# Effects of feed additives on gut microbial diversity of *Clostridium perfringens* challenged broilers

by

**Armann D. Reynecke**

Submitted in partial fulfilment of the requirements for the degree

**Magister Scientiae Agriculturae**

**MSc(Agric) Animal Science: Animal Nutrition**

in the Faculty of Natural and Agricultural Sciences

Department of Animal and Wildlife Sciences

University of Pretoria

Pretoria

October 2019

Supervisor: Dr. C Jansen van Rensburg

Co-supervisor: Prof. E van Marle-Köster

## Declaration

I, Armann Reynecke, hereby declare that this thesis, submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University.

_____

Armann Reynecke
Pretoria
21/10/2019

## Acknowledgements

*This thesis is dedicated to my beautiful bride and our future children.*

I would like to thank the following organisations and people:

Cargill and all the relevant personnel involved. This includes but is not limited to Stephanie Ladirat, Jean de Oliveira, Ils Van-Crombruggen and Ken Finley.

Professor Este van Marlë-Koster and Doctor Christine Jansen van Rensburg for their patience, guidance and wisdom.

Dewald Steyl for his selfless assistance and dear friendship.

My collection of parents for their ever steadfast love and support, both emotionally and financially.

My wife, Micheline, for her patience, understanding, encouragement and assistance in every way.

Finally, the God of the invisible, the Champion of all creation, the Holy Trinity. He who knows me completely, yet loves me eternally and renews His grace for me every day.

"Amid the eternal silences
God's endless Word was spoken;
None heard but He who spake,
And the silence was unbroken.

O marvellous! O worshipful!
No song or sound is heard,
But everywhere and every hour
In love, in wisdom, and in power,
The Father speaks His dear Eternal Word."

*Frederick W. Faber*

## Abstract

The interactions between host and gut microbiota are a rapidly developing field with a plethora of implications. Recent developments in identification and quantification methods of gut microbes allow for a better understanding of said interactions. For the local broiler production industry, studying the microbiome of the gastrointestinal tract (GIT) is imperative to ensure ease of transitioning into an antibiotic-free feeding practice. *Clostridium perfringens* infections are a major burden for the global poultry industry and can lead to necrotic enteritis (NE). The economic losses induced by NE in the South African broiler industry are estimated to be $68.81 million per annum. In this trial, day-old Ross 308 broiler males were randomly placed in an environmentally controlled house and subjected to eight dietary treatments with 12 replicate pens each containing 23 birds. The antibiotic growth promoter (AGP), zinc bacitracin (ZB), was used as the positive control, and three additional feed additives were used either alone or in combination. The three feed additives are classified as a direct fed microbial (DFM), an essential oil mixture (EO), and finally a mixture of EOs and organic acids (OAs). To induce *C. perfringens* infection, birds received a coccidial vaccine (Immunocox, Ceva) of 10x the prescribed dosage at 10 days of age and were orally inoculated with a broth of *C. perfringens* at 14 days of age. Both ileal and caecal digesta samples were collected at 21 and 35 days of age. Bacterial 16S rRNA sequencing was performed on these samples using a customized chip containing 100 selected intestinal bacteria. Age had the most significant effect on microbial abundance in both the ileum and the caeca. The dominant bacterial phylum regardless of age was *Firmicutes*, followed by *Bacteroidetes* and *Proteobacteria*. The ileum samples showed that microbial diversity increased with age, whereas the caecal samples revealed a reduction in diversity in the older samples. For the 35-day samples, the DFM and EO treatments increased ileal *Lactobacillus* and *Lachnospiraceae* respectively when compared to the negative control (no additives). Increased *Lactobacillus* and *Lachnospiraceae* are beneficial for broiler health and production. No significant differences were observed between the positive control (AGP) and the other treatment groups except for the EO + AGP combination group. Further research on the microbiome of the chicken's intestinal tract is necessary.

# Contents

# List of Tables

# List of Figures

# List of Abbreviations

| | |
|---|---|
| AGP | Antibiotic growth promoter |
| AMEn | Nitrogen-corrected apparent metabolisable energy |
| ANOVA | Analysis of variance |
| ANS | Autonomic nervous system |
| BSH | Bile salt hydrolases |
| CFU | Colony-forming unit |
| CLA | Conjugated linoleic acid |
| CNS | Central nervous system |
| DFM | Direct fed microbial |
| DNA | Deoxyribonucleic acid |
| EC | European Commission |
| EDTA | Ethylenediamine tetraacetic acid |
| ENS | Enteric nervous system |
| EO | Essential oil |
| FAO | Food and Agricultural Organisation of the United Nations |
| FCR | Feed conversion ratio |
| FOS | Fructooligosaccharides |
| G+C | Bacterial guanine and cytosine content |
| GHG | Greenhouse gas |
| GIT | Gastrointestinal tract |
| GOS | Galacto-oligosaccharides |
| ME | Metabolisable energy |
| NE | Necrotic enteritis |
| NSP | Non-starch polysaccharide |
| OA | Organic acid |
| OUT | Operational taxonomic unit |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PRR | Pattern recognition receptors |
| rRNA | Ribosomal ribonucleic acid |
| SCFA | Short-chain fatty acid |
| SGLT | Sodium-glucose transport protein |
| Spp. | Species |
| TE | Tris EDTA |
| TCR | T-cell receptor |
| TOS | Transgalacto-oligosaccharides |
| USA | United States of America |
| ZB | Zinc bacitracin |

# *Chapter 1 – Introduction and aim*

## 1.1 Introduction

The global livestock sector is responsible for providing 1.3 billion people with an income and food for at least 800 million food-insecure people, not to mention the rest of the world that has food security (Herrero *et al*., 2013). Although the livestock sector is clearly imperative for global food security, it has a few drawbacks. It is the main reason for the reduction in global biodiversity, it utilizes 33% of the total land surface of the world, almost 30% of humanity's freshwater footprint relates to animal products and livestock is responsible for 8 – 15 % of global greenhouse gas (GHG) emissions (Herrero *et al*., 2011; O'Mara, 2011; Broom *et al*., 2013; Gerbens-Leenes *et al*., 2013; Herrero *et al*., 2013; Hyland *et al*., 2016).

It is expected that the world population will reach 9.1 billion people in 2050. This will require a significant increase of 70% of global food production, meaning an additional 200 million tons of meat (FAO, 2009). Herrero *et al.* (2013) reported the influence of biomass use, feed efficiencies, production and GHG emissions of global livestock, based on data pertaining to 28 regions, eight livestock production systems, four animal species, and three livestock products and results indicated that the production of pork and poultry are the most efficient of the production systems evaluated. In a study done by Peters *et al.* (2014), major livestock products in agricultural production systems were investigated in terms of feed conversion ratios (FCR). The authors found the feed conversion ratios of the livestock products based on carcass weight to vary from 2.6 to 23.75. The lowest value is that of broiler meat and the highest value that of beef. Feed conversion ratio is an expression of feed consumed per unit of weight gained (Hanset *et al*., 1987) and is a well-known indicator of animal/ feed efficiency. With regards to land use, production of 1 kg of chicken, pork and beef required 8.1-9.9 $m^2$, 8.9-12.1 $m^2$ and 27-49 $m^2$ of land, respectively (De Vries & De Boer, 2010). Meissner *et al.* (2014) stated that "The bulk of the increased global demand will have to come from intensive pig and poultry systems…", thereby highlighting not only the value of broiler meat for future food security, but also the expectation of the responsibility carried by the broiler industry.

*Clostridium perfringens* is a pathogenic bacteria and is one of the primary causes of necrotic enteritis (NE), a worldwide poultry disease (Caly *et al*., 2015). The disease is responsible for major economic losses for the poultry industry. The study performed by Skinner *et al.* (2010) estimated the cost of subclinical NE to amount to at least 7 US cents per broiler. The latest broiler production statistics for South Africa show that 983 million broilers were slaughtered during 2018 (SAPA, 2018). This implies an estimated cost of $68.81 million for the year due to the effects of NE in the South African broiler industry. *C. perfringens* induced NE can lead to depressed water and feed intake, reduced growth and sudden death with mortality rates of up to 50% (Cravens *et al*., 2013; Caly *et al*., 2015). Although the intestinal tract of healthy chickens contain small numbers of *C. perfringens*, it is necessary that the bird be exposed to certain risk factors before NE becomes prevalent (Wu *et al*., 2014). These risk factors need to allow an increase in the number of *C. perfringens* bacteria in order to cause NE (Antonissen *et al*., 2016). *C. perfringens* and NE is thus a highly relevant topic of study in current poultry production and research.

Antibiotics have commonly been used worldwide as a growth promoter as well as for treatment or prevention of *C. perfringens* induced NE (Caly *et al*., 2015). However, the increasing consumer awareness of

antibiotics has led to the widespread concern about horizontal gene transfer of pathogenic microbiota that are resistant to antibiotics used in human medicine (Gaggia *et al.*, 2010; Huyghebaert *et al.*, 2011). This resulted in the use of antibiotic growth promoters (AGPs) being banned by the European Commission (EC) on the 1st of January 2006 (EC Regulation No. 1831/20031). AGPs are systematically being phased out of animal production systems due to this increased awareness of antibiotic resistance. The mechanism of action of AGPs is to directly manipulate the microbial diversity of the gut by inhibiting bacterial growth (Gonzalez & Angeles, 2017). Animal scientists and meat producers worldwide now face the challenge of finding or developing alternatives that are financially competitive with antibiotics as well as effective, sustainable and safe. Several alternatives are being researched and applied quite effectively throughout the broiler industry. The use of probiotics, prebiotics, exogenous enzymes and vaccinations are some of the most well-known and proven alternatives for antibiotics (Huyghebaert *et al.*, 2011; Caly *et al.*, 2015). If one intends to find viable alternatives to AGPs, especially for *C. perfringens* challenged broilers, a better understanding of how the microbiome of broilers are affected by various feed additives could be invaluable.

Microbiota colonise the intestine of vertebrates in abundant and diverse numbers and influence the health and performance of these animals (Yin *et al.*, 2010). These microbial populations have certain functions in the chicken of which some are known and understood whilst others still require further studies. Diversity and function of microbes are no new concept or idea to research, but their relevance and importance in birds remain mostly unknown (Bjerrum *et al.*, 2006; Kohl, 2012). Establishment of healthy gut microbiota is essential for proper immune and intestinal development (Brisbin *et al.*, 2011; Rinttila & Apajalahti, 2013). Probiotics can be used to ensure an effective establishment of the bacteria early on in the life of chicks and might eliminate the future use of antimicrobials (Cisek & Binek, 2014). Microbiota are a current and relevant topic, not only in animal health but to a great degree in human health as well due to gut microbiota that play a role in immune homeostasis and regulation of predisposition to diseases (Cenit *et al.*, 2014).

Accurate and sufficient identification of microbes was a constraint when only culture-dependant methods were available but culture-independent methods radically changed this. The use of molecular markers such as 16S rRNA allow for accurate identification of species and establishment of phylogenetic relationships (Janda & Abbott, 2007). It is recommended, however, that culture-dependant methods still be integrated into conjunction with culture-independent methods when analysing microbial diversity.

## 1.2 Aim of study

The aim of this study was to assess the ileal and caecal microbiome, using 16S rRNA sequencing methods, of *C. perfringens* challenged broilers that received different feed additives and an AGP, alone and in combination. The objectives for this study were as follows:

1. To assess the impact of three feed additives on ileal and caecal microbiota of *C. perfringens* challenged broilers and compare their effect with an AGP.
2. To assess the impact of various combinations of feed additives and AGP on ileal and caecal microbiota of *C. perfringens* challenged broilers.
3. To evaluate how the microbiota diversity changes from 21 days to 35 days of age in broilers.

## 1.3 Hypotheses

**Null Hypothesis:** The three feed additives will have no effect on the microbiota of the ileum and/ or caeca of broilers challenged with *C. perfringens*.

**Alternate Hypothesis:** The three feed additives will have an effect on the microbiota of the ileum and/ or caeca of broilers challenged with *C. perfringens*.

**Null Hypothesis:** The three feed additives' effect on the microbiota from the ileum and/ or caeca of broilers challenged with *C. perfringens* will differ significantly from that of the AGP.

**Alternate Hypothesis:** The three feed additives' effect on the microbiota from the ileum and/ or caeca of broilers challenged with *C. perfringens* will not differ significantly from that of the AGP.

**Null Hypothesis:** The microbiota diversity of the ileum and/ or caeca of broilers challenged with *C. perfringens* will differ between 21 days and 35 days of age.

**Alternate Hypothesis:** The microbiota diversity of the ileum and/ or caeca of broilers challenged with *C. perfringens* will not differ between 21 days and 35 days of age.

# *Chapter 2 – Literature review*

## 2.1 Introduction

Developments with regards to sequencing technology are quite recent and the interest in the interaction between host and gut microbes is profound. The multitude of studies performed on the human gut microbiota dwarfs the amount of scientific papers on broiler gut microbiota. In this literature review, several key topics are addressed in an attempt to highlight the relevance of this study and to develop a better understanding of the establishment and function of gut microbiota and its importance for animal production systems. Additionally, in order to stay relevant with the objective of this study, literature on *C. perfringens*, feed additives and microbial identification methods are also reviewed.

## 2.2 Establishment of gut microbiome

The gut microbiome is defined as the sum of the genomes of all microbiota present in the gastrointestinal tract (GIT) (Wang & Kasper, 2014). The commensal microbiota is a diverse and dynamic system and has been referred to as a "super organism" (Aziz *et al*., 2013). The microbiome has recently been receiving more attention as an important part of healthy gut functionality. Gut development (Blake & Suchodolski, 2016), immune function (Kohl, 2012) and provision of dietary nutrients (Rinttila & Apajalahti, 2013) are some of the functions that underline the importance of the gut microbiota, and will be discussed later within this chapter.

Several theories are involved in the early establishment of gut microbial populations. From microbiome research done in humans, the idea of a sterile gut at moment of birth was only recently challenged. Research now shows that the uterine environment in fact is not free of bacteria, hence colonisation might begin before birth (Greenhalgh *et al*., 2016) Research indicate that the initial diversity is low and then gradually increases throughout the development of the infant (Clemente *et al*., 2012). The GIT of the human infant is continually colonised after birth by a plethora of microorganism species after which the density, or microbial load of the GIT reaches a plateau at weaning (Benson *et al*., 2010). The development of the gut microbiota population composition appears to be highly influenced by the environment to which the infant is exposed to. This theory is supported by a study done by Yin *et al.* (2010) which states that human individuals do not share a common core microbiota. However, research done by Benson *et al.* (2010) states that the "core measurable microbiota" behave as a "complex polygenic trait" (or quantitative trait). This implies that the composition of the core measurable microbiota is partly under the control of the host's genome, and is thus also heritable. In the late 1990's, Agnes Wold, a professor of clinical bacteriology, proposed that a delay in the bacterial colonisation of infants in Sweden was the possible reason for lower immune tolerance (Willyard, 2011). Since then several hypotheses have been developed and researched with regards to gut microbiota and their functions, not only in humans but in livestock as well.

A number of scientific papers consider the alimentary tract of the chicken to be sterile at hatch and that microbial colonisation is also initiated at hatch (Chambers & Gong, 2011; Rinttila & Apajalahti, 2013; Cisek & Binek, 2014; Stanley *et al*., 2014a; O'Callaghan *et al*., 2016). This theory seems reasonable since the chick is not exposed to any environmental microbes prenatally. This theory was developed by studies using culture-based methods. New technology in culture-independent methods enabling the use of molecular markers have recently invalidated the idea of a germ-free chick at hatch (Deeming, 2005; Pedrosa, 2009). Kizerwetter-Świda

& Binek (2008) initially found that microbial colonisation only started during hatch but, further reports indicated gram-positive cocci present in certain embryos during the final stages of incubation, and that *Enterococcus* spp. were most common. The presence of gram-positive cocci found in egg albumen and yolk is also proof of the ability of certain microbes to penetrate the egg shell before hatch. Deeming (2005) found microbiota to be present in the yolk sac of one-day-old chicks while Pedrosa (2009) made use of molecular and microscopic techniques to search for microorganisms in the intestine of an 18-day old chick embryo. Results from Pedrosa (2009) revealed the presence of a diversified microbiome already present within the intestinal tract after the $16^{th}$ day of incubation and concluded that "the chick microbiota came first".

The development of the microbiome is the process by which the commensal gut microbial population grows, diversifies and stabilises and is referred to as "ecological succession". After hatching, the bacterial concentrations increase rapidly. Studies reported bacterial densities in the proximal and distal intestine that reached $10^8$ and $10^{10}$ cells per gram of digesta in only 1 day after hatching (Chambers & Gong, 2011; Rinttila & Apajalahti, 2013; Cisek & Binek, 2014). In less than one week, microbiota levels can reach a maximum of $10^9$ cells per gram of ileal digesta and $10^{11}$ cells per gram of caecal digesta (Rinttila & Apajalahti, 2013). Initially, the gastrointestinal tract (GIT) of the day-old chick is colonised by facultative aerobes (microbes that ferment in the presence of oxygen) such as *Enterobacteriaceae*, *Lactobacillus* and *Streptococcus* that dominate the ileum (Rinttila & Apajalahti, 2013). In the caeca, anaerobic (microbes that ferment in the absence of oxygen) gram-negative and gram-positive non-spore forming rods and cocci (including species of clostridia) have been identified (Torok *et al.*, 2011). These anaerobes and gram-positive cocci populations should increase during the lifetime of the broiler until it reaches a plateau at 28 days of age (Mountzouris *et al.*, 2010). The concentrations of gut microbial populations will be maintained at constant levels due to unavailability of open attachment sites on the mucus layer and epithelial cells of the intestine as bacteria compete for sites of attachment and nutrients and cannot survive unless bound to a specific site (Cisek & Binek, 2014). The initial colonisation of the gut with aerobes like *Lactobacillus* is due to the positive reduction potential of the intestinal environment at hatching, meaning it can oxidise aerobes. This is soon altered to more reducing conditions as bacteria consume available oxygen. In turn, this facilitates subsequent growth and colonisation of anaerobes (Rinttila & Apajalahti, 2013).

The importance of the establishment of the gut microbiome in the chick's gut is often overlooked. *In ovo* administration of chemical compounds started back in the 1980's when chicks were vaccinated *in ovo* for Marek's disease (Sharma & Burmester, 1982). In this study, they found that embryonically vaccinated chicks showed better resistance to disease challenge compared to chicks that were vaccinated post-hatch. This discovery led to the furtherance of *in ovo* administration of biologics such as nutritious compounds and immunostimulants. Although *in ovo* administration can help with the establishment and development of a healthy gut environment, a current area that needs to be exploited and optimised is the method of deliverance. This involves age, location of injection, volume and dose (Roto *et al.*, 2016).

## 2.3 Functions and interactions of gut microbiota

The microbiome (genes encoded by the microbiota) has several functions with regards to metabolism and immunity. All these functions are related to gastrointestinal health and overall health of the animal. Commensal intestinal bacteria can be altered with the use of direct fed microbials (DFMs) in order to stimulate a more favourable microbiome. Benefits associated with these microorganisms include assisting with digestion, synthesis of dietary compounds, gastrointestinal development (Chambers & Gong, 2011), processing of toxins (Kohl, 2012), inflammatory immune responses, host energy metabolism, synthesis of vitamins and competitive exclusion of pathogens (Rinttila & Apajalahti, 2013; Cisek & Binek, 2014).

Cisek & Binek (2014) referred to the microbiome as "vital to many aspects of host physiology". O'Callaghan *et al.* (2016) referred to the microbiome as "virtual endocrine organ" and stressed its importance in domestic and farm animal endocrinology. The gut microbiome has a broad and not yet fully understood platform of action but mostly functions and interacts through two main components namely nutrition and immunity. Current knowledge reveals that there is an ongoing interaction between the host and the commensal gut microbiota (Rinttila & Apajalahti, 2013). Understanding and exploiting how the microbes interact with the host is imperative for improving broiler health and production.

The gut microbes provide several nutritional benefits to the host. Microbiota are known for their ability to assist with the digestion of dietary compounds that are indigestible by the host (Cisek & Binek, 2014). Microbes have the ability to ferment some of these non-digestible energy sources such as non-starch polysaccharides (NSPs) and produce short-chain fatty acids (SCFAs) (Rinttila & Apajalahti, 2013; Cisek & Binek, 2014; O'Callaghan *et al.*, 2016). The production of SCFAs are one of the main mechanisms through which microbes affect the host's health and nutrition. These end products from carbohydrate and protein fermentation provide the host with a source of energy and increase the efficiency of utilisation of dietary energy, therefore increasing nitrogen-corrected apparent metabolisable energy (AMEn) (Evans *et al.*, 2013; Cisek & Binek, 2014). The major SCFAs produced are butyrate, acetate and propionate (Kelly, 2010; Evans *et al.*, 2013; Pan & Yu, 2014) and their main site of synthesis and absorption is the proximal large intestine (O'Callaghan *et al.*, 2016). This provides the host with a source of energy that would otherwise have been lost. Many animals are adapted to utilise this function to their maximum benefit. Animals like ruminants have large fermentation chambers where microbes digest carbohydrates and produce SCFAs as an energy source to the host (Van Soest, 1994). In ostriches, it has been found that up to 75% of their metabolisable energy (ME) requirement is supplied through microbial fermentation in the hindgut (Swart *et al.*, 1993; Kohl, 2012). With regards to broilers specifically, good bird performance is associated with caecal bacterial communities that degrade cellulose and resistant starch (Stanley *et al.*, 2014a).

In addition to providing the host with energy, SCFAs also have an effect on the development and health of the GIT of poultry. The major SCFAs produced from microbial fermentation is known as an energy source of the gut epithelial cells (colonocytes) and stimulates their growth and proliferation (Chambers & Gong, 2011; Rinttila & Apajalahti, 2013; Cisek & Binek, 2014; Pan & Yu, 2014; O'Callaghan *et al.*, 2016). In turn, this results in increased villi height and intestinal weight and therefore improved absorption. Butyrate and other SCFAs are imperative for normal gut development and maintenance (Chambers & Gong, 2011). In addition to the SCFAs produced by microbes mentioned earlier, microbiota also produce fatty acids like conjugated linoleic

acid (CLA), which seems to be associated with reduced fat in animals and has also been shown to have anti-carcinogenic effects (Cisek & Binek, 2014).

Gut microbes are able to ferment proteins, amino acids and other nitrogenous compounds like uric acid (Cisek & Binek, 2014). Proteins that escape digestion in the small intestine are fermented once carbohydrate sources are depleted and also provide energy substrates to the host, although of a lesser energetic value (Rinttila & Apajalahti, 2013; Cisek & Binek, 2014). The fermentation of proteins and amino acids result in the formation of potential toxins and carcinogens. These compounds include phenols, indoles, ammonia and amines (Rinttila & Apajalahti, 2013; Qaisrani *et al.*, 2015). Uric acid formed through the mixture of urine and faeces at the cloaca (via retrograde peristalsis) might also be catabolised to ammonia that is absorbed (Kohl, 2012; Pan & Yu, 2014). These processes contribute to levels of circulating toxic compounds like ammonia. In addition to the toxic effect of these compounds, they also tend to increase the gut pH which promotes proliferation of pathogenic microorganisms (Rinttila & Apajalahti, 2013). Some of the dietary nitrogen reaching the hindgut is incorporated into microbial protein and can be a source of amino acids for the host. However, most of the microbes reside in the caeca that have no ability to digest and absorb protein and are thus excreted in the faeces. Microbial protein can only be utilised where coprophagy is possible and microbial protein can be digested in the proximal gut (Pan & Yu, 2014).

Vitamins are synthesised by gut microbiota and may serve as a vitamin source for the host (Rinttila & Apajalahti, 2013; Cisek & Binek, 2014; Pan & Yu, 2014; Cox, 2016; O'Callaghan *et al.*, 2016). Vitamin B seems to be the most prominently synthesised vitamin by bacteria (LeBlanc *et al.*, 2013). Germ-free animals have been observed to require higher dietary amounts of vitamin B and K than naturally raised animals (Hooper *et al.*, 2002). Although gut microbes have the ability to synthesise these vitamins, many of it is lost in the faeces since they cannot be absorbed in the caeca (Hooper *et al.*, 2002). Evidence for this is seen in a greater vitamin requirement in chickens housed in wire cages where coprophagy is prevented (Vispo & Karasov, 1997).

Microbes have the ability to assist with nutrient absorption (Rial, 1984; Kelly, 2010; Kohl, 2012). It was found that gnotobiotic mice inoculated with one microbial species showed 2.6 times increased intestinal expression of the SGLT-1 protein than the germ-free animals (Hooper *et al.*, 2001). The SGLT-1 protein is a sodium-glucose transporter protein and is thus evidence that the gut microbiome can influence nutrient absorption.

In contrast, the host-microbe interaction also means that the host provides some nutrients to the intestinal microbes. It is important however to stress that the diet is the ultimate source of substrates for host and microbial metabolism (Hooper *et al.*, 2002; Chambers & Gong, 2011). The commensal microbiota in the lower intestine derive their main source of carbon and energy from diet-derived complex carbohydrates not degraded in the small intestine along with dietary protein and protein derived from pancreatic enzymes and gastrointestinal secretions. In terms of function, the microbes degrade and process large quantities of substances derived from the host, for e.g. mucins and sloughed epithelial cells (Hooper *et al.*, 2002; Rinttila & Apajalahti, 2013). The goblet cells of the gut produce mucins which serve as a source of nitrogen and energy for certain commensal and pathogenic microorganisms (Pan & Yu, 2014). Besides the general mutualistic symbiotic relationship, there seems to be some degree of competition for nutrients between the host and microbiome (Pan & Yu, 2014). This is minimal in healthy birds where microbial nutrient utilisation is limited

due to the low pH and rapid passage rate of the small intestine and most digestible nutrients are absorbed by the host. However, some circumstances might promote bacterial overgrowth and thus increase the degree of competition for nutrients between host and microbiome (Pan & Yu, 2014).

Although colonisation of the gut increases the integrity of the epithelial wall, it may also inhibit nutrient absorption (Kohl, 2012). Birds have decreased intestinal surface to accommodate for flight and thus rely more on paracellular absorption. The colonisation of microbes inhibits the ability of the intestinal wall to absorb nutrients as efficiently as possible due to decreased surface area exposed for absorption. Studies with germ-free chickens have indicated that microbe colonisation decreases total absorption of glucose and vitamins (Kohl, 2012). Despite the theoretical basis of this drawback, Wostmann *et al.* (1983) found that conventionally reared animals have a 30% lower energy intake requirement to maintain their body weight than their germ-free counterparts.

Bile salts are derived from cholesterol within the host. One of its most important functions is its assistance with lipid absorption. Bile salts, also referred to as bile acids, associate with fat molecules and form micelles which can then be absorbed by the host. The surfactant nature of bile acids also gives them antimicrobial properties thus controlling the growth of bacteria within the small intestine (Evans *et al.*, 2013). Certain microbiota residing in the hindgut produce specialised enzymes called Bile Salt Hydrolases (BSH) which deconjugate the bile acids that escape reabsorption in the terminal ileum. Modification of the primary bile acids produce secondary bile acids which are partially reabsorbed and partially excreted (Evans *et al.*, 2013; Blake & Suchodolski, 2016).

