 4.1: Pie chart showing the percentages of the most predominant phyla in layer samples (A); stacked bar chart showing Cumulative taxonomic composition at Phylum level across all five treatments(B) and; Prevalence of all observed phyla taxa across all treatments (C).

Cumulative relative abundance at the genus level showed that *Lactobacillus* (93 %) which is associated with the phylum Firmicutes was the most predominant genus across all the treatment groups, followed by *Bacteroides* (4 %), which is associated with the phylum Bacteroidetes (**Figure 4.2**). There was a significant increase in beneficial bacteria such *as Lactobacillus*, *Bacteroides*, *Blautia*, and *Enterococcus*. This increase was observed as the inclusion level of BSF larvae meal in the diet increased as compared to the control treatment group. In T5 which had 100 % BSF, the levels of beneficial bacteria increased considerably. However, some reads corresponding to possible clinical pathogenic bacteria such as *Campylobacter*, *Clostridia*, *Staphylococcus*, and *Streptococcus* were present mostly in T4 and T5 though at very low amounts of less than 1 % which are permissible according to the feed safety guidelines by Kenya Bureau of Standards (KEBS)**.** In T3, the clinical pathogens were very minimal making the beneficial bacteria to be in surplus (**Figure 4.2**).

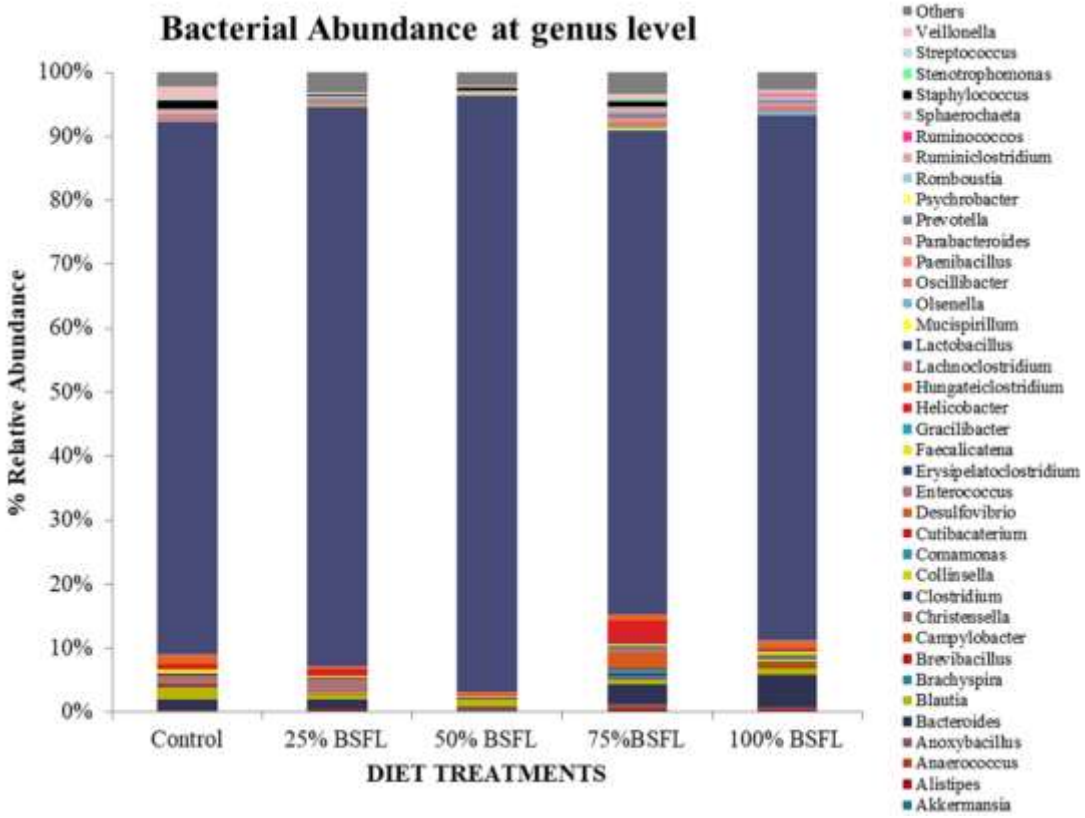


Figure 4.2: Cumulative abundance composition of bacteria OTUs identified at genus level across the different dietary treatments in layers.

Lactobacillus species was the most abundant bacteria that dominated all parts of the chicken gut including the esophagus, crop, proventriculus, gizzard, small intestines, large intestines, and the ceca. The ceca had the most microbial diversity followed by the crop and these two parts were dominated by the phylum Firmicutes, whereas the small intestines and esophagus had the least diversity and were colonized by Proteobacteria (**Figure 4.3**)

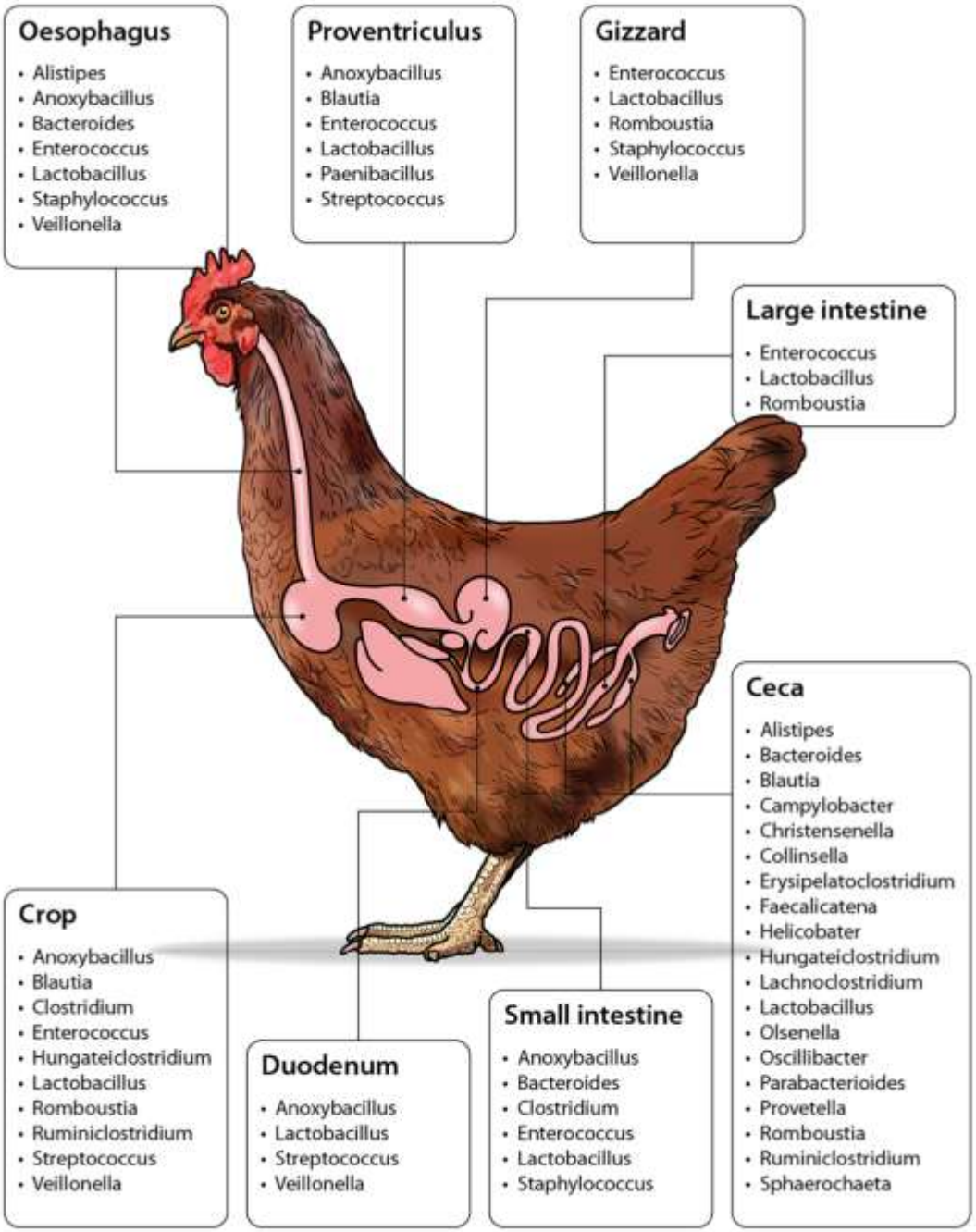


Figure 4.3: Bacteria genera identified across the entire gut segments in layer chicken fed different diets.

4.1.2 Alpha and Beta diversity

Alpha diversity assessed by the Shannon index showed that diet T5 which had 100 %BSFL was the most diverse diet treatment with a Shannon index of 8.0 while T3 and

T4 which had 50 and 70 % BSFL, respectively, had the least diversity of 2.1 (**Figure 4.4**). The Chao1 richness index showed that T5 (100 %BSFL) had the highest species richness and the most abundant read count with over 600 species identified.

