

INTESTINAL MICROBIOTA AND THE PATHOGENESIS OF DYSBACTERIOSIS IN BROILER CHICKENS

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Intestinal microbiota and the pathogenesis of dysbacteriosis in broiler chickens

Abstract

The complex microbial community of the gastrointestinal tract plays an important role in an animal's health by aiding digestion, producing nutrients, protecting against pathogens and in the maturation of the host immune system. A balanced intestinal microbiota is therefore essential for the performance of all food producing animals. Dysbacteriosis (DB) is a poorly understood digestive condition of poultry increased in incidence following the EU wide ban on the use of antimicrobials as growth promoters. It is proposed that DB is an imbalance in the intestinal microbiota; however this is yet to be proven. The aim of this thesis was to explore in detail the role of gut microbiota and their metabolic activity in the development of DB. Gaining a better understanding of DB will aid development of novel management strategies without relying on antimicrobial intervention.

Culture-independent microbial profiling using PCR-DGGE revealed an altered microbiota in the small intestinal and caeca in DB affected birds with increased presence of members of the bacteroidetes and clostridia. Specific broiler breeds have been implicated with dysbacteriosis onset; significant changes in intestinal microbiota were observed relating to host genotype. Diet impacts upon the intestinal microbiota and increasing protein density was found to alter the composition of the microbiota leading to an increase in members of the bacteroidetes. Birds fed a vegetarian diet demonstrated improved enteric health and altered ileal microbiota in comparison to birds fed a standard diet. Metabolite profiling via ^1H NMR revealed altered biochemistry in the GI tracts of DB affected birds. The results show bile acid deconjugation and malabsorption of nutrients by the host thus explaining the growth depression seen in affected birds.

This thesis reveals previously unknown aspects of DB and provides a firm foundation for future work in elucidating more information on the mechanisms behind the condition and subsequent control strategies.

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To my family

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1. General Introduction

1.1. Introduction

Poultry production in the UK has risen dramatically over the past 50 years. Compared to the 5 million birds produced in 1953, the UK is currently producing in the region of 860 million chickens *per annum* of which 805 million are broilers and early figures from the Department for Environment, food and Rural Affairs (DEFRA) this year suggest this number is approaching 1,000 million. Chicken meat constitutes 40% of the primary meat market in the UK with sales grossing at £1.3 billion per annum. As of June 2008 there were approximately 168 million chickens on the ground in the UK of which almost 110 million were broiler chickens housed across approximately 31,000 broiler units (5% of these units are organic and free range operations) (72, 80-82). The vast majority of broiler chickens in the UK are raised conventionally in large purpose-built sheds which house tens of thousands of birds at any one time. The birds are placed in the sheds after hatching where they are housed until slaughter. Decades of research and genetic selection by broiler breeding companies has resulted in a modern broiler chicken that is a far cry from the traditional broiler chicken of 50 years ago. By establishing a breeding chain where the parent breeding stock have a high egg yield with optimal hatchability breeding companies aim to produce a bird on the broiler farm able to meet the demands of the consumer, the grower and the supermarkets. Many different criteria are considered when selecting birds to be included in the breeding programme; ultimately producers aspire to rear a broiler chicken which has a strong skeletal structure and cardiovascular system, robustness to cope with the challenges faced during the growth period, resistance to disease and importantly the ability to efficiently convert food into muscle. A typical broiler chicken today is ready for slaughter at around 35-40 days of age and will weigh around 2 kg. Due to their rapid growth rates the chickens require around 3.5-4 kg of feed during this growth period. In order to meet the high consumer demand for poultry meat, birds are grown under intensive farming conditions which can make them more susceptible to disease and enable a more rapid spread of disease due to the intimate living conditions (211). However, with improved biosecurity and

ventilation combined with vaccine usage and improved knowledge of disease prevention and management, intensive farming remains an extremely successful operation.

Up until recent years the addition of growth promoting antimicrobials to the feed of food-producing animals as a means to enhance feed efficiency was common practice worldwide (152). The mode of action of antibiotic growth promoters (AGPs) is thought to be due to the effects on the bacterial communities residing in the intestinal tract of all animals. The AGPs cause an overall reduction in bacterial numbers within the gastro intestinal (GI) tract of the birds, resulting in more nutrients being available for absorption by the host. The reduction in bacterial numbers also results in lower production of bacterial metabolites (which can suppress growth), thus a growth promoting effect is seen (87, 407). The first recorded evidence of these effects dates back to the 1940s where Moore *et al.* (274) discovered that streptomycin, when added to feed, enhanced the growth of chicks with no toxic side effects. This discovery was echoed a few years later by Stokstad and Jukes (369) while investigating inexpensive sources of vitamin B12 as a supplement for poultry feed. They were using the by-products of chlortetracycline fermentation as a source of vitamin B12 and found that the birds fed on this diet exhibited an increase in growth rate in comparison to birds fed on alternative diets. This increase could not be attributed to the vitamin B12 alone and later it became clear that this was due to traces of the antibiotic in the feed ration supplementing growth. This phenomenon was investigated further by Libby and Schiable (227) where over a four year period they demonstrated that continuous feeding of antibiotics to birds resulted in an increase in weight compared to control birds. Subsequent studies with cattle and pigs showed similar effects (74, 75, 234). During this period farmers developed confinement rearing which was the first step towards intensive farming. This style of farming enhanced the risk of certain diseases, but the inclusion of AGPs in the diet helped to control these diseases by reducing or preventing the growth of deleterious bacteria in the GI tract of the birds. A wide variety of antimicrobial preparations soon became available and as they became cheaper the usage of them became more widespread (152).

In recent years however, there has been growing public concern that the medical and veterinary professions have been overusing antimicrobials and that this has contributed to the emergence of drug resistant bacteria (20, 152, 184). Investigations involving chickens administered with AGPs have highlighted the presence of drug resistant strains of *Enterococcus faecium* and other potentially pathogenic enterococci; the dominance of these drug resistant strains was shown to diminish once the antimicrobials were removed from the birds' diet (44, 90, 158). In a move to combat the ever increasing risk of drug resistant bacteria, growth promoting antibiotics have been withdrawn from farming practices across the EU (383). The ban was initiated in 1999 and came into full force in 2006 (EC Regulation No. 1831/2003). This move has however been met with some criticism from veterinarians and animal health experts due to the adverse impact on the health and welfare of animals and on industry economics. Recent reports from the EU and the US have shown that therapeutic antibiotic usage has been on the increase since the removal of AGPs. In the UK, between the years of 2002 and 2008 there was an increase in therapeutic antibiotic use in poultry from 13 tonnes to 18 tonnes (410) and this increase is thought to be largely due to the reduction of AGP administration. Despite improvements in animal welfare and housing on farms, the poultry industry is dealing with increased cases of enteric disease such as necrotic enteritis (NE) and non-specific bacterial enteritis (dysbacteriosis); this can lead to secondary problems such as pododermatitis and hock burn which in part may be due to degradation of the litter covering the floor of the chicken houses (49, 105, 146, 175, 280). There is a general consensus within the industry that the shift in legislation regarding AGPs has resulted in an increase in enteric problems. In order to pursue novel management strategies and develop natural alternatives to AGPs, a better understanding of the mechanisms involved in the development of enteric disease is required. Alternatives to AGPs should either reduce nutrient availability to the intestinal microbiota, improve host immunity against unfavourable organisms or enhance the dominance of the beneficial species of bacteria of the intestinal microbiota (23). However there is a need to first understand the dynamics of the relationship between the host, the intestinal microbiota and the diet.

1.2. The Avian GI tract

The gastrointestinal (GI) tract of animals and birds is a specialised tube running from the mouth to the anus, of which the primary purpose is the conversion and digestion of food into its basic components for absorption and utilisation by the host (450). The early embryological development of the different regions of the GI tract is fundamentally similar developing cranially to caudally giving rise to a common gross structure of an inner mucous membrane lining and an outer muscular layers separated by connective tissue (147, 345). However, once the adult alimentary tract has fully developed it is a highly organised and segregated structure comprising of distinct regions namely the oesophagus, stomach, small intestine (duodenum, jejunum & ileum), and large intestine (caecum, colon and rectum). Each of these regions has a varying histological and anatomical structure designed for their individual functions in the digestive process (59, 397)

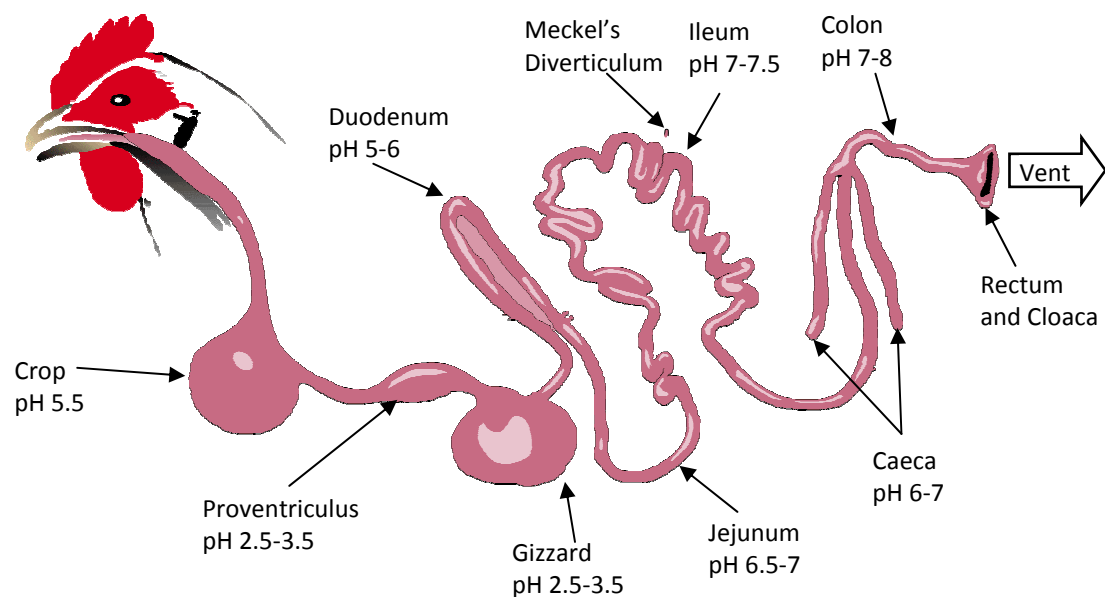


Figure 1.1 Schematic drawing of the GI tract of a chicken, with approximate pH values (Artwork by Paul Pople, IFR)

The anatomy of the avian GI tract (Figure 1.1) demonstrates a variety of evolutionary adaptations to reduce body mass for flight (96), and these adaptations make the avian GI tract different to that of mammals. Firstly, birds have a lightweight beak which is used in the prehension of food and as they lack teeth they are unable to masticate and as a result food is swallowed whole. On swallowing,

the food is stored in the crop before passing into the stomach (148). The crop is an extension of the oesophagus and the inner surface is covered with a thick layer of non-secretory stratified squamous epithelium (129, 264). The food remains in the crop for up to 6 hours where it undergoes bacterial fermentation predominantly by members of the *Lactobacillus* genus; this marks the beginning of the digestion process (18, 264). From the crop the food passes into the proventriculus and then swiftly into the gizzard. These regions make up two independent regions forming the glandular and muscular parts of the avian stomach respectively (148). The mucosa of the proventriculus secretes acid resulting in a low pH environment in both the proventriculus and the gizzard (360). The inner surface of the gizzard is lined by a very tough koilin layer and the outer surface is comprised of thick muscle which lends itself to the function of mechanically grinding the digesta (17). It is not uncommon for wild birds to selectively include pieces of grit or stone in their diets to aid the grinding in the gizzard (293). On leaving the gizzard the digesta passes into the small intestine which is comprised of the duodenum, jejunum and the ileum. The regions of the small intestine are not as clearly defined as those of the foregut. The duodenum and jejunum meet at the caudal aspect of the duodenal loop and it is widely accepted that the region defined by Meckel's Diverticulum (Figure 1.2) marks the junction of the ileum and jejunum (148). Meckel's Diverticulum is a vestigial remnant of the attachment of the yolk sac during development forming a distinctive and easily recognisable landmark along the small intestine (29).

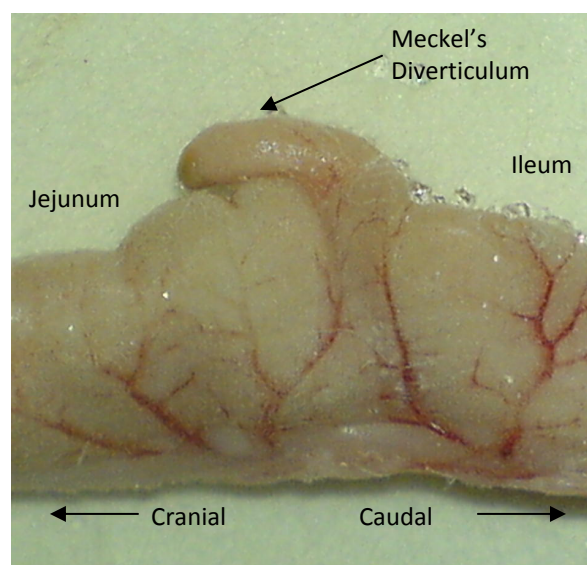


Figure 1.2 Photograph of a section of broiler intestine showing Meckel's Diverticulum at the junction of the ileum and jejunum

Within the small intestine the digesta is mixed with bile salts from the gall bladder and enzymic secretions from the pancreas consisting of proteinases, amylases and lipases. In addition, the mucosa of the small intestine is secretory, producing mucus and digestive enzymes which when combined with its high surface area, due to the presence of villi and microvilli, makes the small intestine the major site of chemical digestion and nutrient absorption. The digesta then passes through the ileocaecal junction into the large intestine; at this point birds have two enlarged caeca which branch out forming two separate blind ended compartments (260). The caeca are thought to be involved in the breakdown of otherwise indigestible plant material and the absorption of water, glucose and volatile fatty acids. The caeca empty every 24-48 hours at which point they are refilled (262), originally it was thought that the caeca drew up contents from the ileum (17) however it has been discovered that caecal filling occurs by retrograde peristalsis where colonic contents are pushed backwards against a meshwork of villi at the opening of the caeca which results in the caecal contents being made up of the finer more particulate matter of the colonic contents (60). From the ileocaecal junction the digesta enters the colon which is very short in comparison to mammals and hence there is very little absorption or digestion; from here the faecal pellet passes into the cloaca where it is mixed with uric acid (the avian product of protein breakdown) and expelled via the vent.

1.3. Intestinal Microbiota

1.3.1. Introduction

Bacteria are one of the most diverse group of organisms on the planet existing ubiquitously in interdependent communities in any given environment (422). These communities are found virtually everywhere in and around our environment, such as in soil, seas, rivers, and both in and on our bodies (419). When faced with the extremes of conditions (such as temperature, pressure and pH), evolution and adaptation have enabled bacteria to prevail and exploit the advantages of living in an environment too extreme for other organisms (173, 343, 412). Consequently higher organisms such as birds and mammals have had to adapt to a world full of

microbes and interact with many different bacteria on a daily basis resulting in symbiotic, competitive and pathogenic relationships (169). All of these interactions are exhibited in the GI tract (450). Along the entire GI tract there is a diverse microbial community comprised of bacteria, yeasts, archaea, ciliate protozoa, anaerobic fungi and bacteriophages (245), commonly referred to as the intestinal microbiota (149). The composition of the intestinal microbiota is highly dynamic and there are spatial shifts in population along each region of the GI tract in relation to the change in environmental conditions of each compartment (169). The intestinal microbiota consists predominately of bacteria, and it has been estimated that the bacterial cells outnumber the host cells by approximately ten to one (150). Original investigations into the intestinal microbiota of humans using culture based techniques suggested that there was in the region of 10^{10} - 10^{12} bacterial cells per gram of faecal matter and colon contents (275, 339). Even during these initial studies microbiologists recognised the difficulties in accurately characterising the hundreds of billions of bacteria that make up the human intestinal microbiota with one review stating that 'the number of bacteria defecated by one person at one time is usually more than 1,000 times greater than the human population of the world' (276). In the 1970s and 1980s it was suggested that the intestinal microbiota of humans contained up to 500 different species of bacteria (339, 354). Since the application of culture-independent molecular profiling techniques, it has become evident that early studies underestimated the diversity of the intestinal microbiota. Recent studies suggest that there are in the region of 1000 species of bacteria in the human intestinal tract and 50-90% of these remain uncultured and uncharacterised (12, 100, 451). Needless to say the intestinal microbiota remains an enigma that microbiologists continually have to develop new and advanced techniques in order to understand.

1.3.2. Intestinal microbiota of the chicken

Despite the presence of a monogastric digestive system the intestinal microbiota of a chicken differs to that of a human; these differences can be explained by differences in gut physiology and dietary intake. Recent studies focusing on poultry have proposed that the GI tract of a broiler chicken is colonised by an estimated

640 species of bacteria from 140 different genera (5). The abundance and diversity of the microbiota varies along the GI tract and predictably the regions which have less tolerable conditions and faster passage of contents have lower numbers of bacteria. It is generally considered that the digestive tract of a newly hatched chicken is sterile and that colonisation begins through contact with environmental microbes. However, the presence of bacteria in the caeca of unhatched embryos has been demonstrated (33). It has been suggested that the bacteria originated from the mother or that the bacteria penetrated the egg shell and then into the developing intestine of the embryo, as microbes have been shown to pass through the egg shell shortly after laying while the shell is still moist (28, 79, 209). A recent study by Kizerwetter-Świda *et al.* (199) demonstrated the presence of bacteria in the caeca, liver and yolk sac of 18 and 20 day old embryos; Figure 1.3 shows microscope images from the study indicating the presence of bacterial cells in the caeca of a 20 day embryo and a newly hatched chick.

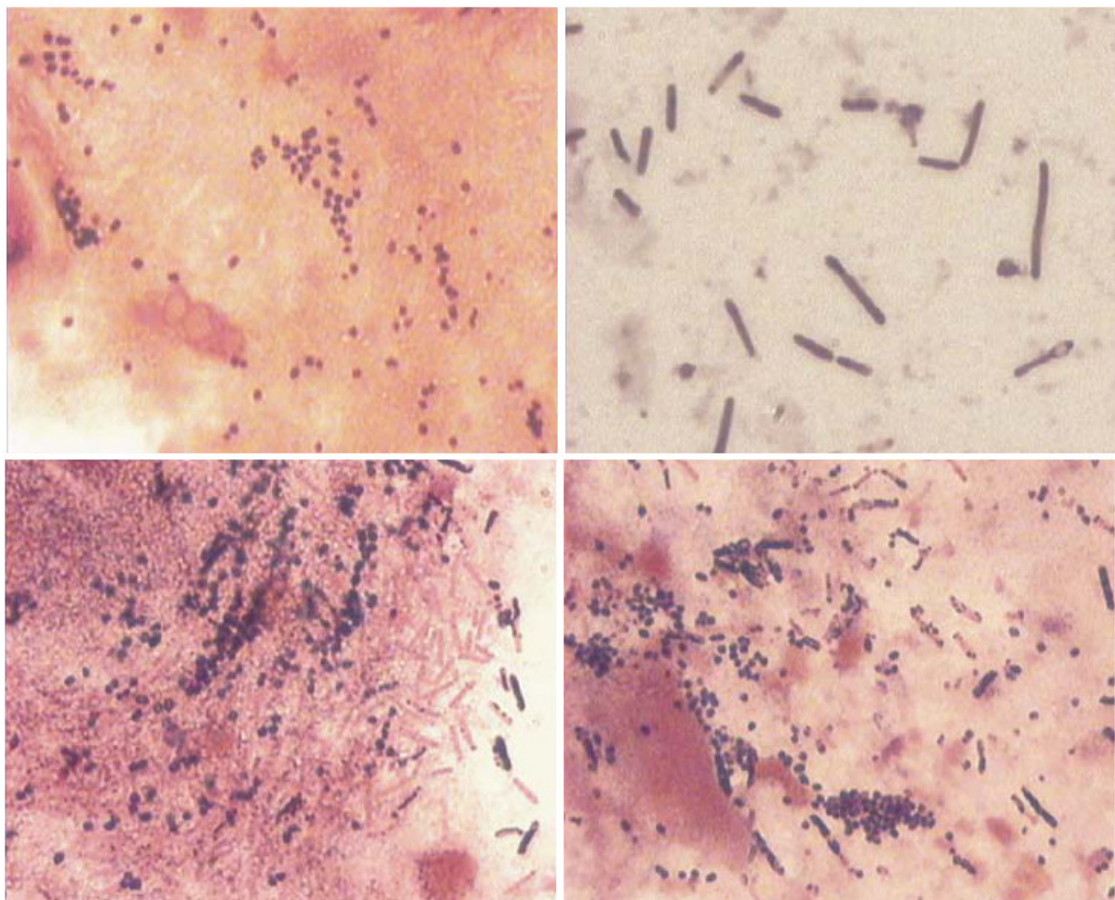


Figure 1.3 Microscope slide images taken from Kizerwetter-Świda *et al.* 2008 (199) showing presence of bacteria in direct smears prepared from caecal contents of 20 day old chicken embryo (top two images) and newly hatched chicken (bottom two images). Gram stain at 1000x magnification.

Despite these findings it is generally considered that the development of the adult intestinal microbiota begins on hatching where bacteria are picked up from the environment, the feed and people handling them post-hatch (396, 398). After one day post-hatch the ileum and caeca are both dominated by bacteria with densities around 10^8 and 10^{10} cells g^{-1} digesta respectively, and after three days these levels increase to 10^9 and 10^{11} cells g^{-1} of digesta respectively (5). Within two weeks the typical adult small intestinal microbiota will be well established and after 30 days the caecal flora will have also developed (2).

The crop harbours a large population of lactobacilli which are attached to the thick epithelia via carbohydrate components of the bacterial cell wall. This association becomes established soon after hatching and will persist through the life of the chicken and the association is resistant to changes in diet and methods of rearing (129). These bacteria ferment the ingesta and produce lactic acid which causes the pH of the crop environment to fall to around 5 (264).

The conditions within the proventriculus are highly acidic providing a less hospitable environment for most bacteria. The gizzard however, despite having a low pH influenced by the proventriculus, does have a substantial population of lactobacilli in the region of 10^8 cfu g^{-1} of ingesta. Enterococci and *Escherichia coli* have also been found in small numbers in this environment (108)

The small intestine has a high rate of peristalsis making it less favourable for bacteria to colonise the lumen. However, there are high levels of lactobacilli within the small intestine with the population stabilising at around two weeks of age (19). Cultivation of small intestinal contents has shown that the majority of bacteria are facultative anaerobes, with *Lactobacillus sp.*, *Enterococcus sp.* and *Escherichia coli* constituting 60-90% of the small intestinal microbiota. Other species found by culture based methods in the ileum and duodenum include obligate anaerobes such as eubacteria, clostridia, propionibacteria and fusobacteria (334).

During the early life of the bird the caeca are dominated by lactobacilli, coliforms and enterococci (267). However, due to the low rate of peristaltic flow of the caeca a more stable and complex microbiota develops over a period of time (408). By four weeks of age the adult caecal flora will have established and the species found include members of the bacteroides, Eubacteria, Bifidobacteria, lactobacilli and clostridia (19, 333).

Culture-independent studies of intestinal microbiota using molecular profiling methods have enabled a greater insight into the nature and complexity of the intestinal microbiota of chickens. It is increasingly evident that the majority of intestinal bacteria within birds and mammals remain uncharacterised. One study suggests that 10% of intestinal bacteria are known species, 35% are unknown bacteria but can be linked to a known genus and the remaining 55% of bacteria are completely unknown (5). Molecular studies have affirmed that within the ileum 70% of the bacterial population is predominately lactobacilli with the remaining 30% consisting of mainly clostridia, streptococci and enterococci (34). The shift in population composition of intestinal microbiota over time has also been demonstrated through molecular methods with significant differences observed in the bacterial profile of birds in different age groups (240). Using denaturing gradient gel electrophoresis (DGGE), Van der Wielen *et al.* (398) demonstrated that the diversity of the intestinal microbiota increases with age, especially within the caeca. In the same study individual birds within a group were shown to have different microbial communities compared to each other, indicating that animals of the same age and breed have unique microbial populations.

1.3.3. Role of the microbiota in health and disease

Within the GI tract there are multiple interactions between the host cells, the intestinal environment, bacterial cells and digesta (34, 450). These interactions emphasise the extremely important role of intestinal microbiota in the health and well being of the host; the manner in which this is achieved is multifactorial and not yet fully understood. The intestinal microbiota dominate the mucosa along the GI tract, forming a protective barrier competing against pathogenic bacteria for

adhesion (167, 231). This principle has a variety of names but is most commonly known as competitive exclusion and has been exploited successfully in human nutrition by including commensal bacteria as probiotic agents in foodstuffs (83, 292). Similar commensal agents have been used as competitive exclusion agents for serious enteric pathogens such as *Salmonella sp.*, *Campylobacter jejuni* and other food borne pathogens in poultry (208, 266, 344). The means by which the intestinal microbiota inhibits the colonisation of pathogenic bacteria is not fully understood but one theory suggests that the microbiota dominate the attachment sites on the mucosal epithelium reducing the opportunity for attachment and colonisation by pathogens (26, 27). Another proposed mechanism is that the intestinal microbiota are able to secrete compounds, including volatile fatty acids and bacteriocins, that either inhibit growth or make the local environment unsuitable for other potential colonisation suitors (346, 398).

Studies using germ-free and gnotobiotic animals have shown that the intestinal microbiota are important in the stimulation and development of the immune system. Not only are the commensal microbiota thought to maintain the gut enteric system in a state of 'alert' by inducing a base level of inflammation but they are also considered to be an important factor in the development of the humoral and cellular immune systems during early life (51). Studies have shown that animals lacking an intestinal microbiota are more susceptible to disease and have poorly developed secondary lymphoid tissues, slower lymphoid cell development and general deficiencies in immunological mechanisms when compared to their wild type counterparts (167, 393). In addition to protection against disease and stimulation of the immune system the intestinal microbiota can influence host growth rates by producing extra nutrients such as short chain fatty acids (SCFA) and vitamins through the fermentation of non-digestible components of the digesta and the endogenous mucus produced by goblet cells (12, 150, 169, 357, 403).

The gastrointestinal microbiota is a highly metabolic entity, as powerful as any organ in the animal body (4), and it has the potential to elicit deleterious effects on the growth rates and performance of the host animal. Firstly there is competition between the host cells and the microbiota for nutrients such as glucose and amino

acids resulting in reduced availability of nutrients from the feed (62, 107, 407). Additionally, bacteria such as lactobacilli deconjugate bile acids leading to poor fat emulsification and thus reduced fat absorption which reduces the nutrients available for the host (107, 130). The products of bacterial metabolism can also have a negative effect on growth due to the production of ammonia and toxic amines from the fermentation of peptides (111, 136). The intestinal microbiota also causes a thickening of the intestinal wall and as a result the rate of nutrient absorption is reduced (46). AGPs reduce the population of bacteria within the intestinal lumen, thus reducing these negative effects leading to less competition for nutrients (152), reduced thickening of the intestinal wall (271) and it is believed that some of the metabolic pathways which produce toxic amines are inhibited (112), thus giving the observed growth promotion due to more energy being available to the host for growth (22).

1.3.4. Factors affecting the GI tract microbiota

There is a delicate balance between the host, the intestinal microbiota, the intestinal environment and dietary compounds. If there is an imbalance in this relationship then disease can occur (4). Microbial populations can be positively or negatively influenced based on the environmental conditions, the age and health state and the dietary intake of the host (5, 131, 240).