There is a clear interaction between the gut microbiome and the diet. Dietary properties have a direct impact on the intestinal environment and hence its microbiome. In humans, a study done by De Filippo *et al.* (2010) revealed that gut microbes adapt to their host's diet and environment. Choct *et al.* (1996) suggested that high levels of non-starch polysaccharides lead to increased digesta viscosity, increased mean retention time and a reduction in nutrient digestibility which favours the growth of anaerobic microbes such as the likes of *C. perfringens*. In contrast, fibre fermentation in the GIT stimulates the growth of certain cellulolytic bacteria which produce SCFAs that lower intestinal pH and therefore promotes a healthy gut microbiome (Jha & Berrocoso, 2015). This idea was first coined by Gibson & Roberfroid (1995) as the "prebiotic effect". Dietary protein source, especially animal protein, favours the growth of pathogens like *Campylobacter spp.* and *C. perfringens.* in the hindgut of the chicken (Pan & Yu, 2014; Qaisrani *et al.*, 2015). Figure 1 below illustrates the interaction between the gut microbiome, infection, intestinal defence and malnutrition.

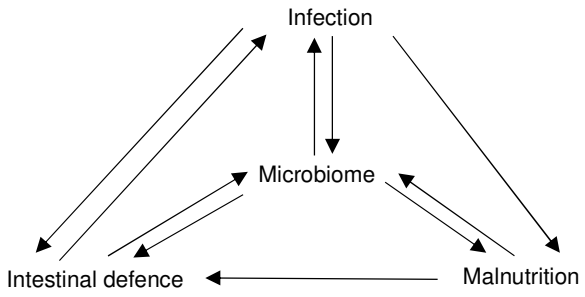



**Figure 2.1** Interaction between nutrition, intestinal defence, infection and the microbiome (Kelly, 2010)

The microbiome interacts closely with host immunity. The gastro-intestinal tract's mucosal surface is the largest bodily surface that has contact with the external environment (Rinttila & Apajalahti, 2013). As mentioned earlier, the establishment and development of the gut microbiome is critical, especially for host immune development (Yin *et al*., 2010; El Aidy *et al*., 2015; Min & Rhee, 2015; Schokker *et al*., 2015). The avian immune system consists of two functional components namely innate immunity and acquired immunity (Chambers & Gong, 2011; Korver, 2012). Acquired immunity functions through a specific response and has "memory". This means that after its first exposure to a pathogenic species, the immune system will develop an appropriate response mechanism for that specific antigen and respond much more rapidly upon the next exposure (Korver, 2012). Innate immunity is regarded as "the first line of defence" and is a non-specific response that try to prevent or remove any pathogenic organisms from interacting with the host (Korver, 2012). Mucosal immunity is based on innate immune function and its ability to differentiate between potential pathogens and harmless antigens (Grenham *et al*., 2011).

The first line of defence in the gut consists of one layer of epithelial cells and specific protein molecules in between them (Fouhse *et al*., 2016). This acts as a physical barrier against pathogenic entry (Min & Rhee, 2015). The epithelial cells can express pattern recognition receptors (PRR) and are responsible for the recognition of pathogenic invasion. The recognition of potential pathogens activates the innate immune system and might result in inflammation and mobilisation of phagocytes that destroy pathogens (Korver, 2012). This highlights the importance of the interaction between the commensal gut microbiota and intestinal development discussed earlier. Acquired or "specific" immunity mentioned earlier consists of T-cells under the control of chemical and cytokine signals (Chambers & Gong, 2011). It is believed that the gut microbiota have the ability to modulate host immune response by having an effect on the endocrine control over the differentiation of the T-cells (Clemente *et al*., 2012). Brisbin *et al.* (2011) found that orally treating chicks with *Lactobacillus acidophilus* resulted in a modulated immune response. Crhanova *et al.* (2011) exploited the effects that natural microbial colonisation and *Salmonella enterica* infection had on immune response. Kohl (2012) stated that the colonisation and diversity of gut microbes have an effect on the development and function of the host's T-cell receptor (TCR) repertoire. Discoveries like these led to great interest in the role that the microbiome has on adaptive immunity, even in humans (Corthier & Doré, 2010; Clemente *et al*., 2012)

Another important function in which microbiota play a role is the complex system known as the brain-gut axis, with gut referring specifically to the microbiota. This system generally includes the central nervous system (CNS), the neuro-endocrine and neuro-immune systems, the sympathetic and parasympathetic arms of the autonomic nervous system (ANS), the enteric nervous system (ENS) and the intestinal microbiota (Grenham *et al*., 2011). This axis involves pathways of communication from brain to microbiome as well as from microbiota on CNS and is hence bi-directional (Wang & Kasper, 2014). Mechanisms of action have been proposed to involve neural, hormonal and immunological routes that influence ENS and CNS signaling with direct and indirect modes of action (Grenham *et al*., 2011). Evidence suggests that the vagus nerve is responsible for the communication observed between the bacteria and the brain (Bravo *et al*., 2012).

One of the primary and most well-known immunological functions of the gut microbiota is competitive exclusion (Chambers & Gong, 2011; Lourenco *et al*., 2012). This points to different interactions between the gut microbes. The potential of a pathogen to successfully infect the host is greatly dependant on its ability to

attach and adhere to the intestinal mucus layer (Stanley *et al.*, 2014a). Competitive exclusion is the process through which microbes that were established first might prevent the further establishment of other microbial species (Chambers & Gong, 2011). There are a few known main mechanisms of action of competitive exclusion. One of the most significant mechanisms is competition for site of attachment on the mucosal surface of the gut wall. The intestinal tract only has enough non-specific receptor sites for a certain number of microbes and thus creates competition between microbes for those sites of attachment and serves as a physical barrier to pathogens (Mead, 2000; Rinttila & Apajalahti, 2013).

Another benefit induced by the SCFAs discussed earlier, is its inhibiting effect on the proliferation of pathogens. This ability mainly functions through two mechanisms: lowering the pH of the intestinal contents and the existence of a certain antagonistic relationship between SCFAs and certain bacteria (Rial, 1984; Mead, 2000; Rinttila & Apajalahti, 2013; Cisek & Binek, 2014; Pan & Yu, 2014). Thus, the production of SCFAs from microbial fermentation serves both a nutritional and immunological role.

Pathogenic proliferation is also prevented by certain microbes through the synthesis of bacteriocins (Bravo *et al.*, 2012; Cisek & Binek, 2014; Yirga, 2015). Bacteriocins are small peptide molecules that can alter bacterial metabolism and might effectively reduce viable pathogens (Rinttila & Apajalahti, 2013; Yirga, 2015). Bacteriocins are also thought to be easily inactivated by endogenous endotoxins or proteolytic enzymes (Rial, 1984).

Microbes require fermentable substrates in order to be able to proliferate. Therefore, microbial organisms are continually in competition with one another for substrates (Rial, 1984; Chambers & Gong, 2011; Cisek & Binek, 2014). Limiting nutrients will inhibit pathogens when native microbes utilise those substrates as well (Mead, 2000). Table 2.1 below provides a summative overview of the functions of the gut microbiome.

**Table 2.1** Summary of functions of the intestinal microbiome (Grenham *et al.*, 2011)

| **Protective functions** | **Structural functions** | **Metabolic functions** |
|---|---|---|
| Pathogen displacement | Barrier fortification | Control of epithelial cell differentiation and proliferation |
| Nutrient competition | Immune system development | Metabolism of dietary carcinogens |
| Receptor competition | Intestinal development | Synthesis of vitamins |
| Production of anti-microbial factors | | Fermentation of non-digestible dietary residue and epithelial-derived mucus |
| Production of SCFAs | | Ion absorption |
| | | Salvage of energy |
| | | Production of SCFAs |

## 2.4 Microbial population dynamics

The gut microbiome is dynamic and the interaction between it and the host is bidirectional (Kelly, 2010; Kohl, 2012). Several attempts have been made to identify what a "normal" chicken gut microbiome should look like. The review performed by Shang *et al.* (2018) summarises the findings of numerous studies as to which microbes are typically present in the GIT of chickens regardless of age, diet and identification and quantification techniques used. Only the findings of the ileal and caecal microbiota are summarised in table 2.2 since this study only focused on the microbiota of these specific anatomical regions.

**Table 2.2** Most commonly abundant bacteria in the GIT of chickens regardless of age, diet and technique differences

| GIT region (abundance per g of content) | Bacterial phyla | Bacteria genera | References |
|---|---|---|---|
| Small intestine (mostly ileum; $10^8$-$10^9$/ g) | Firmicutes/ Low G+C, Gram positive bacteria | *Enterococcus*, *Clostridium*, *Lactobacillus*, *Candidatus Arthomitus*, *Weisella*, *Ruminococcus*, *Eubacterium*, *Bacillus*, *Staphylococcus*, *Streptococcus*, *Turicibacter*, *Methylobacterium* | Lu *et al.*, 2003; Lumpkins *et al.*, 2010; Xiao *et al.*, 2016; Siegerstetter *et al.*, 2017; Kumar *et al.*, 2018 |
| | Cytophaga/ Flexibacter/ Bacteroides/ High G+C, Gram positive bacteria | *Bacteroidetes*, *Flavibacterium*, *Fusobacterium*, *Bifidobacterium* | |
| | Proteobacteria | *Ochrobaterium*, *Alcaligenes*, *Escherichia*, *Campylobacter*, *Hafnia*, *Shigella* | |
| | Actinobacteria/ Cyanobacteria | *Corynebacterium* | |
| Caeca ($10^{10}$-$10^{11}$/ g) | Methanogenic Archaea (0.81%) | *Methanobrevibacter*, *Methanobacterium*, *Methanothermobacter*, *Methanosphaera*, *Methanopyrus*, *Methanothermus*, *Methanococcus* | Lu *et al.*, 2003; Saengkerdsub *et al.*, 2007; Qu *et al.*, 2008; Sergeant *et al.*, 2014; Xiao *et al.*, 2016; Siegerstetter *et al.*, 2017; Kumar *et al.*, 2018 |
| | Firmicutes/ Low G+C, Gram positive bacteria (44-56%) | *Anaerotruncus*, *Ruminococcus*, *Faecalibacterium*, *Lachnospiraceae*, *Bacillus*, *Streptococcus*, *Clostridium*, *Megamonas*, *Lactobacillus*, *Enterococcus*, *Weisella*, *Eubacterium*, *Staphylococcus*, *Streptococcus* | |
| | Bacteroides/ Cytophaga/ Flexibacter/ High G+C, Gram positive bacteria (23-46%) | *Bacteroidetes*, *Alistipes*, *Fusobacterium*, *Bifidobacterium*, *Flavibacterium*, *Odoribacter* | |
| | Actinobacteria | *Corynebacterium* | |
| | Proteobacteria (1-16%) | *Ochobacterium*, *Alcaligenes*, *Escherichia*, *Campylobacter* | |

There are several factors that influence the commensal gut microbiota population. The recent literature review of Kers *et al.* (2018) summarised the main effects as chicken development (age), type and breed, sex, housing, biosecurity level, litter, feed access and climate and geographical region. With regards to age, Lu *et*

*al.* (2003) found significant differences between the caecal populations of chickens at three different age intervals (first week of age, second to fourth week of age, and seventh week of age). Similarly, they also found the ileum to have unique community structures at 3 and 49 days of age. Lumpkins *et al.* (2010) claimed that the bacterial community of broilers changed with age regardless of their genetic lines. Another study indicated that the ileal microbial community of broilers "matured" during days 15 to 22 after hatch and saw an increase in the relative abundance of *Lactobacillus salivarius* and clostridia, while observing a decrease of *Lactobacillus reuteri* (Ranjitkar *et al*., 2016).

The disruption of the gut microbiota correlated with the onset of NE is well documented (Stanley *et al*., 2012; Stanley *et al*., 2014b; Wu *et al*., 2014; Antonissen *et al*., 2015a; Kim *et al*., 2015). The review performed by Antonissen *et al.* (2016) summarises the microbial shifts induced by the NE predisposing factors as shown in table 2.3 below.

**Table 2.3** Effects of necrotic enteritis factors on gut microbiota (Antonissen *et al*., 2016)

| Microbial shift | Source |
|---|---|
| Reduction of segmented filamentous bacteria | (Stanley *et al*., 2014a; Kim *et al*., 2015) |
| Alterations of lactic acid-producing bacteria | (Stanley *et al*., 2012; Wu *et al*., 2014) |
| Decrease of butyrate producers | (Wu *et al*., 2014) |

This sort of change would typically result in a reduction of *Candidatus*, a segmented filamentous bacteria which is part of the *Lachnospiraceae* family and plays an important role in the modulation of the host's immune system (Thompson *et al*., 2013; Antonissen *et al*., 2016). The presence of the *Lachnospiraceae* family has also been shown to be correlated with improved FCR values (Stanley *et al*., 2016). With regards to the lactic-acid producing bacteria, Stanley *et al.* (2012) reported that *C. perfringens* infection increased the abundance of *Lactobacillus crispatus*, *Lactobacillus pontis*, *Lactobacillus ultunese*, and *Lactobacillus salivarius,* all at the expense of *Lactobacillus johnsonii* and *Lactobacillus fermentum*. The supplementation of *Lactobacillus* strains in broiler diets have been associated with increased body weight gain and better FCR values (Kalavathy *et al*., 2003). As indicated in table 2.4, one of the predisposing factors for NE is feeding high protein diets which usually involves the addition of fishmeal, allowing for the proliferation of *C. perfringens* (Drew *et al*., 2004). As shown by the study of Wu *et al.* (2014), high protein or fishmeal diets result in a decrease of butyrate producing bacteria such as those from the *Ruminococcaceae* and *Lachnospiraceae* families.

## 2.5 Clostridium perfringens

Songer (1996) referred to *C. perfringens* as possibly the "most widely occurring pathogenic bacterium" whilst also indicating that the bacteria is the primary cause of enteric diseases of clostridial nature in animals. *C. perfringens* is naturally present in the GIT of animals as part of the normal commensal gut microbiota and can also be found in the environment (Songer, 1996; Vierheilig *et al*., 2013). *C. perfringens spp.* produce four major toxins (α, β, ε and ı) and is classified into five toxinotypes (A, B, C, D and E) (Petit *et al*., 1999). Another important characteristic of *C. perfringens* is its inability to produce 13 essential amino acids, and is also one of the most rapidly growing bacterial pathogens (Shimizu *et al*., 2002). The 13 lacking essential amino acids are obtained *in vivo* from the action of *C. perfringens* enterotoxin (CPE), which is the prominent reason for common

food poisoning in humans (Brynestad & Granum, 2002). The metabolism of *C. perfringens* bacteria is related to its virulence through the VirR/VirS system that controls genes involved with virulence as well as genes involved with energy synthesis and nutrient acquisition. These various genes allow *C. perfringens* to proliferate via degradation of host cells for nutrients, while producing toxins and causing typical "flesh-eating" symptoms of NE (Ohtani & Shimizu, 2015).

In an attempt to develop a consistent method for inducing NE in poultry for future experimental purposes, Cooper & Songer (2010) found that inoculating birds with *C. perfringens* type A elicited typical NE symptoms in more than 75% of the challenged birds. NE develops when certain predisposing factors allow for the proliferation of *C. perfringens* (Williams, 2005). These predisposing factors are summarised in table 2.4 below as described in the review article by Antonissen *et al.* (2016). A review performed by Moore (2016) highlights the main effects of NE on broiler chickens and summarises it as the collection of changes to the gut and immune system, disruption of microbiota and proliferation of pathogenic *C. perfringens* strains.

**Table 2.4** Summary of predisposing factors of necrotic enteritis that allow proliferation of *C. perfringens* (Antonissen *et al.*, 2016)

| Predisposing factor | Source |
|---|---|
| High dietary indigestible, water-soluble NSPs | (Kaldhusdal & Skjerve, 1996; Craven, 2000; Annett *et al.*, 2002) |
| High dietary protein content | (Kaldhusdal & Skjerve, 1996; Shimizu *et al.*, 2002; Drew *et al.*, 2004) |
| Coccidial pathogens | (Van Immerseel *et al.*, 2004; Allaart *et al.*, 2013) |
| Feed contaminated with *Fusarium* mycotoxins | (Antonissen *et al.*, 2015a; Antonissen *et al.*, 2015b) |

## 2.6 Antibiotic growth promoters and alternative feed additives

Feed additives are additional ingredients to the macro raw materials of an animal's diet. They can assist by directly supplementing the essential nutrients that are lacking in the existing diet, or they increase feed utilisation of the other feed ingredients and therefore improve animal growth and performance (Wenk, 2000). Feed additives are often a subject of scrutiny among consumers of animal products, especially in developed countries. A general lack of a public scientific understanding of animal nutrition and its vital role in world food supplies makes for a society that is susceptible to common misconceptions and misunderstandings.

Feed additives can function on many different physiological and molecular ways. One of the most relevant interactions is the effect of the feed additive on the host's commensal intestinal microbiome (Wenk, 2000). Antibiotic Growth Promoters (AGPs) are included in animal diets at sub-therapeutic levels to improve growth and feed conversion efficiency via destroying or inhibiting bacterial growth (Huyghebaert *et al.*, 2011; Pan & Yu, 2014; Cox, 2016; Gonzalez & Angeles, 2017). Including AGPs in livestock diets have been part of animal nutrition practice for more than 60 years (Cox, 2016), and are now beginning to phase out due to widespread concern over build-up of antibiotic resistance (Kim *et al.*, 2016). Although the modes of action of AGPs are not entirely clear, some of its proposed mechanisms include: 1) Inhibition of pathogenic bacteria (Pan & Yu, 2014; Kim *et al.*, 2016); 2) reducing microbial nutrient utilisation (Huyghebaert *et al.*, 2011); and 3) reducing the intensity and frequency of subclinical infections (George *et al.*, 1982). It is important however to mention that Coates *et al.* (1963) found that growth was not affected when germ-free birds received antibiotic

supplement. This provides compelling evidence for the principle that AGPs primarily function through the modification of the gut microbiome.

Probiotics are live fed cultures of microorganisms that beneficially alter the host's gut microbiome (Huyghebaert *et al.*, 2011; Caly *et al.*, 2015). Probiotics, also referred to as direct-fed microbials (DFMs), have been shown to benefit the production of several livestock species with the most significant effects when they were administered to animals that experience stressful gut conditions such as weaning, lactation or rapid dietary changes (Chaucheyras-Durand & Durand, 2010). Probiotics are used in ruminants as well as monogastric species and mostly function through improving intestinal development, morphology and metabolism as well as having some antimicrobial properties (Caly *et al.*, 2015). The antimicrobial properties of some probiotics make it a very attractive alternative to antibiotics, especially when considered to prevent bacterial diseases (Lutful Kabir, 2009). There are also a large number of studies showing the potential of certain microbial strains with anti-*C. perfringens* activity (Caly *et al.*, 2015). Probiotics have become an increasingly popular public preference, especially compared to antibiotics. This effect can be seen in figure 2.2 below which indicates the interest in online search terms "Antibiotic" and "Probiotic" over time since 2004 as provided by Google Trends.

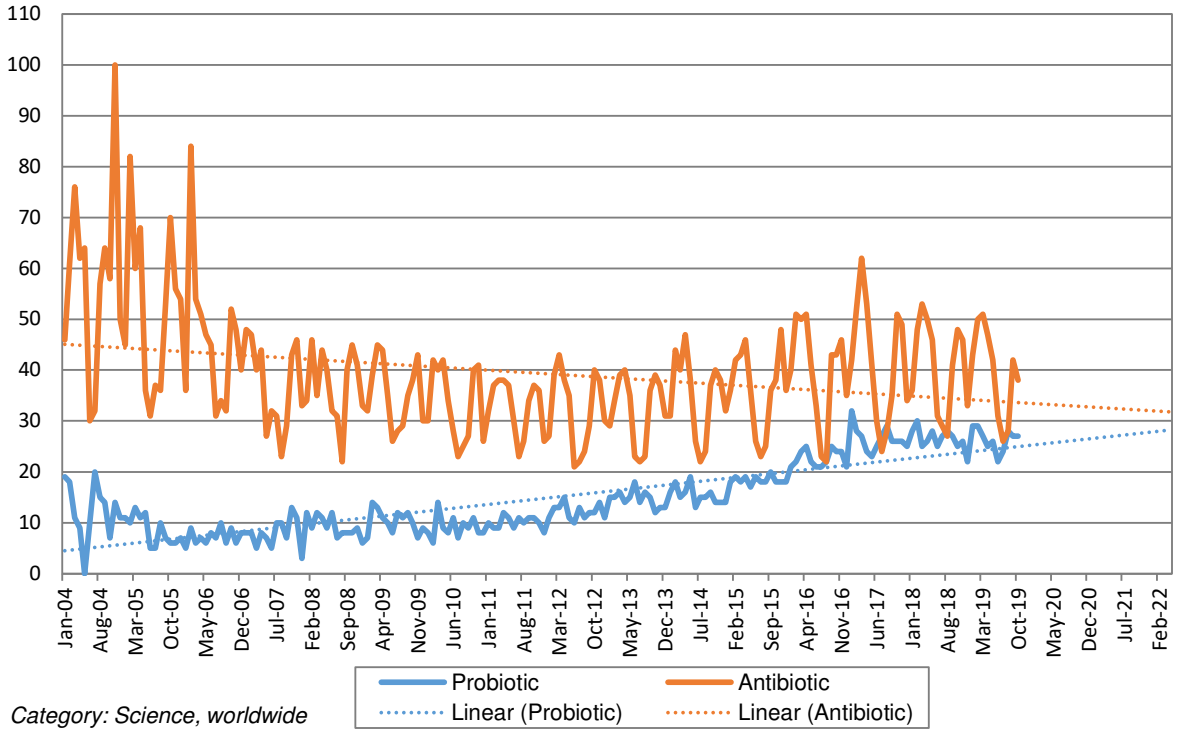



**Figure 2.2** Online interest in search terms "Probiotic" and "Antibiotic" - Data source: Google Trends (https://www.google.com/trends)

Essential oils are plant derived compounds. They are secondary metabolites believed to be involved in a plant's defense system and has antimicrobial properties (Diaz Carrasco *et al.*, 2016). Essential oils not only have antimicrobial activities but some are multifunctional and act as an antioxidant and/or endogenous enzyme stimulator. Examples of plant extracted essential oils include lialol from coriander, carvacrol from oregano, cinnamaldehyde from cinnamon and thymol from thyme (Huyghebaert *et al.*, 2011).

Organic acids are another feed additive shown to have beneficial effects on broiler performance. They are abundant throughout nature and exist with a variety of physico-chemical properties. Organic acids can be fed as individual acids or as a mixture of several acids to elicit antimicrobial activities in a similar fashion as that of AGPs (Van Immerseel *et al*., 2006; Khan & Iqbal, 2016). One of the flagship characteristics of organic acids is their ability to change from undissociated to their dissociated form, allowing them to cross microbial cell membranes and (after dissociation) disrupt microbial metabolism (Adams & Hall, 1988; Van Immerseel *et al*., 2006). Different organic acids of differing lengths and differing levels of saturation will dissociate at different pH levels, giving each organic acid a set target range of antibacterial activity (Huyghebaert *et al*., 2011).

Another category of feed additives that can manipulate the intestinal microbiota populations is called prebiotics. Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the intestinal tract" (Gibson & Roberfroid, 1995; Huyghebaert *et al*., 2011). Most promising prebiotics are nondigestible oligosaccharides which include, but is not limited to, fructooligosaccharides (FOS), galactooligosaccharides (GOS), transgalacto-oligosaccharides (TOS), and lactulose (Gaggia *et al*., 2010).

## 2.7 Identification and quantification of gut microbiota

To be able to study the role and function of gut microbiota, accurate identification of microbes is essential. Since 1885, when Bacterium coli had first been described by Escherich (Sankar *et al*., 2015), culture-based techniques were used to investigate microbial species while they mainly focused on identifying pathogenic species. Culture-based methods involve the controlled growth of a microorganism in predetermined culture media under controlled laboratory conditions. A major breakthrough in the 1970s was when scientists started to apply anaerobic conditions to the culture of gut microorganisms, enabling the identification of more microbes (Sankar *et al*., 2015). Culture-based methods are limited by their design. Previous culture-dependant studies revealed that a mere 10 to 60% of bacteria residing in the caeca grew in culture media (Zhu *et al*., 2002). This could have resulted in underestimations of the microbial diversity in most of the previous culture-based studies done on the avian gut (Kohl, 2012). A case study performed by Vaz-Moreira *et al.* (2011) found that when compared with culture-independent methods, culture-based methods provide lower coverage of bacterial diversity in a freshwater sample. Other limitations with using culture dependent methods also include time and money needed, lack of sensitivity, and the competitive ease of use and efficiency of culture independent methods (Sankar *et al*., 2015).

Culture independent methods or more specifically, rapid nucleic acid sequencing, are one of the most revolutionising discoveries in the identification and quantification of microorganisms (Patwardhan *et al*., 2014; Sankar *et al*., 2015). These methods rely on the use of molecular techniques such as using marker genes. Genetic markers include genes or DNA sequences that can be used to identify individuals or species, with several different genetic markers available (Patwardhan *et al*., 2014). Patwardhan *et al.* (2014) described several candidate molecular markers which can be categorised into nuclear ribosomal genes, mitochondrial genes and chloroplast genes. In the 1970's Carl Woese and co-workers identified that 16S rRNA can be used as a phylogenetic marker (Namsolleck *et al*., 2004). For studying phylogenetic relationships, ribosomal RNA is considered as the best target since it is universal and is composed of highly conserved as well as variable domains and is not easily affected by horizontal gene transfer (Janda & Abbott, 2007; Vetrovsky & Baldrian,

2013). The disadvantage of the 16S rRNA gene is that it is highly conserved, meaning it does not evolve at the same rate in all organisms (Janda & Abbott, 2007; Choi *et al.*, 2015). This will limit its ability to accurately distinguish closely related populations and may result in the underestimation of the diversity of the population (Namsolleck *et al.*, 2004). The 16S gene is also present in all bacteria, making it the ideal gene for bacterial taxonomy and identification of non-culturable bacteria (Patwardhan *et al.*, 2014). Figure 2.3 below illustrates a basic overview of the procedure when using 16S rRNA for identifying microbes.

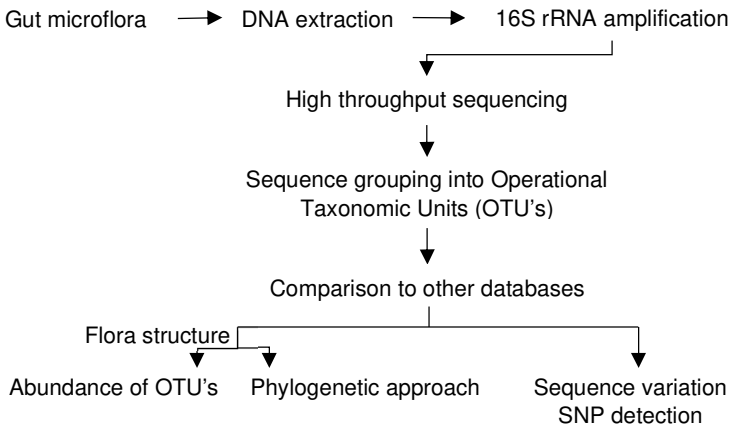



**Figure 2.3** Flow chart depicting the steps involved in deciphering microbial communities based on 16S rRNA sequencing (Sankar *et al.*, 2015)

The development and use of culture-independent methods (16S rRNA sequencing) should not eliminate or replace culture-dependent methods, but should be used in conjunction with each other. Culture-dependent methods should still be an integral part of diversity studies due to the information they provide on the properties and requirements of the isolated strains (Bjerrum *et al.*, 2006). The steps involved in 16S rRNA sequencing of microbes are described in figure 2.3.