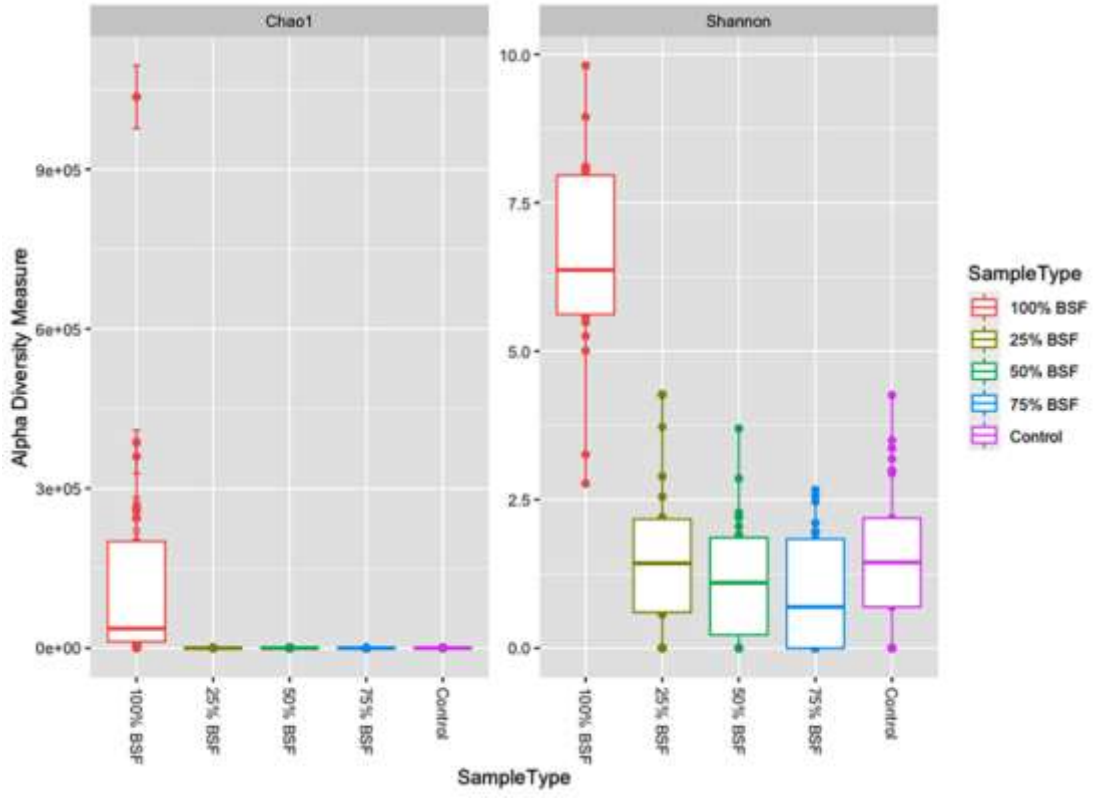


Figure 4.4: Alpha diversity measure using Shannon index and Chao1 richness index showing layer samples according to dietary treatments.

Beta diversity which was calculated using the unweighted unifrac distance method indicated that diet treatment did not adversely affect the diversity of the microbial communities which all appeared to cluster together. Only slight differences were observed in the microbial composition between the different dietary treatments. However, some outliers were observed in bacteria OTUs from T5 (100 %BSFL) which clustered separately and this may have been caused by poor-quality reads (**Figure 4.5**)

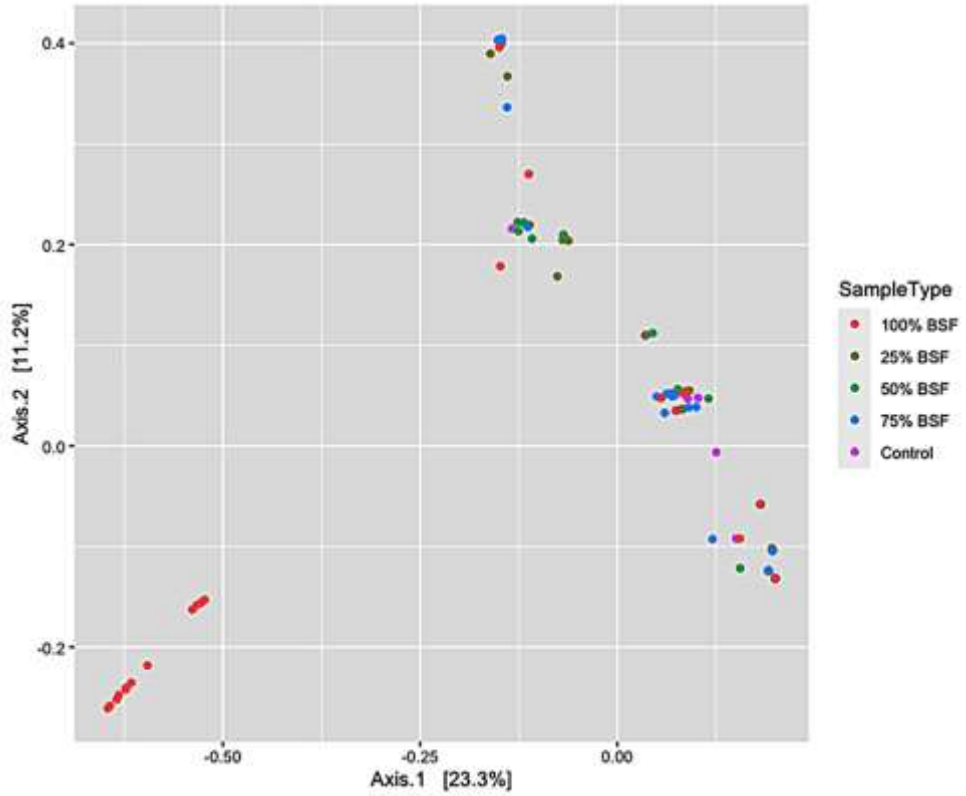


Figure 4.5: Beta diversity PCoA plot based on unweighted unifrac distance method between the different dietary treatments. [PERMANOVA] R-Squared: 0.11509; P-value: 0.001.

A Venn diagram showed that 48 bacteria genera were common across all five treatments. The 100 %BSFL treatment (T5) had the most unique genera totaling 290 (**Figure 4.6**).

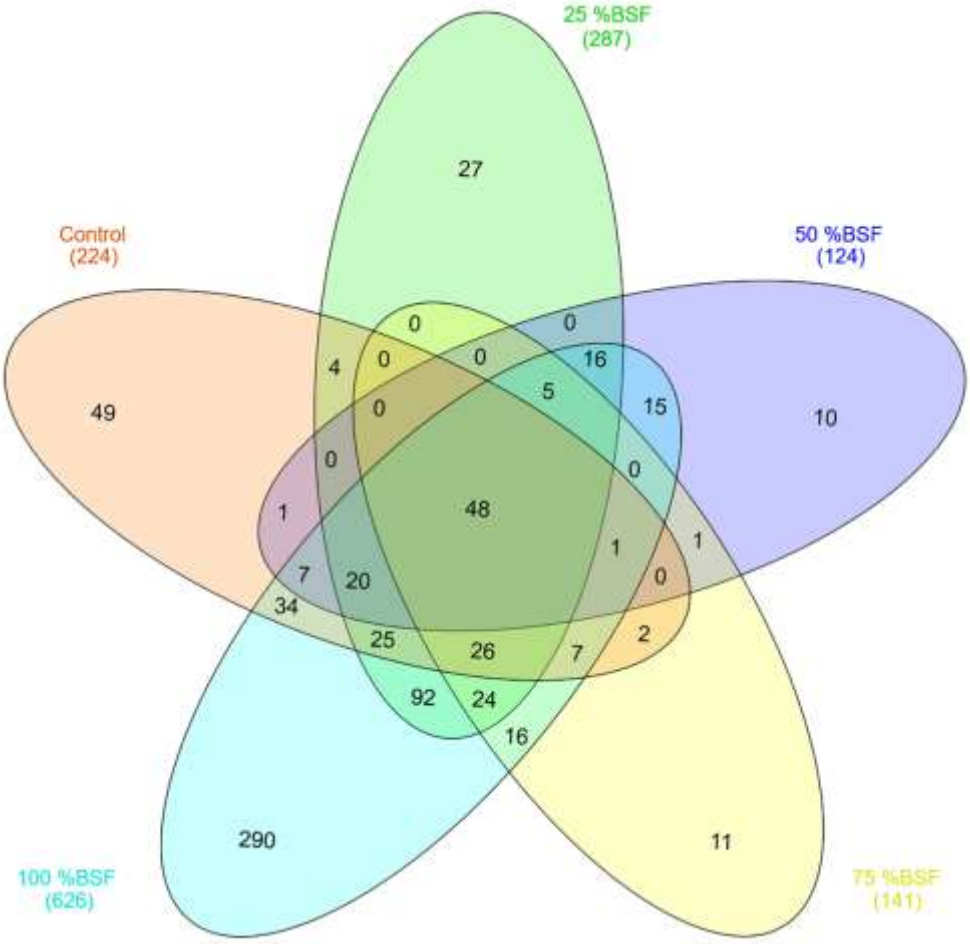


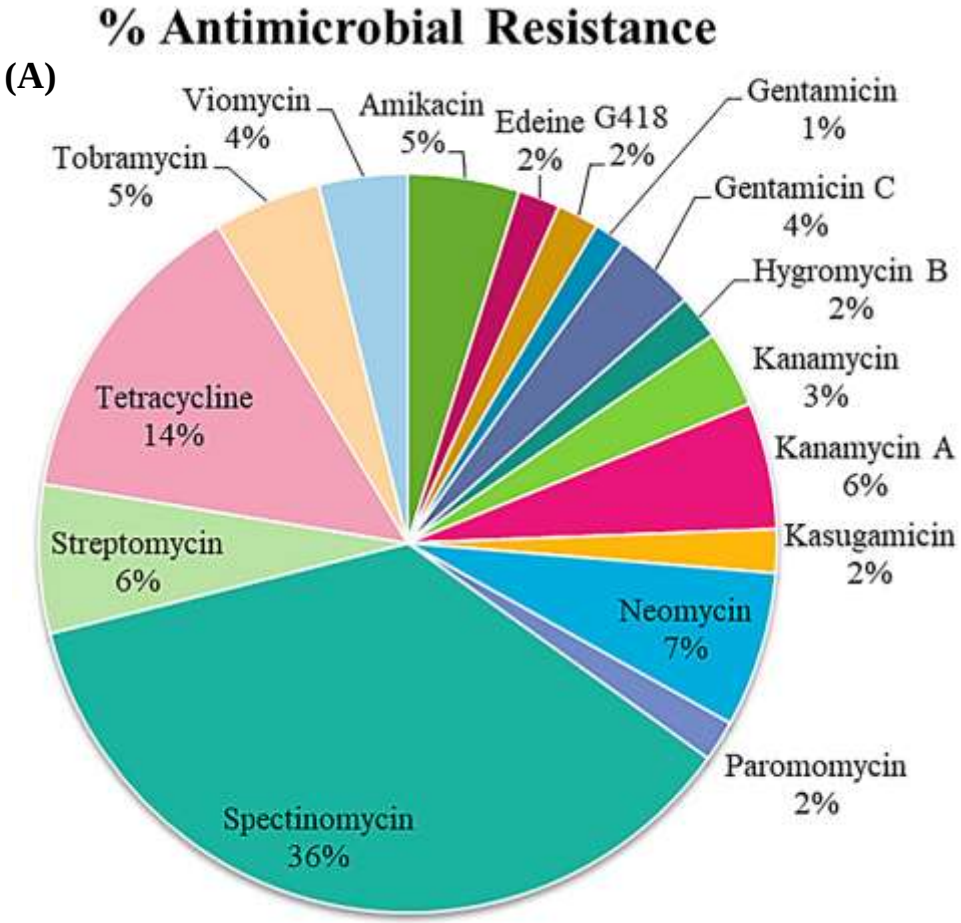
Figure 4.6: Venn diagram showing the identified shared and unique OTUs between the different treatments in layers.