As mentioned previously intensive farming practices included subtherapeutic doses of antibiotics in the diets of animals for two reasons, they promote growth and they can prevent the onset of endemic diseases (152). However, the emergence of drug resistant bacteria along with pressure from consumers, supermarkets and government bodies to reduce the drugs used in food-producing animals has created a need for 'natural' alternatives to boost performance and prevent disease spread (20, 106, 432). In the hunt to find alternatives to AGPs there have been many attempts to manipulate the intestinal microbiota with varying degrees of success. Prebiotics and probiotics are popular methods for manipulating the intestinal microbiota. They are defined as 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal

microflora that confers benefits upon host well-being and health' (140) and 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (128) respectively. Many studies have shown the benefits to the health of broilers administered with either probiotics, prebiotics or a mixture of both (synbiotics) (9, 10, 58, 162, 224). Despite probiotic supplements showing potential in laboratory trials, success varies in the 'real world' of a commercial broiler unit. This is likely due to the complexity of the relationship between the host and the intestinal microbiota and the wide array of factors which can influence the composition of the intestinal microbiota and these must be appreciated when trying to manipulate the intestinal microbiota.

Intestinal bacteria derive most of their energy from dietary compounds and thus diet has a major influence over the bacterial populations (5, 73). As individual bacterial species have different nutritional demands and preferred substrates it is possible to influence the gut microbiota through dietary change (84, 244, 265). Various studies have looked at the effects of dietary components on gut bacterial populations. Using %GC analysis, Apajalahti *et al.* (6) demonstrated, the effects of different diets on the intestinal microbiota of birds. The results indicated that the addition of wheat to the diet stimulated the growth of bacteria with 50%-55% GC content and suppressed those with a GC content of 60%-69%, indicating a shift in microbial populations. Populations of lactobacilli and coliforms were shown to increase in response to diets containing wheat and barley in contrast to maize based diets (255). One study by Knarreborg *et al.* (202) showed that dietary fat source influenced the ileal microbiota with the lactobacilli population and *Clostridium perfringens* being most strongly affected by the changes. Furthermore, changing the dietary fat source from soy oil to tallow resulted in an increase in the total number of anaerobes in the intestinal microbiota and this result correlated with an increase in gut transit time (76). This allows the microbiota of chickens to be modulated by altering the diet and including specific components (essential oils, oligosaccharides, enzymes and specific carbohydrate sources), designed to boost growth and make the conditions within the intestinal tract more favourable for specific commensal bacterial groups (300, 362, 438).

Another major factor affecting the gastrointestinal microbiota is the environment in which the birds are kept and the management associated with them. Poor hygiene predictably results in an increase in enteric disease and associated problems with ensuing wet litter (45, 161, 366). The litter on which the birds are placed is undoubtedly a source of bacteria for the birds, and thus is a potential source of pathogenic bacteria; therefore it is essential that good litter management is followed on farms (123).

The health status of the birds plays a vital role in the modulation of intestinal microbiota. The immune system of the host (235) and integrity of the intestinal wall (150) prevent the entrance of bacteria into the sterile tissues around the intestinal tract. In the event of these mechanisms failing or being put under pressure from a disease challenge, opportunistic pathogens are able to invade tissues and cause infection (4). The immunocompetence of an animal can be diminished if the animal is exposed to stress factors such as heat, handling and transportation, overcrowding or placement in new housing. One response to stress is an increase in the release of compounds such as cortisol, adrenaline and noradrenaline (256). These hormones have a dampening effect on the immune system which can result in an opportunity for infection to occur (352). It has been found that the growth of certain pathogenic enteric bacteria, such as *Escherichia coli* O157:H7, *Salmonella enterica* and *Yersinia enterocolitica*, is stimulated by noradrenaline (122); this is thought to be a major factor in the increased incidence of diarrhoea in cattle following transportation and introduction to a new environment (16, 232) and this could certainly be a factor in other animal species. Suzuki *et al.* (372) demonstrated that overcrowding and heat stress, very commonly seen in intensive poultry farming, has a significant impact on the microbiota of chickens.

Host genotype appears to play a vital role in the maintenance of a healthy intestinal environment. This phenomenon is still not fully understood but it is thought to be related to host mediated factors influencing the enteric environment and the inhabitants therein. An extensive study in poultry by van der Wielen *et al.* (398) demonstrated that individual birds had a unique microbial composition. These

findings have been mirrored in studies performed in humans (448, 449), pigs (355) and mice (386, 395). Within the animal industry a great deal of research is focused on host resistance to disease, and animals found to have increased resistance to certain enteric diseases are included into selective breeding programs (as reviewed in 367).

In addition to the influences of host genotype, age has been indicated as a factor influencing the composition of the intestinal microbiota (171). Culture-independent molecular profiling techniques have shown that as birds age there is an increase in the diversity and complexity of the bacterial populations within the intestinal microbiota where older and younger birds are kept under the same conditions (398). Lu *et al.* (240) illustrated that ileal and caecal microbiota of broiler chickens had a significantly similar microbiota at three days of age but after two weeks these subpopulations had progressed into significantly different communities. The changes of the gut microbiota with age is likely to be associated with a variety of factors including a change in diet as the animal ages, maturation of the immune system, changes in environmental influences through time and an increase in the interplay with other animals which would expose individuals to a greater repertoire of bacteria.

1.4. Dysbacteriosis

1.4.1. Description

The term dysbacteriosis was first coined in the mid 1990s to describe a digestive imbalance in poultry thought to be associated with a number of shifts in the mechanics and conditions of animal production (Barry Thorp, Personal communication). Healthy birds possess a natural resistance to infection by potentially pathogenic micro-organisms (273). The interactions between the host and the intestinal microbiota play a significant role in this phenomenon (235, 341). It is thought that the changes seen in farming practices have influenced the natural disease resistance characteristics which can affect animal health. The key aim with all food producing animals is to obtain good growth rates and performance through food conversion efficiency. Intestinal integrity and health is essential for this (429).

Intestinal health is heavily reliant upon the acquisition and maintenance of a balanced intestinal microbiota, and this has become one of the key topics in European poultry husbandry.

Dysbacteriosis has been on the increase within the intensive broiler industry, particularly following the ban on the use of AGPs. Little is understood about the condition and it has been broadly described as an overgrowth of the intestinal microbiota leading to non-specific enteritis (261). Onset is usually between 20 – 30 days of age and it is thought to be triggered by changes in diet, poor management and overcrowding (42, 198). There is a change in the composition of the intestinal bacteria of birds suffering from this condition. Consequences include malabsorption of feed and deterioration of litter quality due to higher moisture content in the droppings of the affected birds.

There are a variety of clinical signs associated with the condition but few are pathognomonic which makes diagnosis difficult. The main symptom associated with the condition is the production of wet faeces, which have a distinctive orange mucoid appearance along with foamy caecal droppings (Figure 1.4). Within the faeces there can be particles of undigested feed indicating a loss of intestinal function. Other symptoms associated with the condition include growth retardation (leading to poor uniformity of growth across the flock), decrease in activity of the birds, a slight increase in mortality, decreased feed intake, and fluctuations in water consumption.



Figure 1.4 Photograph of typical faecal samples produced by a broiler suffering from dysbacteriosis. (Image taken from Aviagen 'Enteric Health in Broilers' technical bulletin 04/2006)

1.4.2. Detection

At present there is no direct diagnostic test for dysbacteriosis; diagnosis is based on visual inspection of the faeces within a broiler house and the condition of the flock along with fluctuations in water and feed consumption. However, there are methods available to measure the water content of faeces and this can be used to give an indication of intestinal integrity thus allowing the condition to be detected early and treatment to commence before the condition spreads. The litter tray and the F3 faecal fluid finder (Elanco Ltd.) are tools by which water content of the faeces can be measured and thus dysbacteriosis detected. The litter tray method enables droppings to be classified as 'dry' or 'wet' by measuring the moisture ring radiating from the faecal pellet (Figure 1.5). If the ring is greater than 0.5 cm the faeces are 'wet' and below 0.5 cm they are 'dry'. The proportion of wet droppings over a given time period can be assessed and the likelihood of the presence of dysbacteriosis can be measured with a higher proportion of wet droppings.

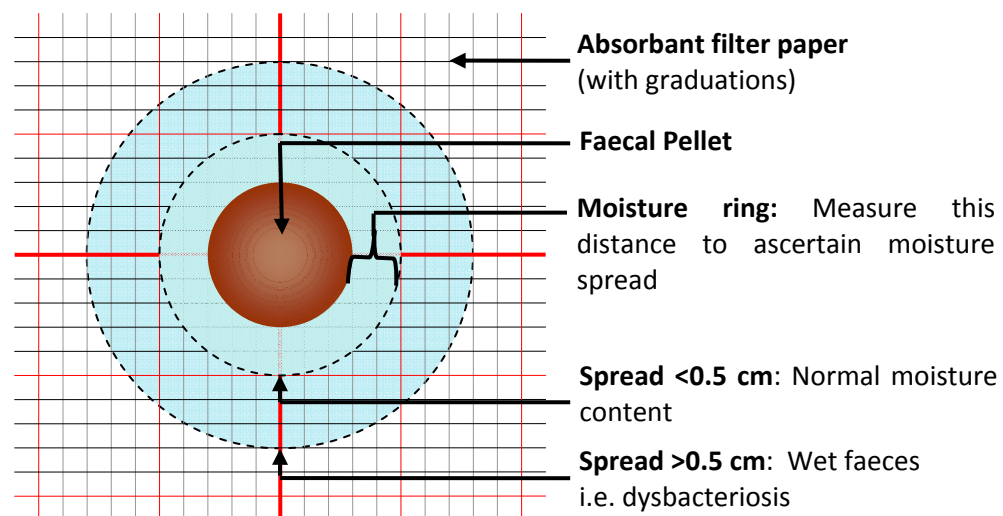


Figure 1.5 Schematic drawing to demonstrate the use of a litter tray to ascertain faecal moisture

The following guidelines are taken from Mortimer (278) describing the use of a litter box as a diagnostic tool. Firstly calculate the proportion of wet droppings daily over a two to three hour period and if the value of the litter box score is less than 20%, wet litter should not be a problem. However, if the litter box score rises above 20%, for two or more consecutive days, then the farm is at risk of wet litter

due to dysbacteriosis. Should the litter box score rises above 50%, for two or more days, then the litter is highly likely to become wet and immediate action is required, possibility in the form of antibiotic intervention.

The faecal fluid finder is a syringe type instrument into which faeces can be placed and the fluid fraction expelled by applying pressure to the faeces (Figure 1.6). This allows comparison of the fluid within a faecal sample to the solid matter by dividing the total fluid volume by the total solid volume to obtain a ratio. The resultant ratio gives an indication of the condition of the intestinal integrity. A ratio of less than 0.5 is normal, 0.5-0.6 indicates destabilisation of intestinal integrity and possible risk of dysbacteriosis. A ratio greater than 0.6 indicates loss of intestinal integrity and high risk of dysbacteriosis. The manufacturer (Elanco Ltd.) suggests monitoring should be carried out by taking 10 fresh faecal samples from around the broiler shed at random twice a day and recording the results.



Figure 1.6 Faecal Fluid Finder used as quick method on the farm for assessing faecal moisture content (Supplied by Elanco Animal Health UK)

When used routinely, these techniques act as an early warning system for the farmer allowing the continual assessment of dropping quality and general litter conditions as an indication for the onset of dysbacteriosis.

1.4.3. Impact of dysbacteriosis on the broiler industry

The impact of dysbacteriosis can be separated into economic and welfare issues. When the birds in a broiler house are suffering from dysbacteriosis the litter becomes very wet which can result in pododermatitis and hock-burn - two types of contact dermatitis resulting from prolonged contact with degrading litter (Figure 1.7). These conditions can result in painful ulceration of the feet and hocks leading to a serious welfare issue if left unchecked and in severe cases breast blisters can develop due to the bird laying down for prolonged periods of time. Illness puts the bird, as with any animal species, under unnecessary stress and discomfort leading to loss of appetite and listlessness. This can ultimately result in reduced growth rates, smaller slaughter weight thus reducing the income for the broiler grower. In addition to the economic loss due to smaller birds there is further economic loss due to treating the condition with antimicrobials. It has been suggested that poor gut health due to conditions such as dysbacteriosis can cost broiler growers 6.8p per bird (119) which, in the current financial climate, when coupled with increasing costs of labour, litter, feed energy for heating and ventilation, is a large financial burden on broiler growers.

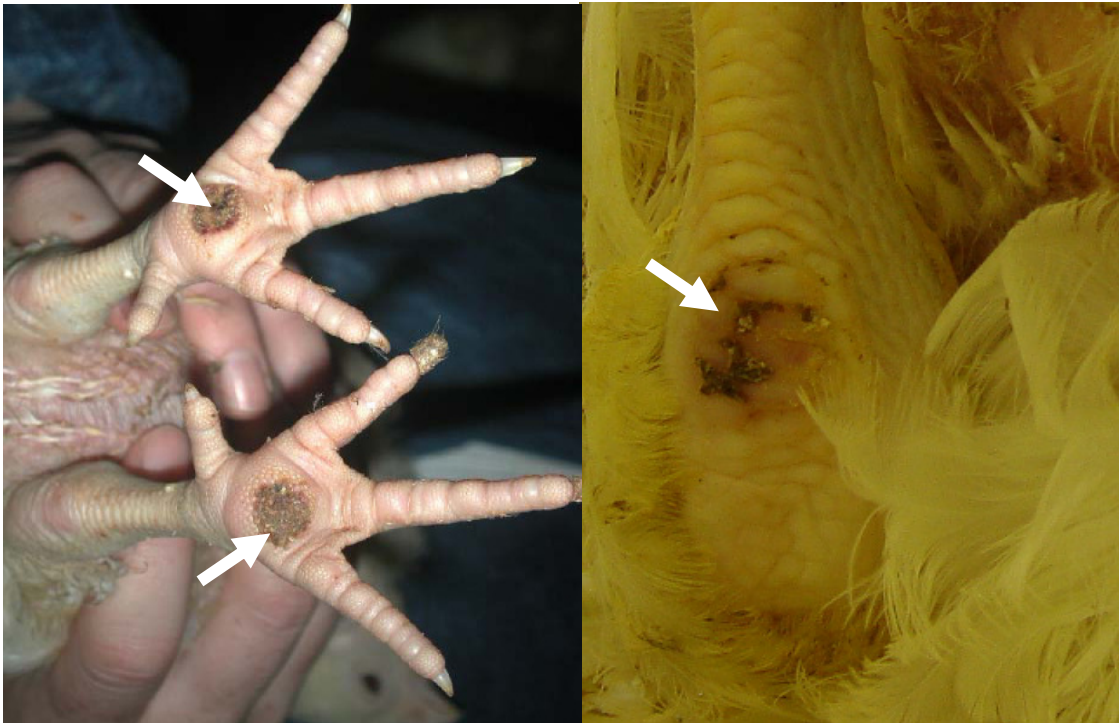


Figure 1.7 Photographs of foot pad dermatitis (left) and hock burn (right), both conditions result from prolonged contact with degrading litter and are of welfare concern. Photographs courtesy of Barry Thorp and Philip Hammond.

1.5. Molecular profiling

1.5.1. Culture based vs. Molecular based microbiology

In order to understand the role and contribution of the intestinal microbiota to enteric health there is a requirement to investigate the species present in the GI tract and how they change in relation to diet, age and disease state. Culture based methods were traditionally used to characterise bacteria from the GI tract, but it is now widely accepted that this approach vastly underestimates diversity (1, 403). It has been shown that selective plating of faecal samples can underestimate the quantity of species actually present (7) and it is believed that an estimated 50%-90% of gastrointestinal microbiota have not yet been isolated in culture (451). Certain species of anaerobic bacteria are slow growing and can take up to two weeks to grow in culture (386), as a consequence either their importance is not fully appreciated or they make culture-based community profiling very time consuming and laborious. Additionally, some bacteria are extremely fastidious and their individual growth requirements have not been simulated successfully *in vitro*. Many culture based assays use selective media to target known bacterial groups this therefore gives *a priori* bias and can result in an unrealistic view of the microbiota present (2, 30, 355, 450).

The advent of molecular profiling methods in the 1980's enabled microbiologists to obtain a more accurate view of the intestinal microbiota without the need for culturing. There are many techniques that utilise molecular technology as a means to monitor microbial populations (Figure 1.8). These include DNA microarrays, fluorescent in situ hybridisation (FISH), dot/slot hybridisation, cloning and sequencing of 16S rDNA genes, quantitative PCR, terminal restriction fragment length polymorphism (TRFLP), temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE) and more recently next generation sequencing (30, 230, 270, 403, 450).

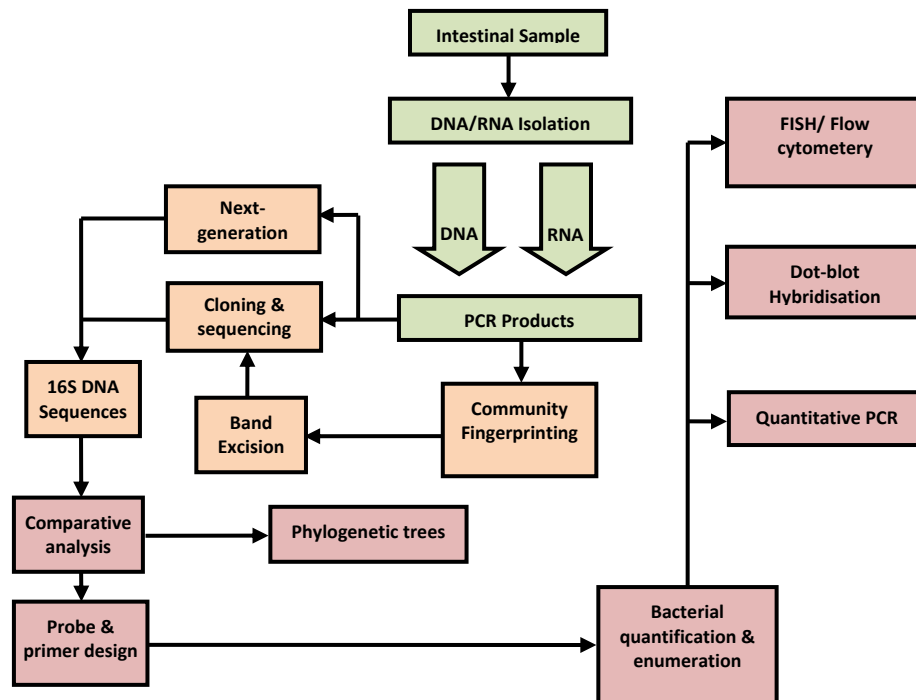


Figure 1.8 Schematic network of molecular techniques and their interactions (adapted from Muyzer and Smalla (86), Vaughan et al.(284) and Zoetendal et al. (403))

When embarking on a molecular profiling investigation, the advantages, disadvantages and limitations of each molecular technique, as outlined in Table 1.1, should be considered so as to ensure the best method is used for the application required.

Method	Main use	Advantages	Limitations
Traditional methods			
Cultivation	Identify and quantify taxa	Provides isolates for further characterization; can focus on recovering strains with desired traits	Incomplete and biased community representation; many types of media needed to maximize species recovery
Microscopy (general stains)	Estimate abundance of all microbes or recognizable types	Quantification without cultivation or broad-range PCR bias ^d can reveal spatial relationships	Cannot distinguish between many taxa with different traits and ecological roles; low throughput
16S rRNA methods			
Oligonucleotide hybridization ^a	Detect and quantify known phylogenetic groups	Can be high-throughput; can reveal spatial relationships; phylogenetic identification of visible cells	Detects only taxa that hybridize to chosen probes; typically 6–18 genus- or family-level groups detected
5' exonuclease PCR ^b	Detect and quantify known phylogenetic groups	Rapid; high throughput	Detects only taxa that hybridize to chosen probes; typically 6–18 genus- or family-level groups detected
Community profiling ^c	Compare communities	Rapid, inexpensive assessment of abundant 16S rRNA sequence variants	Broad-range PCR bias ^d ; additional work needed to identify groups represented in profiles; hard to compare analyses done at different times
16S rRNA sequencing	Phylogenetic identification of microbes; generates data for other 16S rRNA-based methods	Identification to strain level; can detect novel taxa; analysis possible at multiple phylogenetic levels	Broad-range PCR bias ^d ; expensive; laborious data analysis

^aIncludes membrane hybridization, fluorescent *in situ* hybridization (FISH) with cells detected by microscopy (for spatial information) or flow cytometry (for high throughput and accurate quantification), and microarrays. Microarrays detect many more taxa simultaneously, but quantification is more difficult and broad-range PCR bias can be an issue.

^bIncludes denaturing gradient gel electrophoresis (DGGE) and similar techniques, and terminal restriction fragment length polymorphism (tRFLP).

^cAlso called quantitative PCR, real-time PCR, or TaqMan assay. PCR is a biochemical amplification of particular DNA sequences.

^dThe broad-range PCR used for profiling, 16S rRNA sequencing and microarrays might not include all taxa or accurately represent their relative abundance, but these problems are reduced relative to those of cultivation approaches.

Table 1.1 Review of methods used for analysis of microbial communities (taken from review by Dethlefsen *et al.* 2006)

The target for many of these molecular profiling techniques is the 16S ribosome and its encoding gene, which is amplified using the polymerase chain reaction (PCR). The role of the ribosome has remained constant over a long time frame and is present in all bacterial species, making it an ideal target for identifying changes in nucleotide sequence in order to infer phylogeny or relatedness (303, 433). The 16S rRNA gene contains both variable regions and conserved regions, allowing the design of PCR primers which target all or specific bacterial DNA. The 16S rDNA gene is approximately 1.5 kbp in length with nine variable regions (V1-V9) with a range of heterogeneity (289, 332, 434, 435). Figure 1.9, indicating the sequence features of the 16S rRNA gene is taken from a review in 2003 by Baker *et al.* (14). In an extensive study performed by Yu & Morrison (444), the V1 region was found to be the most variable, followed by V9 and then by V3. At present there are around one million 16S rDNA gene sequences available in databases for comparison, which means species identification or at least phylogenetic placement is possible (65, 452). In the same study by Yu & Morrison (444) it was found that after using different primer combinations targeting different hypervariable regions of the 16S ribosomal gene that the primer set amplifying the V3 region alone gave the best results regarding separation, number and intensity of amplicons when used with DGGE.

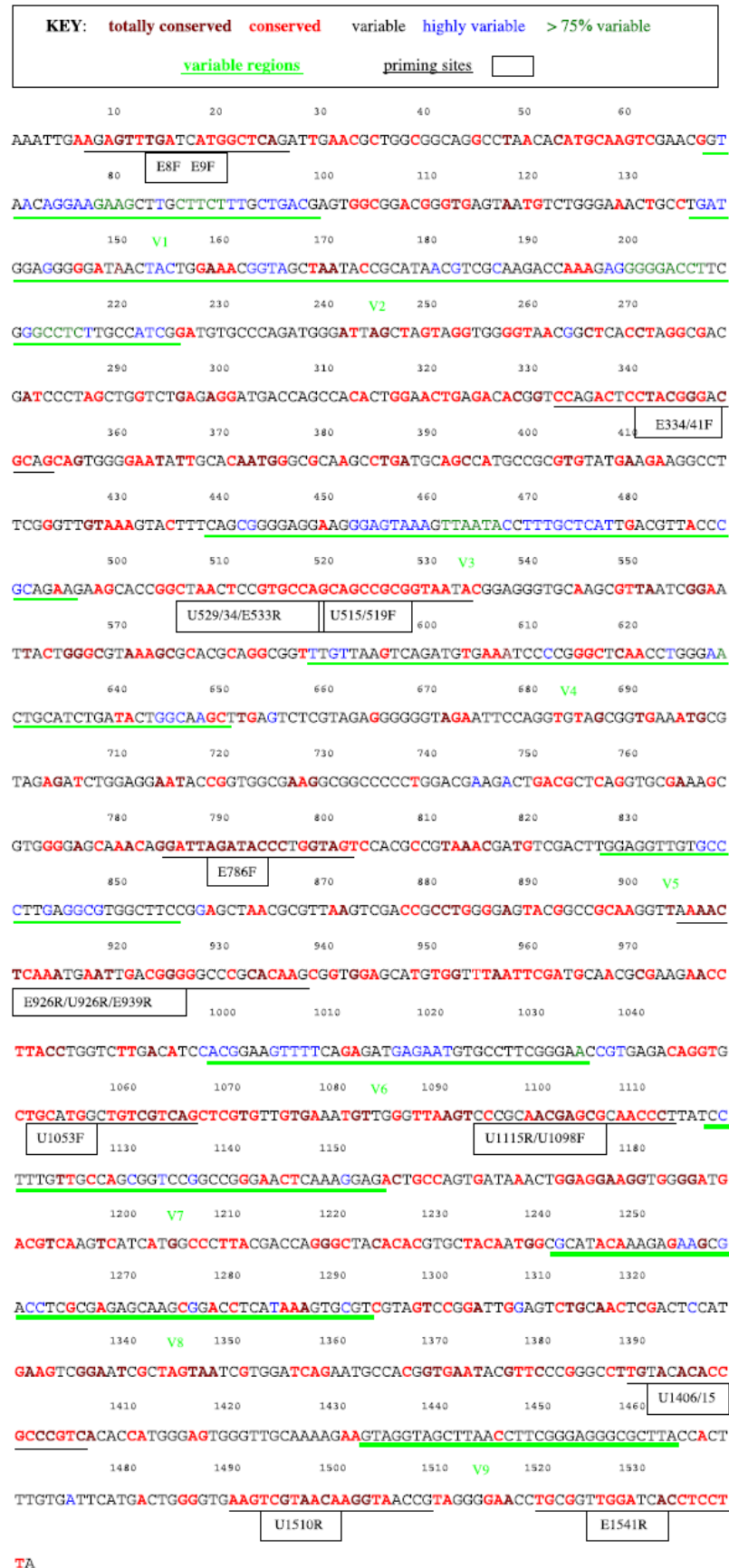


Figure 1.9 *E. coli* 16S rRNA gene sequence annotated with bacterial “universal” priming sites and variable regions V1–V9. The sequence is colour coded to indicate bacterial sequence variability (taken from Baker *et al.* (14))

1.5.2. Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) is a profiling technique that can be used to compare and monitor changes in microbial populations. Despite the significant amount of preparatory and optimisation work required, it can provide a realistic view of the microbial diversity within a mixed population (115). DGGE was originally developed for use in human health studies by Fischer and Lerman (118) to detect point mutations in genes relating to diabetes. By utilising the 16S rDNA sequence variations, DGGE was later adapted for defining the microbial population of biofilms and microbial mats by Muyzer *et al.* (283). The technique has now been tailored to encompass the study of a wide range of microbial environments such as soil (226), rivers and seas (41, 189), clinical samples (225, 257) and food products (110, 268, 313). DGGE can be used to compare changes in microbial communities from one animal to another but also used to track temporal changes in bacterial communities over a time period (116, 117). This has in turn provided a great means of investigating the gastrointestinal microbiota, and has become one of the most widely used methods in characterising the microbial ecology of the GI tract. Many studies have applied this technique effectively to the gastrointestinal microbiota of humans (91, 160, 247, 272), cattle (203, 421), dogs (371, 401), pigs (67, 222, 356), and poultry (165, 176, 304, 398, 446).

DGGE involves the separation of DNA fragments of equal length in a polyacrylamide gel which contains an ascending gradient of denaturants (urea and formamide) (Figure 1.10). The separation is based on the decreased electrophoretic mobility of partially melted DNA molecule compared to that of helical DNA. When double stranded DNA reaches its denaturing point in the gel it will virtually stop progressing through the gel. (283, 284) The denaturing point of DNA is related to the relative concentration and distribution of nucleotides; those sections with a higher proportion of the tri-hydrogen bonded bases (Guanine and Cytosine) have a higher melting point than those with a higher number of the bi-hydrogen bonded bases (Thymine and Adenosine) (143, 442). The addition of a GC rich sequence (GC clamp) on the 5' end of one of the primers is used during PCR this prevents total dissociation of the dsDNA. In theory two DNA fragments of different nucleotide

sequence will have a different denaturing point and thus stop moving at different points in the gel (220, 287, 444). In theory all the sequence variations of the original PCR reaction can be detected on the gel using DNA stains (285, 286, 349) but practically this is not always the case.