A study done at the Ohio State University on the microbiome of chickens and turkeys attempted at generating a phylogenetic diversity census of the microbiome of chickens and turkeys. It made use of 3184 different 16S rRNA gene sequences to identify 12 phyla of bacteria in the chicken. This resulted in identifying Firmicutes as the most predominant phylum in the chicken gut (accounting for almost 70% of all the bacterial sequences of chicken origin). The second and third most predominant phyla were identified as Bacteroidetes (12.3% of bacterial sequences) and Proteobacteria (9.3% of bacterial sequences). Other phyla were present but were represented by a small number of OTU's. In total, 117 genera of bacteria were identified with most genera belonging to the phyla Firmicutes, Bacteroidetes and Proteobacteria (Wei *et al.*, 2013). Some studies have reported the presence of members of the domain Archaea in the chicken caecum (Kohl, 2012). They are much less abundant with approximately $10^5$-$10^7$ cells per gram of digesta in the caeca and their DNA only contribute more or less 1-2% of all the genetic information in the caeca (Cisek & Binek, 2014).

## 2.8 Conclusion

The gut microbiota of broilers have several functions pertaining to broiler health, growth and performance. Better understanding the dynamics of the gut microbiota of broilers might not only lead to healthier and more efficient birds, but also increase the safety and sustainability of their production. Evidence suggest that the establishment of gut microbiota occur before hatch and the colonisation of the gut will plateau as substrates and sites of attachment become less available. The gut microbiota has protective, structural and metabolic functions. *C. perfringens* is a naturally occurring gut microbe in animals that produce toxins and contribute to enteric diseases of clostridial nature. An AGP is a feed additive that functions through altering the gut microbiome. Other feed additives that could be alternatives to AGPs include DFMs, essential oils, organic acids and prebiotics. Rapid nucleic acid sequencing methods such as 16S rRNA allow for the more complete identification and quantification of microorganisms.

# *Chapter 3 - Materials and Methods*

## 3.1 Introduction

This study was carried out at the Hatfield experimental farm of the University of Pretoria, South Africa and consisted of two parts namely part one: growth trial and part two: sample collection for the current study. The trial was done in collaboration with a student performing the growth study and growth parameters were not reported here. The trial and all the procedures involved were approved by the Animal Ethics Committee (AEC) of the University of Pretoria (EC008-16).

## 3.2 Experimental design (part one and two)

Prior to placing the day-old chicks, the broiler house was washed, disinfected, and pre-heated to the comfort zone of the chicks at 36ºC ambient temperature and at least 34ºC floor (litter) temperature. Litter was spread on the floor of the pens to absorb moisture and to help with insulation from the concrete floor. Day-old male Ross chicks were placed according to a completely randomised block design into 96 pens with 23 birds per pen. Therefore, there were 12 replications of the eight treatments. Male birds were selected in order to eliminate growth and performance differences between male and female birds, which differ significantly (Howlider & Rose, 1992).

**Table 3.1** Pen and treatment (Trt) distribution within blocks

| Block 4 | | | | Block 5 | | | | Block 6 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Trt 6 Pen37 | Trt 4 Pen38 | Trt 5 Pen39 | Trt 3 Pen40 | Trt 4 Pen41 | Trt 2 Pen42 | Trt 3 Pen43 | Trt 1 Pen44 | Trt 2 Pen45 | Trt 8 Pen46 | Trt 1 Pen47 | Trt 7 Pen48 |
| Trt 2 Pen25 | Trt 8 Pen26 | Trt 1 Pen27 | Trt 7 Pen28 | Trt 8 Pen29 | Trt 6 Pen30 | Trt 7 Pen31 | Trt 5 Pen32 | Trt 6 Pen33 | Trt 4 Pen34 | Trt 5 Pen35 | Trt 3 Pen36 |
| | | | | | | | | | | | |
| Block 1 | | | | Block 2 | | | | Block 3 | | | |
| Trt 7 Pen13 | Trt 5 Pen14 | Trt 6 Pen15 | Trt 4 Pen16 | Trt 5 Pen17 | Trt 3 Pen18 | Trt 4 Pen19 | Trt 2 Pen20 | Trt 3 Pen21 | Trt 1 Pen22 | Trt 2 Pen23 | Trt 8 Pen24 |
| Trt 3 Pen1 | Trt 1 Pen2 | Trt 2 Pen3 | Trt 8 Pen4 | Trt 1 Pen5 | Trt 7 Pen6 | Trt 8 Pen7 | Trt 6 Pen8 | Trt 7 Pen9 | Trt 5 Pen10 | Trt 6 Pen11 | Trt 4 Pen12 |

A lighting programme consisting out of 23 hours of light and one hour of dark was provided to the chicks during the first week of life, to stimulate normal daily feed and water intake. Thereafter, the length of daylight was reduced to 16 hours of light according to the Ross' Broiler Management Guide.

At day nine, birds were given a coccidial vaccine (Immunocox, Ceva) at 10 times the normal dose rate to cause mucosal damage and make them more prone to *C. perfringens* infection (Cravens *et al*., 2013). At day 14, each individual bird was orally inoculated with one mL of broth containing *C. perfringens* type A species at a concentration of 10 to the power of eight colony forming units (CFU)/mL (Knap *et al*., 2010). The coccidial vaccine contains spores of *Eimeria maxima* which is shown to be an inducing factor of NE (Wu et al., 2010). The *C. perfringens* produces several toxins that damage the intestinal tract (Jiang *et al*., 2009).

### 3.3 Dietary treatments

Birds were fed the same basal diet for pre-starter (0-7 days), starter (7-21), grower (21-28), finisher (28-33) and post-finisher (33-35) with only the feed additive(s) differing between treatments. The pre-starter and starter were fed as crumbles, while the grower and finisher were fed as pellets. The basal diet resembled a typical maize-soya based commercial feed used in South Africa, which adheres to Ross' guidelines, and contained AxtraPhy as a phytase (table 3.3). No carbohydrase or coccidiostat was included. The trial involved the use of four different feed additives including the AGP. These are as follows:

1. Zinc Bacitracin 15% m/m (ZB; an antibiotic growth promoter) (Ceva Animal Health, South Africa, Reg. No. G1070 (Act 36/1947))
2. Biacid Nucleus (an essential oil (EO) product) (Cargill, Minnesota, USA). Department of Agriculture, Forestry and Fisheries (DAFF) import permit (no 11/1/391) for trial purposes (date of permit: 08/02/2016)
3. Biacid (an essential oil (EO) and organic acid blend) (Cargill, Minnesota, USA)
4. Clostat Dry (a three strain *Bacillus* probiotic product) (Kemin, South Africa; Reg. no. V21583)

The inclusion levels of the abovementioned feed additives for each of the eight treatments are indicated in table 3.2 below.

**Table 3.2** Inclusion levels of tested feed additives in all eight dietary treatments

| **Treatment** | **Inclusion level (g/ton)** | | | |
|---|---|---|---|---|
| | Zinc bacitracin® | Biacid Nucleus® | Clostat® | Biacid® |
| Negative control | 0 | 0 | 0 | 0 |
| Zinc bacitracin | 333 | 0 | 0 | 0 |
| Biacid Nucleus | 0 | 100 | 0 | 0 |
| Biacid | 0 | 0 | 0 | 1000 |
| Clostat | 0 | 0 | 500 | 0 |
| Biacid Nucleus + Clostat | 0 | 100 | 500 | 0 |
| Biacid Nucleus + zinc bacitracin | 333 | 100 | 0 | 0 |
| Biacid + zinc bacitracin | 333 | 0 | 0 | 1000 |

The eight dietary treatments were therefore as follows:

1. Negative control (no additives)
2. Positive control (zinc bacitracin as an AGP)
3. Biacid Nucleus (mixture of essential oils)
4. Biacid (essential oils and organic acid blend)
5. Clostat (DFM)
6. Biacid Nucleus + Clostat
7. Biacid Nucleus + AGP
8. Biacid + AGP

The basal diet's ingredient inclusion levels are indicated in table 3.3 below.

**Table 3.3** Ingredient inclusion levels (%) of the basal diet for each feeding phase

| Ingredient | Phase | | | |
|---|---|---|---|---|
| | Pre-starter | Starter | Grower | Finisher |
| Maize (yellow) | 58.50 | 64.00 | 69.63 | 73.73 |
| Soybean oilcake (46.5%) | 33.63 | 28.50 | 20.27 | 16.70 |
| Sunflower oilcake (36%) | 2.00 | 2.50 | 3.00 | 3.00 |
| Gluten 60 | 1.00 | 0.93 | 3.00 | 3.00 |
| Lysine (Sinth 78%) | 0.28 | 0.28 | 0.33 | 0.36 |
| Methionine (DL 98%) | 0.26 | 0.24 | 0.16 | 0.15 |
| Threonine (98%) | 0.06 | 0.05 | 0.03 | 0.04 |
| Soybean oil | 0.67 | 0.50 | 0.97 | 0.80 |
| Feed lime (fine) | 1.74 | 1.55 | 1.42 | 1.28 |
| Mono-dicalcium phosphate (Ws>70%) | 0.93 | 0.61 | 0.45 | 0.24 |
| Salt (fine) | 0.25 | 0.25 | 0.16 | 0.16 |
| Sodium bicarbonate | 0.36 | 0.28 | 0.33 | 0.33 |
| Phytase (Axtra Phy 1000 FTU's) | 0.01 | 0.01 | 0.01 | 0.01 |
| *Broiler starter premix (3kg/t) | 0.30 | 0.30 | 0.00 | 0.00 |
| **Broiler grower premix (2.5kg/t) | 0.00 | 0.00 | 0.25 | 0.00 |
| ***Broiler finisher premix (2kg/t) | 0.00 | 0.00 | 0.00 | 0.20 |

*The broiler starter premix provided the following per kg of the diet: Vit A 12 000 IU, Vit D3 4000 IU, Vit E 60mg, Vit K3 4mg, Vit B1 4mg, Vit B2 9mg, Vit B3 60mg, Vit B5 15mg, Vit B6 5mg, Vit B9 2mg, Vit B12 0.025mg, Biotin 0.2mg, Mn 100mg, Fe 70mg, Zn 60mg, Cu 20mg, Co 0.5mg, Iodine 2mg, Se 0.3mg.
**The broiler grower premix provided the following per kg of the diet: Vit A 11 000 IU, Vit D3 4000 IU, Vit E 60mg, Vit K3 3mg, Vit B1 2mg, Vit B2 8mg, Vit B3 50mg, Vit B5 13mg, Vit B6 5mg, Vit B9 1.7mg, Vit B12 0.025mg, Biotin 0.13mg, Mn 100mg, Fe 40mg, Zn 50mg, Cu 15mg, Co 0.4mg, Iodine 1mg, Se 0.3mg.
**The broiler finisher premix provided the following per kg of the diet: Vit A 10 000 IU, Vit D3 3000 IU, Vit E 60mg, Vit K3 3mg, Vit B1 2mg, Vit B2 7.5mg, Vit B3 50mg, Vit B5 13mg, Vit B6 5mg, Vit B9 1.5mg, Vit B12 0.025mg, Biotin 0.12mg, Mn 100mg, Fe 40mg, Zn 50mg, Cu 15mg, Co 0.4mg, Iodine 1mg, Se 0.3mg.

Values calculated according to the feed composition are shown in table 3.4 below.

**Table 3.4** Calculated nutrient composition (g/kg) of the basal diet for each feeding phase

| Calculated nutrient values | Phase | | | |
|---|---|---|---|---|
| | Pre-starter | Starter | Grower | Finisher |
| Moisture | 106.50 | 106.89 | 105.73 | 106.05 |
| Metabolisable energy, MJ/kg | 11.50 | 11.75 | 12.30 | 12.50 |
| Crude protein | 220.37 | 201.86 | 182.84 | 169.78 |
| Crude fat | 35.74 | 35.29 | 40.71 | 39.93 |
| Crude fibre | 34.43 | 35.36 | 35.44 | 35.36 |
| Ash | 59.36 | 51.90 | 44.23 | 39.18 |
| Calcium | 10.40 | 9.06 | 8.14 | 7.22 |
| Total phosphorous | 5.93 | 5.15 | 4.58 | 4.04 |
| Total lysine | 13.60 | 12.28 | 10.70 | 10.02 |

Representative feed samples were taken from each of the basal feeds. These four feed samples were analysed at Nutrilab (Department of Animal and Wildlife Science, University of Pretoria) according to the proximate analysis system for their nutritional content (table 3.5). This analysis estimates seven fractions in the feed including dry matter, ash, crude protein, crude fibre, ether extract (lipid content), calcium and total phosphorus. Dry matter and ash were analysed according to AOAC's official method of analysis (AOAC,

2000g, Official method of analysis 942.05). Moisture content was determined as per AOAC's official method of analysis (AOAC, 2000d, Official method of analysis 943.01). The Leco FP-428 (Leco Corporation, 3000 Lakeview Avenue, St. Joseph, MI 49085-2396) was used to estimate the nitrogen content of the feed and the method used was according to the AOAC's official method of analysis (AOAC, 2000f, Official method of analysis 988.05). The crude fibre fraction was determined using the AOAC's method of analysis (AOAC, 2000e, Official method of analysis 962.09) as was crude fat (AOAC, 2000a, Official method of analysis 920.39). The AOAC's official method of analysis was used to determine the feed content for calcium (AOAC, 2000b, Official method of analysis 935.13) and phosphorus (AOAC, 2000c, Official method of analysis 965.17).

**Table 3.5** Analysed nutrient values (g/kg) per "as fed" basis for each phase of the basal diet feed

| Nutrient | Pre-starter | Starter | Grower | Finisher |
|---|---|---|---|---|
| Dry matter | 891.0 | 893.0 | 886.0 | 898.0 |
| Crude protein | 224.0 | 201.0 | 185.0 | 180.0 |
| Crude fat | 36.0 | 32.7 | 35.0 | 30.0 |
| Crude fibre | 37.2 | 38.0 | 43.2 | 42.2 |
| Ash | 55.0 | 45.5 | 41.2 | 36.8 |
| Calcium | 8.2 | 7.8 | 7.0 | 6.6 |
| Phosphorous | 6.2 | 5.0 | 4.2 | 4.0 |

### 3.4 Collection of digesta

Birds were weighed on a weekly basis from placement until slaughter. Birds were weighed per pen on all weighing days except for days 21 and 34 when all birds were weighed individually. At day 21 and 35, two birds from each pen, closest to the pen's average weight, were randomly selected for evisceration.

In an enclosed area protected from direct sunlight and wind, table surfaces and all equipment were sterilised with 70% ethanol before, and in between, each sampling. The digesta samples were taken from the middle of the ileum as well as from the half of the caeca proximal to the ileo-caecal junction. The digesta of the two birds from the same pen was emptied into a single container for ileal digesta and a single container for caecal digesta. Therefore, one pooled sample representing each anatomical region was collected for each pen on each sampling day, resulting in a total of 400 samples.

Digesta of each anatomical part was mixed in a sterilised container to create a homogenous mixture of each pen and were split in two, placed into separate sterilised containers and immediately put in a freezer which would have inhibited any further microbial activity. These duplicates from each pen were then stored in a freezer at -40°C and -20°C, respectively. Replications were stored in different freezers in case samples were lost or damaged during storage or shipment.

### 3.5 DNA extraction

Each pen's digesta sample, one from ileum and one from caeca, was used for DNA extraction in the Animal Genetics Laboratory at the Department of Animal and Wildlife Sciences at the University of Pretoria, using the protocol provided by Cargill (Cargill Inc. R&D Centre Europe, Vilvoorde, Belgium, 2017) as included in appendix A. Extracted DNA samples were selected at random to spot-check effectiveness of DNA extraction protocol via gel electrophoresis as shown by figure 3.1 below.

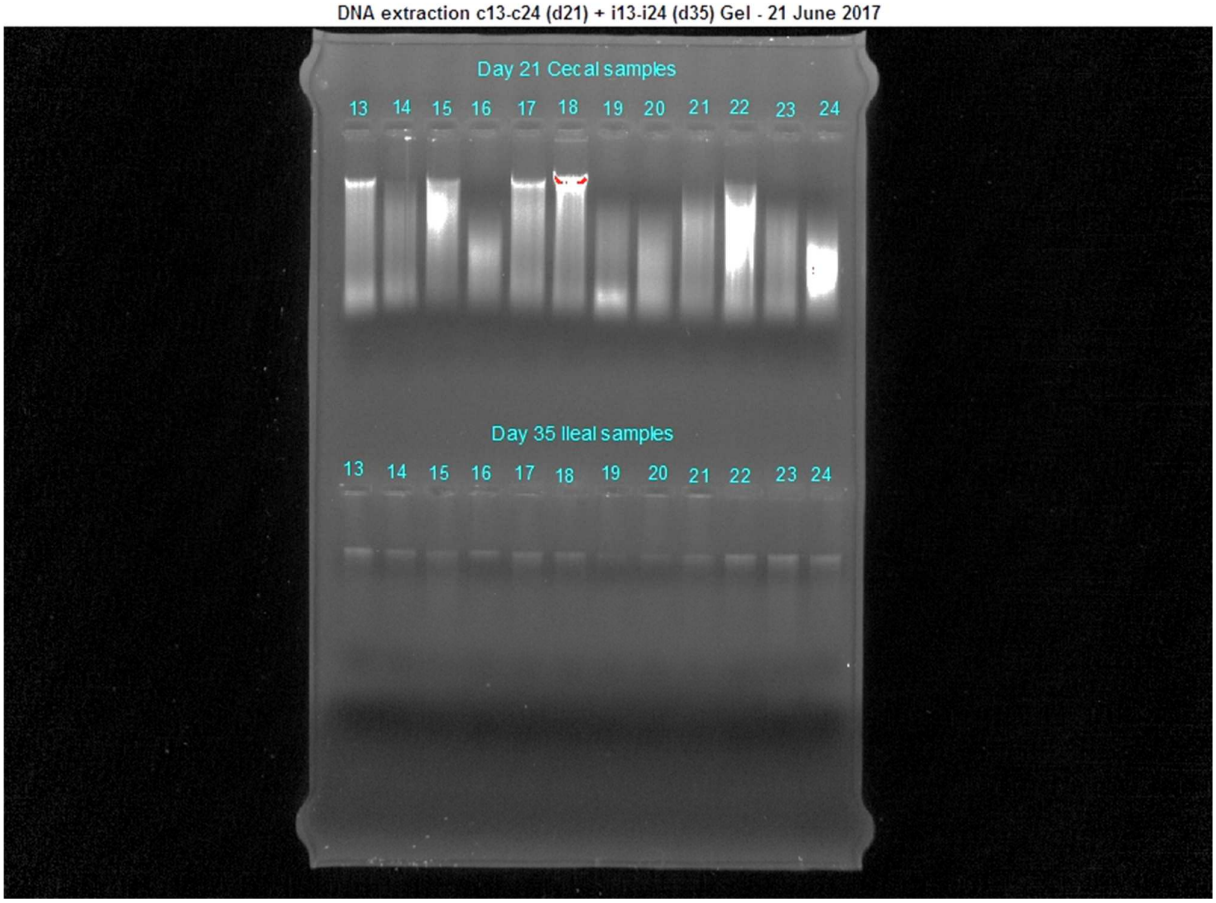



**Figure 3.1** Gel electrophoresis photo of DNA samples during spot-check procedure

These DNA samples were shipped to Cargill in Vilvoorde, Belgium for the Polymerase Chain Reaction (PCR) and sequencing of the 16S rRNA. Bacterial 16S DNA was enriched by PCR including a fluorescent labelled primer. Each labelled DNA was hybridised on a customised microarray chip (Cargill Inc. R&D Centre, Vilvoorde, Belgium, 2017) containing probes for 100 intestinal bacteria previously selected as biomarkers for broiler growth and pathogen presence. The fluorescence of each probe was read by a special camera device and converted into an intensity value used to determine semi quantitatively the presence of each of those bacteria and compare samples according to experimental design.

### 3.6 Data analyses

The raw intensity data for each probe on each microarray chip was compiled and submitted for data quality control and statistical analyses using JMP® Genomics 9.0 software (SAS Institute Inc., Cary, NC, USA, 2015). The selected data treatment to reduce chip-to-chip variation was to standardise it to a shifting point of 1.0. ANOVA was performed on the standardised data using a mixed model where all variables were classed. Feed additive and age and the interaction additive x age were used as fixed effects, chip and block were used as random effects. Caeca and ileum samples were analysed separately. The standardised mean differences were compared at a 5% false discovery rate, which resulted in a –log10 p-value cut-off of 3.832 for caeca and 3.318 for ileum. All pair wise comparisons were used to produce volcano plots. Differences were also used for cluster and PCA analysis, as well as to create a heat map. Parallel plots for the interactions between additives across different ages were also created for selected bacteria probes to facilitate pattern identification.

As mentioned previously, a total of 400 samples were collected and stored for DNA extraction. During the sequencing and/ or data analyses, 52 samples in total were excluded due to poor DNA quality. For the ileum, 194 out of 200 samples were analysed and 155 out of 200 caeca samples were analysed.

# *Chapter 4 – Results*

## 4.1 Introduction

In this chapter statistical results in the form of graphs and figures were presented. Samples representing two sampling locations namely the ileum and the caeca were analysed for microbial diversity. Results are presented first for ileal samples, followed by results for the caeca. In table 4.1 a summary of the samples analysed are presented.

**Table 4.1** Number of samples statistically analysed per anatomical location for each sampling day

| Sampling location | Samples from 21-day old chicks | Samples from 35-day old chicks |
|---|---|---|
| Ileum | 94 | 100 |
| Caeca | 69 | 84 |

For the bacteria measured, table 4.2 below illustrates the composition of each anatomical location's microbiota at phylum level for each sampling day. Across all sample days for both locations, the most abundant phyla among those measured were *Firmicutes*.

**Table 4.2** Composition of bacterial phyla per sampling day and anatomical region in percentages

| Phyla | 21-day Ileum | 35-day Ileum | 21-day Caeca | 35-day Caeca |
|---|---|---|---|---|
| Actinobacteria | 5.51% | 5.51% | 5.59% | 5.58% |
| Bacteriodetes | 9.19% | 9.05% | 10.53% | 11.06% |
| Firmicutes | **73.14%** | **73.47%** | **71.14%** | **70.01%** |
| Fusobacteria | 1.84% | 1.83% | 1.85% | 1.86% |
| Proteobacteria | 9.40% | 9.24% | 9.88% | 10.56% |
| Unclassified | 0.92% | 0.90% | 1.01% | 0.93% |

## 4.2 Diversity of microbiota in the ileum

Out of the total of 200 samples, 6 did not amplify and were excluded from the statistical analysis. Due to between chip/ sample variability, data had to be standardised to allow for a better comparable distribution curve. The standardisation process was effective in fitting the chip density to a normal curve and centre it on 0 with a dispersion of 1. Figure 4.1 below illustrates the chip density before standardisation and figure 4.2 after standardisation.

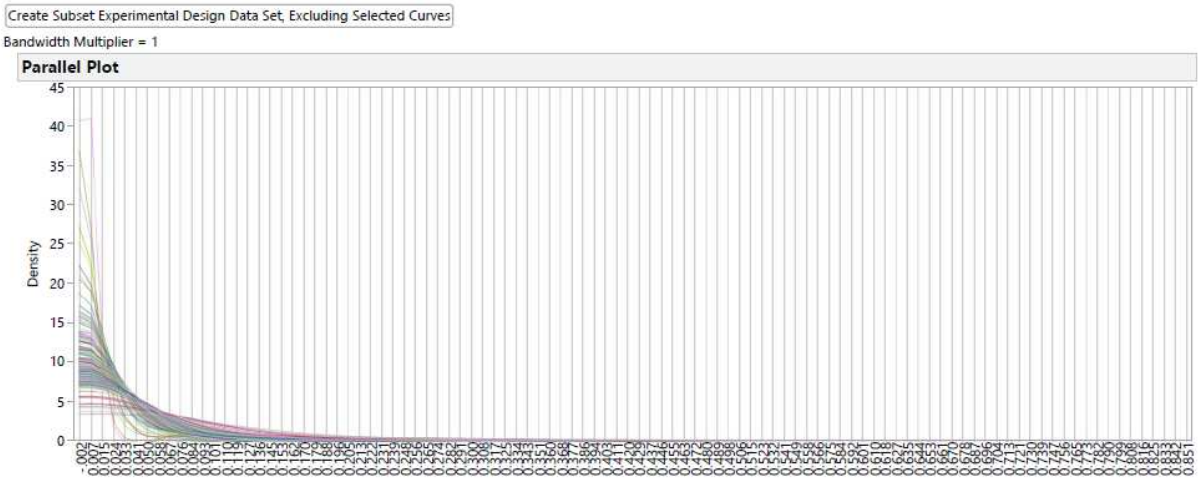



**Figure 4.1** Distribution curve of microbial density of all ileal samples before standardisation

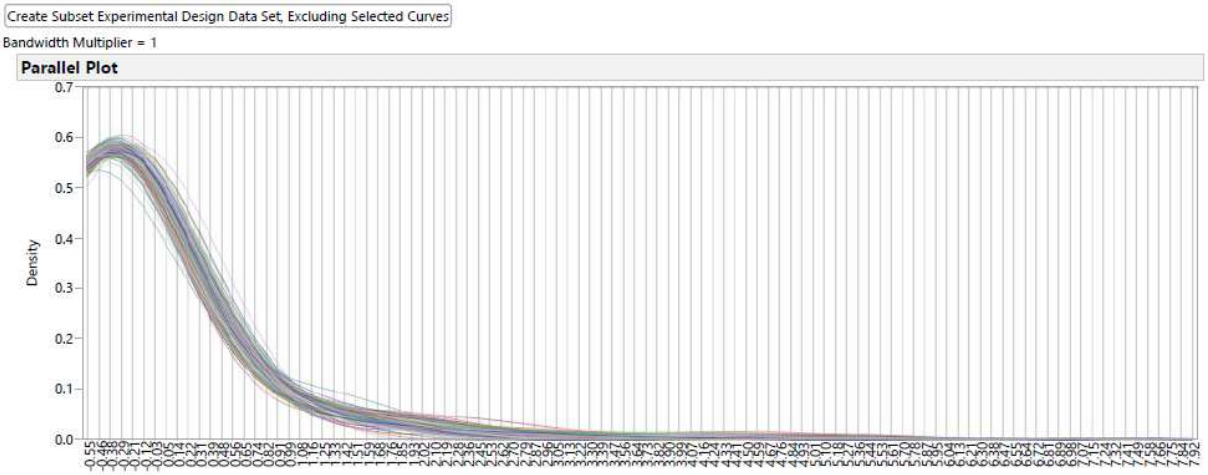



**Figure 4.2** Distribution curve of microbial density of all ileal samples after standardisation

The ileal results below start with a dendrogram showing the relative microbial abundance of the significant probes (from the customised chip) between the different groups of factors tested (age, treatment and age x treatment interaction). Figure 4.3 is provided with a scale and clusters the most similar groups together, highlighting the effect of similar groups of factors on the microbial abundance of the relevant probe.

Figure 4.3 is the dendrogram of the ileal relative microbial abundance of all significant bacterial probes for all treatments, age groups and interactions of treatment and age. The clear effect of age on the establishment of gut microbiota in the ileum is visible. The older birds had relatively less of all bacterial species from *Bacillus pumilis* to *Holdemania*, when compared to the younger 21-day old birds. The one exception is the 21-day old group treated with the combination of Biacid Nucleus (BN) and zinc bacitracin (ZB) that was clustered together with the 35-day groups. Species of note that were found within these that decreased from day 21 to day 35 are several *Clostridium spp., Bifidobacterium* and *Salmonella*. From *Enterococcus hirae* to the bottom which are mostly *Lactobacillis spp.*, the older 35-day old birds had relatively more in their ileum than the 21-day old birds.