4.1.3 Antimicrobial resistance

Antimicrobial resistance genes from the 16S layer samples were identified where 48 CARD genes were generated from WIMP - ARMA workflow in the EPI2ME ONT cloud with read alignment accuracy to CARD of 74.2 % **(Table 4.1).** The total reads analyzed were 357,516 with 305,283 reads being aligned. These genes conferred resistance to 16 antibiotics belonging to different groups including aminoglycosides and pentapeptides. From this dataset, genes conferring resistance to spectinomycin were the most abundant with about 36 % reads, followed by tetracycline with 14 % reads (**Figure 4.7A)**. The cumulative abundance composition of the antibiotic resistance showed that resistance read counts reduced as the BSFLF concentration

increased. Treatment (T1) had the highest amount of resistance reads while T4 and T5 had the least reads (**Figure 4.7B**).

(A)



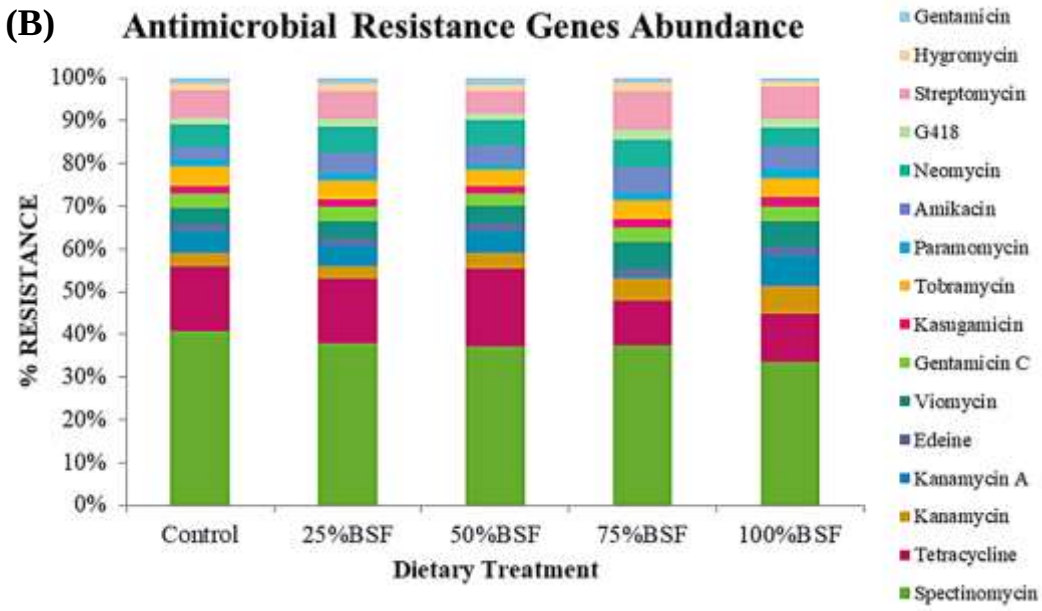


Figure 4.7: Pie chart showing the overall percentages of the conferred resistance antibiotics (A); cumulative composition of antimicrobial resistance genes identified in bacteria taxa present (B).

Table 4.1: Antibiotic resistance genes found in the bacterial communities of layer chicken as obtained from comprehensive antibiotic resistance database (CARD)

Taxon	Gene	Conferred resistance
Salmonella enterica subsp. Salamae	*Salmonella enterica serovar Typhimurium* 16S rRNA mutation in the rrsD	Spectinomycin
Neisseria Meningitidis	*Neisseria meningitidis* 16S rRNA mutation	Spectinomycin
Escherichia Coli K-12	*Escherichia coli* 16S rRNA mutation in the rrsH gene	Spectinomycin
	Escherichia coli 16S rRNA mutation in the rrsC gene	Kasugamicin
	Escherichia coli 16S rRNA mutation	Edeine
	Escherichia coli 16S rRNA mutation in the rrsB gene	Kanamycin A, Tetracycline, G418, Streptomycin, Spectinomycin, Paromomycin, Tobramycin, Gentamicin C
	Escherichia coli 16S rRNA mutation in the rrnB gene	Tetracycline, Spectinomycin, Streptomycin
Propionibacterium acnes	*Propionibacterium acnes* 16S rRNA	Tetracycline
Helicobacter pylori 26695	*Helicobacter pylori* 16S rRNA	Tetracycline
Mycobacterium tuberculosis H37RV	*Mycobacterium tuberculosis* 16S rRNA mutation	Viomycin, Kanamycin, Amikacin, Streptomycin
Mycobacterium	*Mycobacterium chelonae* 16S rRNA mutation	Neomycin, Amikacin, Gentamicin C Tobramycin, Kanamycin A
Chlamydia psittaci 6BC	*Chlamydophila psittaci* 16S rRNA mutation	Spectinomycin
Mycobacterium abscessus	*Mycobacterium abscessus* 16S rRNA mutation	Kanamycin, Neomycin, Amikacin Tobramycin, Gentamicin
Pasteurella multocida 36950	*Pasteurella multocida 16S rRNA mutation*	Spectinomycin
Mycobacterium smegmatis str.MC2 155	*Mycobacterium smegmatis* 16S rRNA mutation in the rrsB gene	Neomycin, Streptomycin, Kanamycin A
	Mycobacterium smegmatis 16S rRNA mutation in the rrsA gene	Neomycin, Hygromycin B, Viomycin
Borrelia burgdorferi	*Borrelia burgdorferi* 16S rRNA mutation	Kanamycin, Spectinomycin, Gentamicin

4.2 BROILERS

4.2.1 Microbial Community Abundance

Minion nanopore sequencing obtained a total of 395,034 raw reads from which 292,100 reads passed the quality filtering. Out these reads, T1 had 100,912 reads, T2 had 72,404 reads, T3 had 38,500 and T4 (control) had 80,309 reads. All samples were rarefied to even sampling depths with an average of 20,000 reads/sample and 984 OTUs identified after quality sampling. The most abundant bacterial phyla observed across all the dietary treatments were Firmicutes (94%), Bacteroidetes (3%), Proteobacteria (2%) and lesser portions of Verrucomicrobia, Actinobacteria, Cyanobacteria and Tenericutes (**Figure 4.8**).

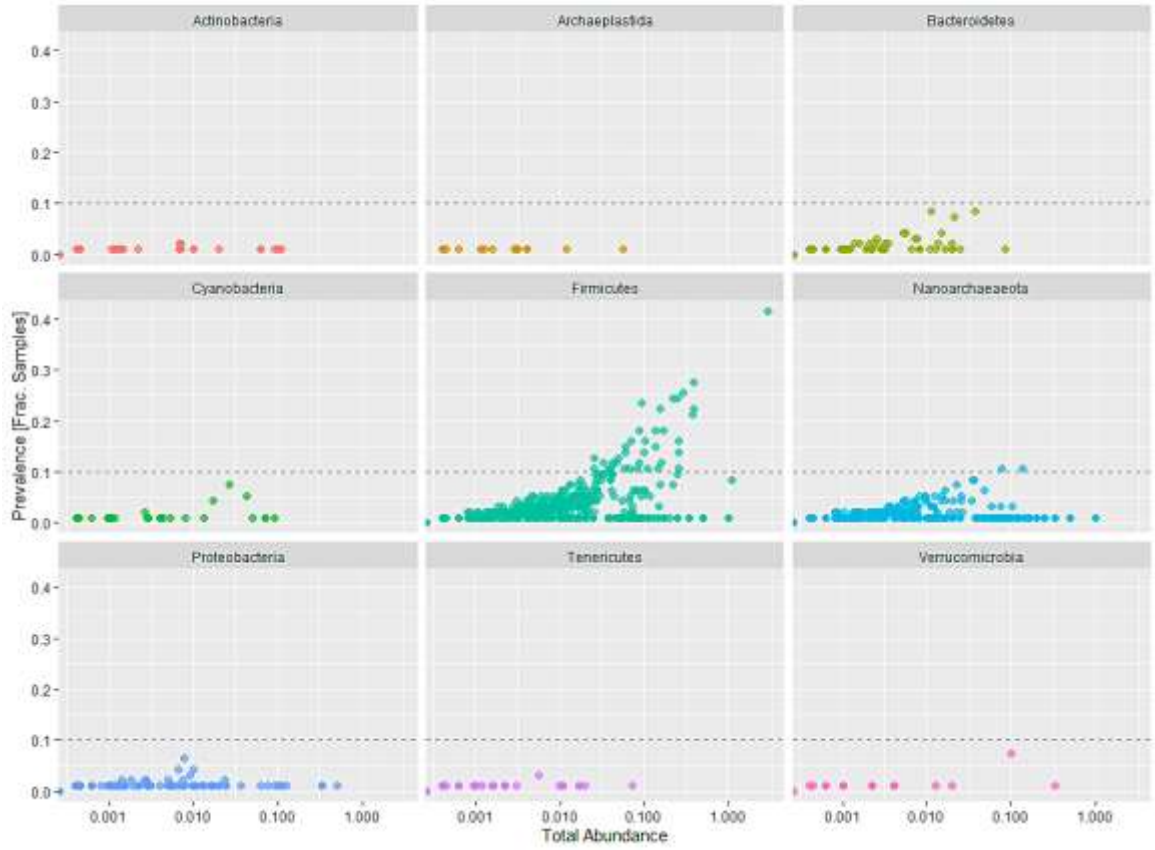


Figure 4.8: Prevalence of the most dominant phyla as observed across all the dietary treatments in broiler samples. Prevalence is shown in relation to total abundance counts.