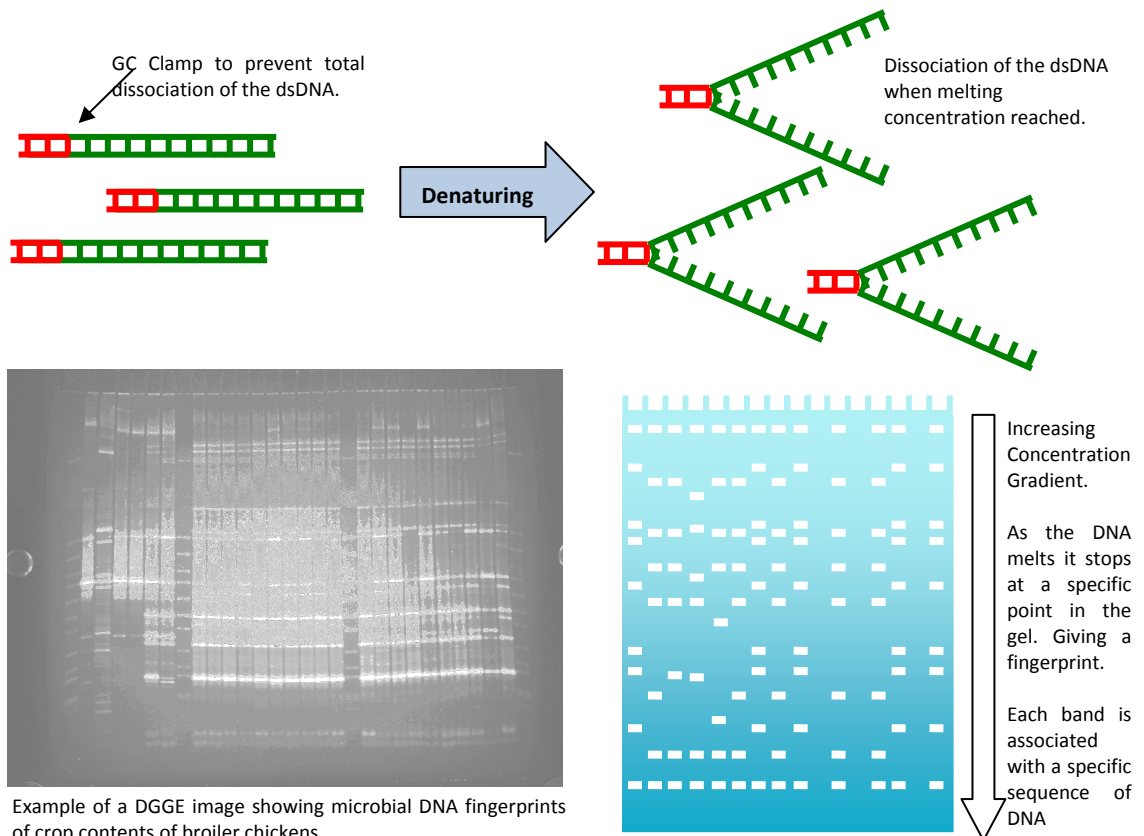


Figure 1.10 Outline of the principles of DGGE.

Farrelly *et al.* (113), demonstrated that bacteria have different sized genomes and different rDNA gene copy number, and this in turn can result in preferential PCR amplification. The copy number of 16S rDNA genes have been documented to range from 1 copy (66) to 14 copies (283), this can understandably cause discrepancies in the projected view of a microbial ecosystem. DGGE analysis can lead to an over estimation of bacterial species present with increased copy number. Microheterogeneity or sequence differences between the copies of the gene sequences within an individual bacterial genome, can also lead to an overestimation of diversity by causing multiple bands (63, 295). In order for DGGE to work successfully it has been shown that the PCR amplicon should be no longer

than 500bp (285). Smaller fragments reduce the phylogenetic information available (284) and thus reduces the ability to distinguish between species. In addition to this some bacterial 16S rRNA sequences could have similar melting properties to those of other bacterial species giving rise to multiple species within a DGGE band and consequently an under estimation of diversity (181). Gel expansion of the excised bands has been suggested as a means to solve this problem, following excision the DNA is run through a shorter denaturant gradient to split any multiple bands (132). Where required, the DNA from the bands is easily extracted from the gel following visualisation by staining using a fluorescent nucleic acid stain such as SYBR Green. One major benefit of DGGE over other molecular methods is that the extracted DNA can be used immediately for downstream processes such as sequencing without the need for cloning (337).

1.5.3. Limitations of non-culture based technologies

The powerful nature of PCR amplification and subsequent molecular techniques now means that previously unknown bacteria within a community can be identified even if they are unculturable or constitute a small portion of the population. However, these techniques are not foolproof and each physical, biological and chemical step involved in molecular analysis is a source of bias, distorting the true nature of the ecological niche being described (394).

The collection and handling of samples both play a crucial role in ensuring that the community profiles obtained are an accurate reflection of the sample site. Rochelle *et al.* (322) demonstrated that samples must be frozen within 2 hours of collection to ensure an accurate species diversity and to prevent enrichment of bacterial groups. The results indicated that samples should ideally be frozen immediately following collection where possible. The extraction of DNA from environmental samples can be another source of error (394). Gram positive bacteria require vigorous conditions in order to release their DNA, thus if these conditions are not met during the DNA extraction procedure their DNA will not be accurately represented in the community profile (109, 284). Environmental samples often contain other biotic and abiotic compounds which can affect PCR efficiency if they

are not removed during DNA extraction (109, 428). Studies to compare DNA extraction methods using commercial kits and non-commercial methods indicate that the QIAamp® DNA Stool Mini Kit provides a reliable, reproducible and efficacious method for extracting DNA from intestinal samples (53, 222, 263).

The PCR reaction itself can be a multifactorial source of bias. Quantitative conclusions can only be drawn from DNA amplification of mixtures of different templates if one assumes the following (taken from 373):

- All molecules are equally accessible to primer hybridisation
- Primer and templates hybridise with equal efficiency
- DNA polymerase extension efficiency is uniform for all templates
- The concentration of template is consistent so exhaustion of substrate is uniform.

There are a variety of factors which can negate the above assumptions. Poor DNA template quality (228), high or low DNA concentration (54), choice of primers and target region of the 16S rRNA gene (373, 444) and an increase in replication cycles (311) can all influence the amplification of the template DNA and result in the formation of chimeric and heteroduplex molecules.

Fromin *et al.* (124) suggest that the best way to ensure accuracy in the comparisons of microbial profiling is to ensure the same standardised methodology for all samples. Thus it can be assumed that any bias will occur uniformly across all sample populations.

1.6. Thesis Outline

Dysbacteriosis is not a well documented condition, in a PubMed search with 'broiler' and 'dysbacteriosis' being the keywords there are no results. If 'broiler' is substituted with 'chicken' in the search there are 3 results for papers published in 1977, 1997 and 1981 relating to studies in Russia on gnotobiotic chicks, but nothing relating to the problems seen with dysbacteriosis in commercial broiler flocks. Poultry text books, manuals and technical bulletins from various publishers and companies in industry comment on dysbacteriosis, but only outlining the condition as a disturbance in the intestinal microbiota resulting in non specific enteritis. It is often noted that the condition is on the increase and is of major economical and welfare importance, but an understanding of the mechanics of the condition is lacking. The fact that dysbacteriosis is seen as an increasing problem in broiler production coupled with the current lack of knowledge available highlights a need to investigate the disease. Consequently the aim of this thesis is to investigate the aetiology of dysbacteriosis and gain insight into the pathogenesis of the condition in an attempt to fill the void in knowledge. As dysbacteriosis is a disturbance to the gut microbiota the most logical place to start is by investigating the intestinal microbiota. A standardised methodology must be in place before embarking on this, to ensure accuracy and uniformity across the samples. **Chapter 2** discusses the core methodologies used in this study and experiments carried out to validate the repeatability and robustness of the techniques used.

The current train of thought is that dysbacteriosis is a disruption of the intestinal microbiota of broiler chickens; however it is not known where along the GI tract this imbalance originates or which bacterial species are involved. **Chapter 3** details investigations comparing the intestinal microbiota of broiler chickens suffering with dysbacteriosis with healthy birds. The aim is to answer three fundamental questions; firstly is there a change in the composition of the intestinal microbiota of broilers suffering from dysbacteriosis, secondly if so where along the GI tract can these changes be seen and lastly, what bacterial groups or species are involved with these changes. Answering these questions will form a foundation on which a structured approach to understanding to dysbacteriosis can be developed.

There is evidence from poultry growers and data from unpublished industrial trials indicating a variety of factors that predispose certain birds to dysbacteriosis. **Chapter 4** investigates the influences of host genetics on the intestinal microbiota of different broiler breeds. This has been identified as a risk factor with certain genetic lines being more susceptible than others. By comparing healthy birds of differing breeds with different susceptibility or resistance to enteric disease, the aim is to ascertain whether or not there are differences in the composition of the intestinal microbiota of the birds that could be linked to dysbacteriosis predisposition.

Chapter 5 looks at the changes in the intestinal microbiota of birds in relation to dietary change diets. It has been found that one of the major factors found to influence gut microbiota is diet; farms with identical feeding regimes where the feed source and composition were the same had little change in gut microbiota profiles, however should the feed source or dietary composition change then there were shifts in the microbiota (6, 176). This means that by manipulating the diet it should be possible to alter the microbial community to a more favourable one (47, 68, 140, 213) and this is the question to be investigated in this chapter.

The metabolic interplay between the host and the intestinal microbiota can be revealed by the composition of metabolites in the contents of the intestinal tract or faeces (338). In **Chapter 6**, by using proton Nuclear Magnetic Resonance (^1H NMR), changes in metabolites in the ileum and caeca are investigated between the dysbacteriosis (DB) affected and healthy birds where a change in bacterial composition of the intestinal microbiota was seen. Metabolite profiling was also used to look for changes in metabolites in the faecal samples of the birds mentioned in chapter 5 where changes in intestinal microbiota were demonstrated between the groups of birds fed on the diets of differing protein source. The aim of performing ^1H NMR analysis is to firstly determine whether the change in microbiota seen in DB birds can be linked to a change in metabolites, thus giving an inclination into possible mechanisms for the onset of dysbacteriosis. Secondly it could be possible to identify metabolites that indicate either a 'good' or a 'bad' enteric environment which would be useful in longitudinal studies.

2. Molecular profiling of intestinal microbiota using PCR-DGGE

2.1. Introduction

This chapter describes the core methodologies common to the majority of experiments in this thesis. Where other techniques have been used or the core methodology has been altered then the protocol will be outlined in the relevant chapter.

DNA based molecular profiling techniques have allowed microbial ecologists to gain more detailed information about the composition and complexity of the intestinal microbiota. Molecular techniques have become increasingly popular since they are not limited by the difficulties of culturing unknown or fastidious bacteria *in vitro* and are thus able to represent microbial populations more completely. There are a variety of different methods in use, each with advantages and disadvantages (Table 1.1); it is therefore necessary to ensure the most appropriate technique is selected for each experimental design.

PCR-DGGE is a popular and well established profiling technique which has been used to profile the microbial communities of a wide range of environment, including the GI tract of birds and mammals as discussed in section 1.5.2. DGGE creates a visual overview of the microbial community at the time of sampling, in the form of a DNA fingerprint rather than phylogenetic or species information. The DNA fingerprint acquired is a representation of the microbial composition of the given sample and therefore can easily be compared with other samples either within the same gel or across gels in order to determine how similar or different two or more samples are. In order to accurately compare the DNA fingerprints of samples with confidence, it is essential to use a standardised protocol on all the samples to ensure accuracy and reproducibility and to limit the potential for handling errors throughout the multi-step PCR-DGGE process. Previous work in the laboratory optimised and standardised the extraction of DNA from intestinal samples, PCR amplification and subsequent DGGE analysis and the standard methodology is outlined in this chapter (387, 388).

2.2. Sample preparation

During the investigations performed in this thesis the intestinal microbiota from different regions of the GI tract of the birds was examined. The crop, gizzard and caeca are distinct regions within the GI tract and are easily identified. The jejunum and ileum are less well defined and therefore a standard protocol was used to identify these regions for sampling. Meckel's Diverticulum (Figure 1.2) is a distinct region along the small intestine and once located provides an ideal landmark as a reference point for sample collection. Ileal samples were taken from a point 10 cm caudal to Meckel's Diverticulum and jejuna samples were taken from a point 10 cm cranial to Meckel's Diverticulum. The colon is relatively short in chickens and samples from this region were taken directly caudal of the ileocaecal junction. As mentioned in section 1.5.3 sample handling can be a source of error when profiling the intestinal microbiota. It was previously demonstrated that in order to prevent enrichment of bacterial groups or distortion of the species diversity due to bacterial growth after extraction from the GI tract, that samples must be frozen within two hours of collection (322), to address this all samples were frozen on dry ice **immediately** on extraction in the field, then transferred to a -20°C freezer for storage until DNA extraction.

2.3. DNA Extraction

There are many different protocols for extracting DNA from environmental samples. In this thesis DNA extraction utilised the QIAamp® Stool Mini Kit (Qiagen, West Sussex, UK) which, in recent studies (53, 222) and previous work in our laboratory (387, 388) has been demonstrated to be the most efficacious in the extraction of DNA from intestinal material when compared to other methods of DNA extraction. The methodology described was used on all samples in this thesis to ensure accuracy for comparison of samples. The kit was used according to the manufacturer's instructions with a few minor alterations, based on experience in our laboratory, to increase the final DNA yield; these alterations are included in the protocol described below. Approximately 150 µl of 710 µm -1180 µm acid washed glass beads (Sigma-Aldrich Ltd., Gillingham, UK) and 500 mg of frozen intestinal

sample were placed in a 10 ml sterile screw-top tube with 5 ml of lysis buffer (ASL) and vortexed vigorously for one minute. Following homogenisation the sample tubes were incubated at 95°C for three minutes, vortexed for 30 seconds and incubated for a further three minutes. Following this 2 ml of the incubated homogenate was transferred to a 2 ml tube and centrifuged at 14,000 rpm for one minute and 1.5 ml of the supernatant transferred to a clean 2 ml tube. The next step in the process involved removal of inhibitors from the sample by adding an adsorption matrix ('Inhibitex' tablet™) vortexing to dissolve the tablet followed by incubation at room temperature for one minute. The sample is then centrifuged for six minutes at 14,000 rpm, the supernatant transferred to a clean 2 ml tube and centrifuged for a further six minutes. Two aliquots of 200 µl of the supernatant were added to two separate tubes containing 15 µl proteinase K followed by addition of 200 µl of AL buffer. The samples were then vortexed briefly and incubated at 70°C for ten minutes. 200 µl of ethanol was added to both tubes and the solutions transferred to the spin column supplied with the kit and centrifuged for one minute at 14,000 rpm to bind the DNA to the silica membrane. The DNA was washed first with 500 µl of wash buffer AW1 and then with 500 µl of wash buffer AW2, and then eluted with 150 µl of pre-warmed (70°C) elution buffer.

Success of the DNA extraction was first checked via agarose gel electrophoresis (0.8% strength gel, TBE buffer) and photography following SYBR Green I staining (Sigma-Aldrich, Gillingham, UK). The quantity and quality of the extracted DNA was quantified using the Nanodrop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

2.4. PCR

For the majority of investigations in this thesis, the variable V3 region of the 16S rRNA gene was targeted to profile the intestinal microbiota of the chickens (Figure 1.9). The general bacterial primers used were GC-341F (5'-**CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC** TAC GGG AGG CAG CAG-3') (GC clamp in bold typeface) and 534R (5'-ATT ACC GCG GCT GCT GG-3') as originally described by Muyzer *et al.* (283). These primers correspond to the positions 341-357 and

518--534 on the *Escherichia coli* 16S rRNA gene giving an amplicon of approximately 233bp in length (primers supplied by Eurofins MWG Operon, Ebersberg, Germany).

PCR amplification was performed using 100 ng of extracted DNA. In addition to the template DNA the reaction mixture contained 1U HotMaster Taq Polymerase (5-Prime, Nottingham, UK), 1x HotMaster buffer, 200 μ M dNTP mixture (Bioline, London, UK), 10 pmol of each primer, 0.1mg/ μ l bovine serum albumin (Roche Diagnostics Ltd., Burgess Hill, UK) and ultra pure water to make the final reaction volume 50 μ l.

PCR amplification was performed using a Thermocycler (Thermo Electron Co., Basingstoke, UK) using the following parameters: 94°C for 2 min., 30 cycles of 94°C for 20 sec, 58°C for 10 sec, 65°C for 20 sec and a final elongation step of 65°C for 10 minutes. The PCR success was confirmed via electrophoresis using 1% agarose gel (wt/vol) and a loading mixture of 1 μ l PCR product, 1 μ l SYBR Green I, 4 μ l loading dye and 4 μ l of ultra pure water. The gel was viewed using a dark reader (Clare Chemical Research, Dolores, USA) and the image captured by digital camera. The PCR products were then purified using SureClean (Bioline, London, UK) in accordance to the manufacturer's instructions with minor alterations with the first incubation step and first centrifugation step both being extended to 20 minutes to increase final DNA yield. The final DNA concentrations were measured using a spectrophotometer (ND1000, Nanodrop Technologies, Wilmington, USA).

2.5. Denaturing Gradient Gel Electrophoresis

2.5.1. Methodology

DGGE was carried out using an Ingeny phorU 2 electrophoresis system (Ingeny, The Netherlands). The amplicons were separated using parallel DGGE in an 8% polyacrylamide gel with a linear gradient of denaturants (urea and deionised formamide) ranging typically from 37% to 58% (100% denaturants is equivalent to 7 M urea and 40% deionised formamide). Gel solutions were prepared to the required concentrations using acrylamide-bisacrylamide (37.5:1) and the required amount of denaturant (Severn Biotech Ltd., Kidderminster, UK), the separate

solutions were added to the appropriate side of the gradient former. Polymerisation of the gel was induced by adding 80 µl 20% Ammonium Persulfate Solution (APS) and 8 µl Tetramethylethylenediamine (TEMED) to each aliquot of denaturants and following the addition of the polymerising agents the gel was poured via the gradient maker using a peristaltic pump (Anachem, UK). Once the gel polymerised an acrylamide-bisacrylamide stacking gel (37.5:1) was added consisting of 0% urea-formamide solution and 80 µl loading dye to make the wells visible for loading. After polymerisation (with 80 µl 20% APS and 8 µl TEMED), 200 ng of the purified PCR amplified DNA was added individually to the wells combined with 3 µl of loading dye. A reference ladder was run alongside the amplicons in multiple wells to assist analysis and calibration of the gel by the gel analysis software. The reference ladder consisted of V3-PCR products generated from the 16S rRNA gene of known enteric bacteria: *Bacteroides fragilis*, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Bacteroides distatoni*, *Lactobacillus acidophilus*, *Enterococcus faecalis*, *Clostridium leptum*, *Campylobacter jejuni* 81176, *Escherichia coli*, *Bifidobacteria angulatum*, *Clostridium perfringens*, *Bifidobacterium infantis*, *Salmonella typhimurium* and *Bifidobacterium longum*.

Once loaded the samples were subject to electrophoresis in 0.5% TAE buffer for 1400 voltage hours at 80V at 60°C, following an initial 200V charge for five minutes to accelerate entry of the DNA into the gel. Following electrophoresis the gels were stained with SYBR Green I (1:20,000 in 0.5% TAE buffer) for 30 minutes, washed in deionised water and the DGGE images captured using the Alpha Digidoc software (Alpha Innotech Co, San Leandro, USA) via a digital camera using dark reader to visualise the DNA.

2.5.2. DGGE gel analysis

The DGGE profile images obtained were analysed using TotalLab TL120 v.2006 (Non-Linear Dynamics, Newcastle, UK). This converts the DNA fingerprints for each lane into a densitometric profile where a variety of functions are applied to analyse the gel. Firstly the software detects the lanes and distorted band shapes can be normalised. Following this, the background noise can be removed from all the

lanes and bands detected using ‘minimum slope’, ‘noise reduction’ and ‘percentage maximum peak’. These parameters determine how pronounced a band should be from the surrounding area, what degree smaller peaks should be ignored and which peaks should be discarded in relation to the size of the largest peak respectively. The values of 100, 5 and 5 respectively were used for the three parameters for the analysis of each DGGE image to ensure consistency. The resultant detected bands were then checked to ensure that fluorescence due to dust or gel artefacts had not been classed as a band; where appropriate these were removed manually.

Once the bands were detected the reference ladder was used to align the lanes in the gel. Each of the 14 bands in the reference ladder is aligned with the corresponding band in other lanes containing the reference ladders. This enables the calculation of the Retardation factor (Rf), a measurement of band position along the lane, which addresses distortions within the gel caused by curvature of the DNA bands and microvariation in gradient across the gel.

Once band distortion, background noise and gel curvature were corrected the bands were aligned from lane to lane. Alignment is based on the Rf values and an Rf tolerance value (in this thesis it was set at 0.05) must be used, the Rf scale ranges from 0 to 1 therefore the tolerance value must lie within this scale. Any two adjacent bands which are equal to or less than the distance of the tolerance value will be aligned and considered to be at the same point in the gel, thus in the case of this study representative of the same phylotype/ribotype.

Once the gel has been aligned band numbers present in each lane of the gel can be quantified and the presence and absence of bands between each lane can be compared to obtain a percentage similarity of shared bands. The similarity was determined using Dice similarity coefficient:

$$S_D = 2n_{AB}/(n_A+n_B)$$

Where n_A is the number of bands in lane A, n_B is the number of bands in lane B n_{AB} is the number of common bands between the two lanes. A value of 1 represents a 100% similarity between the lanes and a value of 0 represents no similarity at all.

The values obtained from this similarity comparison can be represented in a dendrogram to observe any grouping or clustering of the samples.

The number of bands on a DGGE image has a direct correlation to the number of the predominant species within a given sample. Comparison of the mean band number between the healthy and dysbacteriosis populations provides an initial assessment of any differences in species richness between the two groups. The samples obtained represent random independent representatives of the overall population of healthy and DB birds therefore an unpaired Students T-Test is appropriate to compare the significance of any changes in mean band number.

The analysis of the DGGE gel creates a matrix based on presence and absence of bands which can be used to perform multivariate techniques, such as principal component analysis (PCA), to reduce the number of variables in a data set while preserving as much of the original information in the data set as possible (196). PCA is a well established method which has been applied to DGGE data to reduce the dimensionality of the data produced (125, 223, 238) by creating synthetic variables (principal components). These principal components are ranked in such an order that the first principal component account for the majority of the variability between the sample groups. This technique was applied to the data generated by the DGGE gel analysis to identify clustering of sample groups which can be related to the biological or experimental factor being investigated.

2.5.3. DGGE Band sequencing

After gel analysis it was sometimes desirable to identify which bacterial species were responsible for DGGE bands observed. One of the benefits of staining DGGE gels with SYBR Green is that the DNA can be excised from the gel and utilised for downstream processes such as DNA sequencing. Bands of interest were excised from the DGGE gels using a sterile pipette tip pushed into the appropriate band on the gel. The pipette tip was then placed in 50 μ l of H₂O and left for 10 minutes to re-suspend the DNA from the gel plug. The DNA was reamplified via the same PCR conditions as described in section 2.4 above using 5 μ l of the excised DNA suspension as a template. Following this the PCR product was subject to DGGE

profiling to check that the correct DNA fragment had been excised and that the DNA obtained was pure as contamination with DNA from within the original sample is common. If the resultant DGGE profile was mixed the band required was picked again and the process repeated. Once a single band was obtained the PCR product was purified using Sure Clean and the BigDye Terminator V3.1 Cycling Kit (Applied Biosystems, Warrington, UK) was used as per the manufacturer's instructions using the primers 341F (5'-CC TAC GGG AGG CAG CAG-3') and 534R (5'-ATT ACC GCG GCT GCT GG -3') for the sequencing reactions. DNA sequencing was carried out using a Hitachi 3100 Avant Genetic Analyzer (Applied Biosystems, Warrington, UK). Sequence results were compared with the Ribosomal Database Project database (<http://rdp.cme.msu.edu/>) for identification of bacterial species.

2.6. Validation of methodology

Brief trials were used to ascertain the repeatability of the DGGE data obtained from intestinal samples, extracted DNA and PCR products stored for a long period of time. This included an assessment of the effect of storage on samples. Figure 2.1 shows a DGGE image from the caeca of two birds with DNA extracted from the contents of the same caeca 12 months apart following cold storage of caecal samples at -20°C. The standard methodology for DNA extraction, PCR amplification and DGGE profiling mentioned in this chapter was used for all the samples. There are no differences in banding patterns between the DGGE profiles, highlighting that storage at -20°C over time does not affect the DNA fingerprint when using DGGE following the methodology used in this thesis. This allowed samples to be collected and processed at different times.

As chickens have two caeca and often one is larger than the other, there could be concern as to which to use when extracting DNA. Figure 2.1 also shows a DGGE image of both caeca from two different broiler chickens, there are some fluctuations in the band intensity, however there is no discrepancy in band presence and absence between the right and left caeca.

Therefore this investigation indicates that it does not matter whether the right or left caecum is used when choosing which to extract DNA from. as found in a previous study by van der Wielen *et al.* (398).

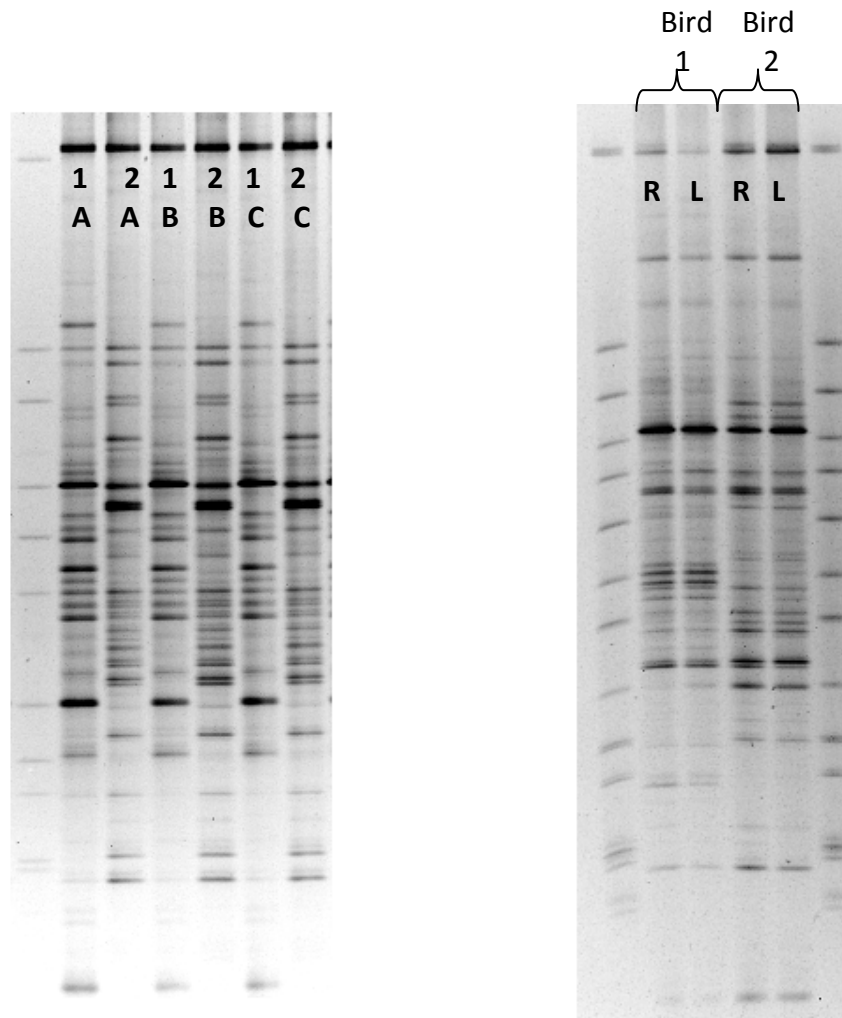


Figure 2.1 The image on the left shows a DGGE profile of the caecal contents of two birds (1&2) with DNA extraction and PCR performed at different time points (A = DNA extraction and PCR performed in January 2006, B = DNA extraction in January 2006 and PCR in January 2007, C = DNA extraction and PCR performed in January 2007). Analysis of the gel image showed 100% similarity between the DNA fingerprints of 1A, 1B & 1C and 100% similarity between 2A, 2B & 2C)

The image on the right shows a DGGE profile of the right (R) and left (L) caeca of two broiler chickens. The outer two lanes are a reference ladder of 14 known enteric bacteria for alignment of the DGGE gel with the analysis software. There was 100% similarity between the right and left caeca of both birds.