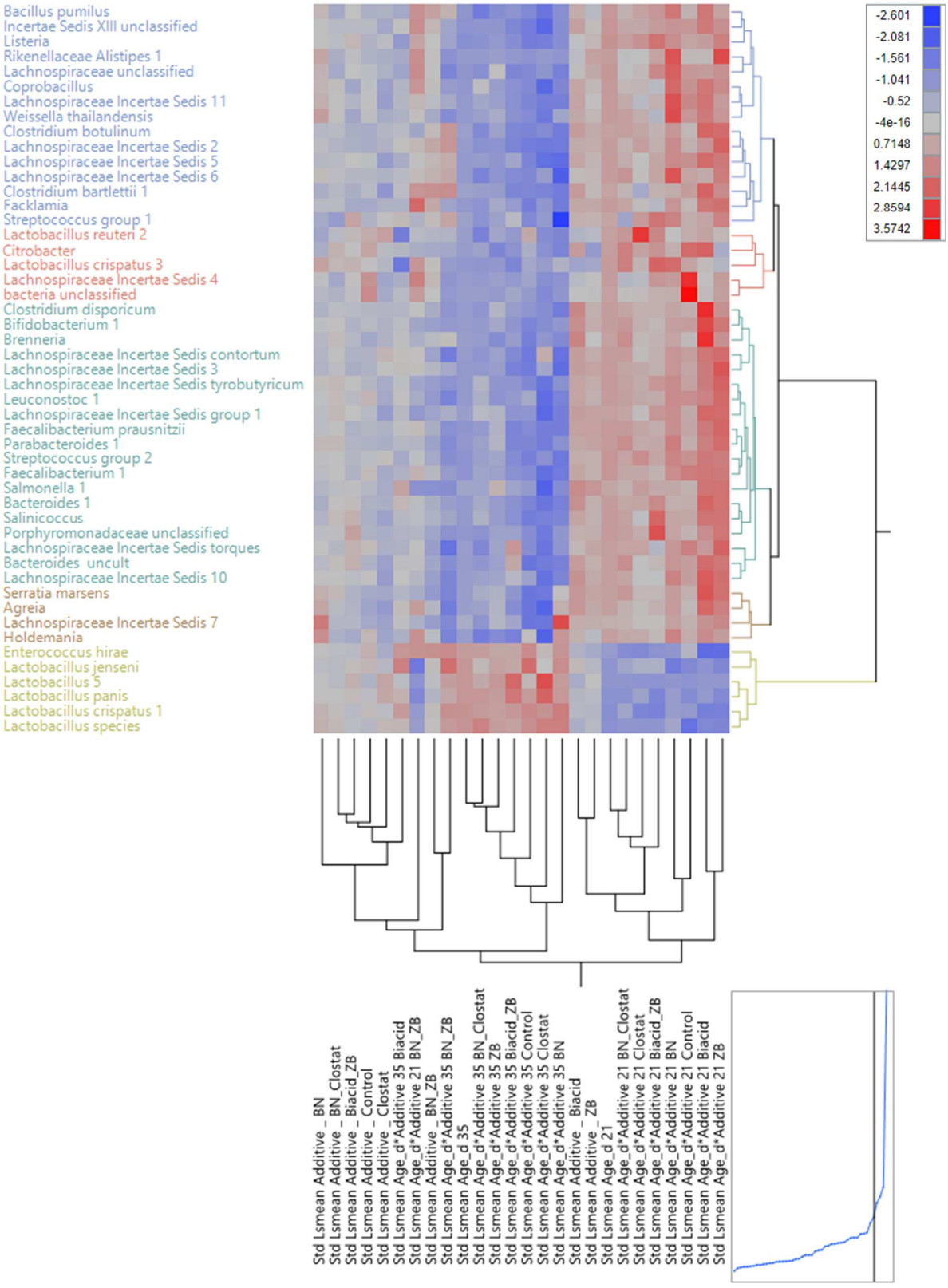



**Figure 4.3** Dendrogram displaying microbial diversity within the ileum for eight treatments at day 21 and 35

Figure 4.4 shows the composition of the ileal samples per age and treatment group at family level as relative abundance percentages. For all the sample groups, the *Lactobacillaceae* family is most abundant, followed by *Lachnospiraceae*.

Following figure 4.4 are all the Volcano plots indicating the differences in relative abundance for the specific microbes between all the categories from the dendrogram in figure 4.3. The p-value cut-off (-log (10)p) for all differences was 3.27 and was indicated by the dotted red line. Any observation above this line is regarded as statistically significant. The x-axis is the difference in relative abundance for a specific microbial probe between the two groups as labelled just below the x-axis. The y-axis is the significance of said difference. Each dot therefore represents a specific microbial probe and derives its colour from the colour that the microbiota are grouped in on the dendrogram in figure 4.3. Volcano plots are used for improved visual demonstration of differences between groups as shown on the dendrogram, and to indicate if these differences are significant. Only the volcano plots that indicate significant differences are illustrated here and the complete set of these significant plots are included in appendix B.

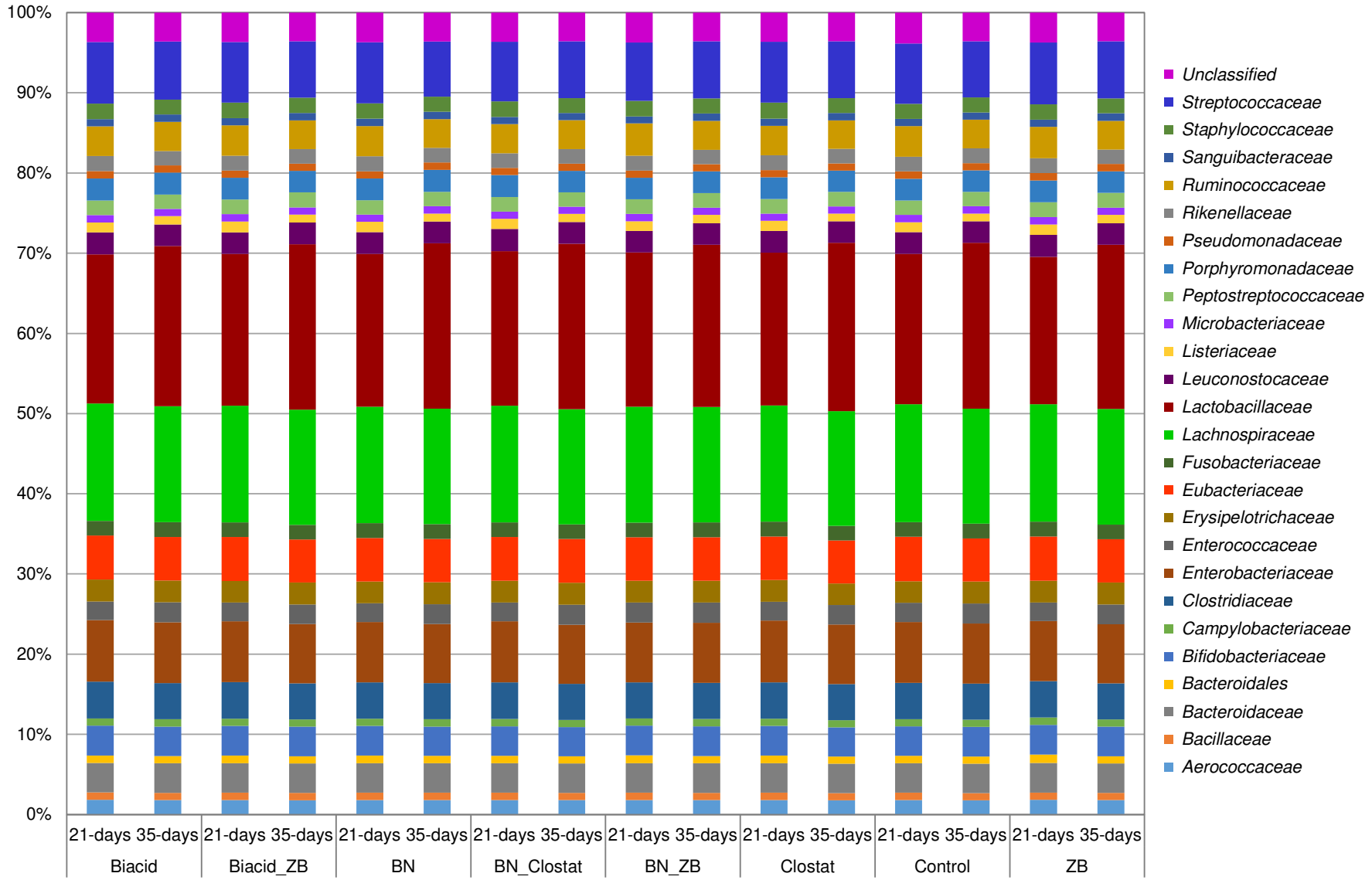



**Figure 4.4** Microbial composition at family level of ileal samples for age and treatment group as relative abundance percentages

Figure 4.5 reveals the degree to which the microbial populations differed between all 21-day and 35-day samples. The 35-day samples in general had significantly more ($P \leq 0.05$) of several *Lactobacillus spp.* and more abundant *E. hirae*. The 21-day samples however had more abundant ($P \leq 0.05$) *Streptococcus group 1 & 2*, *Listeria*, *Lactobacillus crispatus 3* and *E. coli* although the *E. coli* abundance differed to lesser degree between the two age groups compared to the other microbial species previously mentioned.

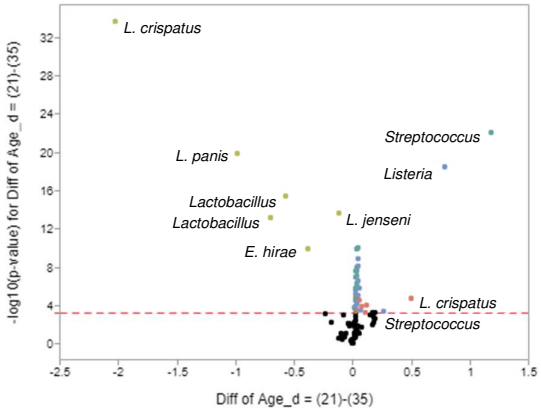
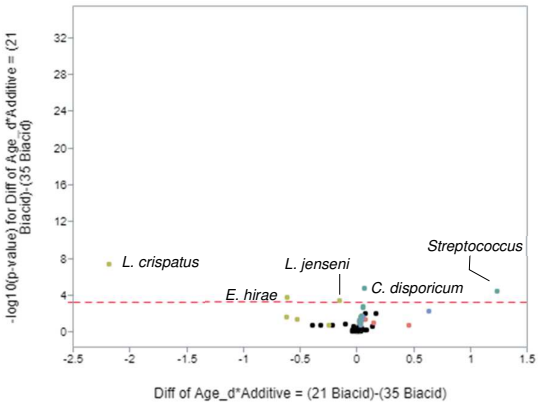
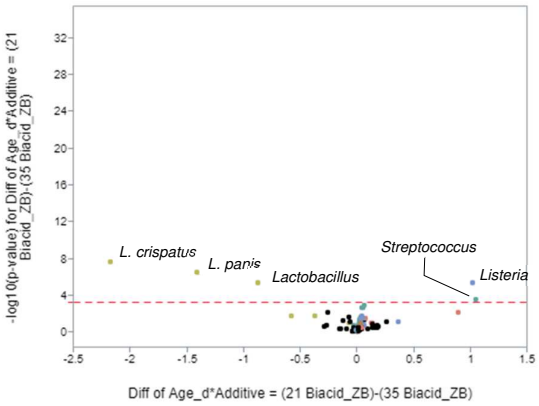



**Figure 4.5** Volcano plot demonstrating significant differences in the ileal microbiome between 21-day old and 35-day old broilers regardless of treatment groups

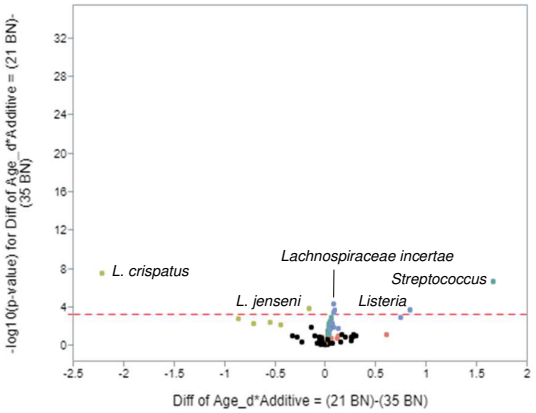
Figure 4.6a reveals how the 35-day old chicks treated with Biacid had more ($P \leq 0.05$) *Lactobacillus crispatus*, *E. hirae* and *Lactobacillus jenseni*. The 21-day samples had more ($P \leq 0.05$) *Streptococcus group 2*. Figure 4.6b reveals more abundant ($P \leq 0.05$) *Lactobacillus spp.* in the 35-day samples treated with Biacid and ZB where as in the 21-day samples *Listeria* and *Streptococcus group 2* were more prevalent. In figure 4.6c it is clear that the samples treated with BN had more ($P \leq 0.05$) *Lactobacillus spp.* in the older birds and more ($P \leq 0.05$) *Streptococcus* and *Listeria* in the younger birds. The only significant differences ($P \leq 0.05$) between the two age groups treated with BN_Clostat as shown in figure 4.6d, are the more abundant *Lactobacillus spp.* in the 35-day samples.



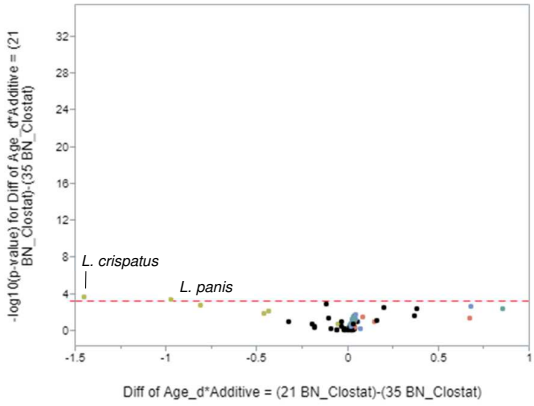

a: Significant difference in abundance for *L. crispatus*, *E. hirae*, *L. jenseni*, *C. disporicum* and *Streptococcus*




b: Significant difference in abundance for *L. crispatus*, *L. panis*, *Lactobacillus*, *Listeria* and *Streptococcus*



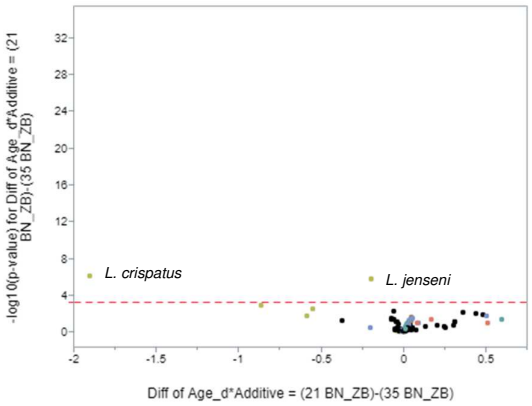

c: Significant difference in abundance for *L. crispatus*, *L. jenseni*, *Lachnospiraceae incertae*, *Listeria* and *Streptococcus*



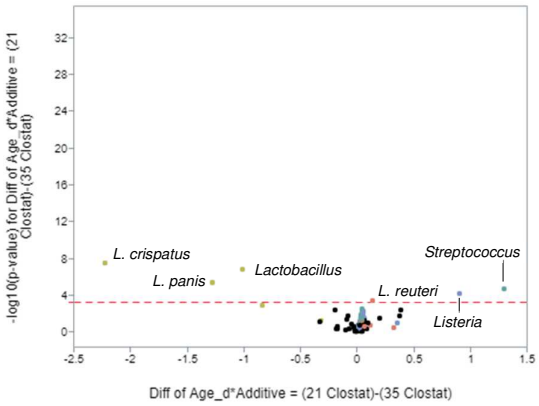

d: Significant difference in abundance for *L. crispatus* and *L. panis*

**Figure 4.6** Volcano plots for differences in the ileal microbiome between 21-day old and 35-day old broilers for the Biacid, Biacid_zinc bacitracin (ZB), Biacid Nucleus (BN) and Biacid Nucleus (BN)_Clostat treatment groups

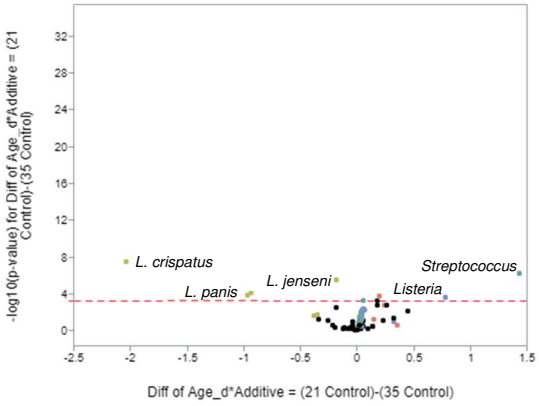
Figure 4.7a shows more abundant ($P \leq 0.05$) *Lactobacillus spp.* in the 35-day samples of the groups treated with the BN_ZB combination. The volcano chart in figure 4.7b reveals how the 35-day samples treated with Clostat had significantly more ($P \leq 0.05$) of 3 *Lactobacillus spp.* and less of *Streptococcus group 2*, *Listeria* and *Lactobacillus reuteri* than the 21-day samples. Figure 4.7c shows that the control group had more abundant ($P \leq 0.05$) *Streptococcus group 2* and *Listeria* in the 21-day samples, 35-day samples had significantly more abundant *Lactobacillus spp.* For the positive control group treated with ZB in figure 4.7d, the volcano chart reveals that *Lactobacillus crispatus and Lactobacillus panis* were more abundant ($P \leq 0.05$) in the 35-day samples compared to the 21-day samples that had significantly more *Streptococcus group 2* and *Listeria*.



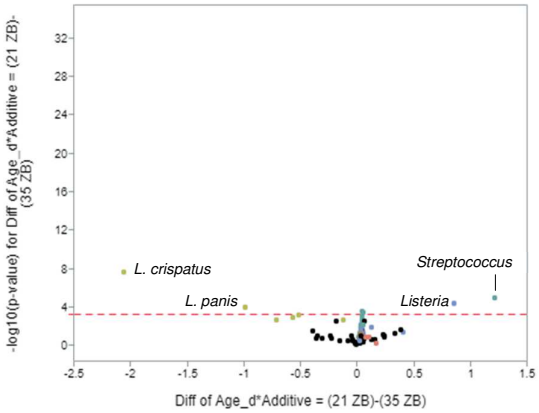

a: Significant difference in abundance for *L. crispatus* and *L. jenseni*




b: Significant difference in abundance for *L. crispatus*, *L. panis*, *Lactobacillus*, *L. reuteri*, *Listeria* and *Streptococcus*



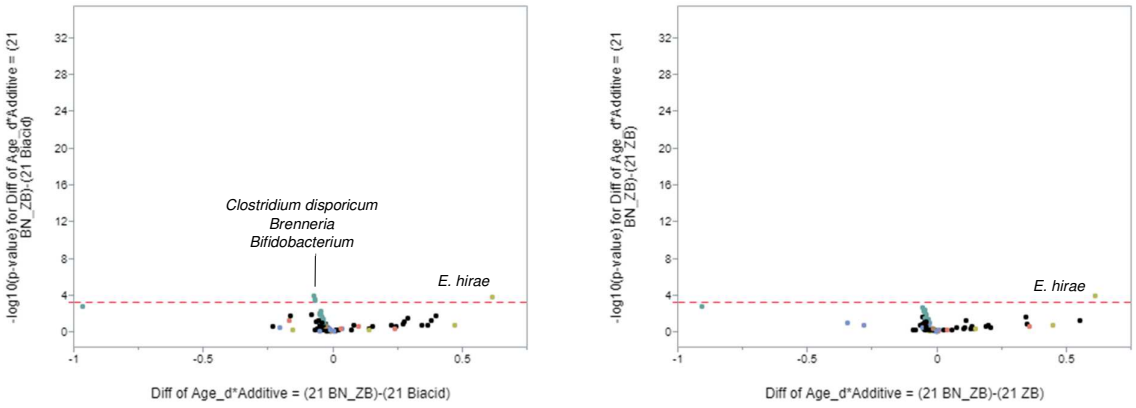

c: Significant difference in abundance for *L. crispatus*, *L. panis*, *L. jenseni*, *Listeria* and *Streptococcus*




d: Significant difference in abundance for *L. crispatus*, *L. panis*, *Listeria* and *Streptococcus*

**Figure 4.7** Volcano plots for differences in the ileal microbiome between 21-day old and 35-day old broilers for the Biacid Nucleus (BN)_zinc bacitracin (ZB), Clostat, Control and zinc bacitracin (ZB) treatment groups

The volcano plots below reflect the differences in microbial populations between all the 21-day sample treatment groups. For most of these comparisons between all the treatment groups of the 21-day samples, no significant differences ($P \leq 0.05$) in microbial abundance for any of the tested chips were found. Only two charts show significant differences. Figure 4.8a shows the differences in microbial abundance between the 21-day samples treated with BN_ZB and Biacid respectively. The group treated with BN_ZB in figure 4.8a had significantly more ($P \leq 0.05$) *E. hirae* and the group treated with Biacid had relatively more abundant *Clostridium disporicum*, *Bifidobacterium* and *Brenneria*. The other one is figure 4.8b, where the 21-day samples treated respectively with BN_ZB and ZB are compared. This volcano plot shows that the BN_ZB treated group had significantly more ($P \leq 0.05$) *E. hirae*.

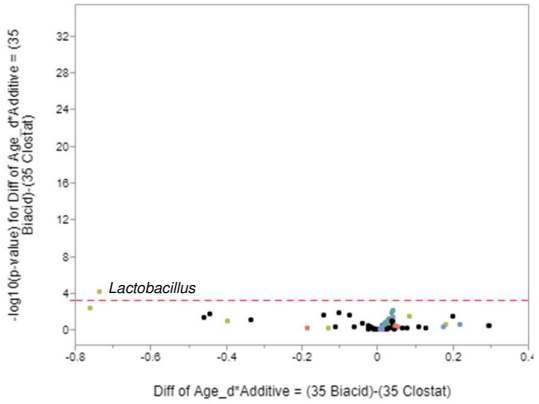



a: Significant difference in abundance for *C. disporicum*, *Brenneria*, *Bifidobacterium* and *E. hirae*

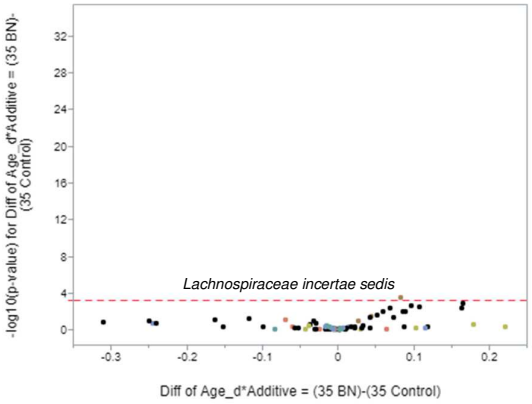
b: Significant difference in abundance for *E. hirae*

**Figure 4.8** Volcano plots for differences in the ileal microbiome between Biacid Nucleus (BN)_zinc bacitracin (ZB) and Biacid as well as between Biacid Nucleus (BN)_zinc bacitracin (ZB) and zinc bacitracin (ZB) for 21-day old old broilers

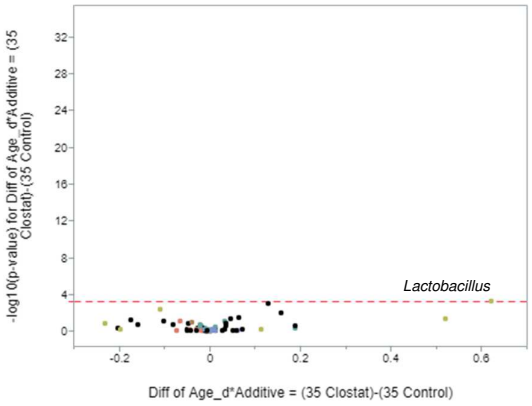
Similar to the 21-day sample group, most of the differences observed between the treatments in the 35-day samples were not statistically significant ($P \leq 0.05$). There were however three comparisons that showed a significant difference. Figure 4.9a shows that the 35-day group treated with Clostat had more ($P \leq 0.05$) *Lactobacillus* than the 35-day group treated with Biacid. Secondly, figure 4.9b reveals how the 35-day sample group treated with BN had relatively more abundant ($P \leq 0.05$) *Lachnospiraceae incertae sedis* than the 35-day control group. Lastly, figure 4.9c below indicates that the 35-day sample group treated with Clostat had significantly more ($P \leq 0.05$) *Lactobacillus* present in their ileum than the 35-day control group. The treatment groups as a whole regardless of age group showed no significant differences ($P \leq 0.05$) between any of the treatment groups for microbial abundance within the ileum.




a: Significant difference in abundance for *Lactobacillus*




b: Significant difference in abundance for *Lachnospiraceae incertae sedis*




c: Significant difference in abundance for *Lactobacillus*

**Figure 4.9** Volcano plots for differences in the ileal microbiome between Biacid and Clostat, Biacid Nucleus (BN) and control and between Clostat and control for 35-day old old broilers

## 4.3 Diversity of microbiota in the caeca

The caecal samples had a greater degree of sample loss with 45 excluded samples and therefore only had 155 available for statistical analysis (table 4.1). During the data quality control, between chip/ sample variability had to be accounted for by standardising the data set. The standardisation process resulted in a more comparable distribution curve, although one outlier was present (see figure 4.11 below). The outlier was removed and, as displayed in the figure 4.12 below, ensured for a distribution curve that was better comparable across all samples.