The phylum Firmicutes significantly increased in T3 compared to T4 **(Figure 4.9)**. The OTUs clustering in the Families of *Enterococcaceae, Lactobacillaceae,*

Ruminococcaceae and *Lachnospiraceae* were predominant in most of the samples **(Figure 4.10A)**. At Genus level, the cumulative relative abundance across the different diets showed *Enterococcus*, *Lactobacillus* and *Ruminococcus* to be the most predominant genre **(Figure 4.10B)**. The genus *Enterococcus* increased in diets that had BSFL inclusion than the control diet and was most abundant in the diet that had T2 while the genus *Ruminococcus* was higher in the control diet than the rest **(Figure 4.10B)**. *Lactobacillus* increased significantly in T1 compared to T4. Across all the gut segments sampled, the genus *Lactobacillus*, *Enterococcus*, *Blautia* and *Alistipes* dominated along this part of the guts. *Lactobacillus* was dominant in the crop, gizzard, duodenum, small intestines, large intestines and cecum. *Enterococcus* was dominant in the oesophagus while *Alistipes* was dominant in the proventiculus **(Figure 4.11).** The highest microbial diversity was observed in the ceca while the lowest diversity was in the duodenum, small intestines and large intestines.

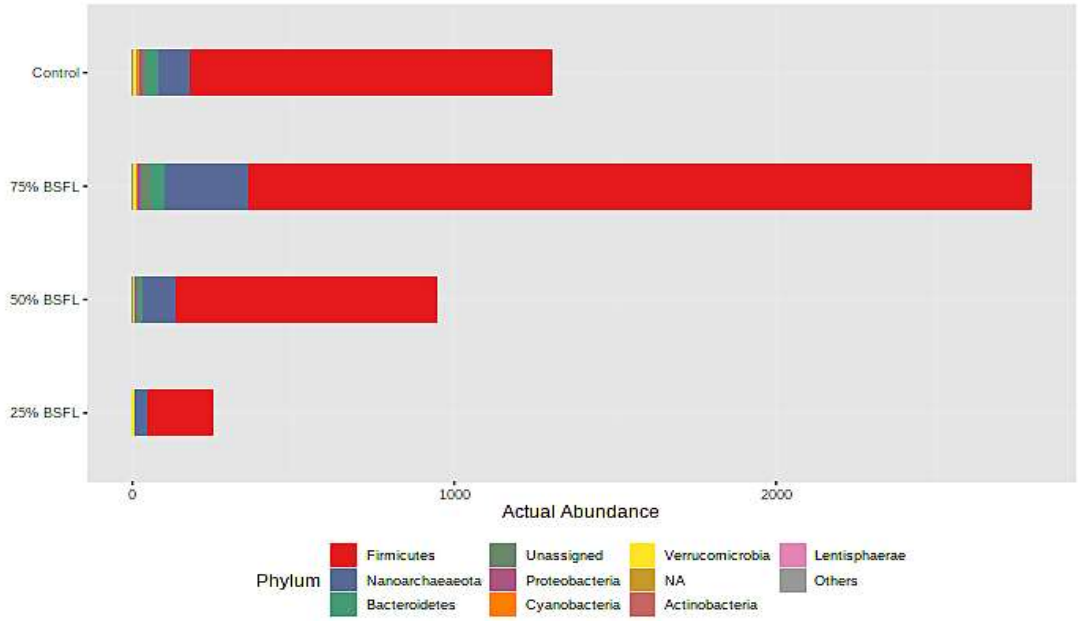
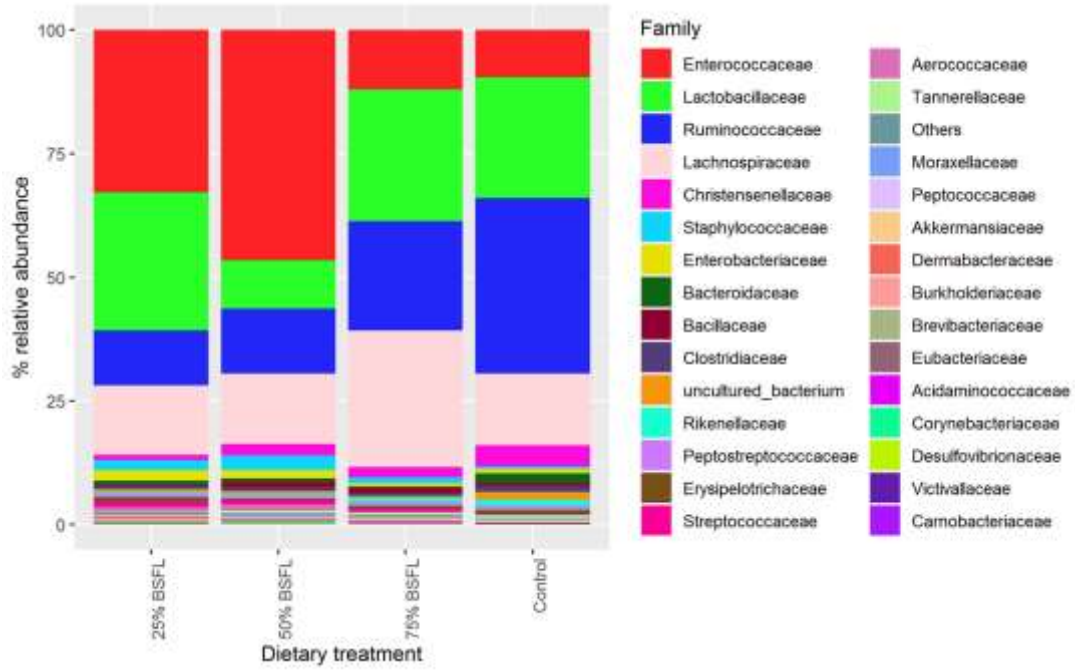


Figure 4.9: Cumulative relative abundance of predominant phyla observed across all the dietary treatments in broiler samples.

(A)



(B)

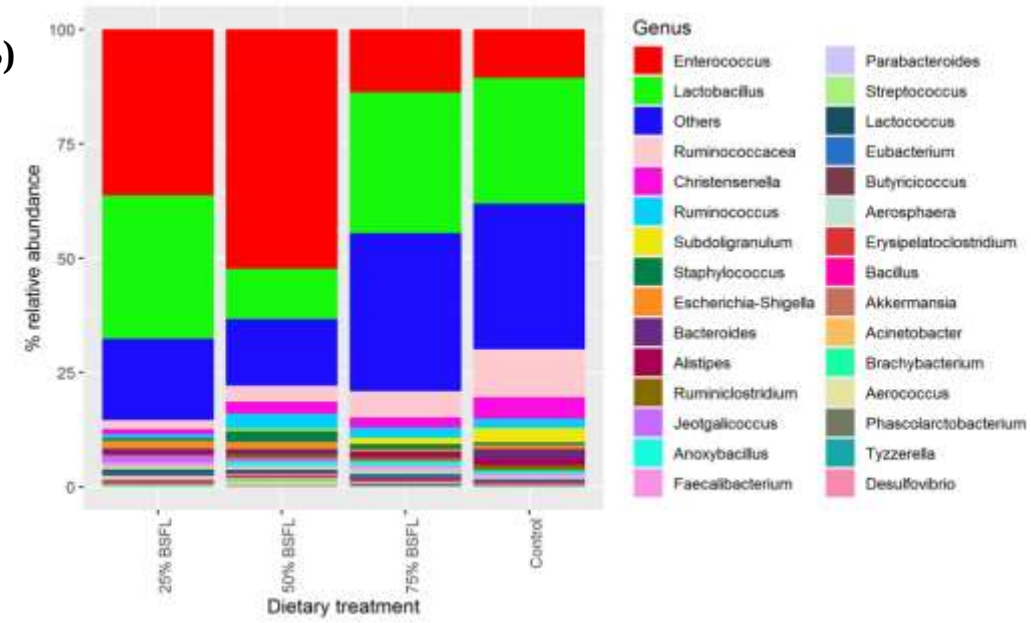


Figure 4.10: Cumulative relative composition of bacteria OTUs at (A) Family level and (B) at Genus level observed across the different dietary treatments in broiler chicken

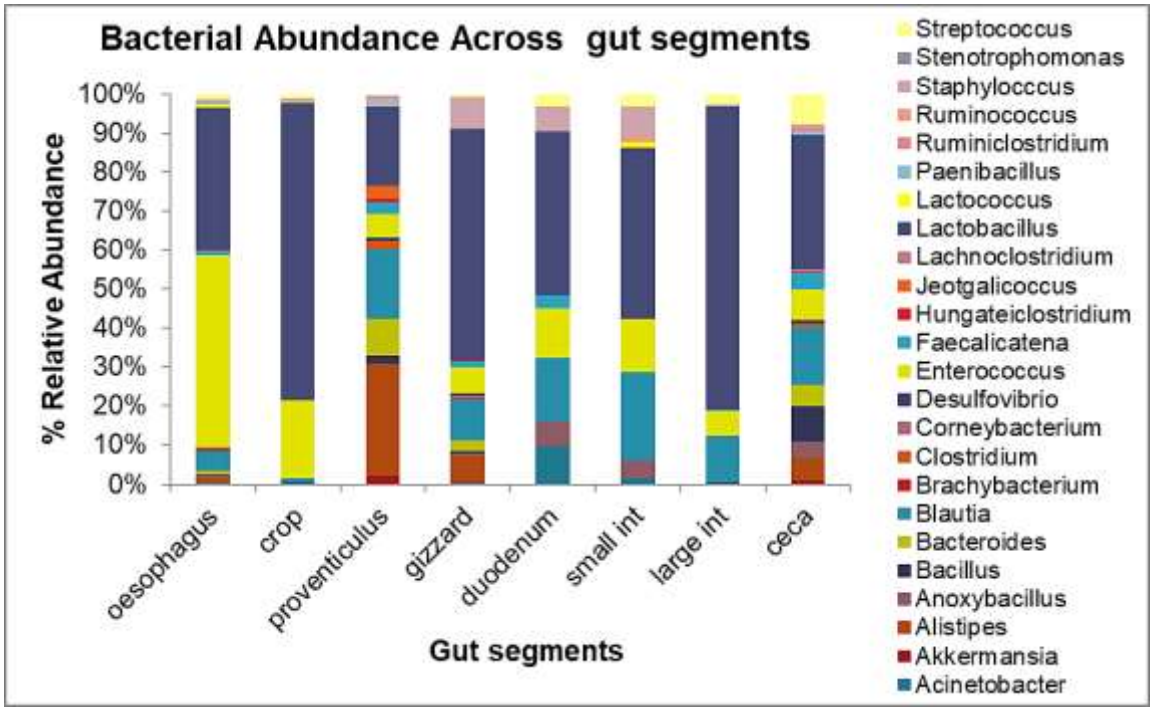


Figure 4.11: Stacked bar graph showing the relative abundance of bacteria across the different broiler chicken gut segments.