3. Investigations into the changes in GI tract microbiota of broiler chickens suffering from dysbacteriosis

3.1. Introduction

Dysbacteriosis (DB) is a digestive disorder of poultry which is a common problem within the intensive broiler industry. The EU legislation involving the restriction of the use of Antibiotic growth promoters (AGPs) is thought to have resulted in an upsurge in the incidence of DB, and it has been suggested that removal of AGPs from the diets of broilers has resulted in an increase in the severity of enteric disease (23). The current strategy for treating dysbacteriosis involves the administration of antibiotics to all the birds in the affected house. This treatment does alleviate the condition but it can return following cessation of the antibiotic therapy. Consequently there are still large quantities of antibiotics being administered to the birds which could be considered to negate the beneficial effects of the AGP ban. With ever increasing pressure on veterinarians and farmers to reduce antibiotic usage there is an urgent need to improve our understanding of dysbacteriosis in order to adopt alternative strategies to prevent and manage the condition.

Before undertaking experimental work there is a need to set out a clear definition for the diagnosis of dysbacteriosis. Unfortunately, there is a lack of good documentation describing the aetiology and diagnosis of it. Currently diagnosis is predominately based on the experience of the veterinarian or farmer involved. Flock affected by the condition have poor uniformity with birds appearing listless with evidence of diarrhoea around the vent of the bird. The most distinctive feature of the condition is the production of orange coloured wet and mucoid faeces along with foamy caecal droppings leading to wet degraded litter. These observations usually form the basis for the initial diagnosis, in addition water intake often but not always fluctuates in affected birds, meaning that on the whole flock level significant fluctuations in water intake can be used as an additional indicator of disease. By the time DB is detected in an affected shed the majority of the birds will be affected therefore the chicken house will be treated as a 'super-organism'

rather than undertaking the unrealistic task of assessing tens of thousands of birds individually. Diagnosis will be based on litter and faecal quality in the whole house and the presenting signs of the birds within the house. For the purposes of the current study fresh faecal samples will be taken from houses identified with suspected dysbacteriosis and faecal 'wetness' assessed using the Faecal Fluid Finder as described in section 1.4.2.

3.2. Comparison of GI tract microbiota of dysbacteriosis affected broilers with unaffected broilers

3.2.1. Introduction

As very little is known about dysbacteriosis the initial aim of this investigation is to ascertain whether or not there are differences in the composition of the intestinal microbiota of broiler chickens from flocks suffering from dysbacteriosis. Secondly, identify the regions of the GI tract demonstrating these changes and then, where possible, the study aims to identify bacterial species or groups involved in these changes. These aims cover the basic questions about the condition and will provide information for further investigations into the aetiology and pathogenesis of the disease. Dysbacteriosis is associated with poor flock uniformity, fluctuations in water intake, deteriorating litter quality and the presence of frothy caecal droppings along with orange wet mucoid faeces (261). Dysbacteriosis was identified in broiler houses on two separate commercial broiler units by veterinary diagnosis and confirmed by measurement of faecal moisture; thus forming two individual case studies.

3.2.2. Case Study One – Swannington (Farm 1)

Dysbacteriosis was provisionally identified by the farmer in a broiler shed housing 20-day old Ross 308 broilers and the diagnosis was confirmed following veterinary inspection. The affected birds had evidence of scour around their vents, they were listless and there was poor uniformity amongst the flock which is indicative of growth retardation of birds within the house. Assessment of faeces from the floor of the house showed a mean faecal fluid to faecal solid ratio of 0.72 indicating

higher than normal fluid (< 0.5 is normal, 0.5-0.6 indicate destabilisation of the gut and >0.6 indicate of a loss of intestinal integrity) in the faeces indicative of a loss of intestinal integrity. There was a high level of capping of the litter in the broiler house. Capping is where the litter mixes with the faeces and pressure from the weight of the birds resulting in hardening of the top layer of litter; it is usually exacerbated by wetter faeces so is often a feature in the event of an enteric problem.

Ten male birds were selected at random from the DB affected flock and euthanised by cervical dislocation. As means of comparison ten healthy control birds were collected at random from an identical chicken house and euthanised in the same manner. In the unaffected house there were no signs of dysbacteriosis, good flock uniformity, no capping of the litter with the moisture content of the faeces being within the normal range (mean fluid to solid ratio of 0.43). The healthy birds were the same age and sex, fed on the same diet, subject to the same management procedures and from the same parent breeding stock as their DB affected counterparts. This was to ensure that the changes seen were likely to be due to dysbacteriosis rather than any other factor.

Immediately following euthanasia, the birds were autopsied and the GI tracts were dissected out, laid on sheets of cardboard and then frozen on dry ice to reduce spoilage and post mortem growth of bacteria within the intestinal lumen. During the post mortem examination the gut morphology of the DB birds was noted to be altered in comparison to the healthy birds. It was consistently noted that the gut contents of the small intestine could be visualised through the gut wall with evidence of gas pockets within the gut lumen. This finding suggests that mucosa of the small intestinal wall was thinner in the affected birds. Additionally there appeared to be increased vasculature within the gut mesentery of some of the DB birds, however it is not clear whether this was caused by post mortem congestion. The whole frozen digestive tracts were taken to the laboratory at the IFR, where they were sectioned into separate anatomical regions: namely crop, gizzard, jejunum (5 cm section taken 10 cm proximal to Meckel's Diverticulum), ileum (5 cm section taken 10 cm distal to Meckel's Diverticulum), colon (5 cm section taken

distal to the ileocaecal junction) and caecum, then stored at -20°C for further analysis.

3.2.2.1. DNA Extraction, PCR-DGGE, DGGE gel analysis and band identification

These were all carried out as described in sections 2.3, 2.4 and 2.5

3.2.2.2. Isolation of bacteria

Initial sequencing did not give a clear indication of the two bacterial species responsible for the bands of interest in the DGGE profiles of the caeca of the healthy and DB birds. The V3 region of the 16S rRNA gene is around 200bp in length thus it is not surprising that for some sequences there is not enough sequence information to get a full species identification. Therefore in order to obtain a more definitive identification of these bacterial species an attempt to culture and isolate these two bacterial species was performed. PCR-DGGE has the capacity to detect members of the intestinal microbial community if they constitute more than 1% of the total bacterial population (284, 448), thus it is possible that the bacterial species that have a greater presence in the intestinal microbiota of the DB birds are present in the intestines of the healthy birds but just at low levels. This theory was tested by attempting to culture these two bacterial species from the caecal contents of two of the DB birds and two of the healthy birds. One gram of caecal contents from each bird was placed into 5ml of PBS (Phosphate Buffered Saline) (appendix I for recipe) containing large glass beads and vortexed for one minute; this was performed in anaerobic conditions. The resultant solutions were serially diluted fourfold due to the large population of bacteria in the caeca. Following this 100 µl of each serial dilution from each caecal sample was spread plated onto bacteroides specific agar (appendix I for recipe). The plates were incubated at 37°C in anaerobic conditions for 5 days, after which 20 colonies were picked from each caecal sample where selection was based on obtaining as many different colony morphologies as possible from each caecal sample to increase the likelihood of isolating the correct bacteria.

The total number of colonies picked was 80 and each one was grown in Brain Heart Infusion (BHI) broth (appendix I for recipe) for 24 hours at 37°C. Each bacterial broth was then streak plated onto BHI agar (appendix I for recipe) and incubated for a further 24 hours at 37°C in anaerobic conditions. Following this, colony PCR was performed on all 80 isolates using the PCR parameters and 341F-GC & 534R primer set as mentioned previously. Following PCR, aliquots of the PCR products were pooled into eight groups of ten and DGGE was performed loading the 8 pools in different wells to compare with the original caecal samples to check for isolation of the correct bacterial species.

3.2.2.3. Results

Mean band number

The number of bands on a DGGE image gives a good indication of the species richness of the predominant species within a given sample. Comparing the mean band number between the healthy and DB birds is an easy way to initially assess if there are any differences in species richness between the two groups. Figure 3.1 shows the mean band numbers for each region of intestine for the healthy and DB birds in case study one. There is a general increase in mean band number in the small intestine and the caeca of affected birds, however this is only significant in the ilea when a Students t-test is applied ($p < 0.05$). In the crop, gizzard and colon there is no difference in the mean band number in the DB birds.

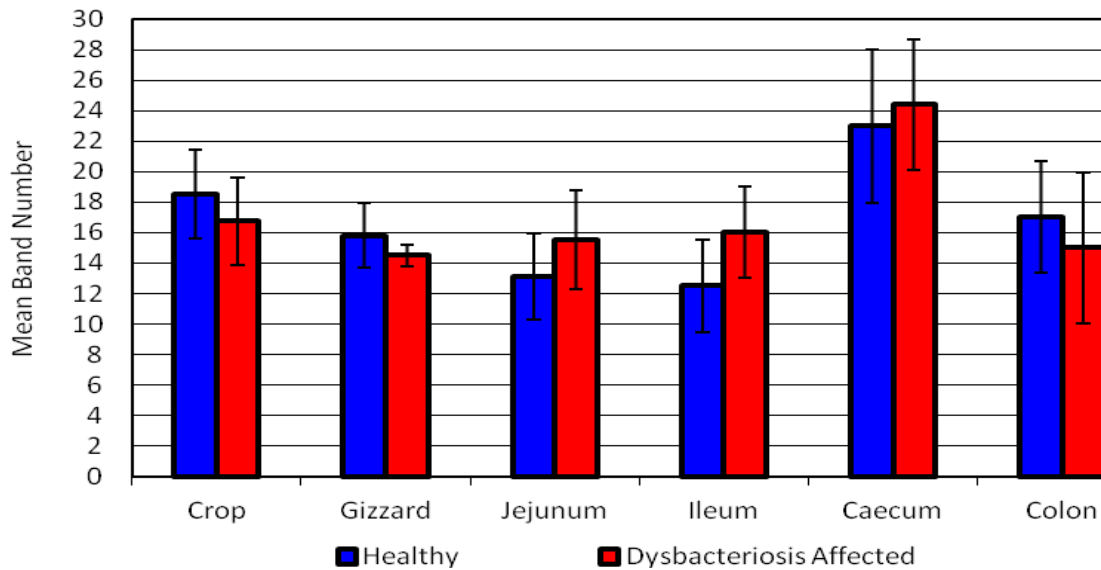


Figure 3.1 Mean DGGE gel band number of each intestinal region following analysis of the DGGE gels from case study one. Only the difference in the healthy and DB affected ilea is significant ($p < 0.05$). Bars represent standard deviation.

Crop and gizzard

Visual inspection of the DGGE images from the crops and gizzards showed no obvious differences in banding patterns between the healthy and DB affected birds (Figure 3.2). Due to problems during extraction of the crops from some of the birds from the first farm, there are only nine healthy bird samples and eight DB affected bird samples for the crop. In both the gizzard and the crop a decrease in mean band number in the DB birds can be seen, however application of an unpaired Students t-test is applied to the data revealed no significant difference ($p > 0.05$) (Figure 3.1). Dice similarity coefficient was applied to the DGGE images and found that there was a general increase in similarity amongst the DB birds than the healthy birds in both the crop and gizzard (Table 3.1, pg 56). Additionally there was a decrease in the mean band pattern similarity when the DB affected bird group was compared to the healthy bird group indicative of a change in the composition of the microbiota. However, when the percentage similarity was plotted into a UPMGA dendrogram to form a similarity hierarchy there was limited separation of the healthy and DB birds (Figure 3.3).

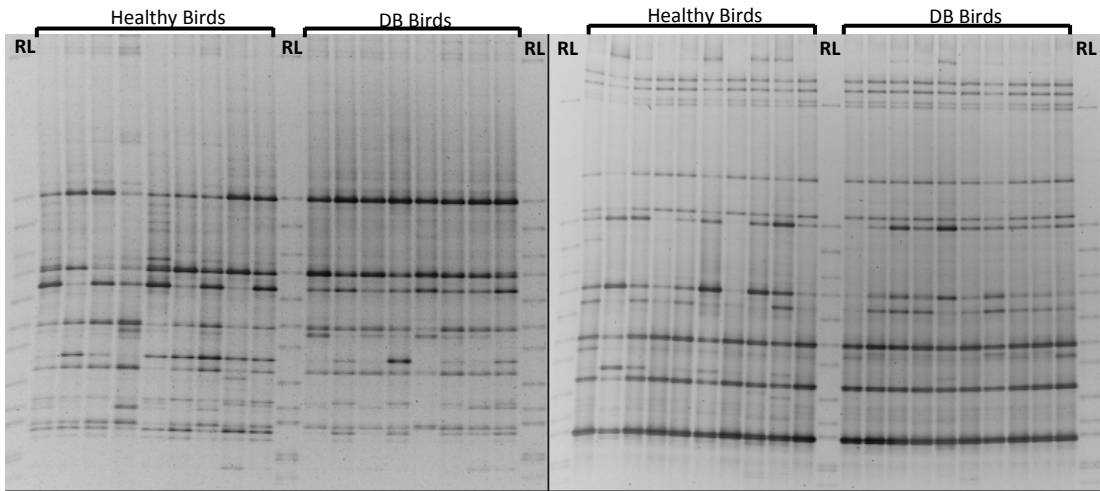


Figure 3.2 DGGE gel visualising DNA obtained from the crops (left images) and gizzards (right image) of healthy and DB birds following PCR amplification of V3 hypervariable region of the 16S rRNA gene. (RL = Reference Ladder as mentioned in section 2.5.1)

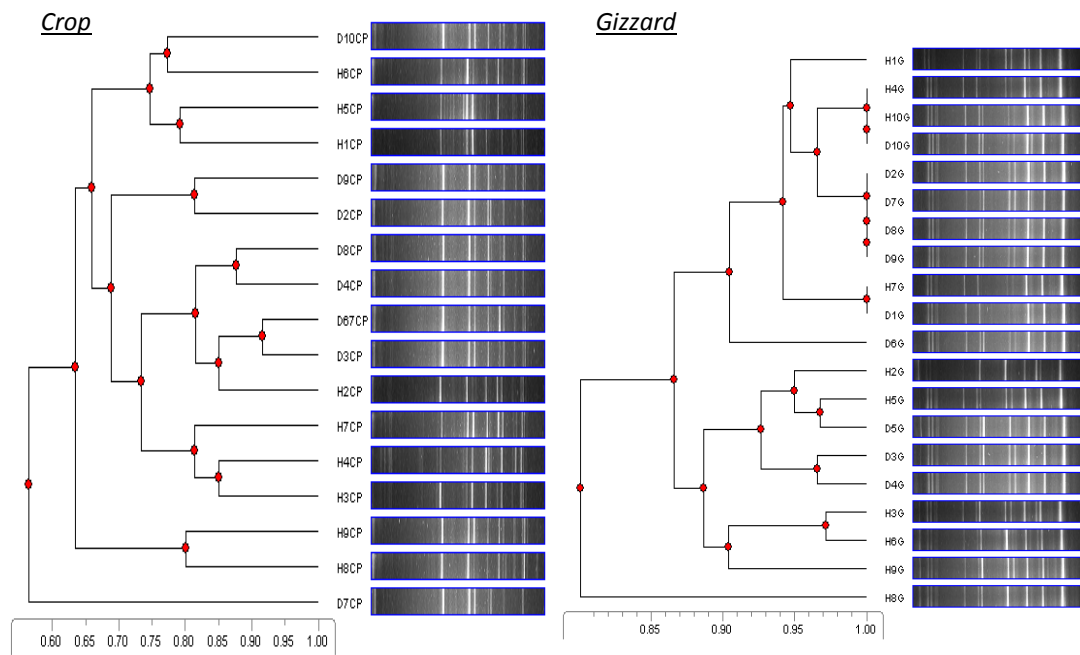


Figure 3.3 Dendrogram of the DGGE lane images in Figure 3.2; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). Limited clustering can really be seen for DGGE images of the crop (CP) or gizzard (G) of the healthy (H) or DB birds (D).

DGGE data is multidimensional and differences between data sets are not always obvious, therefore principle component analysis (PCA) was applied to the DGGE data for the crop and gizzard. By applying PCA to the data set the multidimensional data is converted into two dimensions based on the major variances (principle components) between the two groups. The PCA scatter plots for the crop and gizzard of the birds from the first farm can be seen in Figure 3.4. The principle components do segregate the data points however they don't definitively separate the healthy from the DB birds. Thus indicating that overall there are no real differences between the microbial communities of the crop and gizzard in the healthy and DB birds.

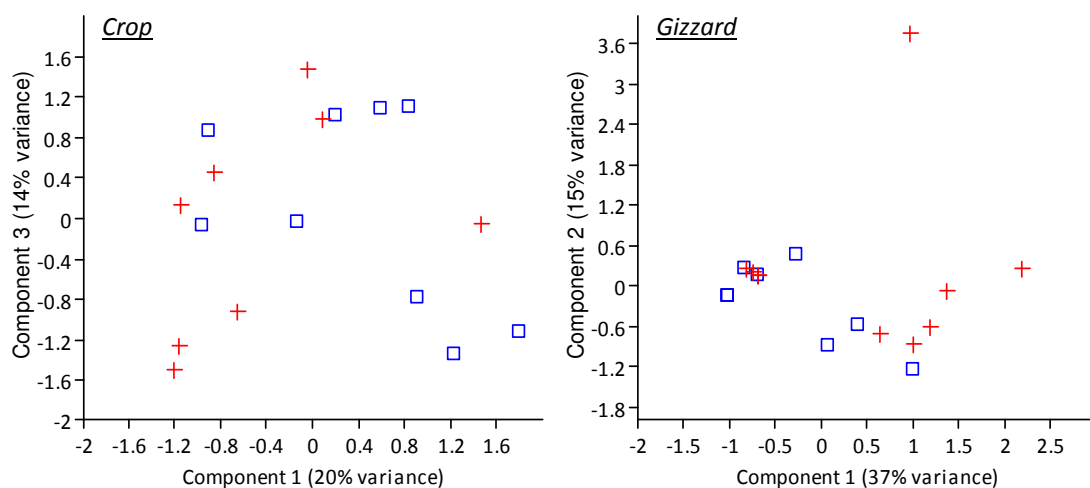


Figure 3.4 Scatter plots following PCA analysis of the crop and gizzard data sets from farm 1. (□ healthy birds, + DB birds)

Visually the crop and gizzard DGGE profiles show a great deal of similarity, which is likely due to the gizzard being fed from the crop via the proventriculus. The gizzard environment is highly acidic and hostile, therefore not an ideal environment for bacterial colonisation hence to see a large number of DGGE bands (representing bacterial diversity) was not expected. The major common bands visible on the DGGE gels were sequenced in order to identify the bacterial community in the gizzard to investigate the common gizzard microbiota. Band excision and

sequencing revealed that many of the bands were actually caused by chloroplast DNA rather than bacterial DNA, meaning that the bacterial community of the the gizzard is not as diverse as implied by DGGE (Figure 3.5). The phenomenon of eukaryotic DNA being co-amplified with prokaryotic DNA is not uncommon. Many studies have demonstrated the amplification of eukaryotic DNA, especially that from mitochondria and chloroplasts, in the presence of general bacterial primers thus potentially giving a less than accurate picture of the true diversity in a sample. The reason for coamplification could be explained the sequence similarity of mitochondria and chloroplasts to prokaryotes and that may be explained by the endosymbiotic theory.

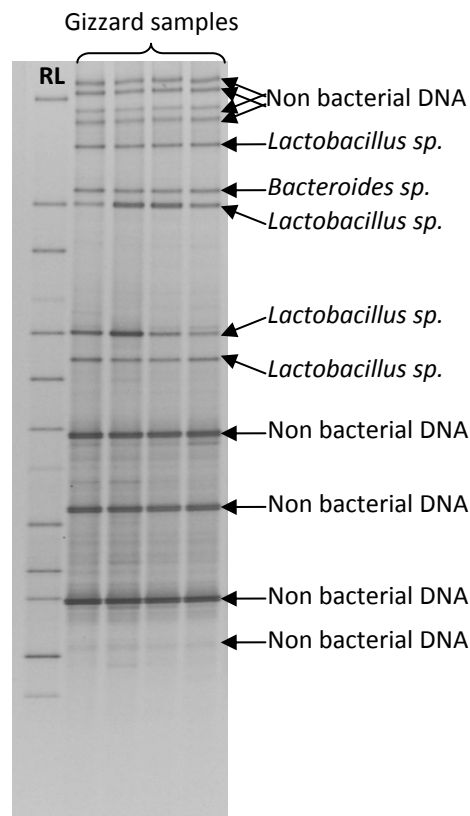


Figure 3.5 DGGE profiles obtained from the gizzard of 4 different chickens from farm one following PCR amplification of V3 hypervariable region of the 16S rRNA gene. The image indicates the bands which are due to bacterial and non bacterial DNA. (RL = Reference Ladder)

One of the key reasons the bacterial 16S rRNA gene is a target for molecular profiling is due to its highly conserved nature throughout evolution, consequently it is highly likely that the mitochondrial and chloroplast ribosomal genes have retained some of this highly conserved genetic coding thus remaining targets for

general bacterial primers (50, 102, 133, 177, 178, 207). The origin of the chloroplast DNA is undoubtedly from the plant matter in the undigested feed present in the gizzard resulting in a large concentration of plant cells in the gizzard contents. Consequently DNA extracted from the gizzard contents will contain a proportion of plant DNA which will be accessible by the primers thus resulting in coamplification and presence on the DGGE gel. As the crop and gizzard have very similar DGGE fingerprints this suggests that the crop profiles will therefore also contain bands produced from coamplification with eukaryotic DNA. This finding provides a very good caveat and further studies with more specific primer sets could be useful in such circumstances.

Jejunum and ileum

The DGGE images obtained from the jejunum and ileum of the birds is shown in Figure 3.6.

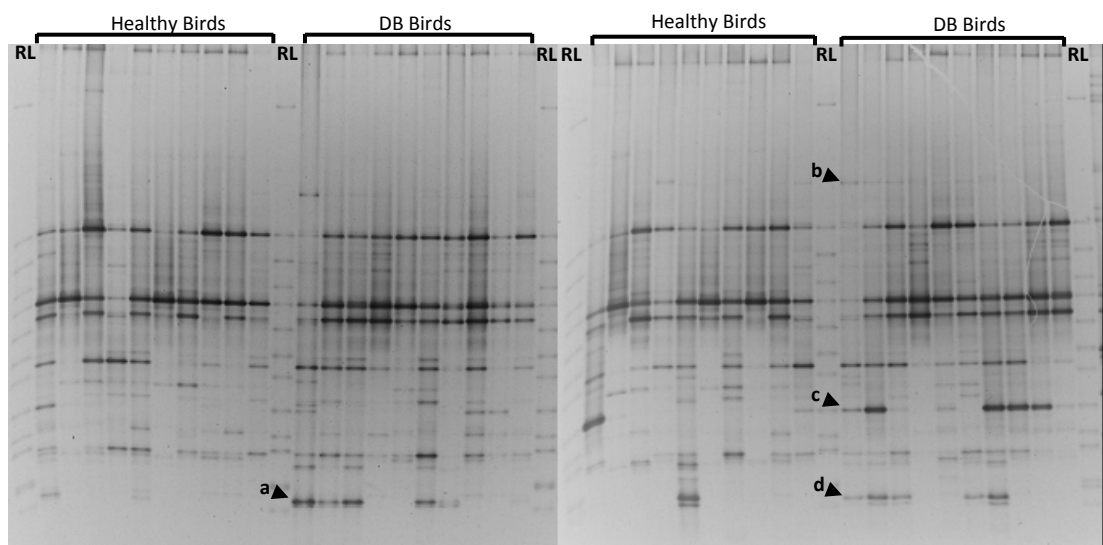


Figure 3.6 DGGE gel visualising DNA obtained from the jejunum (left image) and ileum (right image) of healthy and DB birds following PCR amplification of V3 hypervariable region of the 16S rRNA gene. Distinct differences labelled, percentage represents sequence match with 16S database a) Uncultured *Clostridium* sp. (100%) b) *Lactobacillus aviarius* (100%) c) *Escherichia coli* (100%) d) same as a) (RL = Reference Ladder) (See appendix II for a table linking all the bacteria identified in this thesis).

Visual inspection of the DGGE gels indicated visual differences between the healthy and DB birds; the bacterial species responsible for these bands were identified by band excision and sequencing (highlighted in Figure 3.6). An increase in mean band

number can be seen in the jejunum and ileum of DB birds (Figure 3.1), this increase is significant in the ileal samples ($p < 0.05$) when an unpaired Student's t-test is applied to the data. Dice similarity coefficient was applied to the DGGE images and found that there was a general increase in similarity amongst the DB birds than the healthy birds in both regions sampled from the small intestine (Table 3.1). When the DGGE profiles of the healthy group were compared with those of the DB affected groups there was a decrease in the mean band pattern similarity indicating a change in band patterning and therefore microbial community between the two groups. When the percentage similarity was plotted on a UPMGA dendrogram to cluster the values into a hierarchy there was separation of the healthy and DB birds (Figure 3.7) revealing a greater degree of segregation of the healthy and DB birds than was demonstrated in the crop and gizzard. PCA was applied to the data set for both the jejunum and the ileum to reveal a distinct separation of the healthy and DB affected bird data points (Figure 3.8)

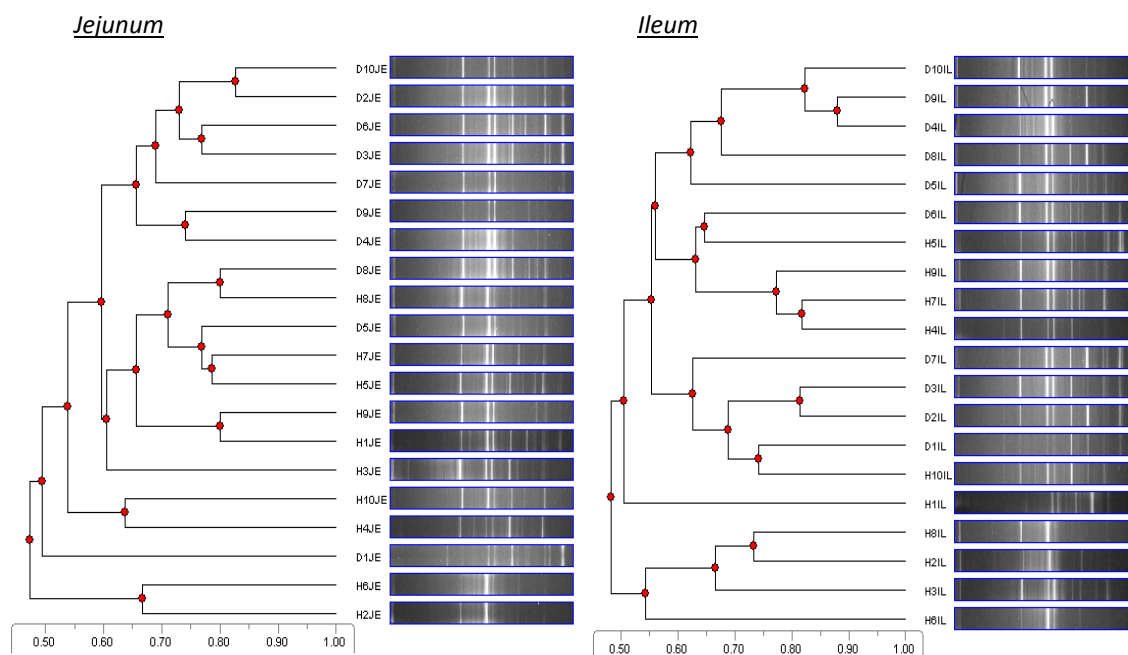


Figure 3.7 Dendrogram of the DGGE lane images in Figure 3.6; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). Clustering of DGGE profiles can be seen for DGGE lanes of the jejunum (JE) and ileum (IL) of the healthy (H) or DB birds (D).