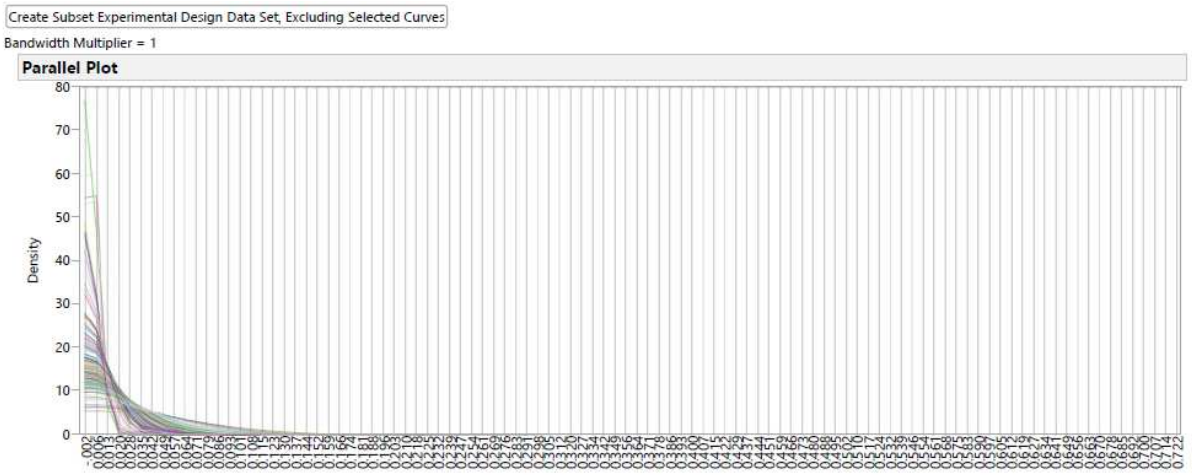



**Figure 4.10** Distribution curve of microbial density of all caecal samples before standardisation

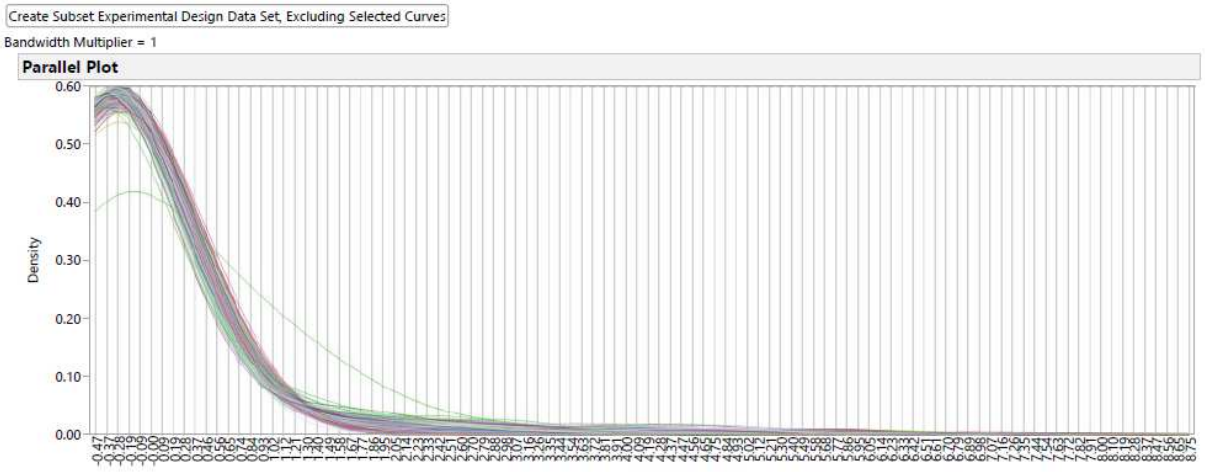



**Figure 4.11** Distribution curve of microbial density of all caecal samples after standardisation

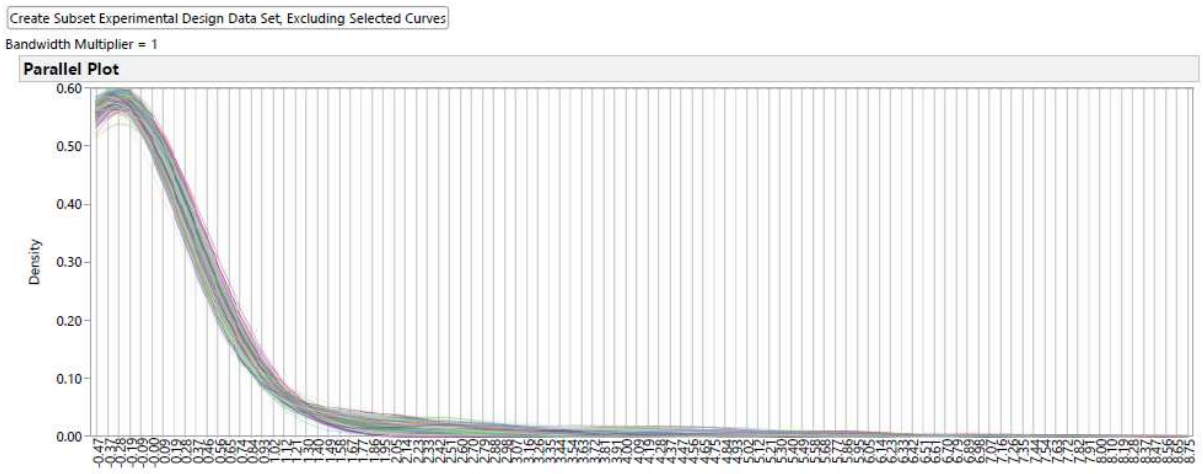



**Figure 4.12** Distribution curve of microbial density of all caecal samples after standardisation and after removal of outliers

The dendrogram in figure 4.13 below, indicates that all additive x age interaction groups are grouped together by age. It seems that all the microbes from *Lactobacillus gasseri 2* at the top to *Holdemania* reduced in relative abundance from day 21 to day 35 samples taken from the caeca. The microbes at the bottom including *E. coli*, *Rikenellaceae alistepes 2* and *Bacteroides dorei* increased in relative abundance in the 35-day samples from the caeca.

Figure 4.14 below the dendrogram shows the microbial composition of the 21-day and 35-day caecal samples per treatment group on a family level. The *Lachnospiraceae* and *Lactobacillaceae* families were the most abundant among the families that were sequenced for.

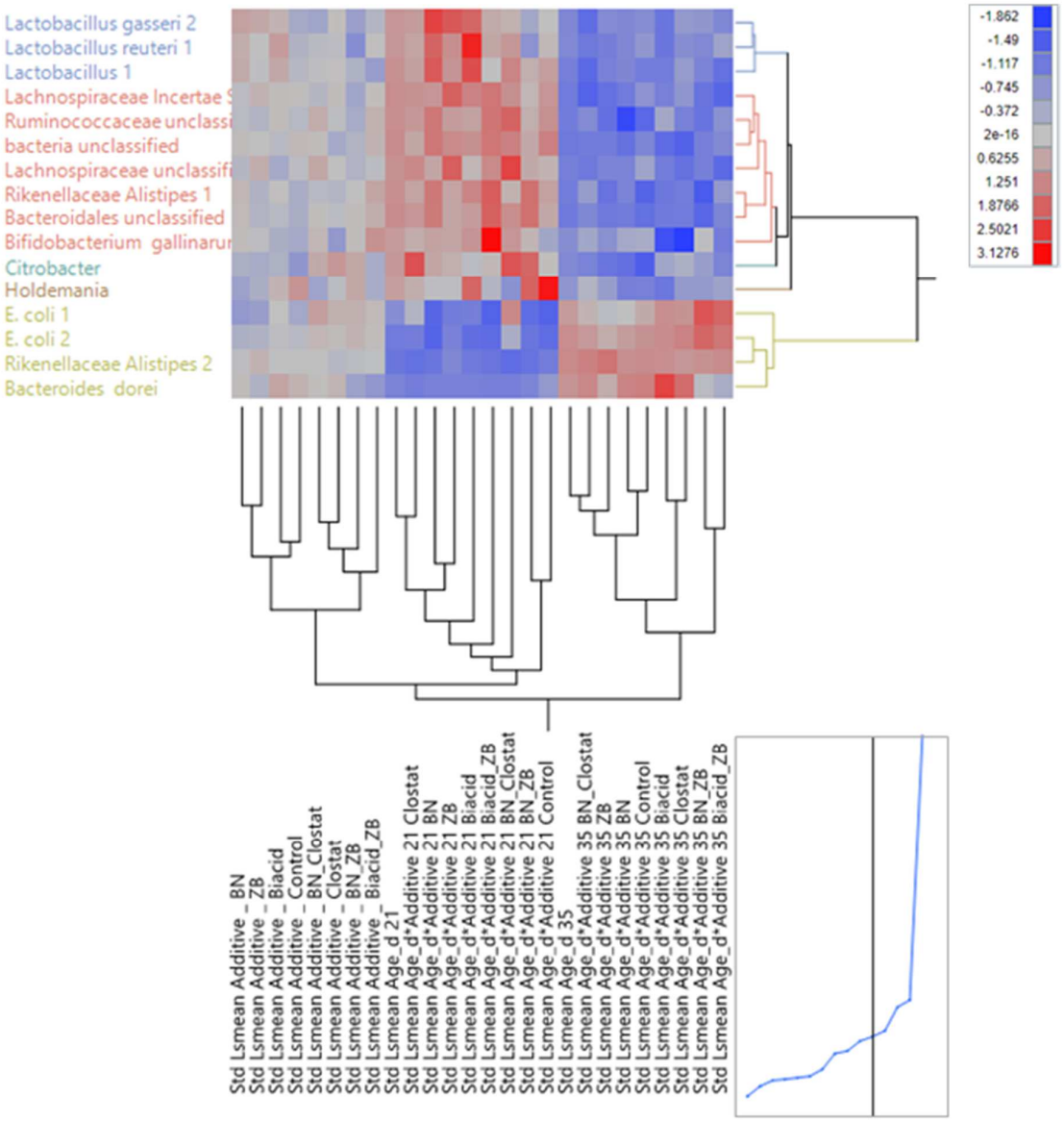



**Figure 4.13** Dendrogram displaying microbial diversity within the caeca for eight treatments at day 21 and 35

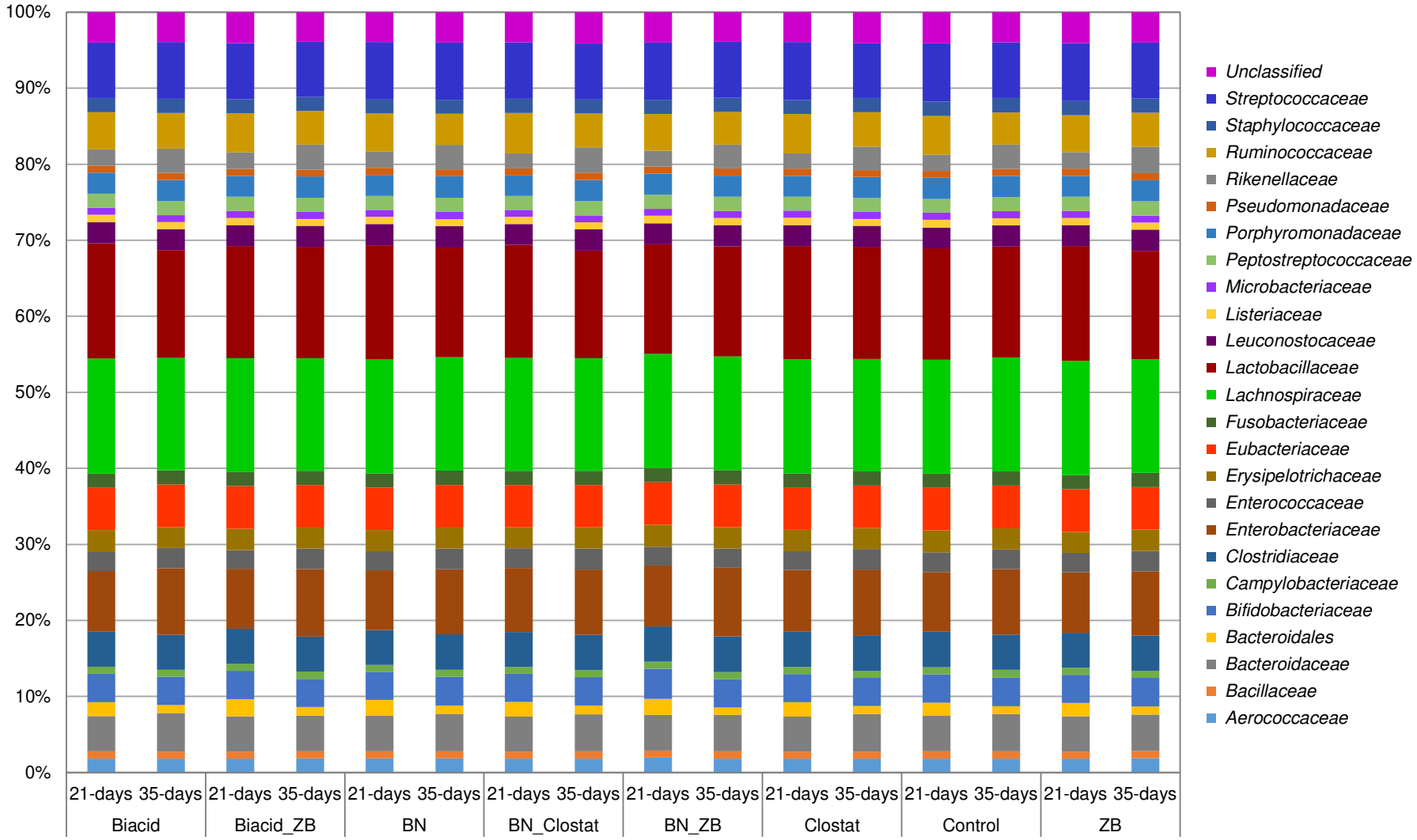



**Figure 4.14** Microbial composition at family level of caecal samples for age and treatment group as relative abundance percentages

Figure 4.15 is a volcano plot showing the difference in relative abundance for microbes between all 21-day and 35-day samples from the caeca. The 21-day samples had significantly more ($P \leq 0.05$) *Bacteroidales*, *Ruminococcaceae*, *Rikenellaceae alistipes 1*, *Lachnospiraceae*, *Lactobacillus spp.*, *Citrobacter*, *Bifidobacterium gallinarum* and *Holdemania*. In contrast, the 35-day samples had significantly more abundant ($P \leq 0.05$) *Rikenellaceae alistipes 2*, *E. coli 2*, *Bacteroides dorei* and *E. coli 1*.

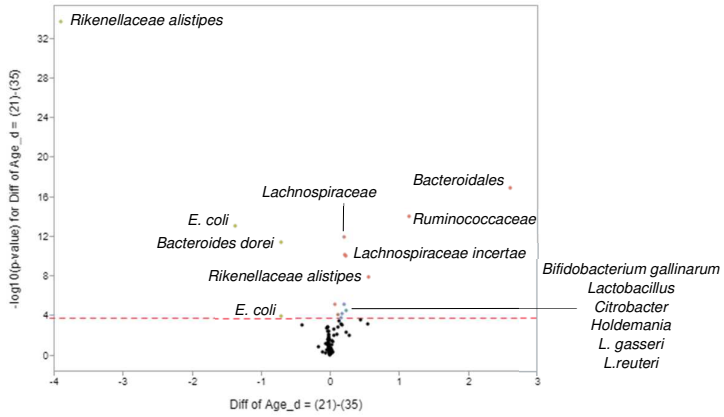
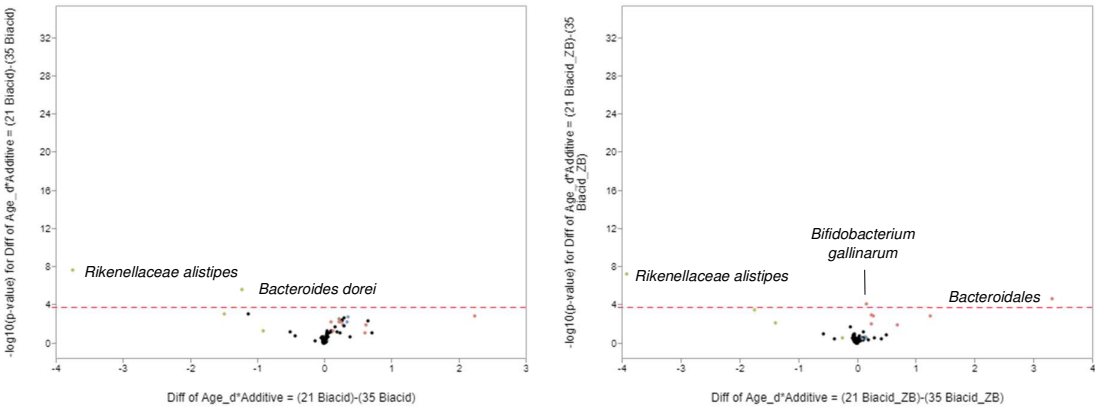


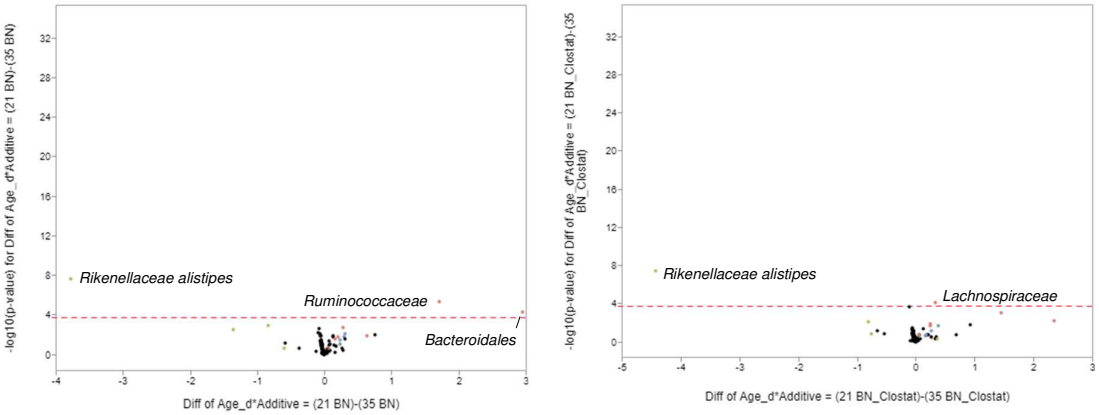

**Figure 4.15** Volcano plot demonstrating significant differences in the caecal microbiome between 21-day old and 35-day old broilers regardless of treatment groups

Figure 4.16a shows that the 35-day samples treated with Biacid had significantly more ($P \leq 0.05$) *Rikenellaceae alistipes 2* and *Bacteroides dorei* than the 21-day group. Figure 4.16b reveals how the 21-day Biacid_ZB group had significantly more ($P \leq 0.05$) abundant *Bacteroidales* and *Bifidobacterium gallinarum*. The 35-day Biacid_ZB group had more ($P \leq 0.05$) *Rikenellaceae alistipes 2*. Figure 4.16c indicates that the 21-day group treated with BN had significantly more ($P \leq 0.05$) *Bacteroidales* and *Ruminococcaceae*. The same treatment's 35-day group had more ($P \leq 0.05$) *Rikenellaceae alistipes 2*. In figure 4.16d it is visible that the 21-day group treated with BN_Clostat had more abundant ($P \leq 0.05$) *Lachnospiraceae* and the same treatment's 35-day group had significantly more ($P \leq 0.05$) *Rikenellaceae alistipes 2*.




a: Significant difference in abundance for *Rikenellaceae allistipes* and *Bacteroides dorei*

b: Significant difference in abundance for *Rikenellaceae allistipes*, *Bifidobacterium gallinarum* and *Bacteroidales*

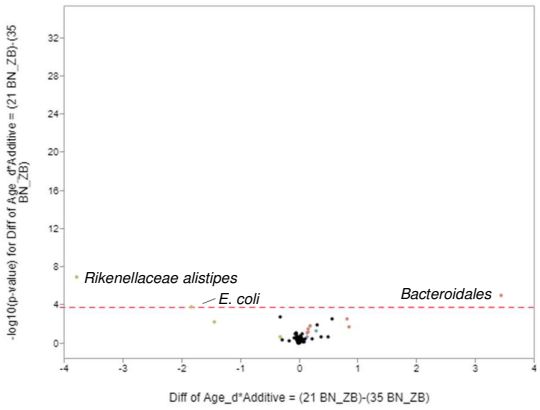



c: Significant difference in abundance for *Rikenellaceae allistipes*, *Ruminococcaceae* and *Bacteroidales*

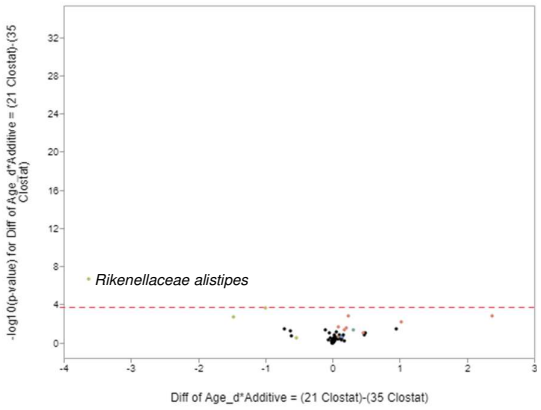
d: Significant difference in abundance for *Rikenellaceae allistipes* and *Lachnospiraceae*

**Figure 4.16** Volcano plots for differences in the caecal microbiome between 21-day old and 35-day old broilers for the Biacid, Biacid_zinc bacitracin (ZB), Biacid Nucleus (BN) and Biacid Nucleus (BN)_Clostat treatment groups

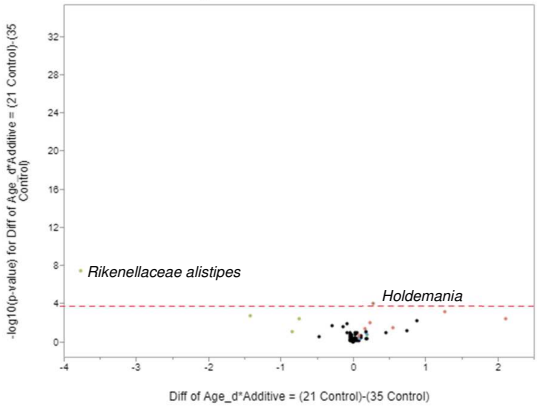
Figure 4.17a shows how the 21-day group treated with BN_ZB had more abundant ($P \leq 0.05$) *Bacteroidales* and the 35-day group had more *Rikenellaceae alistipes 2* and *E.coli*. In figure 4.17b, the *Rikenellaceae alistipes 2* probe was more abundant in the 35-day group treated with Clostat. For the control group, as shown in figure 4.17c, there were significant differences ($P \leq 0.05$) in relative abundance for two of the microbial probes. The 21-day group had more *Holdemania* whereas the 35-day group had more abundant *Rikenellaceae alistipes 2*. In figure 4.17d significantly more ($P \leq 0.05$) *Lachnospiraceae* in the 21-day group and more abundant *Rikenellaceae alistipes 2* in the 35-day samples were observed.



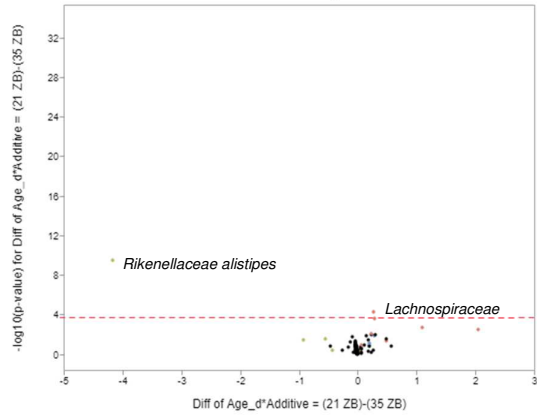

a: Significant difference in abundance for *Rikenellaceae allistipes*, *E. coli* and *Bacteroidales*




a: Significant difference in abundance for *Rikenellaceae allistipes*




c: Significant difference in abundance for *Rikenellaceae allistipes* and *Holdemania*




d: Significant difference in abundance for *Rikenellaceae allistipes* and *Lachnospiraceae*

**Figure 4.17** Volcano plots for differences in the caecal microbiome between 21-day old and 35-day old broilers for the Biacid Nucleus (BN)_zinc bacitracin (ZB), Clostat, Control and zinc bacitracin (ZB) treatment groups

No significant differences ($P \leq 0.05$) were observed for relative abundance of the probes tested between any of the treatment groups for 21-day samples from the caeca. From the differences in relative abundance of microbial probes between all the treatment groups of the 35-day samples, all but one showed no significant differences ($P \leq 0.05$). The only one to reveal a significant difference was the group treated with Biacid that had more ($P \leq 0.05$) *Bacteroides dorei* than the group treated with Biacid_ZB and is shown in figure 4.18. For the comparison between all treatment groups from the caecal samples regardless of age, no significant differences ($P \leq 0.05$) were observed for any of the microbial probes.

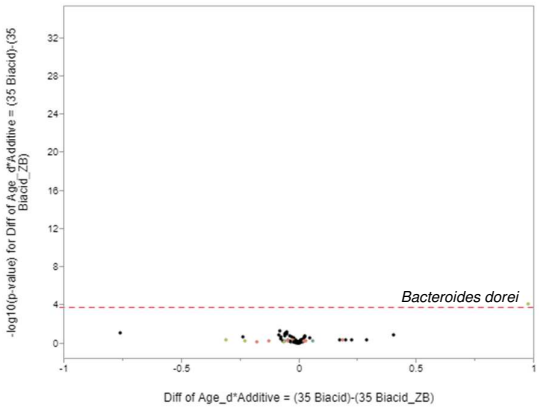



**Figure 4.18** Volcano plot for differences in the caecal microbiome between the Biacid and Biacid_zinc bacitracin (ZB) treatments of 35-day old broilers

# *Chapter 5 – Discussion*

## 5.1 Introduction

The microbiome is a complex system interacting with host infection, intestinal defense and nutrition. The recent development of 16S rRNA sequencing resulted in a vast increase of possibilities for microbiome studies. However, the field of rapid nucleic acid sequencing technology is still new and the amount of data available on broiler specific studies is limited, even more so for broilers challenged with *C. perfringens* (Feng *et al*., 2010). Microbiome studies using next generation sequencing allow for more accurate analysis of communities of microbiota, enabling scientists to better understand the role and interaction of certain microbiota with other variables and improve animal production efficiency and sustainability. The literature reviewed in this study highlighted the interaction of age, environment and diet with the gut microbiome and that the gut microbiomes composition and function is imperative for broiler health and production (Stanley *et al*., 2014a; Choi *et al*., 2015). *C. perfringens* is a naturally occuring pathogen that causes enteric diseases in livestock worldwide, often leading to severe production losses and economic drawbacks, especially in poultry (Van Immerseel *et al*., 2004).

The use of AGPs have decreased globally as widespread concern of antibiotic resistance grew to a point where world governments have banned its use. AGPs are used to improve broiler health and production and are shown to function via directly manipulating the commensal gut microbiota. Alternative feed additives include DFMs, essential oils, organic acids and prebiotics of which all function through an interaction with the gut microbiome (Huyghebaert *et al*., 2011). Knowing how the feed additives tested in this study (as possible AGP alternatives) impact the gut microbiota of broilers inoculated with *C. perfringens*, will provide the industry with scientific evidence with regards to AGP-free feeding practices. From the results obtained, the discussion will address these elements as appropriately as possible.

## 5.2 Microbial diversity of the ileum and caeca

Among the samples for both locations and both age groups, the *Firmicutes* phylum accounted for more than 70% of the bacteria sequenced followed by *Bacteroidetes* and *Proteobacteria* of which each represent almost 10%. These results coincide with the reviews done by Oakley *et al.* (2014) and Shang *et al.* (2018) who reported on literature identifying *Firmicutes* as the dominant phylum in the chicken's GIT. The *Firmicutes* phylum harbours most of the butyrate producing bacteria (Louis & Flint, 2009; Vital *et al*., 2014). Butyrate is a SCFA that improves the host's nutritional status and gut development (Chambers & Gong, 2011) whilst also exhibiting inhibitory effects against pathogens such as *Salmonella* and *C. perfringens* (Van Immerseel *et al*., 2005; Timbermont *et al*., 2010).

Studies done on humans indicate that the ratio between *Firmicutes* and *Bacteroidetes* is correlated with weight, where the *Firmicutes*/*Bacteroidetes* ratio is shown to be significantly higher in obese individuals and significantly lower during weight loss (Ley *et al*., 2006). *Bacteroidetes* is a major member of animal gut microbiota and is shown to have a mutualistic symbiotic relationship with its animal host since both parties benefit from the interaction (Bäckhed *et al*., 2005; Thomas *et al*., 2011). Similar to *Firmicutes*, the *Bacteroidetes* phylum also contributes toward metabolic pathways within the gut. The study done by Polansky

*et al.* (2015) indicated that *Bacteroidetes* microbiota were capable of producing enzymes for propionate production as well as enzymes such as xylose isomerase and others required for polysaccharide degradation.

The third most abundant phylum among the samples from this study was the gram-negative *Proteobacteria* which is regarded as the largest and most diverse phylum of bacteria (Kersters *et al.*, 2006). Although *Proteobacteria* are typically present in low numbers, pathogens found in the human gut such as *Brucella*, *Rickettsia*, *Escherichia* and *Salmonella* are classified as part of this phylum (The Human Microbiome Project, 2012). The review performed by Shin *et al.* (2015) found several studies revealing a correlation between the abundance of *Proteobacteria* and the host's ability to maintain a stable and balanced community of gut microbiota, suggesting that an increased prevalence of the phylum could be a potential marker for diagnosing dysbiosis and disease risk.