4.2.2 Alpha and Beta Diversity

Alpha diversities were assessed by Shannon and Chao 1 indices and the diet with 75% BSFL (T1) inclusion recorded the highest diversity according to Shannon index and the highest bacterial OTU richness according to Chao 1 index. The diet treatment with 25% BSFL (T3) inclusion had the lowest diversity **(Figure 4.12)**. However, this difference was not statistically significant (*P-value >0.05*).

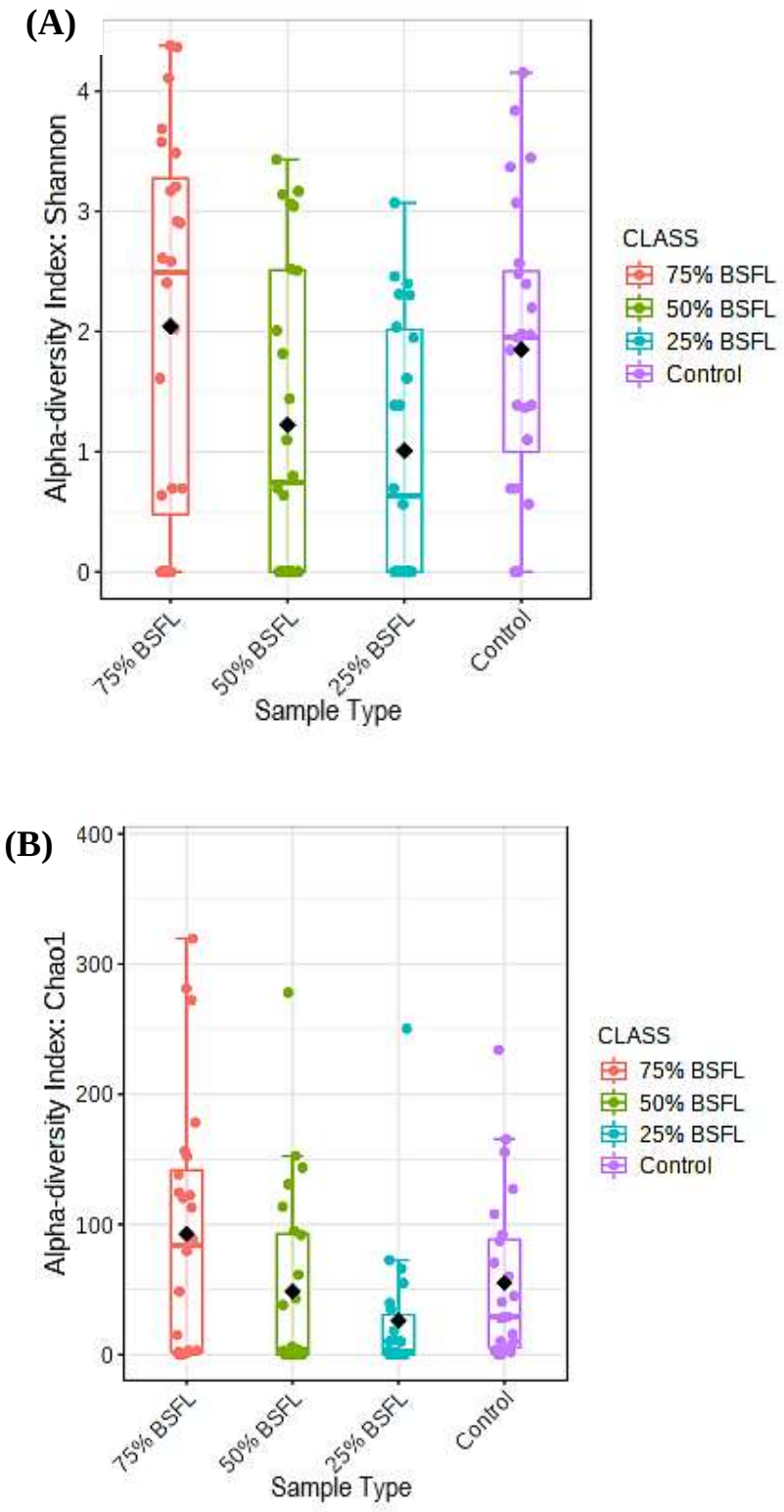
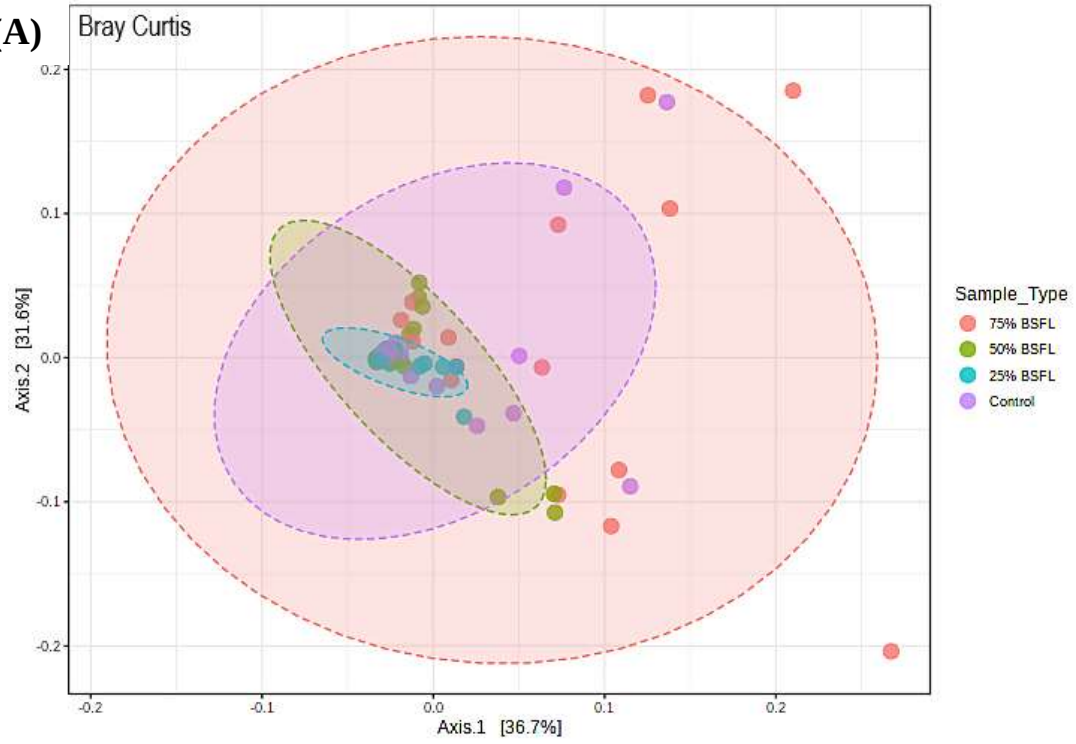


Figure 4.12: Alpha diversities measure by (A) Shannon index and (B) Chao1 index.

Beta diversity calculations showed that very little changes were observed in the abundance of microbial communities present in the samples. All samples shared almost similar communities regardless of diet treatment. When phylogenetic distances were considered via both Bray Curtis **(Figure 4.13A)** and unweighted unifrac distances **(Figure 4.13B),** the bacterial communities clustered together regardless of diet treatment with some outliers identified. This similarity was confirmed by PERMANOVA (*p-value > 0.05*)

(A)



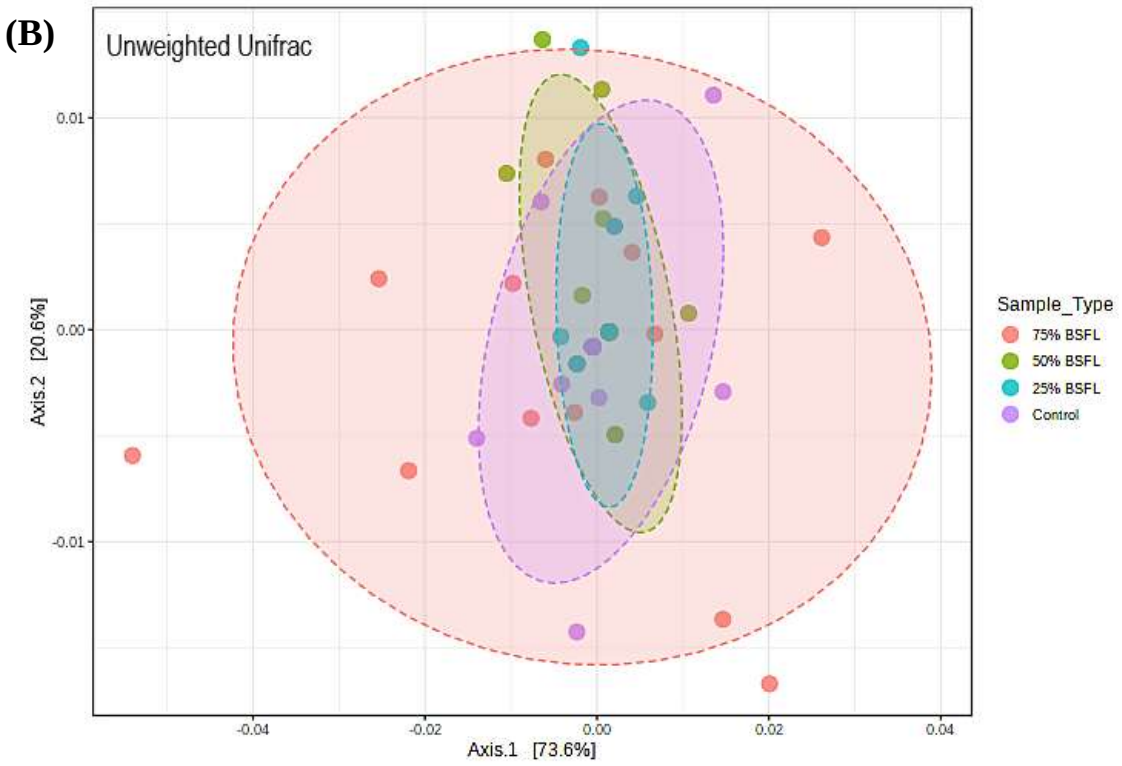


Figure 4.13: Beta diversity PCoA plot based on (A) Bray Curtis distance and (B) unweighted unifrac distance dissimilarity method between the different broilers dietary treatments. [PERMANOVA] R-Squared: 0.049; P-value > 0.001.