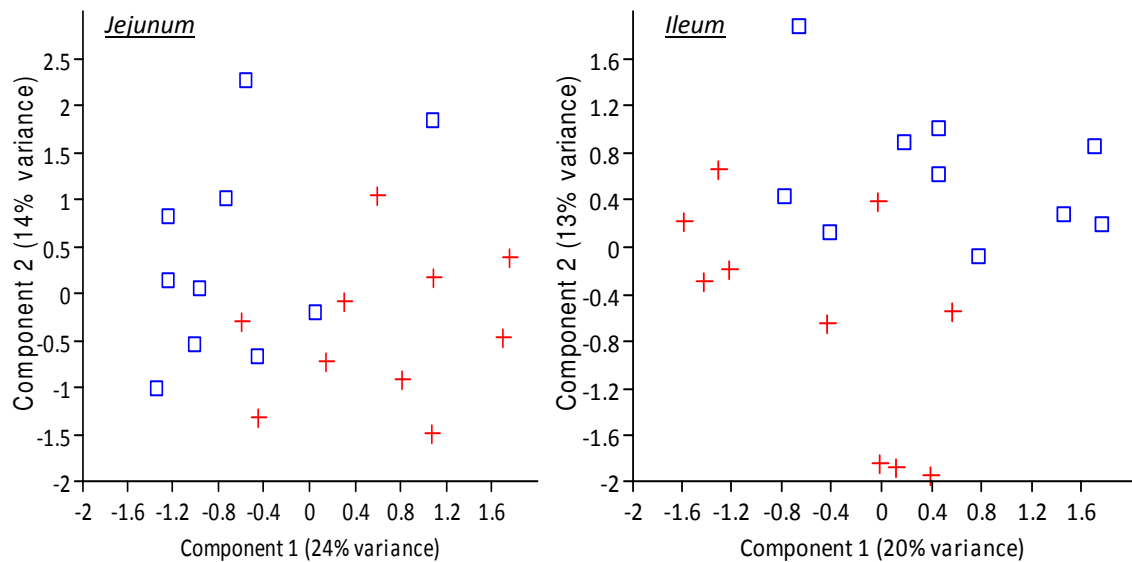


Figure 3.8 Scatter plots following PCA analysis of the jejunal and ileal data sets from farm 1. (□ healthy birds, + DB birds). Segregation of data points for the healthy and DB birds can be seen in both plots indicating differences in microbiota.

The results from the small intestine indicate a change in microbiota with an increase in the presence of *Lactobacillus aviarius*, *Escherichia coli* and an uncultured clostridial species in the DB birds. Clostridial species along with *E. coli* are common members of the resident microbiota of healthy birds which can sometimes cause disease and as such they are often regarded as opportunistic pathogenic bacteria. The results from the small intestine gives the first indication that the birds suffering from dysbacteriosis have an imbalance in the resident intestinal microbiota with an increased species richness which is occurring in the majority of DB birds.

Colon

The DGGE image obtained from the colon samples did not reveal any major visual differences between the healthy and DB birds (Figure 3.9). Construction of a UPMGA dendrogram of the colon data did not reveal any distinct clustering of the healthy and DB birds (Figure 3.10). Similarly PCA analysis of the samples did not reveal any distinct segregation of the healthy and DB affected data points (Figure 3.11), thus indicating that there are no significant changes in the composition of the colonic microbiota of the DB birds.

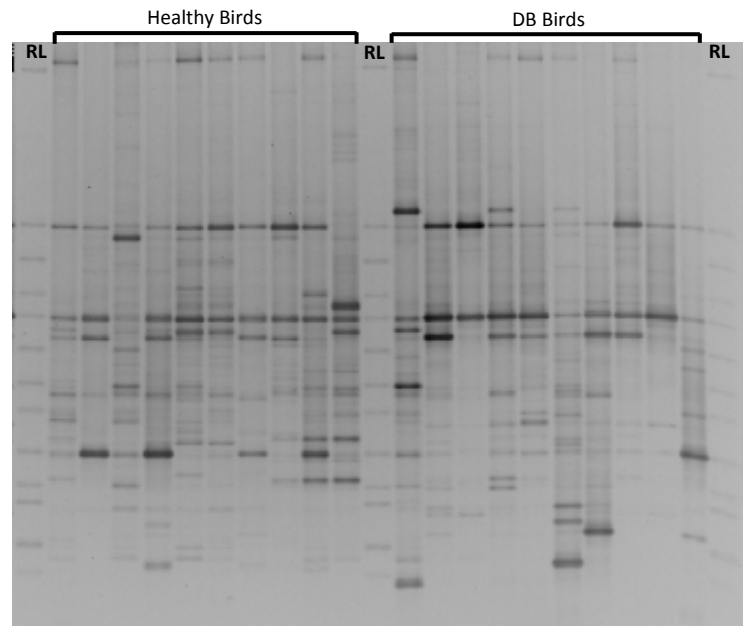


Figure 3.9 DGGE gel visualising DNA obtained from the colon of healthy and DB birds following PCR amplification of the V3 hypervariable region of the 16S rRNA gene (RL = Reference Ladder).

There is a reduction in the mean band number in the DB birds (Figure 3.1) which could be explained by the increased faecal output due to the DB birds having diarrhoea causing more frequent defecation. This would reduce the time the faecal bolus would be present in the colon potentially reducing time for certain members of the colonic microbiota to grow thus altering the species richness.

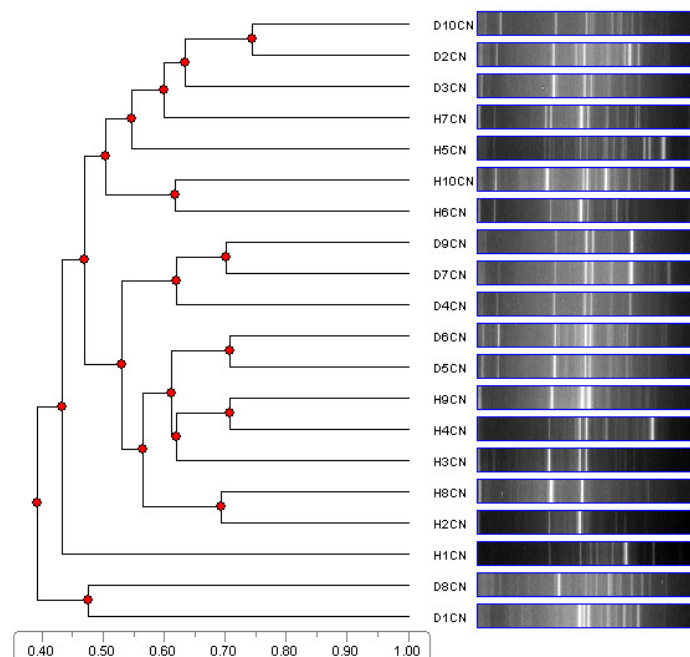


Figure 3.10 Dendrogram of the DGGE lane images in Figure 3.9; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). There is limited clustering of DGGE profiles for the DGGE lanes from the colon (CN) of the healthy (H) and the DB birds (D).

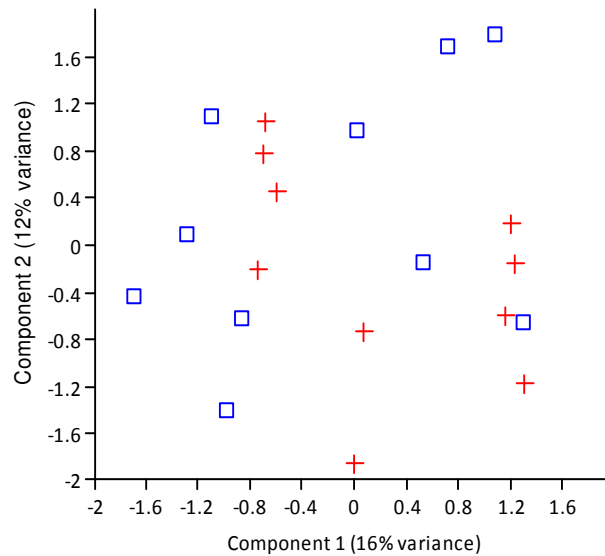


Figure 3.11 Scatter plot following PCA analysis of the colon DGGE data set from farm 1. (□ healthy birds, + DB birds). There is no segregation of the data points to distinguish the healthy and DB birds, indicating there is no overall change in microbial composition of the colonic microbiota in the DB birds.

Caecum

The caecal profiles demonstrated the most obvious differences between the healthy and DB birds with two dominant bands being present in the majority of DB birds and absent in the DGGE profiles of the healthy birds (Figure 3.12). There was a general increase in mean band number in the DB birds, however this increase was not significant when a Students t-test was applied to the data set ($P>0.05$). The dendrogram constructed from the caecal DGGE lane percentage similarities showed distinct separation of the healthy and DB birds (Figure 3.13), thus indicating a difference in the composition of the intestinal microbiota in the DB birds. PCA analysis also demonstrated separation of the healthy and DB birds (Figure 3.14) thus it can be concluded that there is variation between the bacterial composition of the caecal microbiota of DB birds and healthy birds.

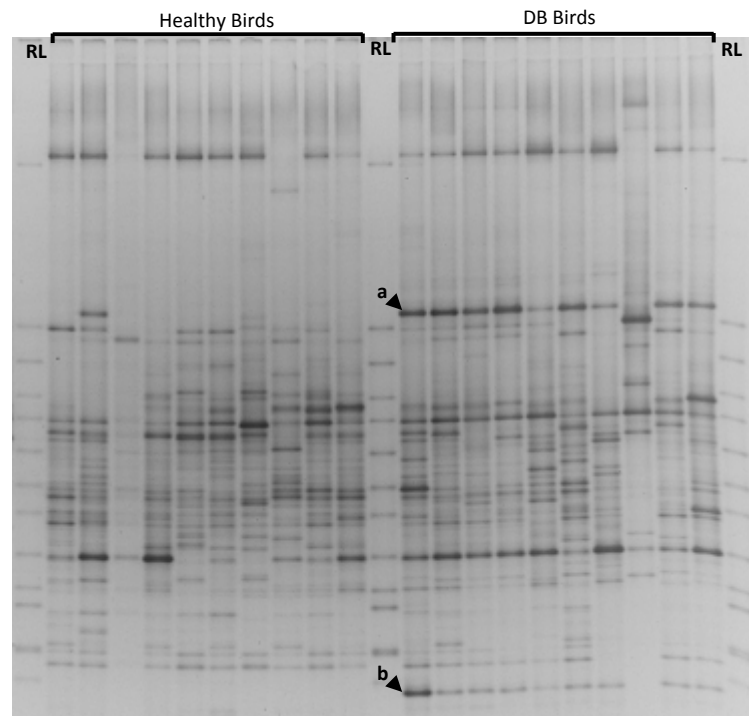


Figure 3.12 DGGE gel visualising DNA obtained from the caeca of healthy and DB birds following PCR amplification of the V3 hypervariable region of the 16S rRNA gene. Distinct differences highlighted a) *Bacteroides dorei* b) *Barnesiella viscericola* (RL = Reference Ladder). (See appendix II for a table linking all the bacteria identified in this thesis).

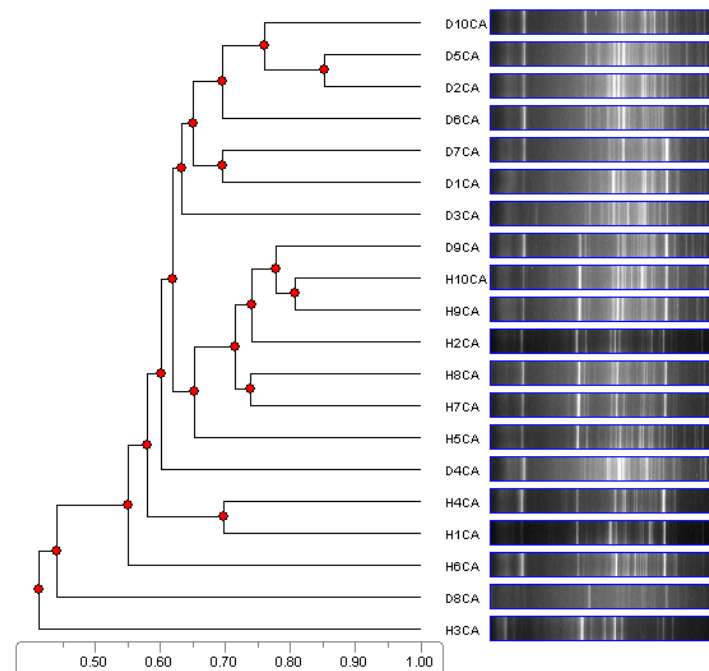


Figure 3.13 Dendrogram of the DGGE lane images in Figure 3.12; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). There is separation of the DGGE profiles for the DGGE lanes from the colon (CA) of the healthy (H) and the DB birds (D).

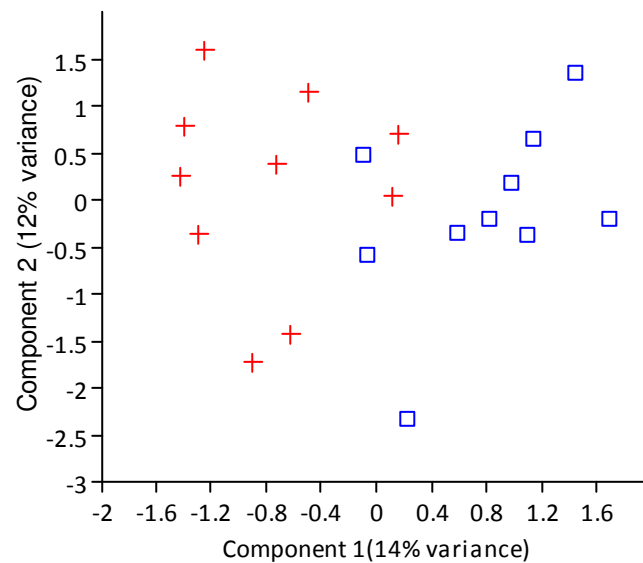


Figure 3.14 Scatter plot following PCA analysis of the caecal DGGE data set from farm 1. (□ healthy birds, + DB birds). There is clear segregation of the healthy and DB affected bird data points indicating a difference in microbial composition of the caecal microbiota between these two groups.

As described in section 3.2.2.2 culturing was performed to isolate the bacterial species responsible for the bands of interest in the caecal DGGE profiles in Figure 3.12. The subsequent DGGE image (Figure 3.15) of the pools of bacterial isolates demonstrated that the two bacterial species responsible for the bands of interest in the DB birds were successfully isolated. These bacterial isolates were visualised using scanning electron microscopy as seen in Figure 3.16. Following successful isolation from the caeca the primers 08F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1391R (5'-GACGGCGGTGTGTRCA-3') (214) were used to PCR amplify a larger fragment of the 16S rRNA gene using the same PCR protocol as previously mentioned in this section with an increased extension time of one minute per cycle to allow for the larger amplicon size.

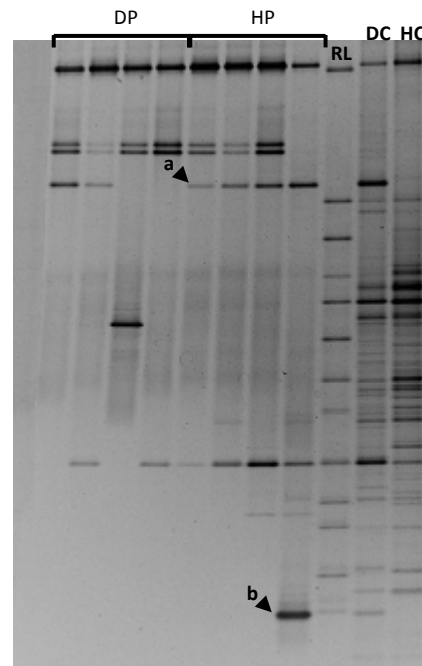


Figure 3.15 DGGE image showing the pooled PCR products from the bacterial isolates from caecal contents. a) *Bacteroides dorei* and b) *Barnesiella viscericola* represent the bands for the successfully isolated bacteria of interest. (HP=Bacteria isolated from healthy caeca, DP=Bacterial isolated from DB affected caeca, HC=Healthy caecal profile, DC=DB affected caecal profile for reference and RL=reference ladder).

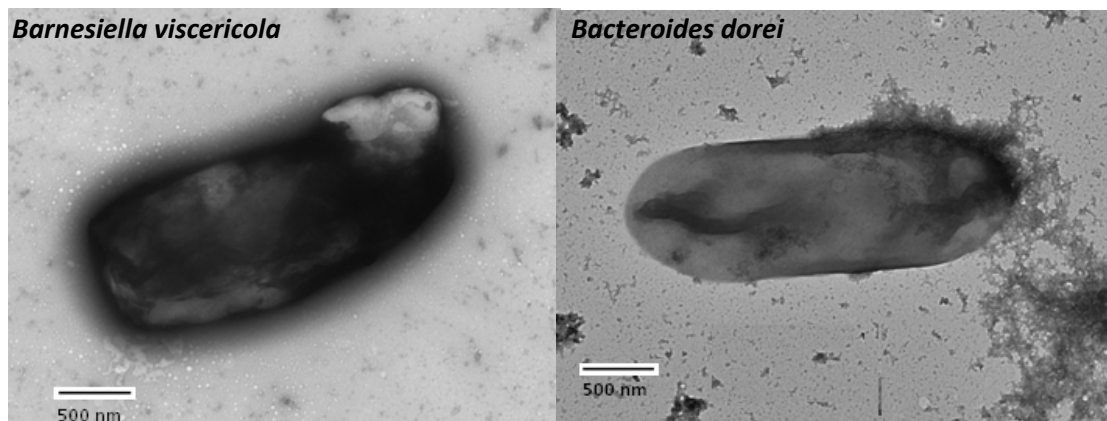


Figure 3.16 Scanning Electron Microscopy of the two isolated bacteria at 6500x magnification. (Microscopy and photography courtesy of Dr Kathryn Cross and Dr Mary Parker, IFR).

Both of these two species of bacteria could be cultured from the healthy caecal contents despite them not showing up on the DGGE profiles, this implies that both these bacterial species are members of the healthy resident microbiota but at a level undetectable by DGGE. This gives an indication that change in composition of the intestinal microbiota in cases of dysbacteriosis is an imbalance of the resident autochthonous microbiota as opposed to an allochthonous species entering the intestinal tract.

Mean percentage band pattern similarities

A high percentage band pattern similarity indicates that the DGGE profiles are alike meaning that there are a higher number of similar bacterial species present in each of the samples being compared. A low percentage similarity indicates that the samples being compared have different DGGE profiles meaning that there is a greater species diversity of the bacteria present in the samples. Table 3.1 shows the mean band pattern similarities for the birds from case study one. There are three comparisons made, firstly the similarity of the healthy birds group (HvH), secondly the similarity of the DB group (DvD) and lastly the healthy bird group compared to the DB group (HvD).

	HvH	DvD	HvD
Crop	68.1%	71.7%	66%
Gizzard	87.2%	92.7%	88.4%
Jejunum	58.8%	65.4%	54.4%
Ileum	54.2%	62.4%	51.8%
Colon	49.2%	50.2%	47%
Caecum	60.3%	61.4%	56.3%

Table 3.1 Mean percentage band similarities for each region of gut for the healthy and DB birds (HvH = healthy group, DvD = DB affected group, HvD = healthy group compared to dysbacteriosis group).

The mean band pattern similarities on the whole suggest that the DGGE profiles between the healthy and DB birds differ, as there is a reduction in percentage similarity when the healthy birds are compared to the DB birds. This reiterates that there are differences in the intestinal microbiota in birds suffering from dysbacteriosis in line with the findings in the DGGE images, dendrograms and PCA plots. An interesting observation here is that there is a greater percentage similarity of DGGE profiles within the DB group of birds than within the group of healthy birds. This suggests a common shift in microbiota in the DB birds causing the differences in DGGE profiles when compared to the healthy bird profiles. The lowest percentage similarities can be found in the colon where there is very little difference in the percentage similarities from group to group; this is likely due to

the different circadian rhythm of each bird in the time and frequency of defecation and caecal emptying all of which will influence the colonic contents at the time of sampling. This indicated that colonic contents are not a good sample type when investigating shifts in microbiota in cases of dysbacteriosis.

3.2.3. Case Study Two – Uphouse Farm (Farm 2)

Dysbacteriosis was diagnosed in a second broiler shed housing 27-day old Hubbard Flex F40 broilers following veterinary inspection. The affected birds demonstrated the same symptoms as the birds examined in case study one with evidence of scour around their vents, listlessness and poor uniformity amongst the flock. In addition to the physical symptoms the affected birds had also demonstrated an increase in water intake on the day that DB was identified. There was capping of the litter and the faeces in the house had a higher than normal fluid content with a mean faecal fluid to faecal solid ratio of 0.68, indicative of a loss of intestinal integrity, with evidence of capping of the litter in the house. Ten affected male birds were selected at random from the DB affected flock and euthanised by cervical dislocation. As with the first study ten healthy control birds were collected at random from an identical chicken house and euthanised by cervical dislocation for comparison. The unaffected birds showed no signs of DB, good flock uniformity, no capping of the litter with the moisture content of the faeces being within the normal range (mean fluid to solid ratio of 0.4). The unaffected birds were the same age, fed on the same diet, subject to the same management procedures and from the same parent breeding stock as their DB affected counterparts.

The GI tracts of the birds were extracted following euthanasia and sampled as per case study one with the same six regions of intestine being taken; namely the crop, gizzard, jejunum, ileum, caecum and colon. The intestinal tracts of the DB birds exhibited the same characteristics as previously seen with visibly thinner gut walls in the small intestine through which the gut contents and gas bubbles could be visualised. There was also increased vascularisation of the mesentery of the affected birds, however as before, it was not clear as to whether or not this was due to *post mortem* congestion.

3.2.3.1. DNA Extraction, PCR-DGGE and DGGE gel analysis

These were all carried out as described in sections 2.3, 2.4 and 2.5

3.2.3.2. Results

Mean band number

As with the DGGE profiles in case study one, the mean band numbers for each region of intestine for the healthy and DB birds were calculated for the birds in this study (Figure 3.17). There is a general increase in mean band number in the small intestine and the caeca of affected birds, however this is only significant in the ilea when a Students t-test is applied ($p < 0.05$). In contrast to the results from case study one in the crop, gizzard and colon there is a general increase in the mean band number in the DB birds, however the differences are not significant.

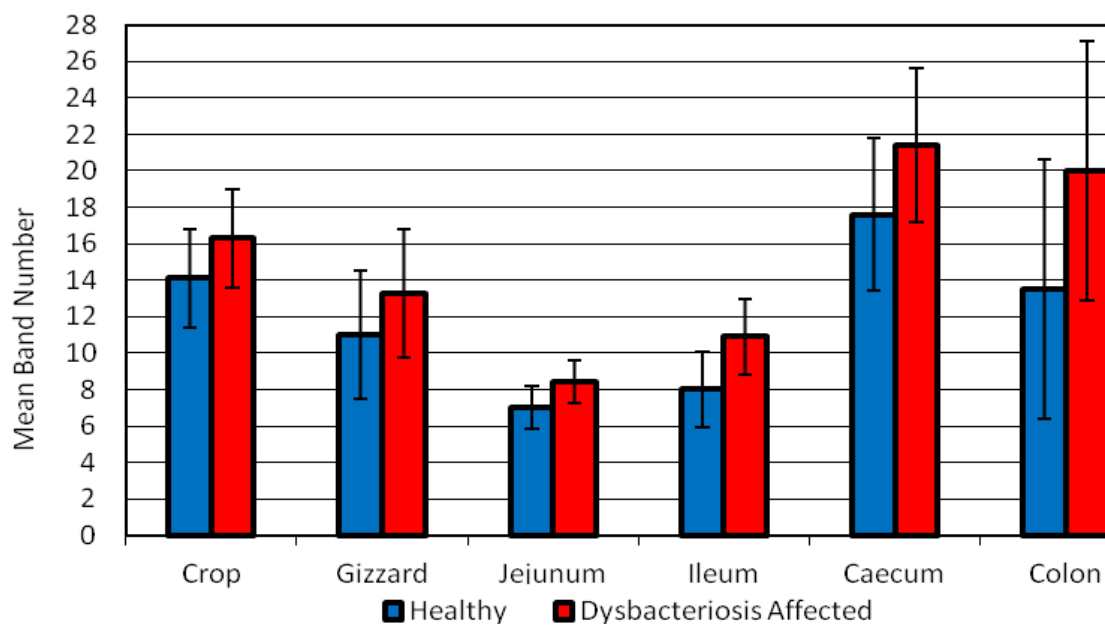


Figure 3.17 Mean DGGE gel band number of each intestinal region following analysis of the DGGE gels from case study two. Only the difference in the healthy and DB affected ilea is significant ($p < 0.05$). Bars represent standard deviation.

Crop and gizzard

The crop and gizzard DGGE profiles from the birds in case study two are shown in Figure 3.18. There is an increase in mean band number in the crop and gizzard profiles of the DB birds, however this was not significant when a Students t-test is applied to the data ($P > 0.05$) (Figure 3.19). This finding is opposite to the finding from the first farm where a larger band number was seen in the healthy birds. It is

difficult to determine the accuracy of the results obtained from the crop and gizzard due to co-amplification of chloroplast DNA during PCR amplification as mentioned previously (page 47).

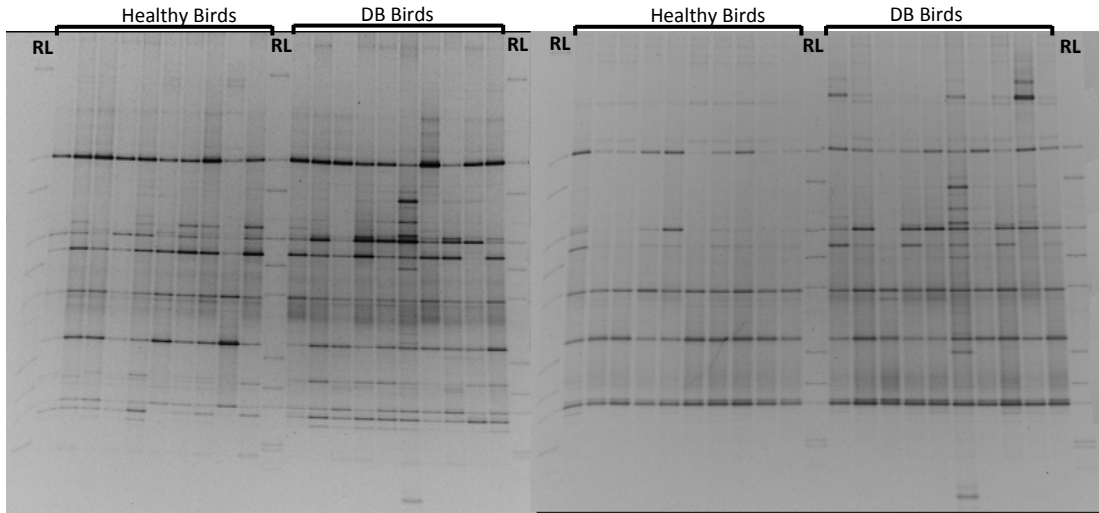


Figure 3.18 DGGE gel visualising DNA obtained from the crops (left images) and gizzards (right image) of healthy and DB birds following PCR amplification of V3 hypervariable region of the 16S rRNA gene. (RL = Reference Ladder)

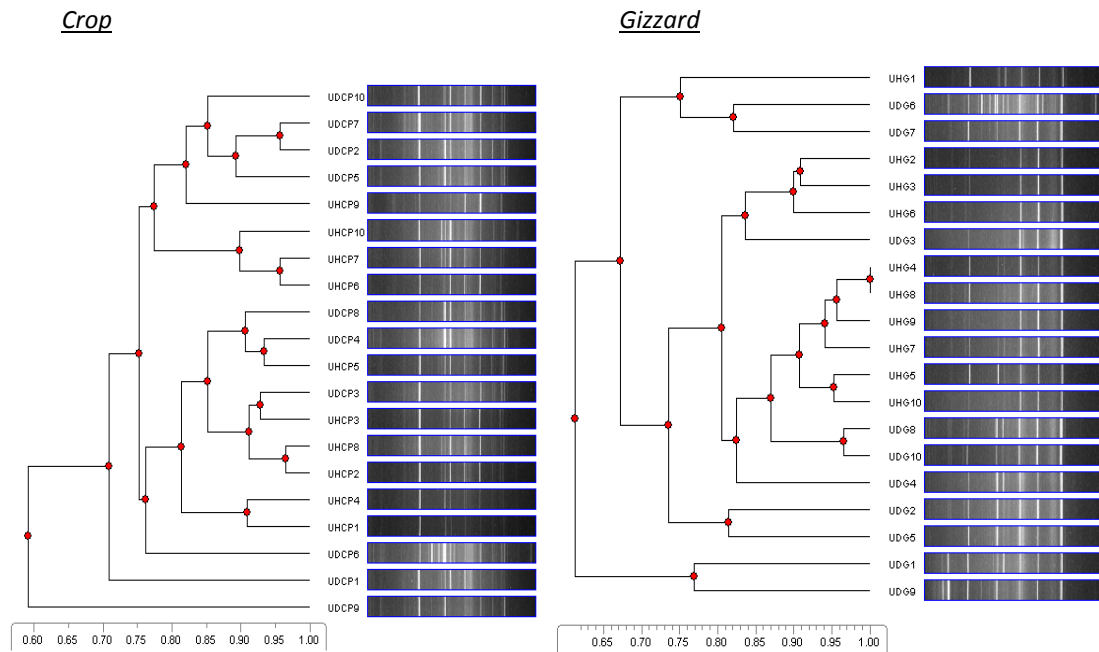


Figure 3.19 Dendrogram of the DGGE lane images in Figure 3.15; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). A degree clustering can really be seen for DGGE images of the crop (CP) or gizzard (G) of the healthy (UH) or DB birds (UD).