### 5.3 Age and anatomical effects

In both the ileal and the caecal results obtained from this study, most of the significant differences ($P \leq 0.05$) in microbiome composition can be attributed to age. This is similar to the results of other studies where chicken development (age) is described as a major contributor to changes in gut microbiome composition (Lu *et al.*, 2003; Gong *et al.*, 2008; Lumpkins *et al.*, 2010; Ballou *et al.*, 2016). The gut microbiome establishes itself by initial colonisation, growth and difersification, and then finally stabilisation (Pedrosa, 2009; Mountzouris *et al.*, 2010; Rinttila & Apajalahti, 2013). Other studies also report the distinctiveness of the microbiota's composition and abundance between each anatomical region of the GIT e.g. the gizzard, the small intestine and the caeca (Shang *et al.*, 2018).

For the ileal samples, all the older birds had more abundant ($P \leq 0.05$) *Lactobacillus spp.* and *E. hirae* and the 21-day samples had more ($P \leq 0.05$) *Streptococcus*, *Listeria* and *E. coli*. This pattern remained up to 35-days of age. From the bacteria relevant in this shift, only *Lactobacillus* is shown to be beneficial to broiler health and production (Kalavathy *et al.*, 2003). Outbreaks of *E. hirae* have been shown to be accompanied by septicaemia and endocarditis, indicating the bacteria's potential pathogenic nature (Chadfield *et al.*, 2005; Kolbjornsen *et al.*, 2011). The *Streptococcus*, *Listeria* and *E. coli* as found in increased abundance in the younger ileal samples are known as pathogenic organisms within gut microbiota (Glaser *et al.*, 2001; Hedegaard *et al.*, 2009; Oakley *et al.*, 2014).

From the caecal results, it was shown that older birds had significantly more ($P \leq 0.05$) *Rikenellaceae alistipes 2*, *E. coli* and *Bacteroides dorei*. The 21-day samples on the other hand had significantly more ($P \leq 0.05$) *Bacteroidales*, *Ruminococcaceae*, *Rikenellaceae alistipes 1*, *Lachnospiraceae*, *Lactobacillus spp.*, *Citrobacter*, *Bifidobacterium gallinarum* and *Holdemania*. This makes it apparent that for the caeca, the younger birds' microbiome was more diverse. The results of other studies where chickens were not challenged with *C. perfringens* showed otherwise and reported an increase in microbial diversity as birds aged (Lu *et al.*, 2003; Ballou *et al.*, 2016). However, the results from this current study indicated that the bacteria that significantly decreased in abundance as the birds aged were prominently butyrate-producing bacteria (Vital *et al.*, 2014; Rivière *et al.*, 2016) and agrees with the findings of Wu *et al.* (2014) that reported a reduction of butyrate producing bacteria in correlation with the proliferation of *C. perfringens*.

These shifts in general imply that even though birds were challenged with *C. perfringens* infection, the gut microbiota was still a dynamic ecosystem susceptible to change. Its ability to change serves as a

foundation for the current feeding practices of feed additives that function primarily through manipulation of the gut microbiome and will be discussed later in this chapter.

The gut microbiome is a dynamic system that varies due to factors induced by the host and the environment (Stanley *et al*., 2014a). The GIT of chicks are colonised as early as during the incubation phase (Kizerwetter-Świda & Binek, 2008; Pedrosa, 2009; Roto *et al*., 2016). Although all chicks will not necessarily be exposed to the same kind of microbes from their environment and diet during early colonisation, the basic physiological condition of the colonised hosts will vary to a lesser degree. Most chicks of the same genetic line exposed to similar feeding and housing conditions will have a similar intestinal environment that the microbes are exposed to when they colonise the intestinal tract (Schokker *et al*., 2015). The young chick's intestinal tract is considered aerobic and therefore should allow for the colonisation of facultative aerobes such as *Streptococcus* and *Enterobacteriaceae* which includes *E. coli* (Rinttila & Apajalahti, 2013).

The results from the 21-day ileum samples of this study had more ($P \leq 0.05$) *Streptococcus* and *E. coli* and are thus in agreement with the results obtained from other studies that similarly found these bacterial groups to dominate the ileum of young broilers (Lu *et al*., 2003; Wise & Siragusa, 2007). The older ileum samples had relatively more ($P \leq 0.05$) of the anaerobic bacteria *E. hirae* which coincides with a number of studies reported this increase with age (Mountzouris *et al*., 2010; Rinttila & Apajalahti, 2013). According to Smyth & McNamee (2008), *E. hirae* infections cause phalomalacia, septicaemia and endocarditis in chickens. Although part of the normal intestinal microbiome, *Enterococci* are considered as "opportunistic pathogens in birds and mammals" (Kolbjornsen *et al*., 2011). Older birds also had more ($P \leq 0.05$) *Lactobacillus spp.* in their ileum, consistent with the findings of Lu *et al.* (2003). *Lactobacillus spp.* are beneficial for improving feed conversion ratio and reducing abdominal fat deposition in broilers (Kalavathy *et al*., 2003). On the contrary, Engberg *et al.* (2000) found that high levels of *Lactobacilli* might be involved with growth depression in broilers mainly due to competition for nutrients with the host and/ or decreased fat absorption via deconjugation of bile acids. In the caeca, the microbiome made a shift from predominantly low G+C, gram positive bacteria (also known as *Firmicutes*) in the younger chicks, to a microbial population that had more abundant high G+C, gram positive bacteria and *Proteobacteria*. Lu *et al.* (2003) also reported that the microbiome of the caeca changed with age, although their study found that the significant change occurred during the first two weeks of life.

This study's results also indicate the distinctiveness of the separate gastrointestinal locations namely the ileum and the caeca, which is in agreement with the studies performed by Gong *et al.* (2007) and Lu *et al.* (2003). From the bacteria on the customised chip, the amount of significant bacterial *spp*. found in the ileum and caecum were 49 and 16 respectively. The ileum has more readily available nutrients compared to the caeca (Apajalahti & Vienola, 2016) and could be a possible explanation for the observation that the ileum had a much more diverse microbial profile when compared to the caecum's microbial profile. This is contrasted by the results of Gong *et al.* (2002) that found the ileum to harbour a less diverse microbial community compared to the caeca. The descriptive study performed by Bjerrum *et al.* (2006) similarly concluded that the caecum was host to a more diverse community of microbiota compared to the ileum.

It is important to note that the sequencing method in the trial for this research was semi-quantitative and was only performed for a specific predefined set of bacteria deemed as significant to broiler health and production. This along with other limitations of current identification techniques could explain the difference observed between this study and those of the authors previously mentioned with regards to the microbial

diversity in the ileum and caecum. The effect of age and anatomical location on the microbiome is therefore a function of the effects and interactions of the physiology and morphology of the host's gastrointestinal environment, and the characteristics of the microbes' metabolism (Shang *et al.*, 2018).

### 5.4 Treatment effects

In this study, DFMs, essential oils, organic acids and prebiotics were tested as replacements for an AGP by observing the effects from the different treatments on the gut microbiota. AGPs have been the general ingredient of choice worldwide to stimulate growth and to prevent bacterial infections from pathogens such as *C. perfringens (Caly et al., 2015)*. Due to build-up of antimicrobial resistance, antibiotics have recently been a subject of scrutiny among public consumers resulting in the ban of its use in Europe (feed additives regulation 1831/2003/EC) since January 2006 (Caly *et al.*, 2015). This has created a domino effect worldwide and led to the increased interest in AGP alternatives such as DFMs, also known as probiotics.

All the feed additives tested in this study work primarily through interacting with or manipulating the host's commensal gut microbiota. Probiotics for example have been shown to contain strains with *in vitro* anti-*C. perfringens* activity (Caly *et al.*, 2015). A study performed by Song *et al.* (2014) found that broilers supplemented with probiotics had increased levels of *Lactobacillus* and *Bifidobacterium* in the small intestine and improved their feed to gain ratio. Probiotics can induce beneficial effects such as improved growth performance and improvement of gastrointestinal health (Bozkurt *et al.*, 2009; Blajman *et al.*, 2014). Other feed additives such as essential oils, organic acids and prebiotics function through inhibiting certain pathogenic bacteria and/ or stimulating the growth of other beneficial bacteria and achieve this through different modes of action (Huyghebaert *et al.*, 2011).

All 21-day samples were compared between treatment groups for the ileum and the caeca respectively and similarly so with the 35-day samples and with all the samples regardless of age. In contrast to age, the different treatments exhibited limited significant differences on diversity of gut microbiota. For the results from the ileum, when comparing all the treatment groups to one another regardless of age, no significant differences ($P \leq 0.05$) were observed for microbial diversity. Due to the effect of age on the microbial profile as discussed earlier, it is important to differentiate and compare the treatment groups within the same age category.

The 21-day samples mostly showed no significant differences ($P \leq 0.05$) between the different treatment groups. There were however two exceptions. The group treated with BN_ZB had more ($P \leq 0.05$) abundant *E. hirae*, which is part of the *Firmicutes* phylum (Gibbons & Murray, 1978; Farrow & Collins, 1985), but had less ($P \leq 0.05$) *Clostridium disporicum*, *Bifidobacterium* and *Brenneria* than the Biacid treated group. It is expected that the treatment including ZB would lead to a significant decrease in certain microbes' abundance. One of the mechanisms of action of AGPs is inhibition of the growth of microbiota (Hughes & Heritage, 2004; Dibner & Richards, 2005). This in combination with the antimicrobial activity of the essential oils (Diaz Carrasco *et al.*, 2016) in the BN could be responsible for the reduction in abundance of the mentioned microbes. These results also imply that the Biacid was more effective in reducing *E. hirae* than the BN_ZB group. This may however also be an effect of the BN_ZB treatment that allows for more abundant *E. hirae* due to the absence or lower abundance of the other microflora mentioned above. The studies previously mentioned indicate that *E. hirae* infection might cause phalomalacia, septicaemia and endocarditis and should be regarded as a

potential pathogen even though it forms part of the normal gut microbiota (Chadfield *et al.*, 2005; Smyth & McNamee, 2008; Kolbjornsen *et al.*, 2011).

With this in mind, the other observed significant difference among the 21-day ileum samples was the BN_ZB treated group where more ($P \leq 0.05$) *E. hirae* was observed compared to the group treated with ZB alone. This highlights the role that the BN has in this combination. This observation is in alignment with the findings of Li *et al.* (2018) who reported that supplementation with essential oils increased the abundance of *Firmicutes* organisms. This is relevant since *E. hirae* is classified as part of the *Firmicutes* Phylum (Gibbons & Murray, 1978; Farrow & Collins, 1985).

Among the 35-day samples, three significant differences were observed. Firstly, the Clostat treated group had more ($P \leq 0.05$) *Lactobacillus* than the negative control group. This is in agreement with the study performed by Song *et al.* (2014) who also found that a supplemental probiotic increased *Lactobacillus* levels in the small intestine. Although Clostat is a probiotic containing a *Bacillus subtilis* strain, Hosoi *et al.* (2000) found that *B. subtilis* can enhance growth and viability of *Lactobacillus* strains. *Lactobacillus* has shown to be beneficial for broiler FCR and body weight gain (Kalavathy *et al.*, 2003). The Clostat group also had more ($P \leq 0.05$) *Lactobacillus* than the group treated with the Biacid. This might be an indication of the antimicrobial properties of the EO + OA mixture as compared to the probiotic effects of the DFM. Essential oils and organic acids have the ability to inhibit the proliferation of certain bacteria by disrupting bacterial cell metabolism and causing cell lysis (Huyghebaert *et al.*, 2011). The third significant difference among the 35-day ileum samples was that the BN group had more ($P \leq 0.05$) of the *Lachnospiraceae* family than the negative control group. A study done by Stanley *et al.* (2016) showed that increased presence of the *Lachnospiraceae* family in the GIT of chickens was correlated to lower (better) FCR values. *Lachnospiraceae* possibly contribute toward efficiency of feed utilisation due to its capacity for butyrate production (Meehan & Beiko, 2014). The BN was therefore able to manipulate the microbiome of the broiler's ileum towards a better profile that could positively impact performance when compared to the control group.

For the caecal samples, comparing all the treatment groups regardless of age also yielded no significant differences ($P \leq 0.05$) for microbial diversity. Within the 21-day age group of samples, no treatment group differed from another with regard to diversity of microbiota. When the 35-day samples were compared, one significant difference was observed. The Biacid treated group had more ($P \leq 0.05$) *Bacteroides dorei* present than the group treated with the combination of Biacid and ZB. The minireview performed by Oakley *et al.* (2014) reported that the microbiota of the chicken's caeca mainly consists of *Firmicutes* and *Bacteroidetes* at the phylum level. It is therefore possible that this observation was due to the ZB's added antimicrobial effect on *Bacteroides dorei*. In humans, *Bacteroides spp.* were shown to be possible inhibitors of *Clostridium difficile* infection (Hopkins & Macfarlane, 2002, 2003). Since the *Bacteroides dorei* was the only significant difference between these two groups, it is clear that providing the ZB as an addition to the Biacid provided no extra benefit to the host's gut microbiota. However, the unit of measurement was relative abundance and it might be possible that absolute microbial numbers changed while ratios of organisms remained relatively constant.

All results from this study was observed from broilers challenged with *C. perfringens*. It is not possible to account for the effect of this challenge or to know its impact on the results since no group were included in this study that were not challenged with *C. perfringens*.

## *Chapter 6 – Conclusion*

The aim of this study was to assess the ileal and caecal microbiome, using 16S rRNA sequencing methods, of *C. perfringens* challenged broilers that received different feed additives and an AGP, alone and in combination, while also evaluating the difference in microbial diversity for the ileum and the caecum between 21 and 35 days of age.

Age had the most significant effect on the microbiome in both the ileum and the caeca. The *Firmicutes* phylum was the most abundant regardless of age, followed by *Bacteroidetes* and *Proteobacteria*. Most of the feed additive effects were non-significant. However, it was observed that the microbiome did differ between some of the treatment groups. Only Clostat and Biacid Nucleus significantly changed the ileal microbiome of the 35-day old broilers when compared with the negative control group. The Clostat and Biacid Nucleus treatments led to increased levels of *Lactobacillus* and *Lachnospiraceae* respectively, of which both are beneficial for broiler feed efficiency and health. Other differences in microbial abundance were observed between 21-day ileal samples of BN_ZB and Biacid, and also BN_ZB and ZB. Among the 35-day ileal samples (in addition to those already mentioned), the Clostat group differed from the Biacid group. From the caecal samples, only the 35-day Biacid and Biacid_ZB groups differed significantly and was probably due to the added antimicrobial effect of the ZB. In comparison with the negative control, Clostat and Biacid Nucleus were able to beneficially manipulate the commensal microbiome of the broilers' ileum.

Although the objectives were accomplished for this study, future studies could gain further insight into the effect of these additives on the gut microbiome if tested on broilers that are not exposed to *C. perfringens* infection. Alternatively, future research could also test the effect of a blend of the DFM, EO and OA on the gut microbiome. This study used the DFM alone and the EO and OA either alone or in combination with the AGP respectively, but no treatment with the three possible alternatives in combination with each other was tested. Studying this could provide insight into possible synergistic effects between those additives as seen by other studies (Bomba *et al.*, 2002; Bozkurt *et al.*, 2009; Abudabos *et al.*, 2017). The beneficial effects of probiotics include improved growth performance and betterment of gastrointestinal health (Bozkurt *et al.*, 2009; Blajman *et al.*, 2014; Song *et al.*, 2014).

The South African broiler industry is moving towards the removal of AGPs from commercial broiler diets. With Europe already comfortably past this point and local middle class consumers rapidly increasing, the pressure will only continue to mount on the local industry to ban the use of in-feed antibiotics. Therefore, more research on the chicken microbiome's role and interaction with its host's health and nutrition is necessary.

## *References*


Abudabos, A.M., Alyemni, A.H., Dafalla, Y.M. & Khan, R.U. (2017). Effect of organic acid blend and Bacillus subtilis alone or in combination on growth traits, blood biochemical and antioxidant status in broilers exposed to Salmonella typhimurium challenge during the starter phase. *J. Appl. Anim. Res., 45*(1), 538-542. doi:10.1080/09712119.2016.1219665

Adams, M.R. & Hall, C.J. (1988). Growth inhibition of food-borne pathogens by lactic and acetic acids and their mixtures. *Int. J. Food Sci. Technol., 23*(3), 287-292. doi:10.1111/j.1365-2621.1988.tb00581.x

Allaart, J.G., van Asten, A.J.A.M. & Gröne, A. (2013). Predisposing factors and prevention of Clostridium perfringens-associated enteritis. *Comp. Immunol. Microbiol. Infect. Dis., 36*(5), 449-464. doi:https://doi.org/10.1016/j.cimid.2013.05.001

Annett, C.B., Viste, J.R., Chirino-Trejo, M., Classen, H.L., Middleton, D.M. & Simko, E. (2002). Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of Clostridium perfringens type A. *Avian Pathol., 31*(6), 599. doi:10.1080/0307945021000024544

Antonissen, G., Croubels, S., Pasmans, F., Ducatelle, R., Eeckhaut, V., Devreese, M., Verlinden, M., Haesebrouck, F., Eeckhout, M., De Saeger, S., Antlinger, B., Novak, B., Martel, A. & Van Immerseel, F. (2015a). Fumonisins affect the intestinal microbial homeostasis in broiler chickens, predisposing to necrotic enteritis. *Vet. Res, 46*, 98. doi:10.1186/s13567-015-0234-8

Antonissen, G., Van Immerseel, F., Pasmans, F., Ducatelle, R., Janssens, G.P., De Baere, S., Mountzouris, K.C., Su, S., Wong, E.A., De Meulenaer, B., Verlinden, M., Devreese, M., Haesebrouck, F., Novak, B., Dohnal, I., Martel, A. & Croubels, S. (2015b). Mycotoxins Deoxynivalenol and Fumonisins Alter the Extrinsic Component of Intestinal Barrier in Broiler Chickens. *J. Agric. Food Chem., 63*(50), 10846-10855. doi:10.1021/acs.jafc.5b04119

Antonissen, G., Eeckhaut, V., Van Driessche, K., Onrust, L., Haesebrouck, F., Ducatelle, R., Moore, R.J. & Van Immerseel, F. (2016). Microbial shifts associated with necrotic enteritis. *Avian Pathol., 45*(3), 308-312. doi:10.1080/03079457.2016.1152625

AOAC. (2000a). Official method of analysis 920.39. *Association of Official Analytical Chemists, Volume I*(17th Edition).

AOAC. (2000b). Official method of analysis 935.13. *Association of Official Analytical Chemists, Volume I*(17th Edition).

AOAC. (2000c). Official method of analysis 965.17. *Association of Official Analytical Chemists, Volume I*(17th Edition).

AOAC. (2000d). Official method of analysis 943.01. *Association of Official Analytical Chemists, Volume I*(17th Edition).

AOAC. (2000e). Official method of analysis 962.09. *Association of Official Analytical Chemists, Volume I*(17th Edition).

AOAC. (2000f). Official method of analysis 988.05. *Association of Official Analytical Chemists, Volume I*(17th Edition).

AOAC. (2000g). Official method of analysis 942.05. *Association of Official Analytical Chemists, Volume I*(17th Edition).

Apajalahti, J. & Vienola, K. (2016). Interaction between chicken intestinal microbiota and protein digestion. *Anim. Feed. Sci. Tech.* doi:http://dx.doi.org/10.1016/j.anifeedsci.2016.05.004

Aziz, Q., Dore, J., Emmanuel, A., Guarner, F. & Quigley, E.M. (2013). Gut microbiota and gastrointestinal health: current concepts and future directions. *Neurogastroent. Motil., 25*(1), 4-15. doi:10.1111/nmo.12046

Bäckhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. & Gordon, J.I. (2005). Host-Bacterial Mutualism in the Human Intestine. *Science, 307*(5717), 1915-1920.

Ballou, A.L., Ali, R.A., Mendoza, M.A., Ellis, J.C., Hassan, H.M., Croom, W.J. & Koci, M.D. (2016). Development of the Chick Microbiome: How Early Exposure Influences Future Microbial Diversity. *Front. Vet. Sci., 3*(2). doi:10.3389/fvets.2016.00002

Benson, A.K., Kelly, S.A., Legge, R., Ma, F., Low, S.J., Kim, J., Zhang, M., Oh, P.L., Nehrenberg, D., Hua, K., Kachman, S.D., Moriyama, E.N., Walter, J., Peterson, D.A. & Pomp, D. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc. Natl. Acad. Sci. U.S.A., 107*(44), 18933-18938. doi:10.1073/pnas.1007028107

Bjerrum, L., Pedersen, K., Engberg, R.M., Jensen, B.B., Leser, T.D. & Finster, K. (2006). Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. *Poultry Sci., 85*(7), 1151-1164.

Blajman, J.E., Frizzo, L.S., Zbrun, M.V., Astesana, D.M., Fusari, M.L., Soto, L.P., Rosmini, M.R. & Signorini, M.L. (2014). Probiotics and broiler growth performance: a meta-analysis of randomised controlled trials. *Br. Poult. Sci., 55*(4), 483-494. doi:10.1080/00071668.2014.931930

Blake, A.B. & Suchodolski, J.S. (2016). Importance of gut microbiota for the health and disease of dogs and cats. *Animal Frontiers, 6*(3), 37. doi:10.2527/af.2016-0032

Bomba, A., Nemcová, R.r., Mudroňová, D. & Guba, P. (2002). The possibilities of potentiating the efficacy of probiotics. *Trends. Food. Sci. Tech., 13*(4), 121-126. doi:https://doi.org/10.1016/S0924-2244(02)00129-2

Bozkurt, M., Küçüky1lmaz, K., Çatl1, A.U. & Ç1nar, M. (2009). The effect of single or combined dietary supplementation of prebiotics, organic acid and probiotics on performance and slaughter characteristics of broilers. *S. Afr. J. Anim. Sci., 39*, 197-205.

Bravo, J.A., Julio-Pieper, M., Forsythe, P., Kunze, W., Dinan, T.G., Bienenstock, J. & Cryan, J.F. (2012). Communication between gastrointestinal bacteria and the nervous system. *Curr. Opin. Pharmacol., 12*(6), 667-672. doi:10.1016/j.coph.2012.09.010

Brisbin, J.T., Orouji, S., Esufali, J., Mallick, A.I., Parvizi, P., Shewen, P.E., Sharif, S. & Gong, J. (2011). Oral treatment of chickens with lactobacilli influences elicitation of immune responses. *Clin. Vaccine. Immunol., 18*(9), 1447-1455. doi:10.1128/CVI.05100-11

Broom, D.M., Galindo, F.A. & Murgueitio, E. (2013). Sustainable, efficient livestock production with high biodiversity and good welfare for animals. *Proc. R. Soc. Biol. Sci. Ser. B, 280*(1771). doi:10.1098/rspb.2013.2025

Brynestad, S. & Granum, P.E. (2002). Clostridium perfringens and foodborne infections. *Int. J. Food Microbiol., 74*(3), 195-202. doi:https://doi.org/10.1016/S0168-1605(01)00680-8

Caly, D.L., D'Inca, R., Auclair, E. & Drider, D. (2015). Alternatives to antibiotics to prevent necrotic enteritis in broiler chickens: a microbiologist's perspective. *Front. Microbiol., 6*, 1-12. doi:10.3389/fmicb.2015.01336

Cenit, M.C., Matzaraki, V., Tigchelaar, E.F. & Zhernakova, A. (2014). Rapidly expanding knowledge on the role of the gut microbiome in health and disease. *B.B.A. Mol. Basis. Dis., 1842*(10), 1981-1992. doi:10.1016/j.bbadis.2014.05.023

Chadfield, M.S., Christensen, J.P., Juhl-Hansen, J., Christensen, H. & Bisgaard, M. (2005). Characterization of Enterococcus hirae Outbreaks in Broiler Flocks Demonstrating Increased Mortality Because of Septicemia and Endocarditis and/or Altered Production Parameters. *Avian Dis., 49*(1), 16-23.

Chambers, J.R. & Gong, J. (2011). The intestinal microbiota and its modulation for Salmonella control in chickens. *Food Res. Int., 44*(10), 3149-3159. doi:10.1016/j.foodres.2011.08.017

Chaucheyras-Durand, F. & Durand, H. (2010). Probiotics in animal nutrition and health. *Benef. Microbes, 1*(1), 3-9. doi:10.3920/bm2008.1002

Choct, M., Hughes, R.J., Wang, J., Bedford, M.R., Morgan, A.J. & Annison, G. (1996). Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. *Br. Poult. Sci., 37*(3), 609-621. doi:10.1080/00071669608417891

Choi, K.Y., Lee, T.K. & Sul, W.J. (2015). Metagenomic analysis of chicken gut microbiota for improving metabolism and health of chickens--a review. *Asian Austral. J. Anim., 28*, 1217+.

Cisek, A.A. & Binek, M. (2014). Chicken intestinal microbiota function with a special emphasis on the role of probiotic bacteria. *Pol. J. Vet. Sci, 17*(2). doi:10.2478/pjvs-2014-0057

Clemente, J.C., Ursell, L.K., Parfrey, L.W. & Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell, 148*(6), 1258-1270. doi:10.1016/j.cell.2012.01.035

Coates, M.E., Fuller, R., Harrison, G.F., Lev, M. & Suffolk, S.F. (1963). A comparision of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. *Br. J. Nutr., 17*(1), 141-150. doi:10.1079/BJN19630015

Cooper, K.K. & Songer, J.G. (2010). Virulence of Clostridium perfringens in an experimental model of poultry necrotic enteritis. *Vet. Microbiol., 142*(3), 323-328. doi:https://doi.org/10.1016/j.vetmic.2009.09.065

Corthier, G. & Doré, J. (2010). A new era in gut research concerning interactions between microbiota and human health. *Gastroen. Clin. Biol., 34*(1), S1-S6.

Cox, L.M. (2016). Antibiotics shape microbiota and weight gain across the animal kingdom. *Animal Frontiers, 6*(3), 8-14. doi:10.2527/af.2016-0028

Craven, S.E. (2000). Colonization of the intestinal tract by Clostridium perfringens and fecal shedding in diet-stressed and unstressed broiler chickens. *Poultry Sci., 79*(6), 843-849. doi:10.1093/ps/79.6.843

Cravens, R.L., Goss, G.R., Chi, F., De Boer, E.D., Davis, S.W., Hendrix, S.M., Richardson, J.A. & Johnston, S.L. (2013). The effects of necrotic enteritis, aflatoxin B1, and virginiamycin on growth performance, necrotic enteritis lesion scores, and mortality in young broilers. *Poultry Sci., 92*(8), 1997-2004. doi:10.3382/ps.2013-03011

Crhanova, M., Hradecka, H., Faldynova, M., Matulova, M., Havlickova, H., Sisak, F. & Rychlik, I. (2011). Immune response of chicken gut to natural colonization by gut microflora and to Salmonella enterica serovar enteritidis infection. *Infect. Immun., 79*(7), 2755-2763. doi:10.1128/IAI.01375-10

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., Collini, S., Pieraccini, G., Lionetti, P. & Hartl, D.L. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U.S.A., 107*(33), 14691-14696.