A Venn diagram showed that 47 bacteria genera were common across all the 4 treatments. The control (T4) treatment had the most unique genera totalling to 105 followed by the 75%BSFL treatment (T1) with 88 unique genera. Most of the OTUs were shared between the control diet (T4) and the 75% BSFL (T1) diet (**Figure 4.14**).

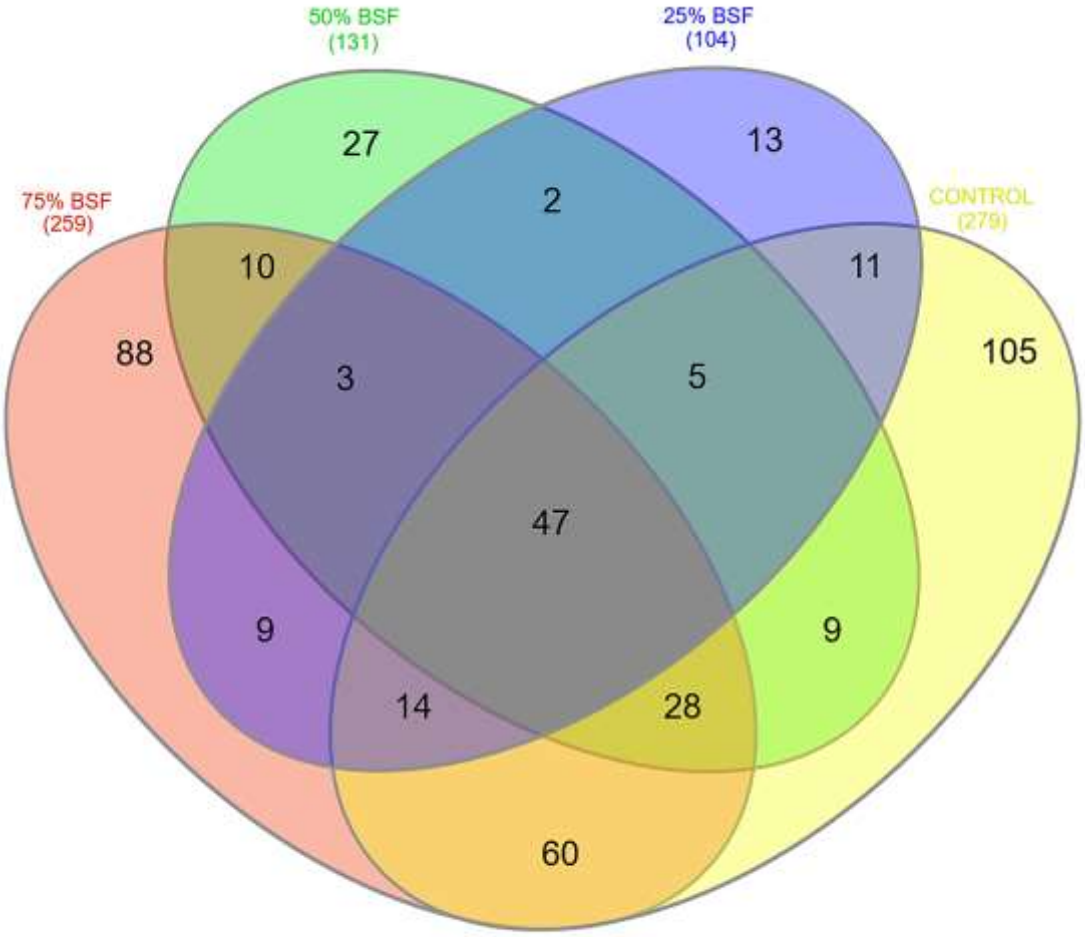


Figure 4.14: Venn diagram showing the unique and shared OTUs between the dietary treatments in broiler samples.

CHAPTER FIVE

DISCUSSION

The use of low-cost insect-based feed as an alternative to the expensive conventional fish/soya bean meal based has gained global research attention recently to improve the sustainability of poultry production (S. H. Khan, 2018, Biasato et al., 2017). The impact of the novel poultry feed on the gut microbiome of chickens has received inadequate research interest. This study aimed to analyze the effects of feeding BSFL-based meal on the gut microbial composition of both the layers and broilers chicken using Oxford Nanopore 16S sequencing. Understanding the modulation of the gut microbial community influenced by factors such as diet is very crucial as an essential component to chicken health (Lin et al., 2018, Kers et al., 2018). In this study, it observed that the partial replacement of conventional fishmeal with BSFL-based diets had a positive influence on the gut microbial communities of the chickens. This report is related to a previous study by Coretti et al, (2017) which reported that inclusion of BSF based meal in laying hens diets did not have a negative effect on their nutrition and gut microbiota composition. High microbial richness and diversity was observed in diets that had high concentrations of BSF, especially in the treatment diet which had 100 % BSF inclusion. Several studies have recently reported that a high bacterial diversity tends to be associated with a healthy gastrointestinal tract which improves the overall health status of poultry (Coretti et al., 2017, Huyben et al., 2019). Therefore, the layer chicken fed BSF in this study may have a healthier gut microbial community since the species richness and diversity was higher than the standard conventional fishmeal diet. Across all treatment diets for the layer chicken, the most predominant bacteria phyla identified included Firmicutes (90 %), Bacteroidetes (7 %), Proteobacteria (2 %), and Actinobacteria (1 %). Lesser portions of Cyanobacteria, Deferribacteres, Spirochaetes, Tenericutes, and Verrucomicrobia were also observed. The findings support previous reports showing Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria to be among the dominant phyla across all parts of the chicken gastrointestinal tract (Kohl, 2012; Oakley et al., 2014; Shang et al., 2018). The phylum Firmicutes represents the most beneficial bacteria classes that are important in both animals and humans including Lactobacillales, Bacillales,

Clostridia, and Veillonellales (Rychlik, 2020). The most abundant genera observed across all the dietary treatments were mainly *Lactobacillus, Enterococcus,* and *Blautia* which are associated with the phylum Firmicutes, and *Bacteroides* which are associated with the phylum Bacteroidetes. However, diets with BSF meal inclusion supported the same microbial communities as the standard fishmeal diet at both phylum and genus levels with only a few taxa changing in abundance showing that the BSFL diets are a suitable replacement for the conventional fishmeal. A significant increase in the lactic acid bacteria (*Lactobacillus, Enterococcus* and *Bacteroides*) was observed in the diets that had a higher concentration of BSF, thus implying BSFLF might have the potential to boost the probiotic activities in the gut of the chicken. Lactic acid bacteria (LABs) have been known to produce bacteriocins which are said to inhibit the pathogenic bacteria in the gut thus they may be used as indicators of a healthy gut (Huyben et al., 2019).

Across the entire gut of the layer chickens, the highest diversity of bacteria was observed in the ceca and crop, while the lowest diversity was recorded in the esophagus, proventriculus, and small intestines. This low density could be because of the short passage time in these parts and the dilution of the digesta in the small intestines (Yeoman et al., 2012). *Lactobacillus,* of the family Lactobacillaceae, was observed as the most dominant taxa in all the dietary treatments and all the gut parts with the highest read counts being recorded in the crop and the cecum. This family of bacteria are beneficial commensals that have been studied extensively in the food and medicine industries concerning both humans and animals (Yan et al., 2017). *Lactobacillus* species being an intrinsic component of the intestinal microflora are now being acknowledged as the most efficacious probiotic candidates. These Lactic acid producing bacteria have been reported to have various health benefits in human health some of which include stimulation of immune responses, anti- cancer activity, prevention and treatment of inflammatory diseases (Toshimitsu et al., 2017), Lactose intolerance alleviation, antimicrobial activity against resistant pathogens and respiratory viral infections (Ayeni et al., 2009). *Lactobacillus* species have been identified to produce lactic and acetic acids which help in lowering the pH value in the gut and competing with potential pathogens for nutrients through direct competitive interactions thus suppressing the colonizing potential of some major

pathogens (Yan et al., 2017). In addition, *Lactobacillus* has been used as an alternative to antibiotic growth promoters in poultry and as feed supplements to improve growth performance (Torok et al., 2011). This may reduce the administration of antibiotics in feed in poultry production systems. Antibiotics have been extensively used as growth-promoting agents in poultry production and this has prompted them to be under intense scrutiny due to their adverse effects such as the increase in emergence of resistant pathogenic bacteria and the risk of zoonotic transfer of resistant pathogens to humans (Vieco-Saiz et al., 2019). *Lactobacillus* is being considered a safe alternative to antibiotics because of its mode of action as a probiotic in animals. In poultry health, probiotics have been identified to have beneficial effects on intestinal histological changes, growth performance, and immunomodulation as well as improving the sensory characteristics of poultry meat (Kabir, 2009). Alayande, (2020) reported in his studies that *Lactobacillus* stimulates the growth of beneficial microbes in animals preventing the colonization of enteric pathogens through their ability to produce antimicrobial substances. These results are similar to a study by Dowarah et al, (2017) who observed that the use of *Lactobacillus* as a probiotic in pig production improved the growth performance with increased growth rates and feed conversion rates. These various studies show that *Lactobacillus* as a probiotic has a great potential as an alternative to antibiotic agents in poultry production. *Bacteroides*, *Enterococcus* and *Blautia* were also identified as abundant beneficial bacteria from our samples and their role in the degradation of polysaccharides to produce short chain fatty acids (SCFAs) such as acetic acid, butyrate, propionate and lactate has been well documented (Rychlik, 2020). These SCFAs are known to reduce undesirable and detrimental bacteria by inhibiting acid-sensitive pathogens and promoting mucosal growth by stimulating gut epithelial cell proliferation. They also act as a source of energy and stabilize glucose levels in poultry (Borrelli et al., 2017).