When Dice similarity coefficient was applied to the data, a reduction in band pattern similarity between the crop and gizzard profiles of the healthy and DB birds could be seen (Table 3.2, pg 67). Despite this reduction there was only limited segregation of the two groups following construction of a dendrogram (Figure 3.19). To complete the analysis of the crop and gizzard samples, PCA was performed to reveal no clustering of the crop data points and a small degree of clustering in the gizzard profiles (Figure 3.20).

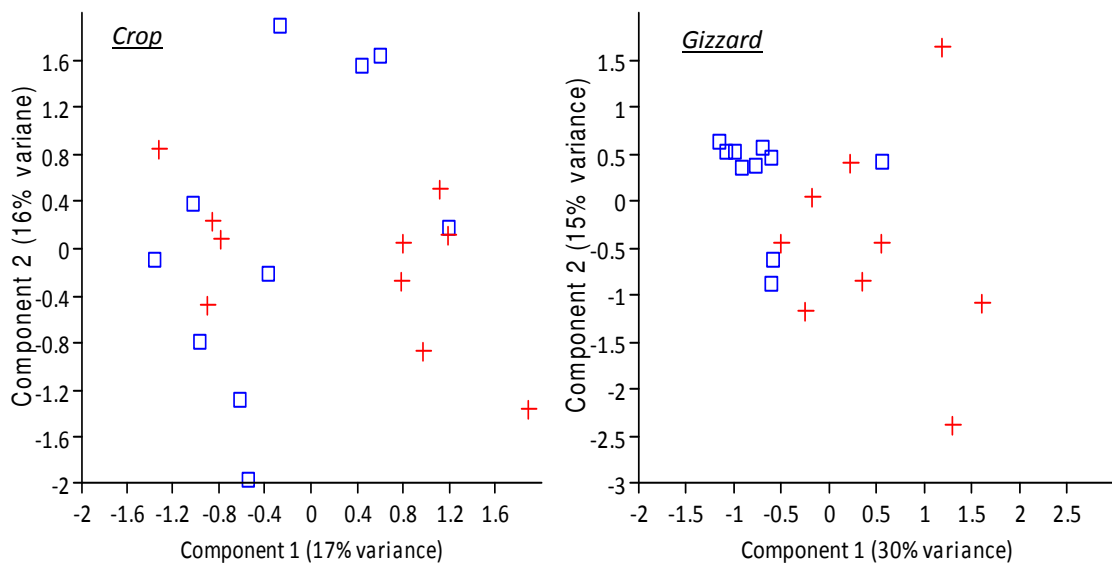


Figure 3.20 Scatter plots following PCA analysis of the crop and gizzard data sets from farm two (□ healthy birds, + DB birds).

The results of the crop and gizzard from both farms are not consistent due to the varying demonstrations of differences in the microbiota between the healthy and DB state. It is probable the co-amplification of chloroplast DNA has interfered with the projection of the true nature of the crop and gizzard microbiota. Therefore to obtain a realistic picture re-examination of the samples using more specific primers to remove the interference of plant DNA is needed.

Jejuna and Ilea

The DGGE images (Figure 3.21) show visual differences in the intestinal microbiota of the jejuna and ilea of DB birds. An increase in mean band number can be seen in both the jejuna and ilea of DB birds and as seen in the results from the first farm this is significant in the ilea ($p < 0.05$) but not in the jejuna (Figure 3.6). The bacterial

species identified show some similarities in the fluctuations of certain bacterial species and groups seen previously i.e. an increased presence of *Lactobacillus aviarius*, a species of bacteroides and a *Clostridia sp.* in the DGGE profiles of the DB birds could be seen, showing consistency with earlier results.

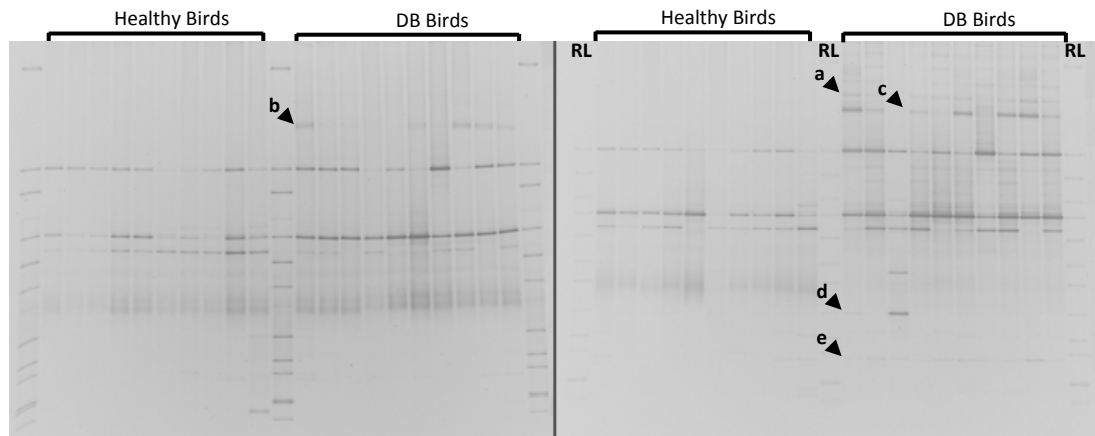


Figure 3.21 DGGE gel visualising DNA obtained from the jejunum (left image) and ileum (right image) of healthy and DB birds following PCR amplification of V3 hypervariable region of the 16S rRNA gene. Distinct differences labelled, percentage represents sequence match to 16S database a) *Bacteroides vulgatus* (98%) b) *Lactobacillus aviarius* (100%) c) *Bacteroides vulgatus* (99%) d) *Escherichia coli* (100%) e) Uncultured *Clostridia sp.* (100%) (RL = Reference Ladder) (See appendix II for a table linking all the bacteria identified in this thesis).

Additionally an increased presence of *E. coli* was seen in the DGGE profiles of a couple of the affected birds, it is not a significant difference between the two groups however, considering the results from farm one, one could propose that the *E. coli* populations in the intestinal tracts of the affected birds from farm two were just starting to increase when DB was identified. Construction of a dendrogram (Figure 3.22) highlighted separation of the healthy birds away from the DB birds. This segregation can be seen further when the data is analysed by PCA (Figure 3.23). The observations from the small intestine of the birds from farm two are consistent with those from farm one, i.e. the birds suffering from DB are demonstrating a difference in microbiota between the healthy and DB birds. There is a general increase in species richness in the small intestine with the visual presence of bands in the DGGE profiles of the DB birds which are absent from the healthy bird DGGE profiles

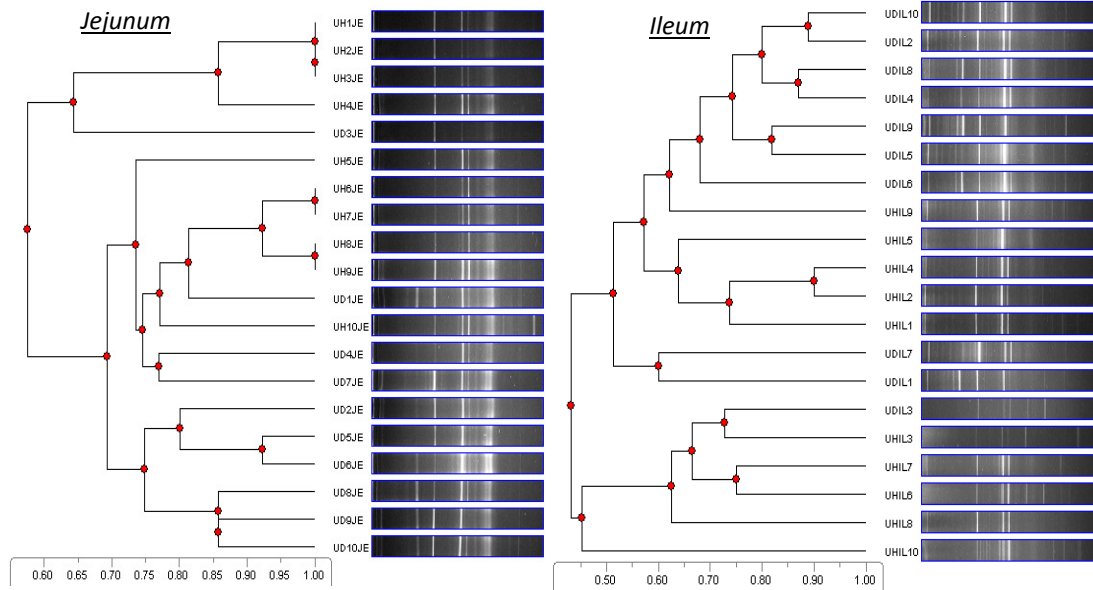


Figure 3.22 Dendrogram of the DGGE lane images in Figure 3.21; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). Clustering of DGGE profiles can be seen for DGGE lanes of the jejunum (JE) and ileum (IL) of the healthy (UH) or DB birds (UD).

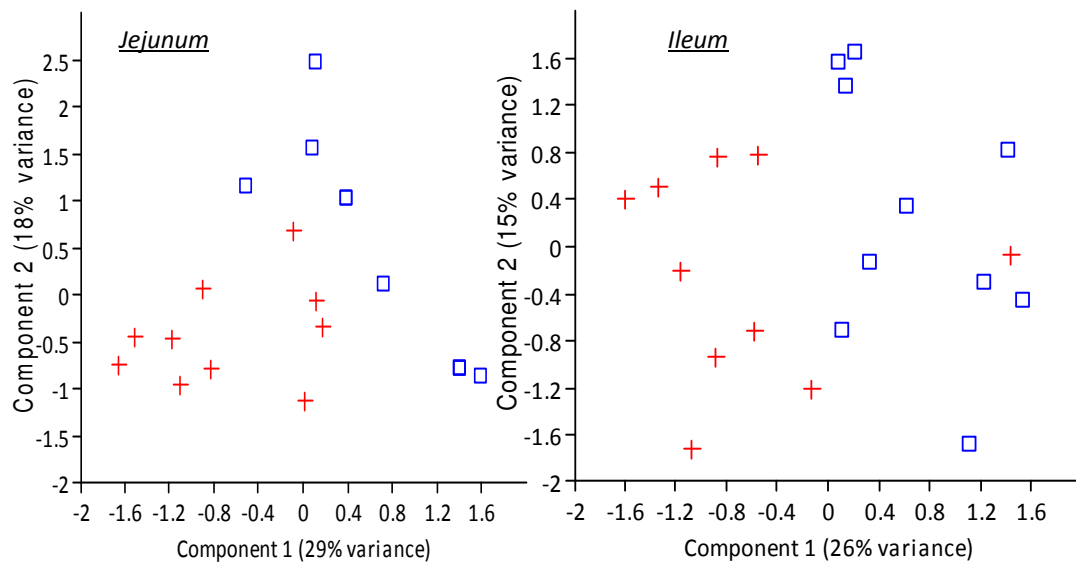


Figure 3.23 Scatter plots following PCA analysis of the jejunal and ileal data sets from farm two. (□ healthy birds, + DB birds). Segregation of data points for the healthy and DB birds can be seen in both plots indicating differences in microbiota.

Colon

Figure 3.24 shows the DGGE image obtained from the colon samples taken from the birds in case study two.

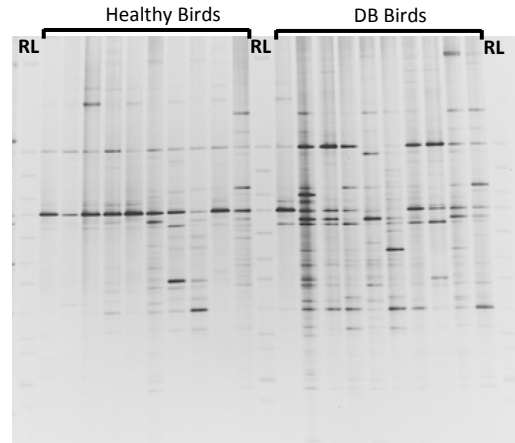


Figure 3.24 DGGE gel visualising DNA obtained from the colon of healthy and DB birds following PCR amplification of the V3 hypervariable region of the 16S rRNA gene (RL = Reference Ladder).

Visual inspection of the gel reveals two things; firstly the DB birds have a greater band number and secondly there are some minor differences in band presence and absence in the top portion of the gel. These differences lead to distinct separation of the DGGE profiles following construction of a dendrogram (Figure 3.25) and analysis with PCA (Figure 3.26).

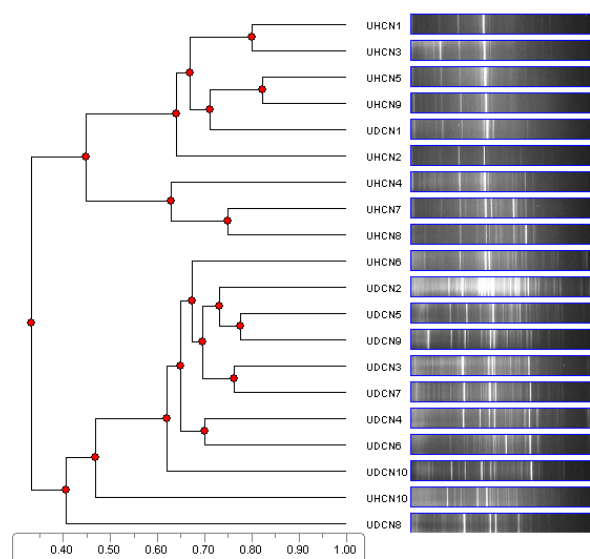


Figure 3.25 Dendrogram of the DGGE lane images in Figure 3.24; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). There is definite separation of DGGE profiles from the colon (CN) of the healthy (UH) and the DB birds (UD).

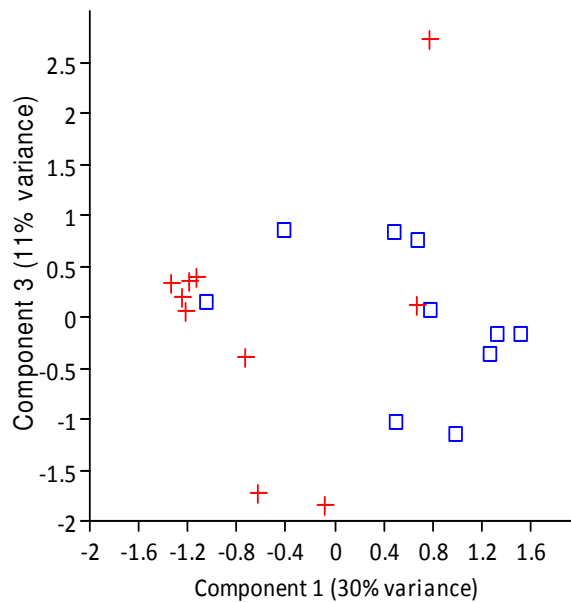


Figure 3.26 Scatter plot following PCA analysis of the colon DGGE data set from farm two. (□ healthy birds, + DB birds). There is segregation of the data points to distinguish the healthy and DB birds, indicating there is a difference in the microbial composition of the colonic microbiota in the DB birds.

This result contrasts with the result seen in the birds from farm one where there was a general decrease in the diversity of the colonic microbiota in the DB birds. As the results from both farms do not concur, it is not clear as to how the colonic microbiota is altered during dysbacteriosis. This finding echoes what is seen in the crop and gizzard where there is a greater mean band number in the DB birds than in the healthy birds. As it is not uncommon for chickens to peck the litter in a chicken house they are likely to swallow faecal material which is likely to influence the crop microbiota and thus the gizzard microbiota. Therefore the increased species richness in the crop and gizzard microbiota seen in the DB birds in study two could be a response to the increased bacterial load in the faeces being expelled by the birds. It is worth bearing in mind that the birds on both farms are different ages and different breeds therefore the differences between the colonic microbiota in the dysbacteriosis state could be linked to either of those factors. Species diversity in the intestinal tract of broilers increases with age (240, 398), therefore one hypothesis could be that when a broiler chicken experiences dysbacteriosis later in life, the increased diversity along the GI tract and further development of the immune system could result in a differential change in microbiota in the colonic environment when dysbacteriosis is experienced.

Caeca

The caecal DGGE image is shown in Figure 3.27 where obvious differences can be seen in the DGGE banding patterns of the healthy and DB birds.

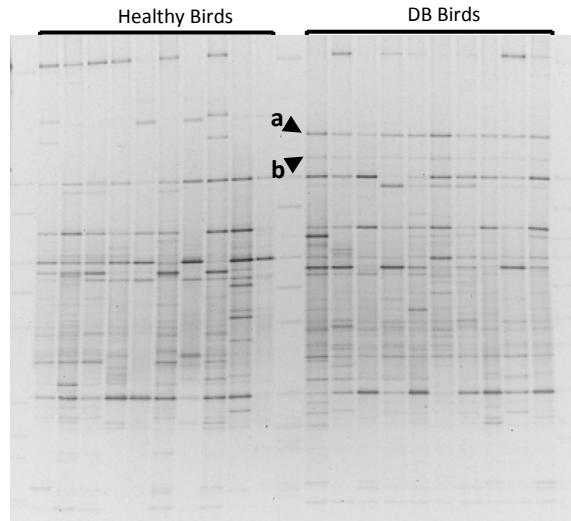


Figure 3.27 DGGE gel visualising DNA obtained from the caeca of healthy and DB birds following PCR amplification of the V3 hypervariable region of the 16S rRNA gene. Distinct differences highlighted and percentage indicated sequence match in 16S database a) *Bacteroides vulgatus* (100%) b) *Bacteroides ovatus* (100%) (RL = Reference Ladder) (See appendix II for a table linking all the bacteria identified in this thesis).

The differences seen in the caeca of the DB birds are similar to those seen in the affected birds in case study one, where there is an increased presence of members of the bacteroidetes. In this case study it is two different species that are responsible for the obvious band pattern differences, namely *Bacteroides ovatus* and *Bacteroides vulgatus*. These bacterial species can be found in the intestinal microbiota of many animal species and are considered to be part of the normal intestinal microbiota. It would appear that, combined with the results from the jejunum and ileum, the birds suffering from DB are experiencing an overgrowth or imbalance of the resident microbiota. Dendrogram construction (Figure 3.28) and PCA analysis (Figure 3.29) clearly segregate the healthy and DB affected groups highlighting that there is a difference in the intestinal microbiota of these birds according to DGGE.

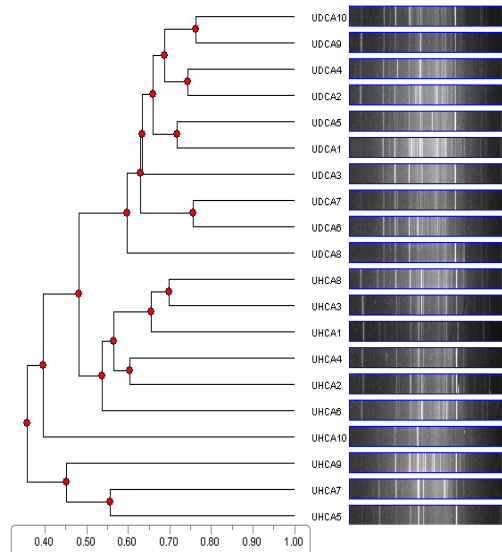


Figure 3.28 Dendrogram of the DGGE lane images in Figure 3.27; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). There is separation of the DGGE profiles for the DGGE lanes from the colon (CA) of the healthy (UH) and the DB birds (UD).

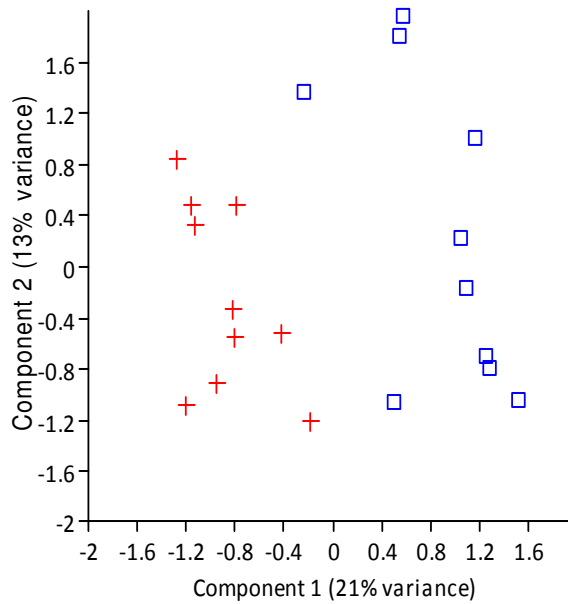


Figure 3.29 Scatter plot following PCA analysis of the caecal DGGE data set from farm two. (□ healthy birds, + DB birds). There is clear segregation of the healthy and DB affected bird data points indicating a difference in microbial composition of the caecal microbiota between these two groups.

Mean percentage band pattern similarities

Table 3.2 shows the mean band pattern similarities for the birds from case study two. As with the first study three comparisons were made; the similarity of the healthy birds group (UHvUH), the similarity of the DB affected group (UDvUD) and the healthy bird group compared to the DB affected group (UHvUD).

	UHvUH	UDvUD	UHvUD
Crop	70.8%	69.3%	63.2%
Gizzard	86.3%	72.4%	69.7%
Jejunum	70.8%	76.4%	65.7%
Ileum	53.6%	61.6%	49%
Colon	48.1%	55.7%	38.7%
Caecum	46.8%	64.5%	42.4%

Table 3.2 Mean percentage band similarities for each region of gut for the healthy and DB birds (UHvUH = healthy group, UDvUD = DB affected group, UHvUD = healthy group compared to dysbacteriosis group)

The mean band pattern similarities on the whole follow the same pattern as with the first case study, showing a reduction in percentage similarity when the healthy birds are compared to the DB birds. Thus implying a shift in intestinal microbiota in the DB affected state. These differences in band pattern similarity are shown in the PCA and dendrogram analyses where separation of the data can be seen in various regions of the gut as in case study one.

3.2.4. Conclusion

At present very little known about DB and it is regarded as a non-specific enteritis associated with an imbalance of the intestinal microbiota. Administration of antimicrobials to birds suffering from DB alleviates the condition (Dr Barry Thorp, personal communication) thus substantiating the proposed involvement of bacteria. Prior to this study there had been no known attempts using PCR-DGGE to characterise the bacterial species involved in the condition or which areas of the

intestinal tract are affected. Therefore the key aims of this investigation were to firstly identify and characterise any differences in intestinal microbiota in DB affected broilers and subsequently identify which regions of the intestinal tract were most affected. The results from DGGE profiling, along with the subsequent dendrogram and PCA analyses, indicated a difference in bacterial composition of the microbiota of DB birds compared to the healthy birds and this is predominately in the small intestine and the caeca. If we consider each farm separately, the DGGE profiles demonstrated an increase in the presence of *Lactobacillus aviarius*, *Escherichia coli* and an uncultured clostridial species in the small intestine of the DB birds from farm one. In the caeca of these birds DGGE profiling indicated an increase in the presence of *Bacteroides dorei* and *Barnesiella viscericola*. In the birds from farm two there is a similar increase in bacterial species in the small intestine where profiling by DGGE showed an increased presence of *Lactobacillus aviarius*, *Bacteroides vulgatus*, an uncultured clostridial species (different to the one seen in farm one) and a limited increase in *Escherichia coli*. As with the birds from farm one, the DB birds had an altered caecal microbiota where a similar increase in the presence of two members of the bacteroidetes namely *Bacteroides ovatus* and *Bacteroides vulgatus* is seen. The results from both farms demonstrated a significant increase in mean band number, thus species richness, in the ileal microbiota and a trend of increased species richness in the jejunum and caeca of affected birds. Despite their absence on the DGGE profiles, it was possible to isolate a number of these bacterial species from the gut contents of healthy birds thus indicating that these bacterial species are part of the birds' resident microbiota but present at a level undetectable by DGGE. This finding indicates that the increase in bacterial species seen using DGGE in the small intestine and caeca of birds with DB is most likely the result of a fluctuation in the growth of certain species of the resident microbiota rather than exogenous bacterial species entering the intestinal tract. Due to the limitations of DGGE it is likely that there are changes in the populations of other bacterial species that aren't detected and more sensitive methods would be required to identify them such as mass cloning and sequencing or next generation high-throughput sequencing. The results from the crop, gizzard and colonic contents reveal conflicting results with the healthy birds

having a trend of greater species richness in the first case study and the DB birds having a trend of greater species richness in the second study. The projected microbial profiles from the crop and gizzard were compromised by the coamplification of plant DNA therefore it is difficult to make any substantial conclusions about these regions of intestine therefore further investigation with more specific PCR primers may reveal differences not shown in this study. The inconclusive results from the colonic samples could be a result of either the age or breed of the birds resulting in a differential response of the colonic microbiota in the DB state. The colonic samples could be influenced by the differing individual circadian rhythms relating to the production of caecal and faecal droppings. Therefore future studies using fresh faeces obtained from the birds before euthanasia or a pool of faecal samples from one bird over a daily period could provide a more consistent means for comparison. However, in line with the initial aims of this part of the investigation it can be concluded that birds suffering from DB have a different intestinal microbiota in the small intestine and caeca when compared to healthy birds. This altered microbiota involves a general increase in species richness in these gut regions, caused by an overgrowth of normally less dominant species of the resident microbiota. However, it is not clear why the overgrowth occurs or the role the bacterial species identified play in the manifestation of DB.