De Vries, M. & De Boer, I.J.M. (2010). Comparing environmental impacts for livestock products: A review of life cycle assessments. *Livest. Sci., 128*(1–3), 1-11. doi:http://dx.doi.org/10.1016/j.livsci.2009.11.007

Deeming, D.C. (2005). Yolk sac, body dimensions and hatchling quality of ducklings, chicks and poults. *Br. Poult. Sci., 46*(5), 560-564. doi:10.1080/00071660500255042

Diaz Carrasco, J.M., Redondo, L.M., Redondo, E.A., Dominguez, J.E., Chacana, A.P. & Fernandez Miyakawa, M.E. (2016). Use of Plant Extracts as an Effective Manner to Control Clostridium perfringens Induced Necrotic Enteritis in Poultry. *Biomed Res. Int., 2016*, 3278359. doi:10.1155/2016/3278359

Dibner, J.J. & Richards, J.D. (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Sci., 84*(4), 634-643. doi:10.1093/ps/84.4.634

Drew, M.D., Syed, N.A., Goldade, B.G., Laarveld, B. & Van Kessel, A.G. (2004). Effects of dietary protein source and level on intestinal populations of Clostridium perfringens in broiler chickens. *Poultry Sci., 83*(3), 414-420. doi:10.1093/ps/83.3.414

El Aidy, S., Dinan, T.G. & Cryan, J.F. (2015). Gut Microbiota: The Conductor in the Orchestra of Immune–Neuroendocrine Communication. *Clin. Ther., 37*(5), 954-967. doi:http://dx.doi.org/10.1016/j.clinthera.2015.03.002

Engberg, R.M., Hedemann, M.S., Leser, T.D. & Jensen, B.B. (2000). Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. *Poultry Sci., 79*(9), 1311-1319. doi:10.1093/ps/79.9.1311

Evans, J.M., Morris, L.S. & Marchesi, J.R. (2013). The gut microbiome: the role of a virtual organ in the endocrinology of the host. *J. Endocrinol., 218*(3), R37-R47. doi:10.1530/joe-13-0131

FAO. (2009). How to Feed the World in 2050.

Farrow, J.A.E. & Collins, M.D. (1985). Enterococcus hirae, a New Species That Includes Amino Acid Assay Strain NCDO 1258 and Strains Causing Growth Depression in Young Chickens. *Int. J. Syst. Evol. Micr., 35*(1), 73-75. doi:doi:10.1099/00207713-35-1-73

Feng, Y., Gong, J., Yu, H., Jin, Y., Zhu, J. & Han, Y. (2010). Identification of changes in the composition of ileal bacterial microbiota of broiler chickens infected with Clostridium perfringens. *Vet. Microbiol., 140*(1), 116-121. doi:https://doi.org/10.1016/j.vetmic.2009.07.001

Fouhse, J.M., Zijlstra, R.T. & Willing, B.P. (2016). The role of gut microbiota in the health and disease of pigs. *Animal Frontiers, 6*(3), 30. doi:10.2527/af.2016-0031

Fujimoto, S., Nakagami, Y. & Kojima, F. (2004). Optimal bacterial DNA isolation method using bead-beating technique. *Memoirs Kyushu Univ. Dep. Of Health Scis. Of Medical Sch., 3*, 33-38.

Gaggia, F., Mattarelli, P. & Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. *Int. J. Food Microbiol., 141 Suppl 1*, S15-28. doi:10.1016/j.ijfoodmicro.2010.02.031

George, B.A., Quarles, C.L. & Fagerberg, D.J. (1982). Virginiamycin Effects on Controlling Necrotic Enteritis Infection in Chickens. *Poultry Sci., 61*(3), 447-450. doi:10.3382/ps.0610447

Gerbens-Leenes, P.W., Mekonnen, M.M. & Hoekstra, A.Y. (2013). The water footprint of poultry, pork and beef: A comparative study in different countries and production systems. *Water Resources and Industry, 1–2*, 25-36. doi:http://dx.doi.org/10.1016/j.wri.2013.03.001

Gibbons, N.E. & Murray, R.G.E. (1978). Proposals Concerning the Higher Taxa of Bacteria. *Int. J. Syst. Evol. Micr., 28*(1), 1-6. doi:doi:10.1099/00207713-28-1-1

Gibson, G.R. & Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr., 125*(6), 1401-1412.

Glaser, P., Entian, K.D., Jones, L.M., Pérez-Diaz, J.C., Vázquez-Boland, J.A., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couvé, E., de Daruvar, A., Dehoux, P., Domann, E., Domínguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Fsihi, H., Portillo, F.G.-D., Garrido, P., Gautier, L., Goebel, W., Gómez-López, N., Hain, T., Hauf, J., Jackson, D., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueño, E., Maitournam, A., Vicente, J.M., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Voss, H., Wehland, J. & Cossart, P. (2001). Comparative Genomics of Listeria Species. *Science, 294*(5543), 849-852.

Gong, J., Forster, R.J., Yu, H., Chambers, J.R., Wheatcroft, R., Sabour, P.M. & Chen, S. (2002). Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. *FEMS Microbiol. Ecol., 41*(3), 171-179. doi:10.1111/j.1574-6941.2002.tb00978.x

Gong, J., Si, W., Forster, R.J., Huang, R., Yu, H., Yin, Y., Yang, C. & Han, Y. (2007). 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. *FEMS Microbiol. Ecol., 59*(1), 147-157. doi:10.1111/j.1574-6941.2006.00193.x

Gong, J., Yu, H., Liu, T., Gill, J.J., Chambers, J.R., Wheatcroft, R. & Sabour, P.M. (2008). Effects of zinc bacitracin, bird age and access to range on bacterial microbiota in the ileum and caeca of broiler chickens. *J. Appl. Microbiol., 104*(5), 1372-1382. doi:doi:10.1111/j.1365-2672.2007.03699.x

Gonzalez, R.M. & Angeles, H.J.C. (2017). Antibiotic and synthetic growth promoters in animal diets: Review of impact and analytical methods. *Food Control, 72*, 255-267. doi:http://dx.doi.org/10.1016/j.foodcont.2016.03.001

Greenhalgh, K., Meyer, K.M., Aagaard, K.M. & Wilmes, P. (2016). The human gut microbiome in health: establishment and resilience of microbiota over a lifetime. *Environ. Microbiol., 18*(7), 2103-2116. doi:10.1111/1462-2920.13318

Grenham, S., Clarke, G., Cryan, J.F. & Dinan, T.G. (2011). Brain-gut-microbe communication in health and disease. *Front. Physiol., 2*, 94. doi:10.3389/fphys.2011.00094

Hanset, R., Michaux, C. & Stasse, A. (1987). Relationships between growth rate, carcass composition, feed intake, feed conversion ratio and income in four biological types of cattle. *Génét. Sél. Évol., 19*(2), 225-248. doi:10.1186/1297-9686-19-2-225

Hedegaard, L., Christensen, H., Chadfield, M.S., Christensen, J.P. & Bisgaard, M. (2009). Association of Streptococcus pluranimalium with valvular endocarditis and septicaemia in adult broiler parents. *Avian Pathol., 38*(2), 155-160. doi:10.1080/03079450902737763

Herrero, M., Gerber, P., Vellinga, T., Garnett, T., Leip, A., Opio, C., Westhoek, H.J., Thornton, P.K., Olesen, J., Hutchings, N., Montgomery, H., Soussana, J.F., Steinfeld, H. & McAllister, T.A. (2011). Livestock and greenhouse gas emissions: The importance of getting the numbers right. *Anim. Feed. Sci. Tech., 166–167*, 779-782. doi:http://dx.doi.org/10.1016/j.anifeedsci.2011.04.083

Herrero, M., Havlík, P., Valin, H., Notenbaert, A., Rufino, M.C., Thornton, P.K., Blümmel, M., Weiss, F., Grace, D. & Obersteiner, M. (2013). Biomass use, production, feed efficiencies, and greenhouse gas emissions from global livestock systems. *Proc. Natl. Acad. Sci. U.S.A., 110*(52), 20888-20893. doi:10.1073/pnas.1308149110

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G. & Gordon, J.I. (2001). Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine. *Science, 291*(5505), 881-884.

Hooper, L.V., Midtvedt, T. & Gordon, J.I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr., 22*(1), 283.

Hopkins, M.J. & Macfarlane, G.T. (2002). Changes in predominant bacterial populations in human faeces with age and with Clostridium difficile infection. *J. Med. Microbiol., 51*(5), 448-454. doi:doi:10.1099/0022-1317-51-5-448

Hopkins, M.J. & Macfarlane, G.T. (2003). Nondigestible Oligosaccharides Enhance Bacterial Colonization Resistance against Clostridium difficile In Vitro. *Appl. Environ. Microbiol., 69*(4), 1920-1927. doi:10.1128/aem.69.4.1920-1927.2003

Hosoi, T., Ametani, A., Kiuchi, K. & Kaminogawa, S. (2000). Improved growth and viability of lactobacilli in the presence of Bacillus subtilis (natto), catalase, or subtilisin. *Can. J. Microbiol., 46*(10), 892-897.

Howlider, M.A.R. & Rose, S.P. (1992). The response of growing male and female broiler chickens kept at different temperatures to dietary energy concentration and feed form. *Anim. Feed. Sci. Tech., 39*(1), 71-78. doi:https://doi.org/10.1016/0377-8401(92)90032-2

Hughes, P. & Heritage, J. (2004). Antibiotic growth-promoters in food animals. *FAO,* 129-151.

Huyghebaert, G., Ducatelle, R. & Van Immerseel, F. (2011). An update on alternatives to antimicrobial growth promoters for broilers. *Vet. J., 187*(2), 182-188. doi:10.1016/j.tvjl.2010.03.003

Hyland, J.J., Styles, D., Jones, D.L. & Williams, A.P. (2016). Improving livestock production efficiencies presents a major opportunity to reduce sectoral greenhouse gas emissions. *Agric. Sys., 147*, 123-131. doi:http://dx.doi.org/10.1016/j.agsy.2016.06.006

Janda, J.M. & Abbott, S.L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J. Clin. Microbiol., 45*(9), 2761-2764. doi:10.1128/JCM.01228-07

Jha, R. & Berrocoso, J.D. (2015). Review: Dietary fiber utilization and its effects on physiological functions and gut health of swine. *Animal, 9*(9), 1441-1452. doi:10.1017/s1751731115000919

Jiang, Y., Kulkarni, R.R., Parreira, V.R. & Prescott, J.F. (2009). Immunization of Broiler Chickens against Clostridium perfringens- Induced Necrotic Enteritis Using Purified Recombinant Immunogenic Proteins. *Avian Dis., 53*(3), 409-415.

Kalavathy, R., Abdullah, N., Jalaludin, S. & Ho, Y.W. (2003). Effects of Lactobacillus cultures on growth performance, abdominal fat deposition, serum lipids and weight of organs of broiler chickens. *Br. Poult. Sci., 44*(1), 139-144. doi:10.1080/0007166031000085445

Kaldhusdal, M. & Skjerve, E. (1996). Association between cereal contents in the diet and incidence of necrotic enteritis in broiler chickens in Norway. *Prev. Vet. Med, 28*(1), 1-16. doi:https://doi.org/10.1016/0167-5877(96)01021-5

Kelly, P. (2010). Nutrition, intestinal defence and the microbiome. *Proc. Nutr. Soc., 69*(2), 261-268. doi:10.1017/S0029665110000108

Kers, J.G., Velkers, F.C., Fischer, E.A.J., Hermes, G.D.A., Stegeman, J.A. & Smidt, H. (2018). Host and environmental factors affecting the intestinal microbiota in chickens. *Front. Microbiol., 9*(FEB).

Kersters, K., De Vos, P., Gillis, M., Swings, J., Vandamme, P. & Stackebrandt, E. (2006). Introduction to the Proteobacteria. doi:10.1007/0-387-30745-1_1

Khan, S.H. & Iqbal, J. (2016). Recent advances in the role of organic acids in poultry nutrition. *J. Appl. Anim. Res., 44*(1), 359-369. doi:10.1080/09712119.2015.1079527

Kim, J., Guevarra, R.B., Nguyen, S.G., Lee, J.H., Jeong, D.K. & Unno, T. (2016). Effects of the Antibiotics Growth Promoter Tylosin on Swine Gut Microbiota. *J. Microbiol. Biotechnol., 26*(5), 876-882. doi:10.4014/jmb.1512.12004

Kim, J.E., Lillehoj, H.S., Hong, Y.H., Kim, G.B., Lee, S.H., Lillehoj, E.P. & Bravo, D.M. (2015). Dietary Capsicum and Curcuma longa oleoresins increase intestinal microbiome and necrotic enteritis in three commercial broiler breeds. *Res. Vet. Sci, 102*, 150-158. doi:https://doi.org/10.1016/j.rvsc.2015.07.022

Kizerwetter-Świda, M. & Binek, M. (2008). Bacterial microflora of the chicken embryos. *J. Anim. Feed Sci., 17*, 224-232.

Knap, I., Lund, B., Kehlet, A.B., Hofacre, C. & Mathis, G. (2010). Bacillus licheniformis Prevents Necrotic Enteritis in Broiler Chickens. *Avian Dis., 54*(2), 931-935.

Kohl, K.D. (2012). Diversity and function of the avian gut microbiota. *J. Comp. Physiol. B, 182*(5), 591-602. doi:10.1007/s00360-012-0645-z

Kolbjornsen, O., David, B. & Gilhuus, M. (2011). Bacterial osteomyelitis in a 3-week-old broiler chicken associated with Enterococcus hirae. *Vet. Pathol., 48*(6), 1134-1137. doi:10.1177/0300985810396513

Korver, D.R. (2012). Implications of changing immune function through nutrition in poultry. *Anim. Feed. Sci. Tech., 173*(1-2), 54-64. doi:10.1016/j.anifeedsci.2011.12.019

Kumar, S., Chen, C., Indugu, N., Werlang, G.O., Singh, M., Kim, W.K., Thippareddi, H. & Venkitanarayanan, K. (2018). Effect of antibiotic withdrawal in feed on chicken gut microbial dynamics, immunity, growth performance and prevalence of foodborne pathogens. *PLoS. ONE, 13*(2), e0192450. doi:10.1371/journal.pone.0192450

LeBlanc, J.G., Milani, C., de Giori, G.S., Sesma, F., van Sinderen, D. & Ventura, M. (2013). Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Curr. Opin. Biotech., 24*(2), 160-168. doi:http://dx.doi.org/10.1016/j.copbio.2012.08.005

Ley, R.E., Turnbaugh, P.J., Klein, S. & Gordon, J.I. (2006). Microbial ecology: human gut microbes associated with obesity. *Nature, 444*(7122), 1022-1023.

Li, Y., Fu, X., Ma, X., Geng, S., Jiang, X., Huang, Q., Hu, C. & Han, X. (2018). Intestinal Microbiome-Metabolome Responses to Essential Oils in Piglets. *Front. Microbiol., 9*, 1988. doi:10.3389/fmicb.2018.01988

Louis, P. & Flint, H.J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett., 294*(1), 1-8. doi:10.1111/j.1574-6968.2009.01514.x

Lourenco, M.C., Kuritza, L.N., Westphal, P., Muniz, E., Pickler, L. & Santin, E. (2012). Effects of Bacillus subtilis in the Dynamics of infiltration of immunological cells in the intestinal mucosa of chickens challenged with Salmonella Minnesota. *Int. J. Poult. Sci., 11*(10), 630-634.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J.J. & Lee, M.D. (2003). Diversity and Succession of the Intestinal Bacterial Community of the Maturing Broiler Chicken. *Appl. Environ. Microbiol., 69*(11), 6816-6824. doi:10.1128/aem.69.11.6816-6824.2003

Lumpkins, B.S., Batal, A.B. & Lee, M.D. (2010). Evaluation of the bacterial community and intestinal development of different genetic lines of chickens. *Poultry Sci., 89*(8), 1614-1621. doi:10.3382/ps.2010-00747

Lutful Kabir, S.M. (2009). The role of probiotics in the poultry industry. *Int. J. Mol. Sci., 10*(8), 3531-3546. doi:10.3390/ijms10083531

Mead, G.C. (2000). Prospects for 'competitive exclusion' treatment to control salmonellas and other foodborne pathogens in poultry. *Vet. J., 159*(2), 111-123. doi:10.1053/tvjl.1999.0423

Meehan, C.J. & Beiko, R.G. (2014). A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. *Genome biology and evolution, 6*(3), 703-713. doi:10.1093/gbe/evu050

Meissner, H.H., Scholtz, M.M. & Palmer, A.R. (2014). Sustainability of the South African Livestock Sector towards 2050 Part 1: Worth and impact of the sector. *S. Afr. J. Anim. Sci., 43*(3), 282. doi:10.4314/sajas.v43i3.5

Min, Y.W. & Rhee, P.-L. (2015). The Role of Microbiota on the Gut Immunology. *Clin. Ther., 37*(5), 968-975. doi:http://dx.doi.org/10.1016/j.clinthera.2015.03.009

Moore, R.J. (2016). Necrotic enteritis predisposing factors in broiler chickens. *Avian Pathol., 45*(3), 275-281. doi:10.1080/03079457.2016.1150587

Mountzouris, K.C., Tsitrsikos, P., Palamidi, I., Arvaniti, A., Mohnl, M., Schatzmayr, G. & Fegeros, K. (2010). Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. *Poultry Sci., 89*(1), 58-67. doi:10.3382/ps.2009-00308

Namsolleck, P., Thiel, R., Lawson, P., Holmstrøm, K., Rajilic, M., Vaughan, E.E., Rigottier-Gois, L., Collins, M.D., de Vos, W.M. & Blaut, M. (2004). Molecular methods for the analysis of gut microbiota. *Microb. Ecol. Health Dis., 16*(2-3), 71-85. doi:10.1080/08910600410032367

O'Callaghan, T.F., Ross, R.P., Stanton, C. & Clarke, G. (2016). The gut microbiome as a virtual endocrine organ with implications for farm and domestic animal endocrinology. *Domest. Anim. Endocrinol., 56 Suppl*, S44-55. doi:10.1016/j.domaniend.2016.05.003

O'Mara, F.P. (2011). The significance of livestock as a contributor to global greenhouse gas emissions today and in the near future. *Anim. Feed. Sci. Tech., 166–167*, 7-15. doi:http://dx.doi.org/10.1016/j.anifeedsci.2011.04.074

Oakley, B.B., Lillehoj, H.S., Kogut, M.H., Kim, W.K., Maurer, J.J., Pedroso, A., Lee, M.D., Collett, S.R., Johnson, T.J. & Cox, N.A. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiol. Lett., 360*(2), 100-112. doi:10.1111/1574-6968.12608

Ohtani, K. & Shimizu, T. (2015). Regulation of toxin gene expression in Clostridium perfringens. *Res. Microbiol, 166*(4), 280-289. doi:https://doi.org/10.1016/j.resmic.2014.09.010

Pan, D. & Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes, 5*(1), 108-119. doi:10.4161/gmic.26945

Patwardhan, A., Ray, S. & Roy, A. (2014). Molecular Markers in Phylogenetic Studies-A Review. *J. Phylogen. Evolution. Biol., 02*(02). doi:10.4172/2329-9002.1000131

Pedrosa, A.A. (2009). Which came first the egg or its microbiota. *The Poultry Informed Professional*(107).

Peters, C.J., Picardy, J.A., Darrouzet-Nardi, A. & Griffin, T.S. (2014). Feed conversions, ration compositions, and land use efficiencies of major livestock products in U.S. agricultural systems. *Agric. Sys., 130*, 35-43. doi:http://dx.doi.org/10.1016/j.agsy.2014.06.005

Petit, L., Gibert, M. & Popoff, M.R. (1999). Clostridium perfringens: toxinotype and genotype. *Trends Microbiol, 7*(3), 104-110. doi:https://doi.org/10.1016/S0966-842X(98)01430-9

Polansky, O., Sekelova, Z., Faldynova, M., Sebkova, A., Sisak, F. & Rychlik, I. (2015). Important Metabolic Pathways and Biological Processes Expressed by Chicken Cecal Microbiota. *Appl. Environ. Microbiol., 82*(5), 1569-1576. doi:10.1128/AEM.03473-15

Qaisrani, S.N., Van Krimpen, M.M., Kwakkel, R.P., Verstegen, M.W.A. & Hendriks, W.H. (2015). Dietary factors affecting hindgut protein fermentation in broilers: a review. *World Poultry Sci. J., 71*(1), 139-160. doi:10.1017/S0043933915000124

Qu, A., Brulc, J.M., Wilson, M.K., Law, B.F., Theoret, J.R., Joens, L.A., Konkel, M.E., Angly, F., Dinsdale, E.A., Edwards, R.A., Nelson, K.E., White, B.A. & Ahmed, N.E. (2008). Comparative Metagenomics Reveals Host Specific Metavirulomes and Horizontal Gene Transfer Elements in the Chicken Cecum Microbiome. *PLoS. ONE, 3*(8). Retrieved from doi:10.1371/journal.pone.0002945

Ranjitkar, S., Lawley, B., Tannock, G. & Engberg, R.M. (2016). Bacterial Succession in the Broiler Gastrointestinal Tract. *Appl. Environ. Microbiol., 82*(8), 2399-2410. doi:10.1128/AEM.02549-15

Rial, D.R. (1984). Interactions among Microorganisms of the Indigenous Intestinal Flora and Their Influence on the Host. *Rev. Infect. Dis., 6*, S73-S79.

Rinttila, T. & Apajalahti, J. (2013). Intestinal microbiota and metabolites--Implications for broiler chicken health and performance. *J. Appl. Poultry. Res., 22*(3), 647-658. doi:10.3382/japr.2013-00742

Rivière, A., Selak, M., Lantin, D., Leroy, F. & De Vuyst, L. (2016). Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut. *Front. Microbiol., 7*, 979-979. doi:10.3389/fmicb.2016.00979

Roto, S.M., Kwon, Y.M. & Ricke, S.C. (2016). Applications of In Ovo Technique for the Optimal Development of the Gastrointestinal Tract and the Potential Influence on the Establishment of Its Microbiome in Poultry. *Front. Vet. Sci., 3*, 63. doi:10.3389/fvets.2016.00063

Saengkerdsub, S., Anderson, R.C., Wilkinson, H.H., Kim, W.-K., Nisbet, D.J. & Ricke, S.C. (2007). Identification and Quantification of Methanogenic Archaea in Adult Chicken Ceca. *Appl. Environ. Microbiol., 73*(1), 353-356. doi:10.1128/AEM.01931-06

Sankar, S.A., Lagier, J.C., Pontarotti, P., Raoult, D. & Fournier, P.E. (2015). The human gut microbiome, a taxonomic conundrum. *Syst. Appl. Microbiol., 38*(4), 276-286. doi:10.1016/j.syapm.2015.03.004

SAPA. (2018). *Broiler industry production report*. Retrieved from http://www.sapoultry.co.za/home/monthly-broiler.php:

Schokker, D., Veninga, G., Vastenhouw, S.A., Bossers, A., de Bree, F.M., Kaal-Lansbergen, L.M.T.E., Rebel, J.M.J. & Smits, M.A. (2015). Early life microbial colonization of the gut and intestinal development differ between genetically divergent broiler lines. *BMC Genomics, 16*(1), 1-13. doi:10.1186/s12864-015-1646-6

Sergeant, M.J., Constantinidou, C., Cogan, T.A., Bedford, M.R., Penn, C.W., Pallen, M.J. & Parkinson, J.E. (2014). Extensive Microbial and Functional Diversity within the Chicken Cecal Microbiome. *PLoS. ONE, 9*(3). Retrieved from doi:10.1371/journal.pone.0091941

Shang, Y., Kumar, S., Oakley, B. & Kim, W.K. (2018). Chicken Gut Microbiota: Importance and Detection Technology. *Front. Vet. Sci., 5*, 254. doi:10.3389/fvets.2018.00254

Sharma, J.M. & Burmester, B.R. (1982). Resistance of Marek's Disease at Hatching in Chickens Vaccinated as Embryos with the Turkey Herpesvirus. *Avian Dis., 26*(1), 134-149. doi:10.2307/1590032

Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S. & Hayashi, H. (2002). Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater. *Proc. Natl. Acad. Sci. U.S.A., 99*(2), 996-1001. doi:10.1073/pnas.022493799

Shin, N.R., Whon, T.W. & Bae, J.W. (2015). Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol, 33*(9), 496-503. doi:10.1016/j.tibtech.2015.06.011

Siegerstetter, S.-C., Schmitz-Esser, S., Magowan, E., Wetzels, S.U., Zebeli, Q., Lawlor, P.G., O'Connell, N.E., Metzler-Zebeli, B.U. & Smidt, H. (2017). Intestinal microbiota profiles associated with low and high residual feed intake in chickens across two geographical locations. *PLoS. ONE, 12*(11). Retrieved from doi:10.1371/journal.pone.0187766

Skinner, J.T., Bauer, S., Young, V., Pauling, G. & Wilson, J. (2010). An Economic Analysis of the Impact of Subclinical (Mild) Necrotic Enteritis in Broiler Chickens. *Avian Dis., 54*(4), 1237-1240. doi:10.1637/9399-052110-Reg.1

Smyth, J.A. & McNamee, P.T. (2008). Staphylococci, streptococci and enterococci *Poultry Diseases* (pp. 191-199): Elsevier.

Song, J., Xiao, K., Ke, Y.L., Jiao, L.F., Hu, C.H., Diao, Q.Y., Shi, B. & Zou, X.T. (2014). Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. *Poultry Sci., 93*(3), 581-588. doi:10.3382/ps.2013-03455

Songer, J.G. (1996). Clostridial enteric diseases of domestic animals. *Clin Microbiol Rev, 9*(2), 216-234. doi:10.1128/cmr.9.2.216

Stanley, D., Keyburn, A.L., Denman, S.E. & Moore, R.J. (2012). Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis. *Vet. Microbiol., 159*(1), 155-162. doi:https://doi.org/10.1016/j.vetmic.2012.03.032

Stanley, D., Hughes, R.J. & Moore, R.J. (2014a). Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Appl. Microbiol. Biotechnol., 98*(10), 4301-4310. doi:10.1007/s00253-014-5646-2

Stanley, D., Wu, S.-B., Rodgers, N., Swick, R.A. & Moore, R.J. (2014b). Differential Responses of Cecal Microbiota to Fishmeal, Eimeria and Clostridium perfringens in a Necrotic Enteritis Challenge Model in Chickens. *PLoS. ONE, 9*(8), e104739. doi:10.1371/journal.pone.0104739

Stanley, D., Hughes, R.J., Geier, M.S. & Moore, R.J. (2016). Bacteria within the Gastrointestinal Tract Microbiota Correlated with Improved Growth and Feed Conversion: Challenges Presented for the Identification of Performance Enhancing Probiotic Bacteria. *Front. Microbiol., 7*, 187-187. doi:10.3389/fmicb.2016.00187

Swart, D., Mackie, R.I. & Hayes, J.P. (1993). Fermentative digestion in the ostrich (Struthio camelus var. domesticus), a large avian species that utilizes cellulose. *S. Afr. J. Anim. Sci., 23*(5/6).

The Human Microbiome Project, C. (2012). Structure, function and diversity of the healthy human microbiome. *Nature, 486*(7402), 207-214.

Thomas, F., Hehemann, J.H., Rebuffet, E., Czjzek, M. & Michel, G. (2011). Environmental and gut bacteroidetes: the food connection. *Front Microbiol, 2*, 93. doi:10.3389/fmicb.2011.00093

Thompson, C.L., Mikaelyan, A. & Brune, A. (2013). Immune-modulating gut symbionts are not 'Candidatus Arthromitus'. *Mucosal Immunology (1933-0219), 6*(1), 200-201. doi:10.1038/mi.2012.91

Timbermont, L., Lanckriet, A., Dewulf, J., Nollet, N., Schwarzer, K., Haesebrouck, F., Ducatelle, R. & Van Immerseel, F. (2010). Control of Clostridium perfringens-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils. *Avian Pathol., 39*(2), 117-121. doi:10.1080/03079451003610586

Torok, V.A., Hughes, R.J., Mikkelsen, L.L., Perez-Maldonado, R., Balding, K., MacAlpine, R., Percy, N.J. & Ophel-Keller, K. (2011). Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. *Appl. Environ. Microbiol., 77*(17), 5868-5878. doi:10.1128/AEM.00165-11

Van Immerseel, F., Buck, J.D., Pasmans, F., Huyghebaert, G., Haesebrouck, F. & Ducatelle, R. (2004). Clostridium perfringens in poultry: an emerging threat for animal and public health. *Avian Pathol., 33*(6), 537-549. doi:10.1080/03079450400013162

Van Immerseel, F., Boyen, F., Gantois, I., Timbermont, L., Bohez, L., Pasmans, F., Haesebrouck, F. & Ducatelle, R. (2005). Supplementation of coated butyric acid in the feed reduces colonization and shedding of Salmonella in poultry. *Poultry Sci., 84*(12), 1851-1856. doi:10.1093/ps/84.12.1851

Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F. & Ducatelle, R. (2006). The use of organic acids to combat Salmonella in poultry: a mechanistic explanation of the efficacy. *Avian Pathol., 35*(3), 182-188. doi:10.1080/03079450600711045

Van Soest, P.J. (1994). *Nutritional Ecology of the Ruminant*: Comstock Publishing Associates.