Notably, some pathogenic reads of the genus *Campylobacter* were identified including species of *Campylobacter jejuni, Campylobacter lari, Campylobacter coli*, and *Campylobacter upsaliensis*. However, these read counts were in very minimal quantities which are among the permissible levels as set by the Standards Projects Committee, developed by (Kenya Bureau of Standards, 2020) on the use of insects for feed. From a recent study conducted by (Tanga et al., 2021) on microbial dynamics of

the black soldier fly reared on different organic substrates, *Campylobacter* bacteria was identified as one of the prevalent species in the gut of unprocessed black soldier flies. In regards to this study, *Campylobacter* reads were observed in the ceca of layer chicken fed on diet 4 (T4) and 5 (T5) which had 75 % and 100 % BSF, respectively, suggesting that black soldier fly may act as a mechanical vector transmitting the pathogenic strains to the chicken through the feed in very high concentrations. *Campylobacter* is associated with the phylum Proteobacteria and they are the main pathogenic bacteria identified in poultry which in very high amounts can cause campylobacteriosis in humans (Fravalo et al., 2015). As a result, we recommend the use of safe and sterile substrates when rearing the BSF for feed to eliminate such contaminants that may have an impact on the advantages of using BSF larvae in poultry production.

The occurrence of antibiotic resistance genes (ARGs) was analyzed which were present in the layer samples even though the chicken were not given any antibiotics during the rearing period. Novel ARGs may be present in poultry even without the administration of antibiotics and this may be brought about by horizontal gene transfer from the environment (Y. Liu et al., 2020). Over the years, ARGs have been reported as an emerging environmental pollutant and this is because soil, sewage, manure, and other animal wastes have been considered reservoirs for ARGs in the environment leading to horizontal gene transfer between the bacteria and the animals (B. Li et al., 2015). The presence of ARGs may have also been brought about by known antibiotic resistance agents such as *Campylobacter, Clostridium,* and *Staphylococcus* which were identified in the ceca of the chicken samples. The cumulative abundance of the antibiotic resistance genes from the samples showed that resistance read counts reduced greatly with an increase in BSF concentrations. This is evident more so in dietary treatments that had 75 and 100 % BSF. Insects have generally been known to be a good source of antimicrobial peptides (AMPs) that act against pathogenic bacteria (Moretta et al., 2020). Recent studies by Spranghers et al., (2018) showed potential antimicrobial agents in weaned piglets fed on BSF prepupal-based meal. Since BSF are reared on organic waste substrates, they may be very rich in AMPs which are bactericidal that control resistant pathogens (Park et al., 2014, Park et al., 2015, Moretta et al., 2020). In this study, the decrease of antimicrobial resistance genes in

diets with high concentrations of BSF could probably have been caused by the presence of AMPs in the BSF meal that might have suppressed the resistant bacteria in the samples. Moreover, the lactic acid-producing bacteria which were found to be in surplus in diets with high concentrations of BSF could have led to the reduction of ARGs read counts in these diets. This is because LABs have been known to produce antimicrobial metabolic compounds that reduce resistant pathogens (Vieco-Saiz et al., 2019). The antimicrobial activities of LABs against resistant bacterial strains have been reported by many authors. A study done by Dec et al, (2018) revealed that *Lactobacillus* isolates from chicken produced active compounds with antagonistic properties against *C. jejuni* and *C. coli*. *Lactobacillus casei* isolated from yogurt and cow milk has been reported to inhibit multi-drug resistant *Shigella* species (Mirnejad et al., 2013). Casey et al, (2007) also reported that *Lactobacillus* species had antimicrobial activity against pathogenic *Salmonella* in pigs. This may indeed lead to a shift away from the use of antibiotics in poultry production. However, further investigation on novel antimicrobial peptides from BSF and the presence of antimicrobial resistance genes needs to be given adequate research attention**.**

In the broilers' study, the effects of combining BSFL and *Desmodium* into the feed ratio on the gut bacteria community when compared to those fed conventional feeds were presented using Oxford nanopore sequencing technology. According to the study findings, a BSFL-based diet can replace fishmeal in broiler diets without negatively impacting their gut microbiome. These results are similar to the findings reported by Biasato et al, (2020) who observed that dietary inclusion of BSFL had a positive influence on the cecal microbiota and mucin composition in broilers. Firmicutes, Bacteroidetes, and Proteobacteria were detected to be the most abundant bacterial phyla across all four diets, and lesser portions of Actinobacteria, Tenericutes, Verrucomicrobia, and Cyanobacteria. In this study, the Firmicutes were predominant over the Bacteroidetes and Proteobacteria and this is in agreement with other previous reports done by (Yeoman et al., 2012; Shang et al.,2018). The gut community was colonized by bacteria families of *Enterococcaceae, Lactobacillaceae, Ruminococcaceae,* and *Lachnospiraceae* and these results are comparable to those reported by (Rinttilä & Apajalahti, 2013) on the intestinal microbiome of broiler chicken. The phylum Firmicutes contains the majority of the beneficial bacteria that

are useful to both animals and humans. Firmicutes play a key role in the relationship between the intestinal bacteria and chicken health. Several members of this phylum are used as probiotics including *Lactobacillus*, *Enterococcus,* and *Faecalibacterium* (Rychlik, 2020). The predominant bacteria genera observed across all treatments in this study were *Lactobacillus* and *Enterococcus.* However, diets with BSFL-*Desmodium* meal inclusion supported almost similar microbial communities as the standard fishmeal diet at both phylum and genus levels with only a few taxa changing in abundance. Across the gut segments, the highest microbial diversity was observed in the distal part at the caecum while the lowest diversity was in the duodenum, small intestines, and large intestines. The microbial composition density in the caecum may have increased due to the long feed retention time while in the other parts, there is low pH and rapid passage of the gut contents (Yeoman et al., 2012). The proximal parts were mainly dominated by the *Lactobacillus* bacteria and lesser portions of *Enterococcus* while the distal part (caecum) was dominated by *Lactobacillus, Blautia*, *Bacillus*, *Enterococcus,* and *Alistipes*. Our findings are in agreement with the previous reports which found that *Lactobacillus* dominated the gut sections whereas the caecum is dominated by bacteria from Firmicutes, Bacteroidetes and Proteobacteria (Oakley et al., 2014, Rychlik, 2020)

A significant increase of the Lactic Acid Bacteria (LAB) such as *Lactobacillus* and *Enterococcus* was observed in the diets that had BSFL inclusion, especially in the diet with 75% BSFL. Studies have reported that high bacterial diversity is regarded to be beneficial to gut health because rich communities are likely to compete with pathogens for resources and colonization, preventing pathogen invasion and infection (Huyben et al., 2019). Therefore, the broiler chicken in the study might have a healthier gut community since they had a higher microbial diversity than those fed a conventional fishmeal diet. The LABs are innocuous microorganisms that can produce bacteriocins, and organic acids like lactic acid, hydrogen peroxide, diacetyl, and carbon dioxide, among other inhibitory agents. Lactic acid bacteria can counter pathogenic microbes through their competitive exclusion mechanism based on competition for binding sites and nutrients, prevention of the pathogens' adhesion, modulation of the host immune system, and reduction of toxin bioavailability (Vieco-Saiz et al., 2019). In animal production, LABs have been known to reduce the risk of infections and intestinal

disorders associated with pathogens (Vieco-Saiz et al., 2019). Therefore, LABs have several advantages as potential probiotics and can be used in place of antibiotics in poultry production. Bacteria genera belonging to *Lactobacillus*, *Enterococcus*, *Bacillus*, *Bifidobacterium*, *Streptococcus*, and *Faecalibacterium* are some of the probiotic species commonly being used and they have been identified to have a beneficial effect on broiler nutrition. Among these desirable benefits include broiler growth performance, modulation of gut microbial communities, pathogen inhibition, immunomodulation, improved meat sensory features, and enhancing broiler meat microbiological quality (Kabir, 2009). *Lactobacillus*, which was prevalent across all diets in the broilers' study, remains the utmost probiotic that has received the most attention in poultry production and animal research. This bacteria genus is predominant in the broiler chicken's gastrointestinal tract and plays an important role in chicken physiology by producing lactic and acetic acids that lower pH in the gut, competing for nutrients and adhesion sites with enteropathogenic bacteria such as *Campylobacter*, *Salmonella*, and *Escherichia coli* (Ahmed et al., 2019) and the excellent tolerance properties to acids and bile salts (L. Wang et al., 2014). Previous studies have reported the potential of *Lactobacillus* to have anti-*Campylobacter* activity against the zoonotic disease campylobacteriosis found in contaminated poultry meat (Dec et al., 2018). In addition, *Lactobacillus* has also been shown to have antimicrobial activity against *Salmonella* in chickens (Ahmed et al., 2019). In pig production, *Lactobacillus* spp. has been reported to promote improved performance, improved immune systems, and control of post-weaning diarrhea among other benefits (Dowarah et al., 2017). In human health, *Lactobacillus* spp. has been reported to have a variety of health benefits in humans, including immune stimulation, anti-cancer activity, the prevention and treatment of inflammatory diseases (Toshimitsu et al., 2017), lactose intolerance relief, antimicrobial activity against resistant pathogens and respiratory viral infections (Mu et al., 2018).