3.3. Comparison of the Lactobacillales population in the crops of dysbacteriosis affected broilers with unaffected broilers

3.3.1. Introduction

The microbiota within the crop of a broiler chicken comprises of predominately lactobacilli which colonise within the first 24 hours post hatch and remain with the chicken throughout its life essentially unaffected by diet, drug treatment or management style. The lactobacilli that colonise the crop form a continuous layer covering the crop epithelium and are involved in the fermentation of feed prior to digestion further down the intestinal tract (127, 129, 396, 398). The initial inoculum of bacteria in the crop of chickens originates from the egg shell and the surrounding

environment. In nature this would be bacteria from the nest substrate and the parental faecal material around the nest, but the environment in a modern hygienic hatchery is a far removed from that of a nest. Consequently the diversity and composition of the bacteria in the hatching tray is likely to be very different to what the birds are exposed to in a more natural environment and this could result in colonisation by bacteria which are not optimal for the broilers. Lactobacilli have, for many years, been considered to be beneficial to health (68, 140, 150, 402) and so the constant seeding of lactobacilli from the crop into the lower intestine can be seen as a continuous 'probiotic' for the broilers. Different strains of lactobacilli have been shown to vary in their ability to attach to the crop or intestinal epithelia which in turn results in a varied ability to colonise the gut (277). One hypothesis as to why some birds develop DB and others do not, is that the birds susceptible to the condition are colonised by strains of lactobacillus which are less protective due to a reduced ability to attach to the intestinal epithelia. This in turn could result in the bird receiving less than optimal protection against fluctuations in gut homeostasis from its resident microbiota. Comparison of the crop microbiota of healthy and DB birds using general V3 bacterial primers gave mixed results with limited differences being seen in the crop and gizzard microbiota of the healthy and DB affected broilers. However, sequencing the major bands from the gizzard profiles revealed that there was coamplification of chloroplast DNA which accounted for the majority of the bands seen on the DGGE profiles. As the crop and gizzard DGGE profiles are very similar is it likely the crop profiles contain many bands derived from plant DNA thus leading to an inaccurate projection of the microbiota in the crop. This investigation aimed to compare the crop microbiota of the healthy and DB birds using PCR-DGGE using primers specific to the lactobacillales, the dominant bacterial group of the foregut of the chicken. These primers are specific to the V3-V4 regions of the lactobacillales 16S rRNA gene thus reducing the likelihood of non-specific binding to chloroplast DNA. The hypothesis to be tested was whether or not there are differences in the composition of the lactobacilli population in the crops of the DB birds compared to that of the healthy birds, and can these differences help explain the pathogenesis of dysbacteriosis.

3.3.2. DNA Extraction, PCR-DGGE, DGGE gel analysis and band identification

These were all carried out as described in sections 2.3, 2.4 and 2.5. However in this study the lactobacillales primers Lac1 (5'-GCAGCAGTAGGGAATCTTC-3') and Lac2-GC (5'-GCCGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCAGGGGATTTCACCGCTACACATG-3') were used (415). Furthermore the DGGE gel gradient was altered to a range of 32.5% to 40% in line with Walter *et al.* (415).

Mean band number

Mean band number is a representation of the bacterial diversity within a sample; comparison of the mean band number of two or more groups can give an indication of changes in bacterial diversity between the groups. Figure 3.30 shows the mean band numbers for the crop lactobacillales populations.

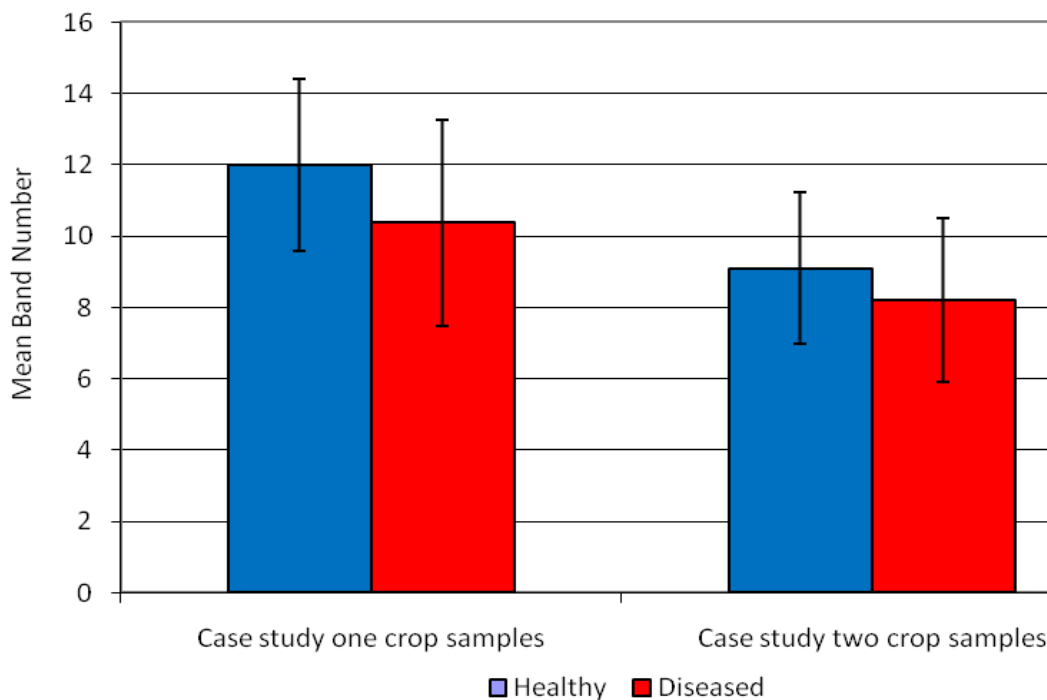


Figure 3.30 Mean DGGE gel band number of the crop DGGE profiles following lactobacillales specific PCR-DGGE. There is a general decrease in the mean band number in the dysbacteriosis state, however these differences are not significant.

Percentage band pattern similarity

Table 3.3 shows the mean band pattern similarities following analysis of the crop lactobacillales DGGE profiling. As with the analysis following PCR-DGGE with general V3 primers, the resultant crop DGGE band pattern similarities from both farms were compared three ways. The profiles from the healthy birds were compared with their healthy flock mates (HvH), the profiles from the DB affected broilers were compared with their DB affected flock mates and then finally the healthy and DB affected bird profiles were compared.

	Healthy v Healthy	DB v DB	Healthy v DB
Farm one crop samples	76.1%	74%	71.9%
Farm two crop samples	74.1%	60%	66%

Table 3.3 Mean percentage band similarities for the crop for the healthy and DB birds following lactobacillales specific PCR-DGGE

The only pattern seen in the mean percentage band similarities is a higher similarity amongst the healthy birds, however following dendrogram construction there is no definitive segregation of the healthy or DB birds suggesting there is no major differences in the lactobacillales composition of the crop microbiota as shown by PCR-DGGE.

DGGE gel analysis

The DGGE images (Figure 3.31) did not reveal any obvious differences in the crop microbiota between the healthy and DB birds. There is some variation in the band presence and absence of some of the birds; however analysis of the data with dendrogram construction (figure 3.32) and PCA (Figure 3.33) did not reveal any convincing segregation between the two groups. This suggests that there is no conclusive evidence of major differences in the lactobacillus composition of the crop microbiota in the healthy or DB birds.

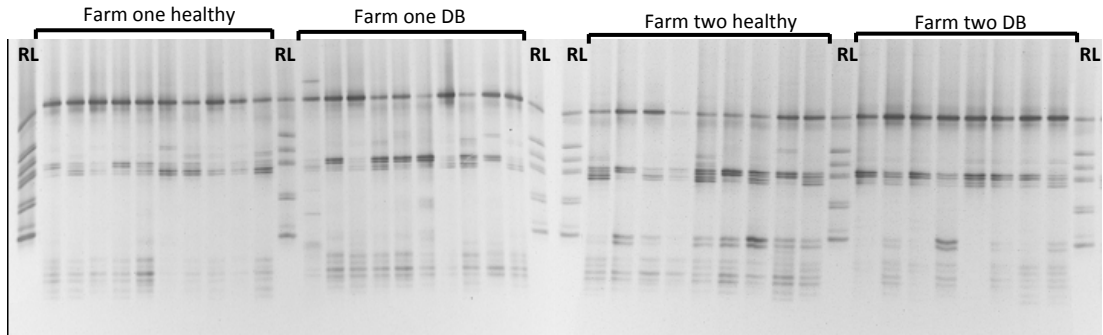


Figure 3.31 DGGE gel visualising DNA obtained from the crops of healthy and DB birds from both case studies following PCR amplification using lactobacillales specific PCR primers (RL = Reference Ladder)

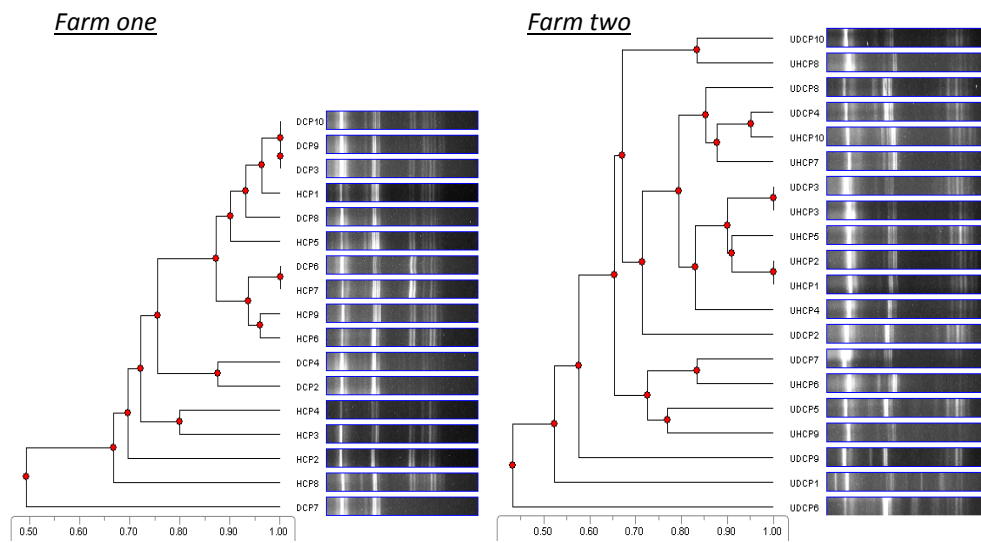


Figure 3.32 Dendrogram of the DGGE lane images in Figure 3.31; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). There is no definitive clustering of the DGGE profiles for the healthy or DB birds from either farm.

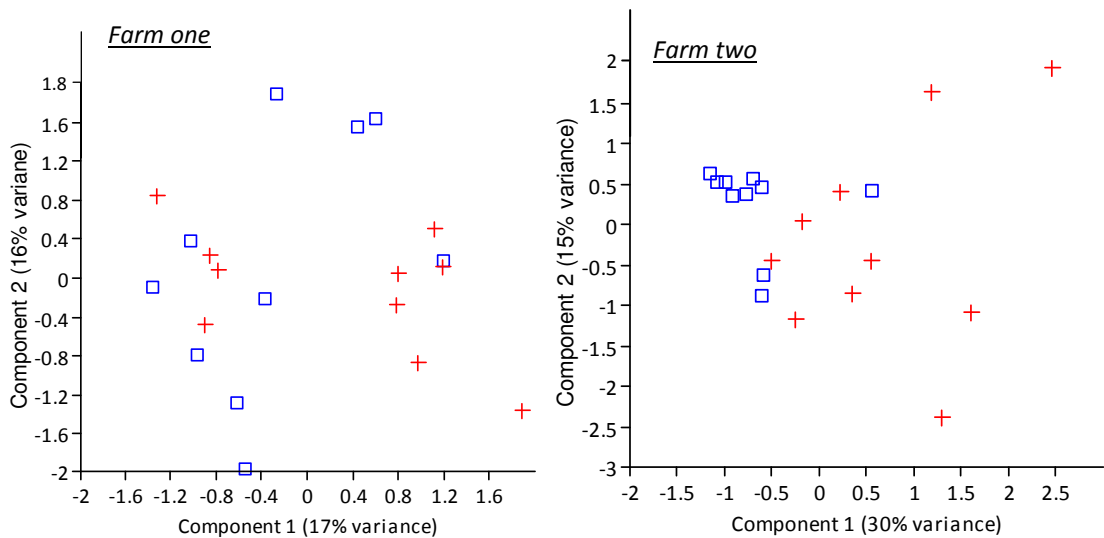


Figure 3.33 Scatter plots following PCA analysis of the crop lactobacillales DGGE profiles from both farms (□ healthy birds, + DB birds). There is no convincing segregation of data points for the healthy and DB birds on both farms.

3.3.3. Conclusion

The aim of this section of the investigation was to re-examine the microbiota in the crops of the healthy and DB birds. The rationale behind this was due to the fact that profiling with general V3 primers revealed coamplification of chloroplast DNA thus distorting the final DGGE images obtained. As members of the lactobacillus genus are the most dominant members of the crop microbiota primers specific to the lactobacillales family were used. The hypothesis was that the birds suffering from DB could have a different composition of lactobacillus species that did not offer optimal protection within the gut leading to DB predisposition. This study did not reveal any conclusive differences in the lactobacillus populations in the crops of healthy and DB affected broilers when DGGE profiling was used.

However, it is reported that there is homology of regions of the 16S rRNA gene of species of lactobacilli and as a result species differentiation is not always possible by DGGE where only a small fragment of the 16S rRNA gene is a target (126, 149, 416). Consequently it is possible that the method of PCR-DGGE used in this study is not sensitive enough to identify differences in the crop microbiota in healthy and DB birds. There have been a variety of approaches used to differentiate species of lactobacillus; these have included lactobacillus group specific PCR and randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) (342), polyphasic approaches involving SDS-PAGE of cellular proteins with RAPD-PCR and amplified fragment length polymorphisms (ALFP) (134) or selective culturing followed by amplified ribosomal DNA restriction analysis (ARDRA) (149). It could also be possible that a mass cloning and sequencing approach would be appropriate to gain more information of the lactobacillus populations in the crop.

3.4. Discussion

The results obtained indicate that in cases of DB there is a difference in the diversity of the intestinal microbiota in the jejunum, ileum and caeca of affected birds when compared to healthy birds. However there is no indication as to how these changes are associated to the onset or manifestation of the condition. The differences in intestinal microbiota in cases of DB appear to be due to an increase in species or

strains of bacteria which are normal residents of gut. Bacteroides, clostridia, anaerobic lactobacilli and *E. coli*, all of which are seen to have increased in numbers in cases of DB, have been all been indicated in small intestinal microbial overgrowth in a variety of mammalian species (153, 154, 246). These bacterial species have been shown to secrete proteases which destroy disaccharidases resulting in a reduction in brush border sucrase and maltase activity. It has also been suggested that *Bacteroides vulgatus* and *Bacteroides ovatus* along with other bacteroides species have the ability to cause damage to the brush border which would reduce the surface area available for absorption, leading to malabsorption (319). The reduction in host disaccharidase activity will result in an increase in disaccharides being available for the microbiota to utilise which could be an explanation for the overgrowth of other members of the microbiota. The community of bacteroides within the intestinal tract of any bird or mammal has a vast repertoire of polysaccharide degrading ability. Fermentation of polysaccharides by intestinal bacteria is one of the key aspects of the symbiotic nature of the intestinal microbiota, as the result is the formation of short chain fatty acids (SCFA) which can be absorbed by the host (120). Dependant on the diet of an animal including poultry, either omnivorous or herbivorous, 10%-70% of an animal's dietary uptake can be in the form of SCFAs derived from bacterial fermentation (25). In addition to the production of SCFA, fermentation of polysaccharides by *Bacteroides sp.* results in the production of bacterial glycans along with mono- and oligosaccharides all of which can be utilised by other members of the intestinal microbiota in bacteria to bacteria mutualism (70, 237, 309, 320, 439). Mucus secreted by the host contains a variety of polysaccharides which can easily be degraded and utilised by members of the bacteroidales (321, 329, 424). In cases of DB there is an increase in mucus production which is evident by the presence of orange mucus present in the faeces. The increase in mucus could act as an increased nutrient source for bacteroides, consequently leading to an increase in the abundance of bacteroides in the DB affected broilers. In the event of increased bacteroides populations in the gut there is likely to be an increase in the provision of glycans and short chain saccharides to other members of the microbiota, thus allowing them to increase in number adding to the overgrowth of intestinal bacteria. One interesting and surprising finding is

the increased presence of *Lactobacillus aviarius* in the DB birds. Generally species of lactobacillus are regarded as beneficial bacteria promoting intestinal health and so one would not necessarily expect to see the increase in a lactobacillus species in the case of enteric disharmony. However there is evidence that lactobacillus species have the ability to cause opportunistic disease including bacteraemia, intestinal inflammation, hepatic abscesses and gangrene, however this is usually in individuals who are immuno-compromised (282, 308, 315, 385). It is possible that the increased presence of *Lactobacillus aviarius* is a consequence of the increase in available carbohydrates in the gut lumen rather than being directly involved with the insult to the enteric environment. Due to the change in bacterial species seen in the DB birds it is likely that there will be a shift in the end products of bacterial fermentation in the gut lumen compared to that within the gut lumen of healthy birds. The change in microbial composition could result in the production of different SCFAs and amines, which could either alter the physiology within the gut lumen or have deleterious effects on the host tissues potentiating the disruption to the enteric environment. It is possible that the changes in bacterial fermentation could provide a window into the interplay between the bacteria and the host within the intestinal lumen in order to give a greater insight into the mechanisms involved in DB. This idea is expanded on later in this thesis.

Dysbacteriosis does have parallels with dysbiosis, which has been described in human patients and is thought to be a precursor to inflammatory bowel disease where there is a shift in microbiota resulting in over stimulation of the gut immune system. However it is not yet known what causes the shift in gut microbiota or the over stimulation of the immune system. Within the intestinal microbiota there is a highly complex community of microbes containing hundreds of species of bacteria co-existing in a balance where the 'friendly' bacterial species play a role in preventing colonisation by pathogens and overgrowth of the putatively harmful opportunistic resident species; it is thought an imbalance in these species can result in inflammation. However the exact relationship between the overgrowth of bacteria and intestinal inflammation remains unclear. There have been a variety of proposed causes for dysbiosis and IBD including genetic disposition, diet,

environment, drug administration and presence of bacteriophages; it is likely that the cause of dysbiosis is multifactorial resulting in a cascade that causes disease and elucidating the aetiology is a current key area of research (69, 219, 301, 336, 377, 378). *Bacteroides spp.*, *Clostridia spp.* and *Escherichia coli*, all of which demonstrate an increased presence in birds with DB, are considered to be opportunistic bacteria and despite being present in healthy individuals they have been implicated in a variety of opportunistic enteric diseases (36, 239, 409). The capacity of these species to cause disease and disrupt the enteric environment can be explained in part by the ability of certain strains of bacteroides, clostridia and *Escherichia coli* to successfully colonise the intestinal mucosa, enhance mucosal permeability, promote bacterial uptake, cross the epithelial barrier and induce the synthesis of proinflammatory cytokines by interacting with epithelial cells and macrophages (77, 201, 325, 326, 420).

As with dysbiosis in humans it is not clear that the differences in bacterial composition that are seen between the healthy and DB birds are a result of DB or whether the affected birds had a different intestinal microbiota composition prior to the onset of disease. It is possible that as the birds were introduced to a different microenvironment when brought onto the farm (i.e. different broiler sheds), that they were exposed to different environmental bacteria leading to differences in the microbiota colonising the gut. Yin *et al.* (443) demonstrated that administering inocula of differing microbial composition to newly hatched chicks resulted in different levels of colonisation in the ileum and caeca in addition to differences in ileal gene expression of the host. An inoculum composing of *Bacteroides*, *Lachnospiraceae* and *Ruminococcus*, was found to influence host genes controlling ion transport, cell cycle and chromosome maintenance in comparison to an inoculum containing *Prevotella*, *Acidaminococcus* and *Dorea*. Further work is needed to determine the importance of these effects on gene expression, however these preliminary data indicates that the initial bacterial community colonising the gut can influence gene expression as well as the maturation of the intestinal microbiota.

Over recent years it has become evident that the intestinal microbiota plays a role in the development and maintenance of the host immune system. The hygiene hypothesis has been proposed as a reason for increased allergy and auto immune disorders in the human population (370). It is suggested that increased cleanliness and a decreased exposure to bacterial antigens early in life inhibits the development and education of the immune system which, in regards to the intestinal microbiota, can lead to a lack of immunotolerance to certain members of the resident microbiota resulting in greater disease potential (195). In a study comparing the intestinal microbiota of piglets reared outdoors in a natural environment to the intestinal microbiota of genetically similar piglets reared in a clear indoor environment, Mulder *et al.* (281) demonstrated the importance of the early-life environment on the development of microbial diversity in the gut. The results highlighted the presence of more firmicutes, especially lactobacilli, in the intestinal microbiota of the outdoor reared piglets. The indoor reared pigs had a significant reduction in the mucosa-adherent lactobacilli species such as *L. reuteri*, *L. delbrueckii*, *L. amylovorus*, *L. johnsonii* and *L. mucosae*. The indoor reared piglets had an increased presence of *Bacteroides sp.* and proteobacteria, which are traditionally viewed as potentially harmful residents of the intestinal microbiota. The study indicated that in a more hygienic environment the piglets developed a different microbiota which one could consider to have a higher proportion of potentially harmful or immunoaggressive bacteria. Interestingly Tsuda *et al.* (389) demonstrated that prior stimulation of antigen presenting cells (APC) with lactobacillus regulates excessive antigen-specific cytokine responses when compared with bacteroides. The findings suggest that lactobacilli contribute to the 'regulated' state of the immune system by down-regulating antigen-specific cytokine production whereas bacteroides induce an 'excessive' state of immune response by stimulating antigen-specific cytokine responses. Therefore in the event of an animal having a higher proportion of bacteroides and reduced number of lactobacilli in their intestinal tract, then their immune system could be in a more aggressive state leading to an excessive immune response when challenged by a novel antigen. This information suggests that the bacterial species that a neonatal

animal comes into contact with in the first few days of life is essential for the correct colonisation and subsequent priming of the immune system.

There is obviously more work to be carried out in order to fully understand why DB occurs and what triggers the onset. This initial study provides information that can be used in the design and implementation of further studies relating to DB to obtain further information about the condition and develop potential management strategies.

4. Comparison of the GI tract microbiota of different genotype of broiler chickens with differing susceptibility to dysbacteriosis

4.1. Introduction

The effects of host genotype on intestinal microbiota have been demonstrated in a variety of species. A study using PCR-DGGE by Zoetendal *et al.* (447) demonstrated that there is a positive correlation between the genetic relatedness of humans and the similarity of the composition of the intestinal microbiota, where monozygotic twins had a more similar microbiota than their respective spouses. However in the same study dizygotic twins demonstrated the same level of similarity to their siblings as the more closely genetically related monozygotic siblings. Turnbaugh *et al.* (391) demonstrated a similar finding using a large scale 16S rRNA gene sequence analysis. It was shown that there was a correlation between host relatedness and microbial composition, however this correlation was the same with siblings who were either dizygotic or monozygotic twins. Furthermore temporal temperature gradient electrophoresis (TTGE) was used by Stewart *et al.* (368) to compare the faecal microbiota of monozygotic twins, dizygotic twins and unrelated pairs. Their results indicated that there was a significantly higher similarity in intestinal microbiota of monozygotic twins than in the dizygotic twins and unrelated pairs. Despite the differences in findings between monozygotic and dizygotic twins it is clear that there is a host genetic influence over the intestinal microbiota of an individual. Furthermore, by transplanting the the intestinal microbiota of adult zebrafish into germ free mice and conversely the intestinal microbiota of adult mice into germ free zebrafish, Rawls *et al.* (317) demonstrated the relative abundance of species changes to resemble the normal composition of the recipient host, although the community lineages of bacterial divisions of the seeding microbiota remaining constant. Thus indicating that selective pressures, distinct to the gut habitat of the host, mould the microbial community in relation to the host species regardless of the initial seeding bacteria. However one consideration to bear in mind is that despite the ability of the gut environment to alter the bacterial community, the strains of bacteria that grow may not be the most appropriate for the recipient host. Following isolation of strains of *Lactobacillus reuteri* from six different host

species (human, mouse, rat, pig, chicken and turkey), Oh *et al.* (296) demonstrated genetic heterogeneity between the six *Lactobacillus reuteri* populations which clearly reflected host origin. Furthermore when all strains were inoculated into gnotobiotic mice the isolates with a rodent origin showed elevated growth and ecological performance; concluding that the evolution of these species is adaptive and together with co-evolution within their host the result is a highly specialised symbiosis. It is highly likely that other bacterial species and strains have similarly evolved with their host. This finding is of great importance when considering potential probiotic species for animal or human use. Despite the lack of data from chicken studies, the mounting evidence of the influence of host genetics on the composition of the gut microbiota could be an explanation for the evidence from the broiler farming community suggesting that certain broiler strains are more susceptible than others to developing DB.

As part of an unpublished industrial trial carried out by Aviagen Ltd. and unrelated to this thesis, different genetic lines of broiler chicken were found more susceptible to a loss of intestinal integrity leading to DB and the onset of wet litter. Secondary to this, the more resistant genetic lines, when housed in pens with the more susceptible genetic lines, exhibited a greater incidence of wet litter. It is possible that the composition of the intestinal microbiota or presence of specific species or strains of bacteria of the healthy bird contribute to the susceptibility to enteric upsets. If this is the case, exposure to the faecal material of the more susceptible birds could induce an enteric upset in the more resistant birds. This investigation aims to compare the intestinal microbiota of four different genetic lines of broiler chicken. In line with the results obtained in Chapter 3 the investigation will focus on the ileal and caecal microbiota of the birds as that is where the majority of changes were seen between the healthy and DB birds.

4.2. Materials and Methods

4.2.1. Sample Selection

Four genetic lines of broiler chickens were used in this experiment two of which were more susceptible to enteric upset (A and X) and two which were more resistant (B and Y). The birds could be separated into further groups; two were commercial broiler lines (X and Y) which are lines grown for meat production and the remaining two were pure breed lines (A and B) used in the selective breeding programmes for commercial lines (Table 4.1). As the aim of this study was to identify bacterial species that could potentially predispose the birds to enteric disease such as dysbacteriosis the birds used for the comparisons were healthy and showing no sign of enteric disease.

	Pure Breed Line	Commercial Broiler Line
Susceptible to enteric upset	A	X
Resistant to enteric upset	B	Y

Table 4.1 Outline of the grouping and codes used for the birds in this particular study.

The birds were obtained from one of the Aviagen Ltd. trials farms and were all housed in the same broiler shed with each group in individual pens with pen mates being the same genotype. The birds were all the same age (21 days), the same sex and fed on the same diet. Ten male birds were collected at random from each pen and euthanised via cervical dislocation. The complete GI tract was removed *post mortem* and frozen immediately on dry ice. Sections were taken from the Ileum (5 cm section, 10 cm caudal of Meckel's Diverticulum) and caecum and stored at -20 °C.

4.2.2. DNA Extraction, PCR-DGGE, DGGE gel analysis and band identification

These were all carried out as described in sections 2.3, 2.4 and 2.5

4.3. Results

Mean band number

The number of bands on a DGGE image is a direct correlation to the species richness within a given sample. Comparing the mean band number between the four groups of birds is an easy way to initially assess if there are any differences in species richness between the groups. Figures 4.1 and 4.2 show the mean band numbers for the ileal and caecal samples of all four groups of chickens respectively.

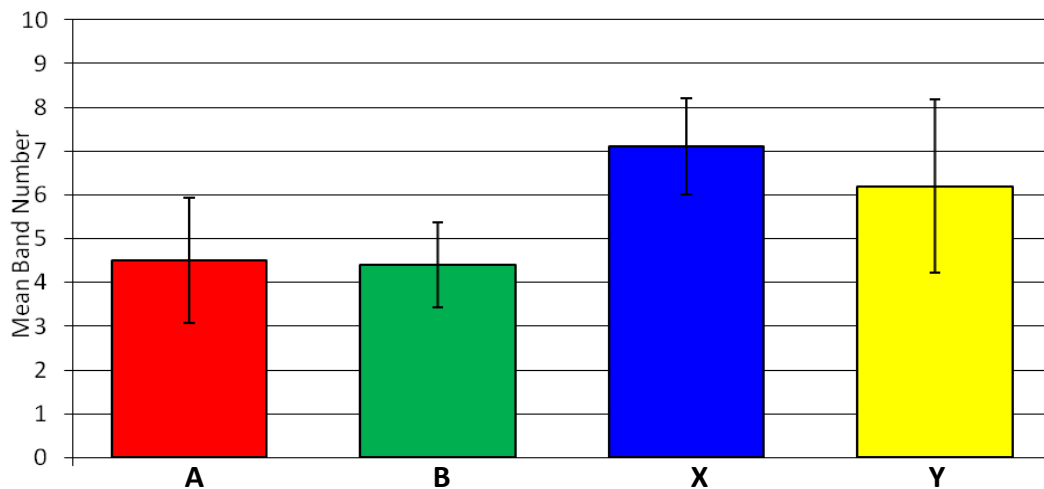


Figure 4.1 Mean DGGE gel band number for the ileal samples from each group of broiler breeds. Significant differences in mean band number can be seen following a t-test between A and X ($p < 0.0003$), A and Y ($p < 0.05$), B and X ($p < 0.0002$) and B and Y ($p < 0.02$) Bars represent standard deviation.