Vaz-Moreira, I., Egas, C.o., Nunes, O.C. & Manaia, C.M. (2011). Culture-dependent and culture-independent diversity surveys target different bacteria: a case study in a freshwater sample. *A. Van Leeuw. J. Microbiol., 100*(2), 245-257.

Vetrovsky, T. & Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS. ONE, 8*(2), e57923. doi:10.1371/journal.pone.0057923

Vierheilig, J., Frick, C., Mayer, R.E., Kirschner, A.K.T., Reischer, G.H., Derx, J., Mach, R.L., Sommer, R. & Farnleitner, A.H. (2013). Clostridium perfringens Is Not Suitable for the Indication of Fecal Pollution from Ruminant Wildlife but Is Associated with Excreta from Nonherbivorous Animals and Human Sewage. *Appl. Environ. Microbiol., 79*(16), 5089-5092. doi:10.1128/aem.01396-13

Vispo, C. & Karasov, W.H. (1997). The Interaction of Avian Gut Microbes and Their Host: An Elusive Symbiosis. In R. I. Mackie & B. A. White (Eds.), *Gastrointestinal Microbiology: Volume 1 Gastrointestinal Ecosystems and Fermentations* (pp. 116-155). Boston, MA: Springer US.

Vital, M., Howe, A.C. & Tiedje, J.M. (2014). Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)genomic Data. *mBio, 5*(2), e00889-00814. doi:10.1128/mBio.00889-14

Wang, Y. & Kasper, L.H. (2014). The role of microbiome in central nervous system disorders. *Brain Behav. Immun., 38*, 1-12. doi:10.1016/j.bbi.2013.12.015

Wei, S., Morrison, M. & Yu, Z. (2013). Bacterial census of poultry intestinal microbiome. *Poultry Sci., 92*(3), 671-683. doi:10.3382/ps.2012-02822

Wenk, C. (2000). Recent Advances in Animal Feed Additives such as Metabolic Modifiers, Antimicrobial Agents, Probiotics, Enzymes and Highly Available Minerals - Review. *Asian-Australas. J. Anim. Sci., 13*(1), 86-95. doi:10.5713/ajas.2000.86

Williams, R.B. (2005). Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. *Avian Pathol., 34*(3), 159-180. doi:10.1080/03079450500112195

Willyard, C. (2011). Gut reaction. *Nature, 479*, 5-7.

Wise, M.G. & Siragusa, G.R. (2007). Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. *J. Appl. Microbiol., 102*(4), 1138-1149. doi:10.1111/j.1365-2672.2006.03153.x

Wostmann, B.S., Larkin, C., Moriarty, A. & Bruckner-Kardoss, E. (1983). Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. *Lab. Anim. Sci., 33*(1), 46-50.

Wu, S.-B., Stanley, D., Rodgers, N., Swick, R.A. & Moore, R.J. (2014). Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens. *Vet. Microbiol., 169*(3–4), 188-197. doi:http://dx.doi.org/10.1016/j.vetmic.2014.01.007

Xiao, Y., Xiang, Y., Zhou, W., Chen, J., Li, K. & Yang, H. (2016). Microbial community mapping in intestinal tract of broiler chicken. *Poultry Sci.*, pew372. doi:10.3382/ps/pew372

Yin, Y., Lei, F., Zhu, L., Li, S., Wu, Z., Zhang, R., Gao, G.F., Zhu, B. & Wang, X. (2010). Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression. *ISME J., 4*(3), 367-376. doi:10.1038/ismej.2009.128

Yirga, H. (2015). The Use of Probiotics in Animal Nutrition. *J. Prob. Health, 03*(02). doi:10.4172/2329-8901.1000132

Zhu, X.Y., Zhong, T., Pandya, Y. & Joerger, R.D. (2002). 16S rRNA-Based Analysis of Microbiota from the Cecum of Broiler Chickens. *Appl. Environ. Microbiol., 68*(1), 124-137. doi:10.1128/aem.68.1.124-137.2002

## *Appendix A*

### DNA extraction protocol

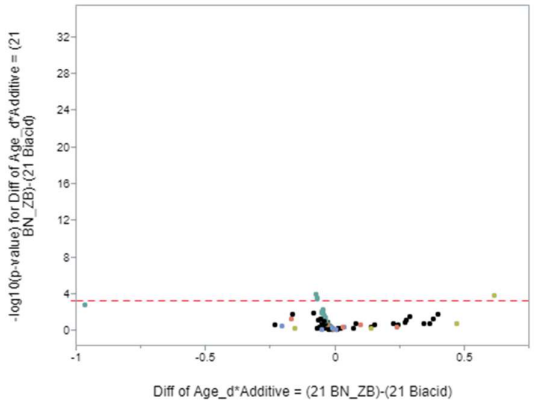
This protocol is designed for the extraction of gut microbiota and for PCR analysis amplifying the 16S rRNA region. Before extraction could commence, the following necessary preparations and mixtures were made beforehand. 100mL Phenol: chloroform: isoamyl alcohol mixture was mixed in the ratio of 25: 24: 1 under a fume extractor hood out of direct sunlight. 1000mL Galleon Lysis buffer was prepared by mixing 44.5mL TE buffer (created by mixing 100mL Tris, pH 8 1M, 20mL EDTA 0.5M and filled up to 1L with distilled water), 50mL 10% SDS and 5mL Triton 100. Galleon binding buffer created by mixing equal parts Buffer AL (from Qiagen DNEasy Blood & Tissue kit) and 100% Ethanol. All pipette tips and tubes were sterilised via an autoclave and all tools and equipment was sterilised with 70% ethanol solution before extraction. All procedures took place within a climate-controlled laboratory.

After the abovementioned preparations were made, DNA extraction could commence with the following procedure. 2mL Screw cap tubes were labelled and placed in order of samples. Samples were removed from the freezer so that they can defrost during this time. 250µL (0.56 grams) of 0.1mm Zirconium beads were weighed into each tube. 400µL Galleon Lysis buffer and 500µL Phenol: chloroform: isoamyl alcohol (pH8) solution were added to each tube. By this time samples have been thawed and 500g of each sample was weighed out into each relevant tube. Tubes were then placed in a bead beater for 3 minutes at top speed to allow cell wall disruption and release of the nucleic acids within the cell's cytoplasm (Fujimoto *et al.*, 2004). After bead beating, tubes are placed in a centrifuge and spun at 10,000 rotations per minute (rpm) for a duration of 5 minutes. New 1.5mL Eppendorf tubes are labelled the same as the 2mL screw cap tubes during this time. The sample mixture tubes are carefully removed from centrifuge so as to not mix the precipitate with the aqueous phase that was formed on the top. Carefully using a pipette, 250µL of the aqueous phase is transferred from each tube to its newly assigned Eppendorf tube. 1µL RNase A (17,500 U) was then added to each sample before they were mixed by vortex and incubated at 37°C for 1 hour. After the incubation period, samples were placed in the centrifuge for 5 minutes at 10,000 rpm to avoid cross-contamination when opening the Eppendorf tubes. 500µL of the pre-mixed Galleon binding buffer was added to each tube and samples were mixed thoroughly by vortex and placed in centrifuge for 1 minute. Using a 1mL pipette tip, this entire sample/ buffer solution was then transferred to the DNEasy spin column (from the Qiagen DNEasy Blood & Tissue kit) placed in a 2mL collection tube. Each spin column was also labelled accordingly. Samples were placed in centrifuge for 1 minute at 11,000 x g and the flow-through and collection tubes were discarderd. Spin columns were placed in new collection tubes and samples were then washed once by adding 500µL of the Qiagen buffer AW1 and centrifuged for 1 minute at 11,000 x g. Flow-through and collection tubes were discarded. Spin columns were placed in new collection tubes and washed again by adding 500µL of the Qiagen buffer AW2 decanting wash solution. Samples were then placed in a centrifuge for 3 minutes at 11,000 x g to also dry the spin column. Flow-through and collection tubes were discarded and spin columns were placed in labelled new 1.5mL microcentrifuge tubes. Elution of DNA was performed by adding 100µL of Qiagen buffer AE to the centre of the spin column membrane. Samples were then incubated for 12 minutes at room temperature and thereafter placed in the centrifuge for 1 minute at maximum speed to collect DNA in

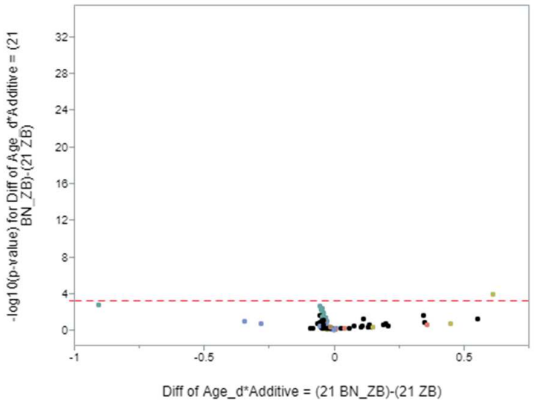
microcentrifuge tubes. Once extraction was completed, DNA samples were immediately placed in a freezer at -40°C to inhibit loss via evaporation or further chemical degradation of DNA if denaturing chemicals remained after washing. Extracted DNA samples were selected at random to spot-check effectiveness of DNA extraction protocol via gel electrophoresis.

## *Appendix B*

### Ileal results (21-days old)

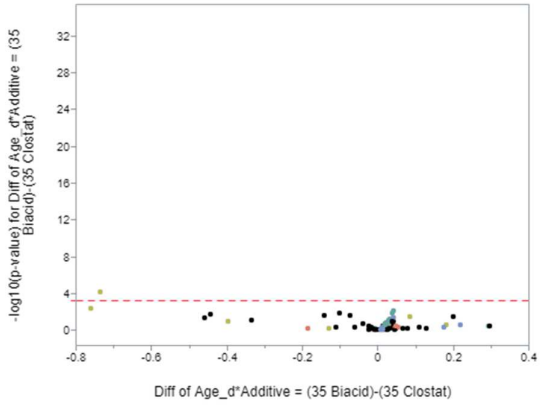



a

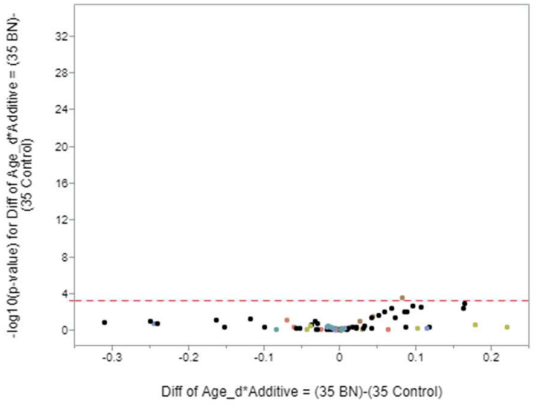



b

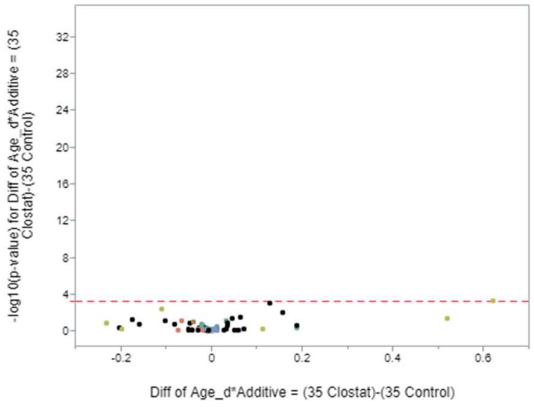
### Ileal results (35-days old)




a

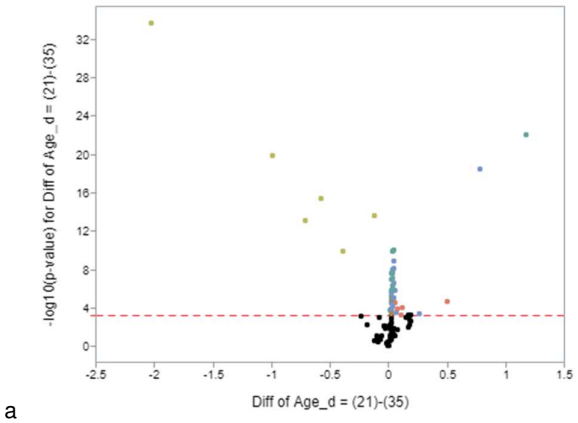



b

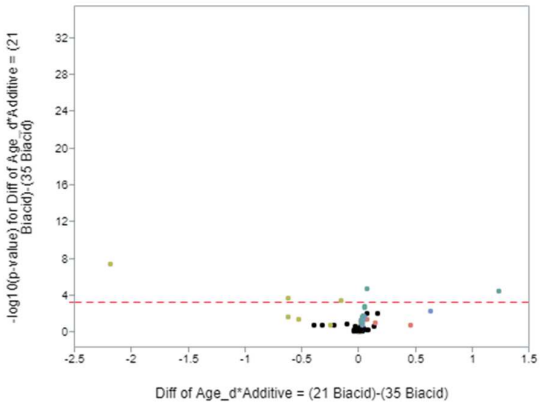



c

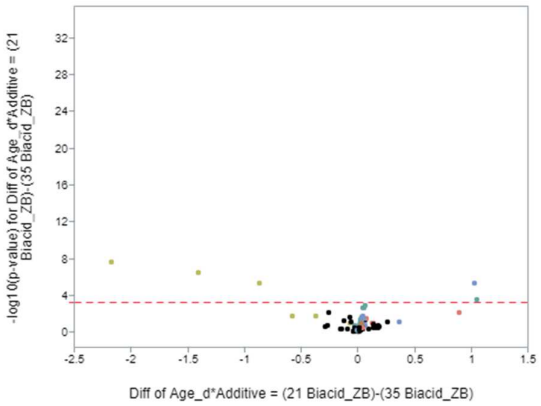
## Ileal results (all treatments)



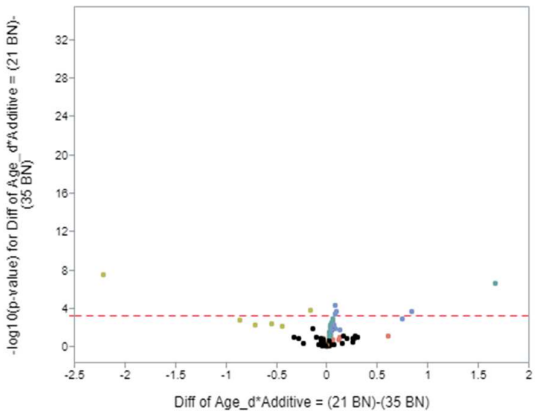

a



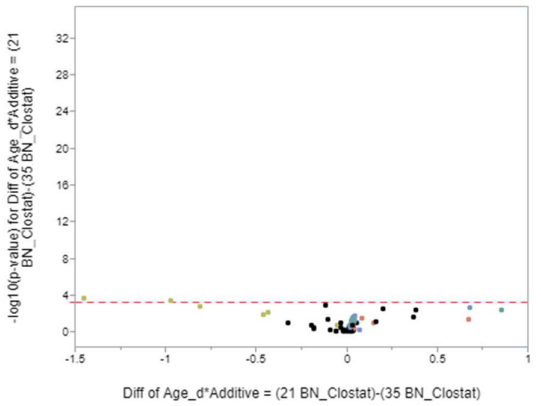

b



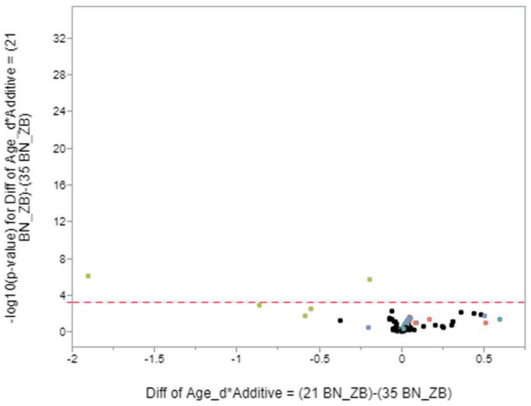

c



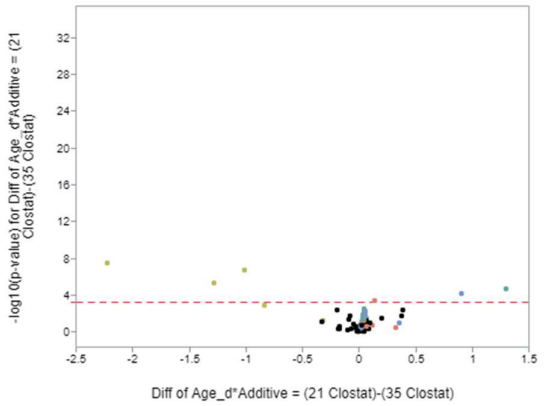

d



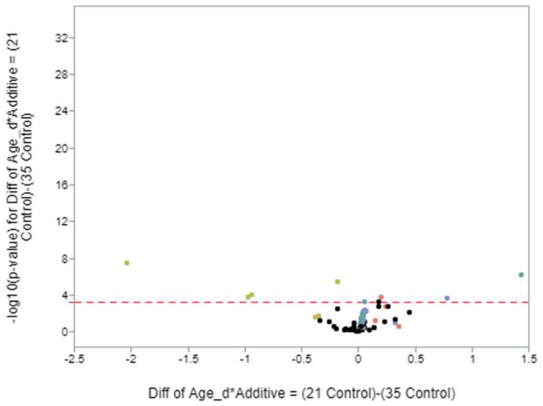

e



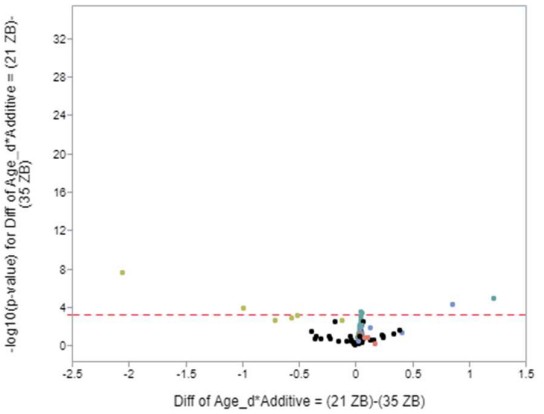

f


-log10(p-value) for Diff of Age_d*Additive = (21 Clostat)-(35 Clostat)
Diff of Age_d*Additive = (21 Clostat)-(35 Clostat)

g

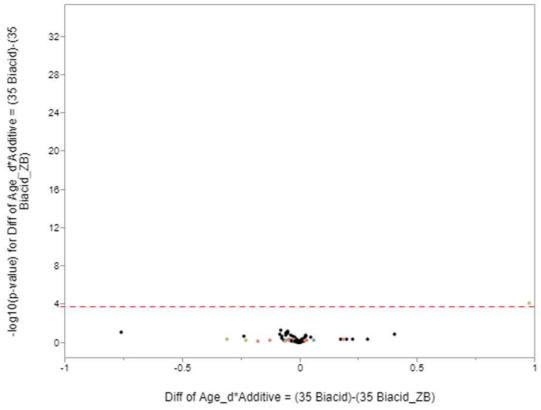

-log10(p-value) for Diff of Age_d*Additive = (21 Control)-(35 Control)
Diff of Age_d*Additive = (21 Control)-(35 Control)

h


-log10(p-value) for Diff of Age_d*Additive = (21 ZB)-(35 ZB)
Diff of Age_d*Additive = (21 ZB)-(35 ZB)

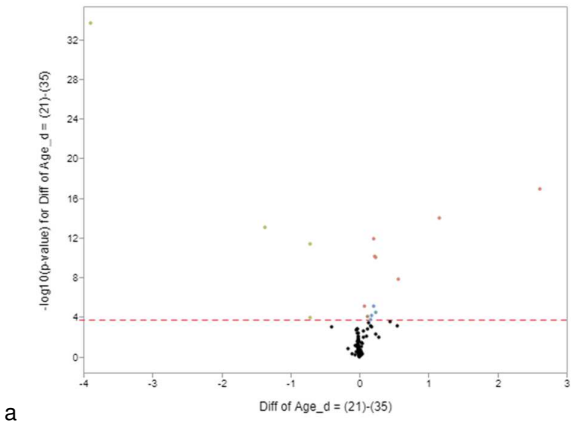
i

## Caecal results (35-days old)

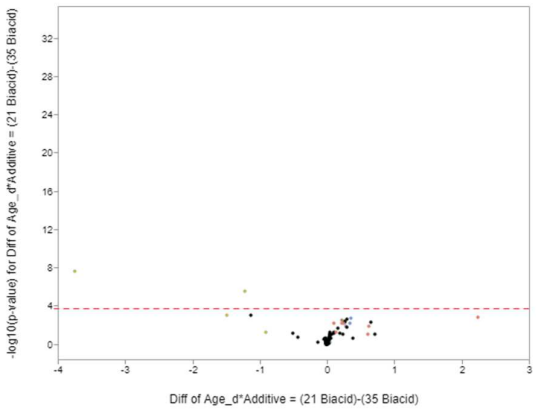



a

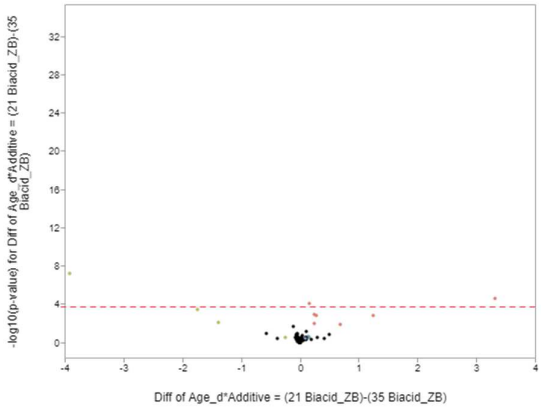
## Caecal results (all treatments)



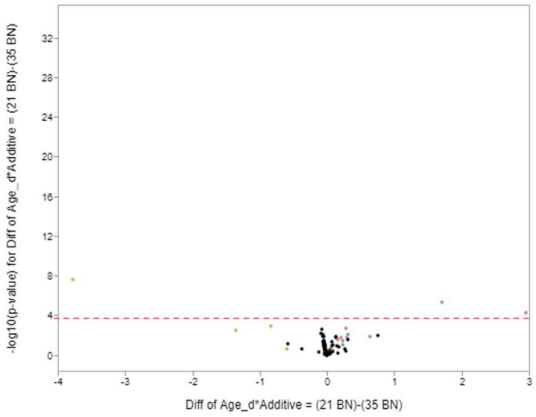

a



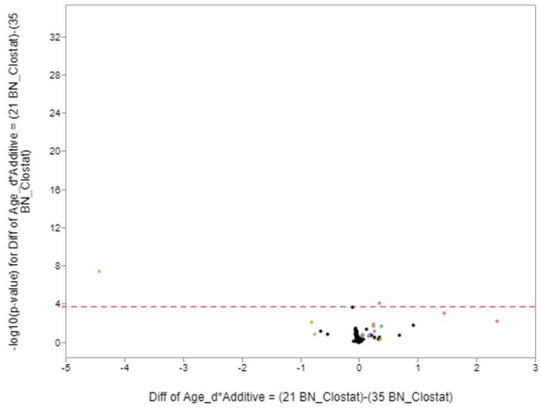

b



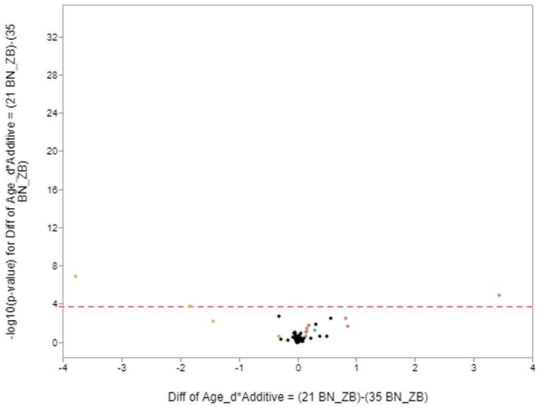

c



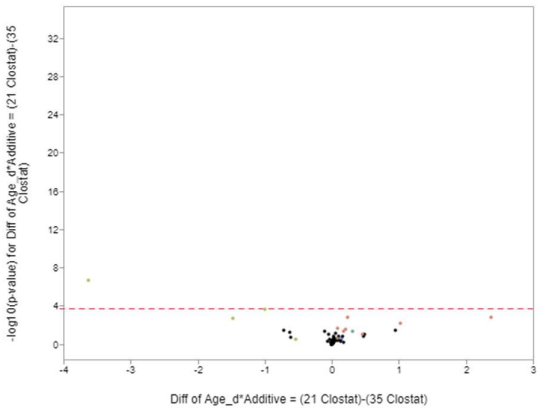

d



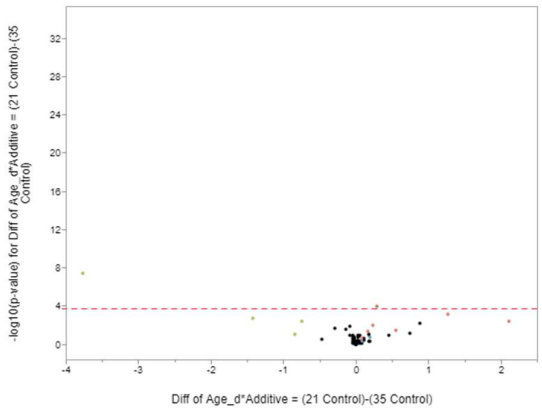

e



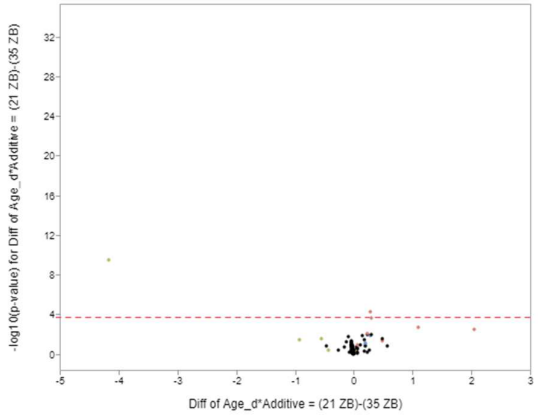

f


-log10(p-value) for Diff of Age_d*Additive = (21 Clostat)-(35 Clostat)
Diff of Age_d*Additive = (21 Clostat)-(35 Clostat)

g


-log10(p-value) for Diff of Age_d*Additive = (21 Control)-(35 Control)
Diff of Age_d*Additive = (21 Control)-(35 Control)

h


-log10(p-value) for Diff of Age_d*Additive = (21 ZB)-(35 ZB)
Diff of Age_d*Additive = (21 ZB)-(35 ZB)

i