According to the broilers' results, it was found that the *Enterococcus* genus was more abundant in diets that contained BSFL compared to the control group consisting of a 100% fishmeal diet. The genus *Enterococcus* which belongs to the family Enterococcaceae could work as a probiotic albeit to a much lesser extent than *Lactobacillus*. Unlike the *Lactobacillus* probiotic strains, reports on the effectiveness

of *Enterococcus* strains as probiotics remain limited. Established probiotic species from this genus include *E.faecalis* and *E. faecium* (Hanchi et al., 2018). In humans, *E.faecium* has been shown to inhibit the growth of pathogens such as *E.coli*, *Salmonella*, *Shigella,* and *Enterobacter* making the strain suitable for use as a treatment for diarrhea, irritable bowel syndrome, immune regulation, and lowering serum cholesterol (Franz et al., 2011). In poultry research, studies have shown that *Enterococcus* as a probiotic has a positive impact on poultry health (Park et al., 2016, Kabir, 2009).In a research study done by (Samli et al., 2007), the effect of *E. faecium* on the growth performance of broiler chickens was investigated and they observed that probiotic treatment considerably improved broiler chick performance in terms of weight gain and feed conversion ratio. Another study on the *E. faecium* strain was also done by (Capcarova et al., 2011) on broilers and observed that it significantly increased the antioxidant status, bilirubin content, serum calcium content, and increased body mass of the broilers. Other potential probiotic bacteria were also identified in the broiler samples including *Faecalibacterium*, *Bacillus,* and *Streptococcus*. All these LABs are being exploited extensively and considered safe alternatives to growth promoters, antibiotics, and feed supplements to improve growth performance (Torok et al., 2011). This may indeed lead to a shift away from the use of antibiotics in poultry production.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The use of insect-based meal as a suitable alternative source of protein for poultry production is rapidly gaining international research attention, but there is limited information that demonstrates the effects of insect-based meal inclusion in chicken diets and its implications on the intestinal microbiome. There is inadequate knowledge on the potential of black soldier fly larvae meal to transfer pathogenic and beneficial microbes present in the rearing substrates through feed and their impact on the gut health of poultry. Therefore, this study focused on the assessment of the gut bacterial community dynamics and their interactions with the chickens when fed on insect-based feeds through metagenomics. From this study:

i. Gut microbial diversities were identified and various bacterial taxa that are of importance in poultry health such as *Lactobacillus* and *Enterococcus* were unraveled. These beneficial taxa could be exploited further in the poultry industry to produce probiotics and prebiotics to improve poultry health and nutrition.

ii. BSFL inclusion in both the broiler diets and layer diets has shown to be a potential and promising feed additive as a replacement for fishmeal and demonstrated the ability to modulate the gut microbial diversity by increasing the wealth of lactic acid bacteria in the chicken. This improves the overall gut health of the chicken.

iii. The use of insect and *Desmodium*-based protein mixture may be a potential and promising protein-rich additive in the chicken diets and the insect-based feed technologies can be promoted alongside push-pull technologies that involve the widespread use of *Desmodium* by over 200,000 smallholder farmers.

iv. Antimicrobial resistance genes were identified in layer chicken and this may be good for the probiotic and commensal bacteria because they may have resistance to antibiotics.

6.2 Recommendations

1. Further research can be done to identify the gut bacteria up to the species level. This will help in further understanding the interactions between the microbes and the host.
2. Further comparative studies may also be done using plant-based proteins and insect-based proteins on other species of chicken such as the indigenous chicken and the free-range chicken.
3. Transcriptomic and metatranscriptomic work should be done to confirm the presence of the antibiotic resistance genes in chickens fed with BSFL which will help in the establishment of novel genes that can be exploited in the biotechnology and poultry industry.
4. The identified probiotics such as *Lactobacillus* and *Enterococcus* can be isolated and further research done to explore their potential use in the poultry industry.

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APPENDICES

Appendix i: Layer vaccination program from day 1 up to 19 weeks

DAY	VACCINE	METHOD
1 (done in the hatchery)	Mareks + IBD- Vaxxitek NCD + IB Live (Vitabron)	Intramuscular injection Spray (done in the hatchery)
15-18	NCD + IB Live	Eye drop/Drinking water
Week 6-8	NCD killed or NCD + IB Live Fowl typhoid	Intramuscular injection Drinking water Intramuscular injection
Week 8-10	Fowl pox Fowl cholera	Wing stab Subcutaneous injection
Week 12-14	Fowl typhoid	Intramuscular injection
Week 16-19	NCD + IB Live Fowl cholera	Drinking water/Spray Subcutaneous injection

Appendix ii: Publication from a peer-reviewed journal

microorganisms

MDPI

Article

Insights into the Gut Microbial Communities of Broiler Chicken Fed Black Soldier Fly Larvae-*Desmodium*-Based Meal as a Dietary Protein Source

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Abstract: The utilization of insect-based diets to improve gastrointestinal function and gut health in poultry is gaining global attention as a promising feed additive. The objective of this study was to determine the optimal inclusion level of the full-fat black soldier fly larvae (BSFL) and *Desmodium intortum* (DI) in broiler chicken diets and to evaluate their impact on the microbial community in the gut. The bacterial communities were characterized using Oxford nanopore sequencing of the full-length bacterial 16S rRNA gene. Four dietary treatments, T1 (25% *DI* + 75% BSFL), T2 (50% *DI* + 50% BSFL), T3 (75% *DI* + 25% BSFL) and T4 (100% fishmeal + 0% *DI* + BSFL), were fed to the broiler chickens for a period of 42 days. Out of the 395,034 classified reads analyzed, the most predominant phyla identified across all the four dietary treatments were *Firmicutes* (94%), *Bacteroidetes* (3%), and *Proteobacteria* (2%). The T1 diet showed the highest alpha diversity and richness according to the Chao1 and Shannon indices. Beta diversity assessment revealed a significant influence of diet on the abundance of the microbiome. There was an increase in beneficial lactic acid bacteria with increasing inclusion of BSFL in the diets. Our findings strongly support the inclusion of BSFL into poultry diet as a promising protein source to reshape the gut microbiota for improved gut health, immune response, and food safety.

Keywords: black soldier fly; gut microbiota; poultry feed; broiler chicken; Oxford nanopore sequencing

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1. Introduction

Current global population trends have anticipated an increase in the human population to about 9.2 billion people by the year 2050, which will increase the global demand for protein, especially poultry meat [1]. Poultry has the potential to bridge the gap between the demand and supply of protein because of its high feed conversion rates [2]. In the tropics, a large number of smallholder farmers contribute significantly to food security by rearing poultry for domestic and local markets [3]. Broiler chicken meat contributes to the daily dietary needs of humans as it contains protein, minerals, vitamins (niacin, riboflavin, and vitamin B6), and other nutrients [4]. Kenya's demand and consumption of broiler chicken meat is expected to hit 165,000 metric tons by 2030, up from 55,000 metric tons in 2000 [5]. This projected increase in demand is based on rapid urbanization, middle-class growth, and ever-increasing global health views promoting poultry products as a better protein source than red meat [6]. Protein is an essential key ingredient and one of the most expensive components required in poultry feed. The most common and widely preferred conventional sources of protein in animal feed include soybean, fishmeal, cotton or sunflower seed cakes, and other plant proteins [7]. However, soybean meal and fishmeal are available in low quantities due to land unavailability for production, global cost fluctuation, human consumption of soybean and fishmeal, and other constraints

Appendix iii: Publication from peer-reviewed journal

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OPEN

Gut microbiota shift in layer pullets fed on black soldier fly larvae-based feeds towards enhancing healthy gut microbial community

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Globally, most gut microbiota-related studies have focused on broilers due to their diverse microbial communities compared to that of layer chicken. However, in Africa few studies have been undertaken despite the increasing benefits to the poultry industry. The utilization of Insect-Based diets to improve the gastrointestinal function and gut health in poultry is increasingly gaining global attention. Here, we evaluated the potential roles of commercial black soldier fly larvae-based feeds (BSFLF) in reshaping the abundance, composition and diversity of the gut microbiota of layer chickens using high throughput Oxford nanopore Minion sequencing of the full length bacterial 16S rRNA gene. Two hundred and fifty ISA Brown layer chicks were reared in pens for a period of 20 weeks. The layer pullets were allotted 5 dietary treatments that were formulated as follows: control diet (T1): 100% FM + 0% BSFL, T2: 25% BSFL + 75% FM; T3: 50% BSFL + 50% FM; T4: 75% BSFL + 25% FM, and T5: 100% BSFL + 0% FM. Sampling was done from the eight major regions including oesophagus, crop, proventriculus, gizzard, duodenum, ileum, large intestines and ceca. Out of the 400,064 classified reads analyzed, the most dominant phyla identified across the feed treatments were Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. The diet treatment with 100% inclusion levels of BSFL showed the highest intra-species alpha diversity and richness according to Chao1 and Shannon index. Intra-species beta diversity assessment revealed that the diet types significantly influenced the abundance of the microbiota, but differences between most abundant taxa were similar. There was increase in abundance of potentially beneficial bacteria (*Lactobacillus*, *Bacteroides* and *Enterococcus*) with increased inclusion levels of BSFLF in layer pullets diets. Across the different gut segments, *Lactobacillus* dominated all the eight regions and the ceca was the most diverse segment. Our findings unravel complex gut microbial shift in laying hen fed BSFLF and therefore underpins the potential roles of beneficial bacteria as promising prebiotics and probiotics in reshaping of the gut microbiota to maintain good gut health.

Insects have proven to be a nutrient—rich ingredient in animal feed, and its sustainable use is gaining momentum worldwide due to the rising costs of major protein sources such as fish meal, soybean, cotton seed cake, among others, which are used in animal feeds[1]. The rapidly increasing human population growth is anticipated to lead to increased demand for animal proteins[2,3]. In Sub-Saharan Africa, domestic chicken production forms the basis of the protein industry with more than 80% of the smallholder farmers contributing significantly to the growth of the poultry industry. But one of the major constrain hampering this industry from attaining its full production potential has been the availability and accessibility to nutritious protein rich feeds[4,5].

Protein is an essential key ingredient in poultry feeds as it is necessary for their growth, body maintenance and high carcass quality[6,7]. Protein alone accounts for over 70% of the total cost of livestock production and lack of such protein supplements continues to limit efficient poultry production[8]. In conventional farming systems,