Using the data from the mean band numbers it was not possible to determine any distinction between the resistant and susceptible birds. However, what does seem apparent is that in the ilea of the pure breed lines of chicken, there were significantly lower band numbers, therefore less species richness, than in the birds of the commercial lines.

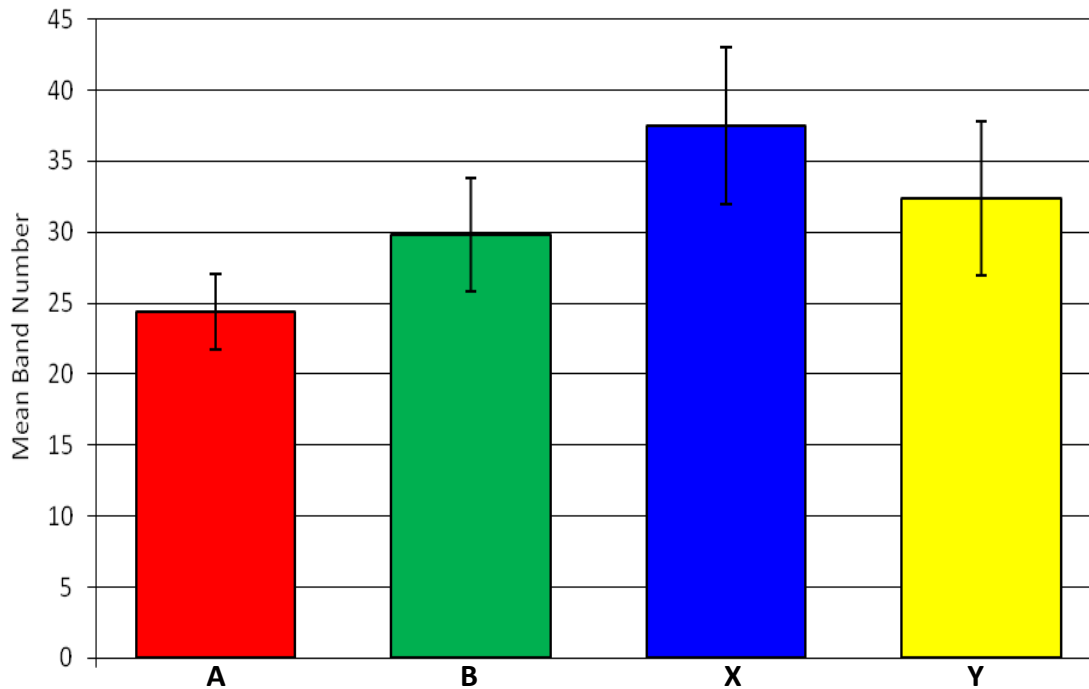


Figure 4.2 Mean DGGE gel band number for the caecal samples from each group of broiler breeds. Significant differences in mean band number can be seen following a t-test between A and B ($p < 0.003$), A and X ($p < 0.00003$), A and Y ($p < 0.0006$), B and X ($p < 0.003$). Bars represent standard deviation.

Similarly the caeca of the commercial broiler lines demonstrate a higher mean band number than the pure broiler lines suggesting that commercial broilers have a greater species richness in their caecal microbiota. As the birds were in the same environment, of the same sex, the same age and fed the same diet this is strong evidence that the differences here are due to host genotype.

DGGE analysis of ileal bacteria

Visual inspection of the DGGE gel obtained from the ileal samples from the four groups (Figure 4.3) revealed limited differences in band distribution however the differences don't appear to differentiate between the susceptible and resistant genotypes. The differences that can be seen are predominately between pure lines (A&B) compared to commercial lines (X&Y), with the latter group having more bands (as seen by the increased mean band numbers shown in Figure 4.1). Dendrogram construction (Figure 4.4) and PCA analysis (Figure 4.5) don't reveal any segregation of the groups according to the DGGE data.

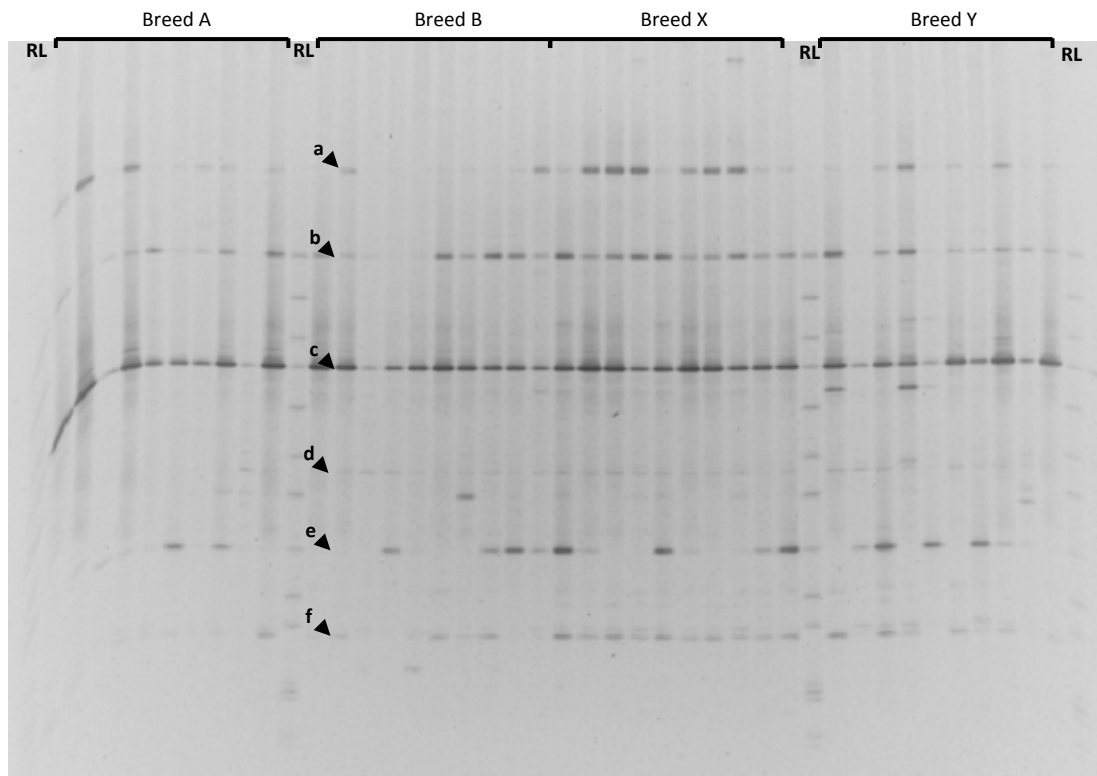


Figure 4.3 DGGE gel visualising DNA obtained from the ilea of the four groups of birds following PCR amplification of the V3 hypervariable region of the 16S rRNA gene. There are limited intragroup and intergroup variations in the bands highlighted. They were identified with band sequencing, percentage indicated sequence match in 16S database a) *Lactobacillus aviarius* (100%) b) *Lactobacillus johnsonii* (100%) c) *Lactobacillus crispatus* or *Lactobacillus fornicalis* or *Lactobacillus helveticus* (100%) d) *Enterococcus sp.* (98%) e) *Escherichia coli* (100%) f) *Clostridium sp.* (98%) (RL = reference ladder). (See appendix II for a table linking all the bacteria identified in this thesis).

The bands identified from the DGGE gel represent a variety of bacterial species commonly found in the GI tract of broiler chickens. A number of them are bacterial species found to have increased presence in DB birds, namely *Lactobacillus aviarius*, *Escherichia coli* and a *Clostridium sp.* Bands “a”, “d” and “f”, which have the closest sequence matches to *Lactobacillus aviarius*, *Enterococcus sp.* and *Clostridium sp.* respectively, show the most variability in presence and absence between the four groups. In the samples from group X the three bands are present in the majority of the birds, however there is reduced presence of bands “a” and “f” in breeds A, B and Y, while band d is absent from the majority of the birds in breed A group.

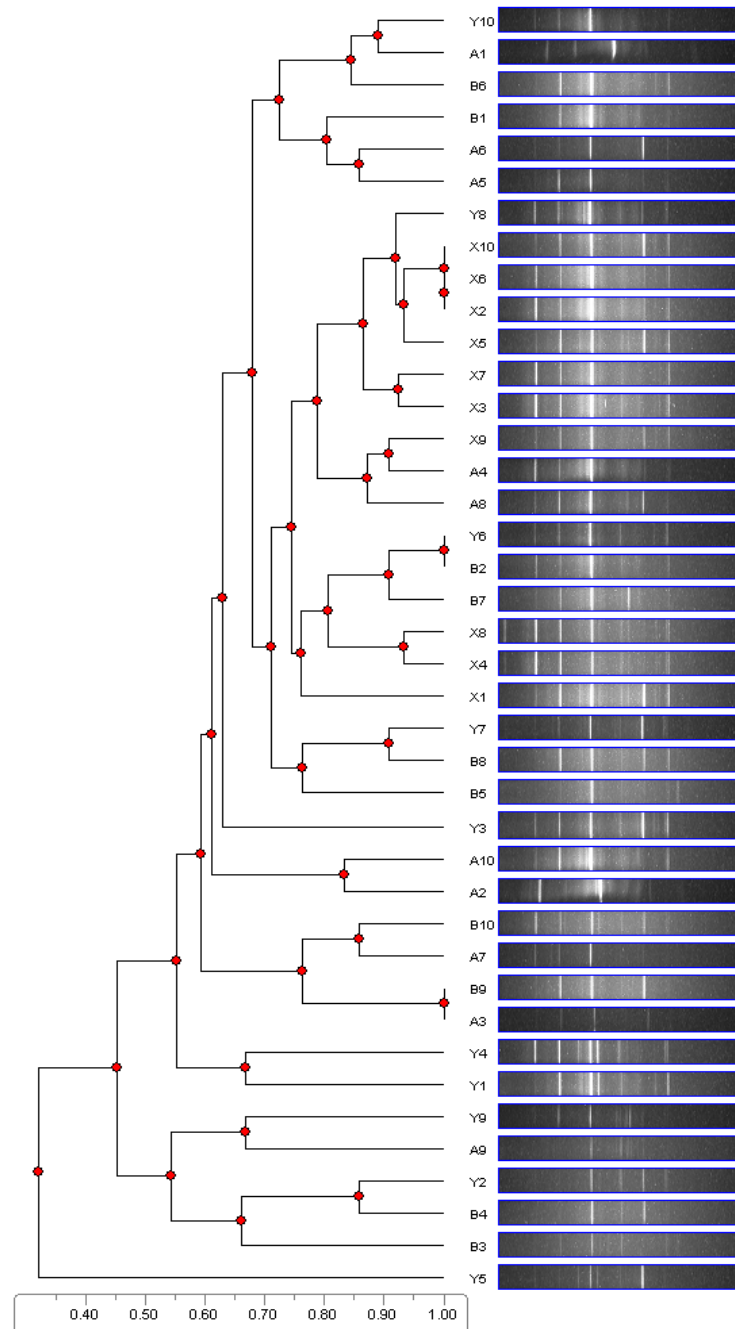


Figure 4.4 Dendrogram of the DGGE lane images from analysis of the ileal samples of the four breeds in Figure 4.3; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). There is very little separation of the DGGE profiles indicating little differences in band pattern similarity.

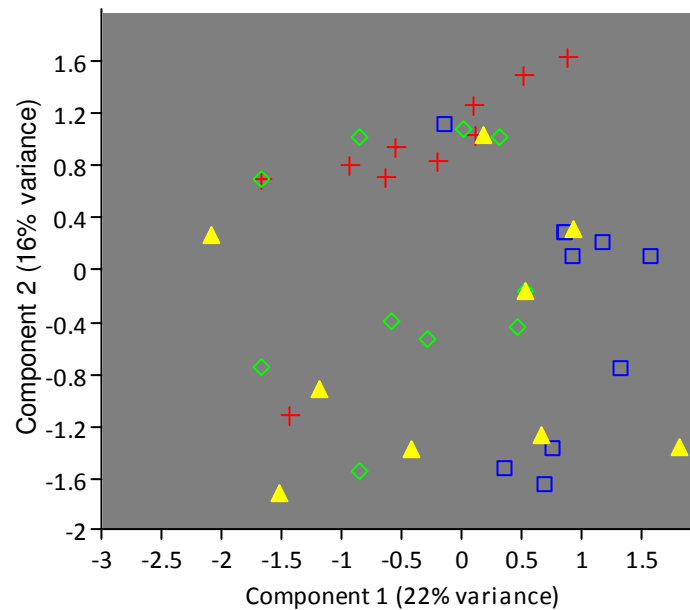


Figure 4.5 Scatter plot following PCA analysis of the ileal DGGE data set from the four genotypes. (+ breed A, \diamond breed B, \square breed X, \blacktriangle breed Y). There is no overall segregation of the data points to distinguish the resistant and susceptible birds.

The results do not reveal any apparent link to the composition of the intestinal microbiota and the breed susceptibility of a bird to DB and wet litter. As the broilers were of the same age, fed the same diet and housed in the same environment it is likely the effect of host genotype that causes the minor group to group variations in band patterns. An interesting observation from the PCA plot is that despite there being no separation of the data points from all four groups, the data points from within breeds A and X (the susceptible breeds) are clustered much closer together than the more resistant breeds indicating that there is less bird to bird variation in bacterial species diversity in these two groups. This is apparent with visual assessment of the DGGE image where there is less variation in the DGGE profiles within A and X. If the host genotype and immune system influence the composition of the microbiota of the birds, it is possible that where there is less variation in the intestinal microbiota composition in a specific genotype of broiler that there is greater homology of the immune system. Taking this further there is the possibility that the same immune response in the event of DB will be elicited by the majority of birds resulting in the susceptibility of the breed to the condition.

DGGE analysis of caecal bacteria

The caecal DGGE image obtained (Figure 4.6) appears to reveal further differences between the commercial broilers and the pure lines, rather than resistant versus susceptible birds.

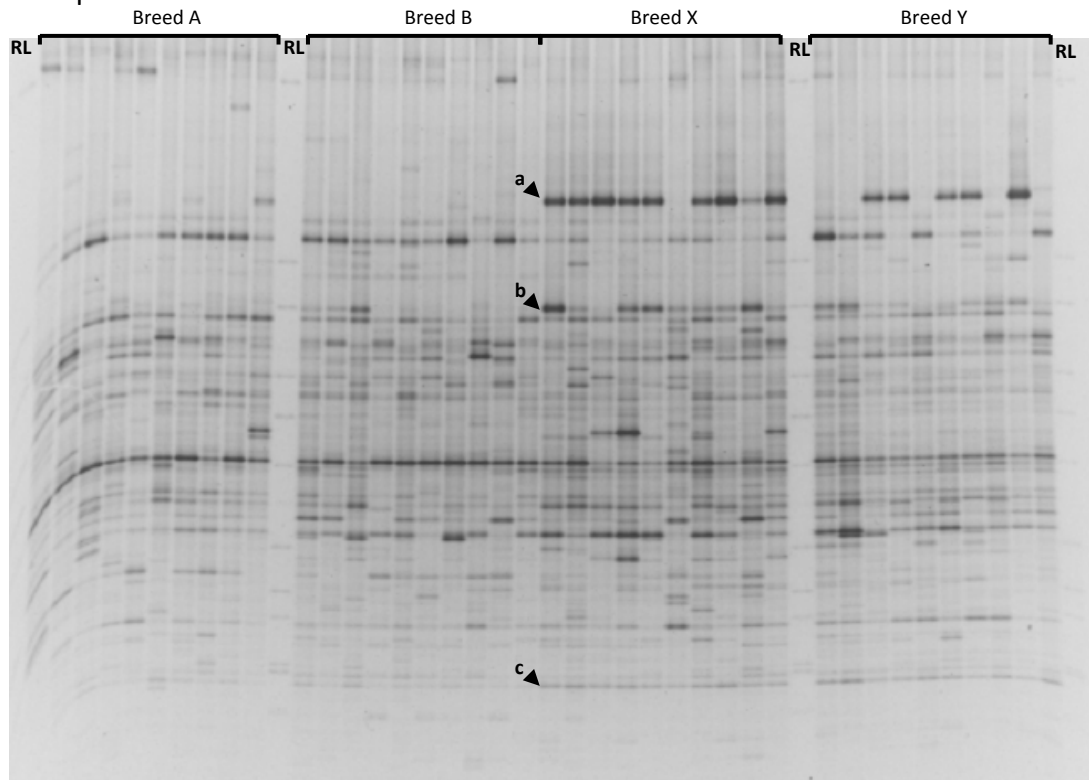


Figure 4.6 DGGE gel visualising DNA obtained from the ilea of the four groups of birds following PCR amplification of the V3 hypervariable region of the 16S rRNA gene. Visual differences are highlighted and was identified with band sequencing a) uncultured *Bacteroides sp.* (100%) and b) *Lactobacillus crispatus* or *Lactobacillus fornicalis* or *Lactobacillus helveticus* (100%) c) *Clostridium sp.* (100%) (RL = reference ladder). (See appendix II for a table linking all the bacteria identified in this thesis).

The most striking differences are highlighted using arrows and band sequencing revealed them to represent an uncultured *Bacteroides sp.* (100% match) and a *Lactobacillus sp.* which had 100% match to several different species, which again emphasises the homogeneity of the lactobacillus 16S rRNA gene. The bands marked as “c” in Figure 4.6 and “f” in Figure 4.3 have the same DNA sequence and correspond to the same *Clostridium sp.* This band appears to be absent in a number of the birds of breed A and has a greater intensity in the two commercial strains of bird. Dendrogram construction revealed clustering of the DGGE lanes but clustering again separated the commercial birds from the pure breed lines rather than resistant vs. susceptible lines (Figure 4.7).

PCA analysis of the DGGE band matrix from the caecal samples reveals clustering of the data points from the four breed lines (Figure 4.8). However in line with what appears to have been the trend throughout this particular study, the data points appear to cluster the commercial broiler breeds away from the pure breed lines.

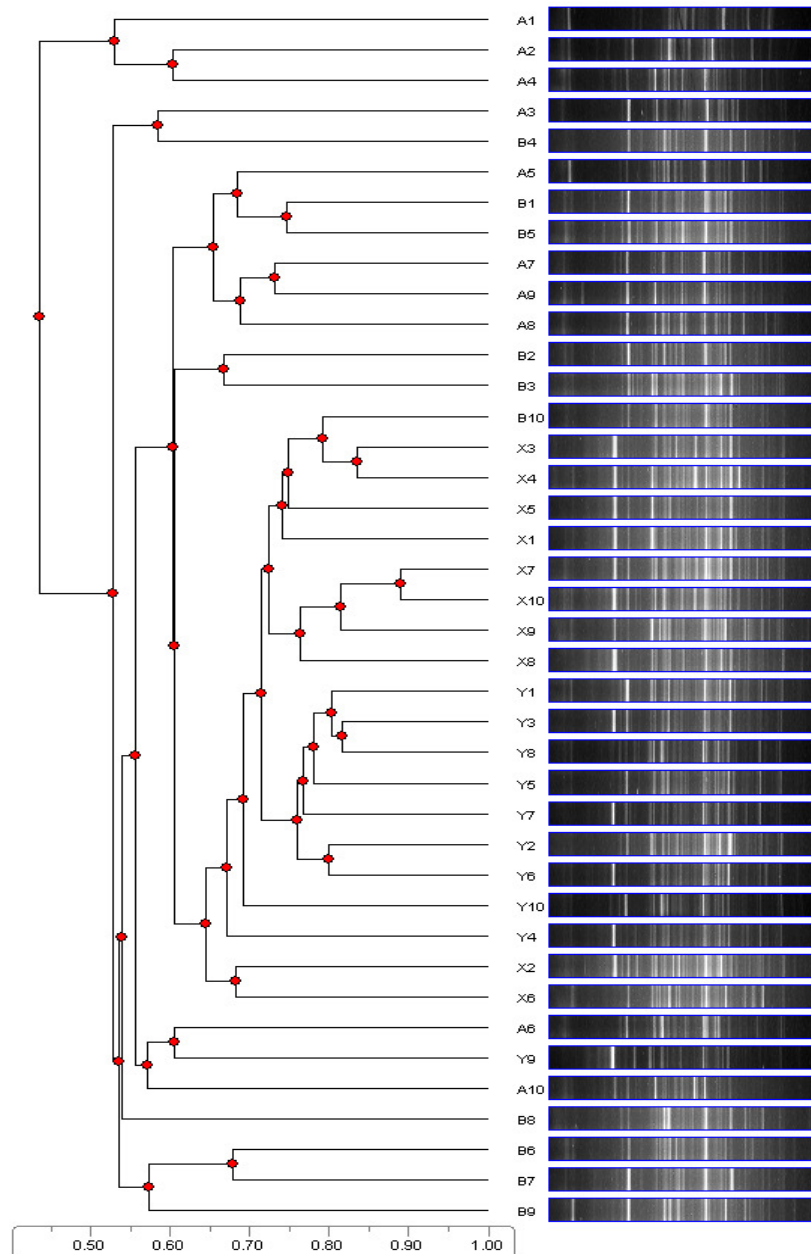


Figure 4.7 Dendrogram of the DGGE lane images obtained from the caecal samples in Figure 4.6; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). The majority of DGGE lanes of X and Y appear to cluster quite tightly in the same region of the dendrogram indicating they are different to the profiles from A and B.

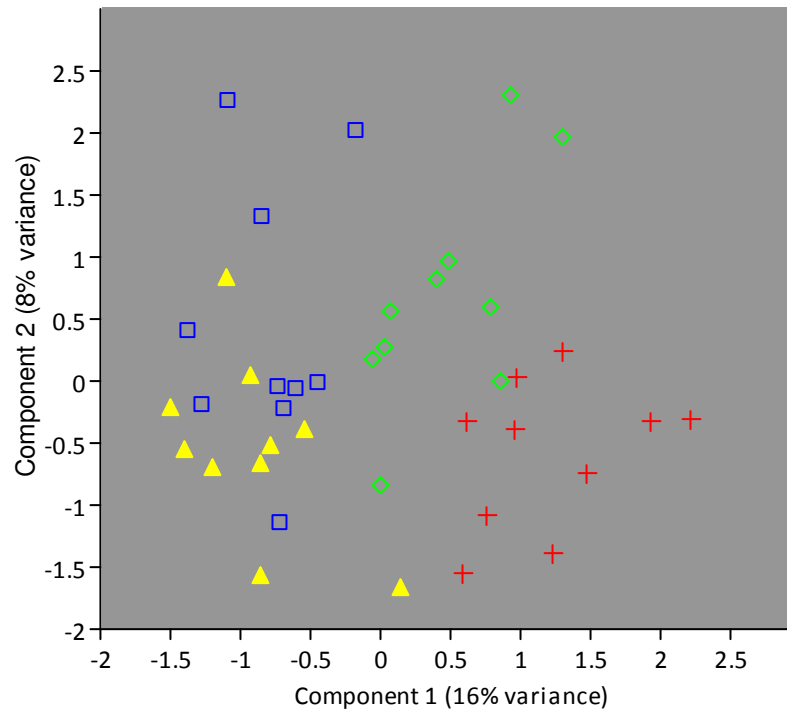


Figure 4.8 Scatter plot following PCA analysis of the caecal DGGE data set from all four breed lines (+ breed A, \diamond breed B, \square breed X, \blacktriangle breed Y). There is no overall segregation of the data points to distinguish the healthy and DB birds, however there is clustering relating to broiler type i.e. the commercial lines (X&Y) being towards the left of the plot and the pure breed lines (A& B) being to the right.

The data points for breeds A and B indicate a degree of separation between the two pure breed lines individually. This could be explained by the significantly higher mean band number in the caeca of breed B (the more resistant pure breed). In terms of resistance to enteric disease, the increased species richness could potentially provide more competition for nutrients within the caecal environment meaning that a bacterial overgrowth is less likely. However, this theory is not supported by the data as both breeds X and Y have a higher mean band number in the caecum with the more susceptible breed X having the larger mean band number. Again these results reiterate the influence of host genotype on the intestinal microbiota, however with regards to susceptibility, it is most likely host specific immune responses to the microbiota or other trigger factors result in the birds developing DB.

4.4. Conclusion

The main aim of this investigation was to compare the intestinal microbiota of broiler genotypes with different resistance and susceptibility to enteric disease leading to wet litter. In light of mounting evidence for the influence of host genetics on the intestinal microbiota this study explored the possibility that there was a common pattern in the composition of the intestinal microbiota in healthy birds susceptible to enteric disease. By doing this there was the potential to implicate commensal bacterial species in the onset of DB giving an insight into the aetiology of the condition. However, analysis of the DGGE images of microbiota from the ilea and caeca did not reveal any significant differences in the profiles of intestinal microbiota that could separate the resistant and susceptible breeds. There were some minor differences in the microbial composition within the ileum of the four groups where specific DGGE bands varied in absence and presence. These differences led to a difference in species richness between some of the groups with commercial broiler breeds X and Y having a significantly higher mean band number when compared to the pure breed lines A and B. Conversely the mean band numbers were not significantly different between A and B or between X and Y, indicating that commercial broiler lines have a greater species richness in the small intestine than pure line birds. A similar difference is seen in the caeca where there is a lower mean band number in the caeca of the pure breed lines compared to the commercial broiler lines. Further analysis of the DGGE data with dendrograms and PCA revealed little clustering or segregation of any of the data points in the ileal microbiota relating to susceptibility or resistance. There was evidence of clustering of the caecal data however the segregation was based on whether the birds were pure breed lines or commercial broiler lines. It would appear that there are no obvious differences or similarities in the composition of the microbiota in healthy birds that can account for their susceptibility to enteric disease. It is possible that potential bacterial species which are involved in susceptibility are present at a level undetectable by PCR-DGGE or that they are a genotype specific strain of a particular bacterial species. The other possibility is that there is a link to total bacterial numbers rather than composition however

alternative methods to PCR-DGGE, quantitative PCR or bacterial cell counting would need to be applied to investigate this.

The results from this investigation demonstrated segregation of the commercial lines and the pure breed lines in terms of the composition of the intestinal microbiota where the commercial lines revealed a greater species richness within the intestinal microbiota. This inevitably gives rise to the question of why there are such differences between the pure and commercial lines; the answer probably lies in the nature of a commercial hybrid bird. Commercial broiler breeds have arisen from the selective breeding of a variety of chicken breeds to obtain the characteristics for optimal broiler production. The result is a specific line of hybrid chicken with a mixture of genes from a wide number of pedigree chicken genotypes. The process of genetic selection, as the name suggests, involves selecting birds for specific phenotypic attributes such as growth rates, feed conversion efficiency, egg laying ability etc. all of which are a result of the genotype of the particular bird. The result is a genetic line of chicken where each individual exhibits the same attributes based on their similar genetic composition i.e. there is limited genetic diversity within each breed line. The mechanisms behind why an individual genotype has a specific intestinal microbiota is still not fully understood and remains a key area of research for gut microbiologists. It is recognised that one of the downfalls of selective breeding for high yielding animals is the detrimental effects on the immune system and disease resistance (210, 316). It is possible that during genetic selection some of the mechanisms involved in maintaining intestinal homeostasis which are controlled by the host genotype are lost or compromised leading to increased susceptibility to enteric disease. It is well documented that there are links between host genotype and resistance to disease which are linked to the host immune system (13, 210, 411, 445). The main cause of differential resistance to infectious diseases is the genetically determined diversity of the immune system and its interactions with many physiological and environmental influences (157). The variation of immune response from one individual to another is linked to differences in the structural and functional diversity of the major histocompatibility complex (MHC), T-cell receptors (TcR), immunoglobulins, cytokines and other

proteins, which are encoded for by multiple and polymorphic genes (89, 157, 267, 445). For a long time, polymorphism of the MHC has been associated with disease resistance (40, 212). There are three classes of MHC in avian species with the MHC class IV being exclusive to birds (192, 335). The region encoding for MHC in chickens is small, at 92kb in length it is 5% of the size of the MHC encoding region of mammals. The nature of MHC expression in birds is very selective, there are 28 haplotypes of MHC in chickens with each haplotype expressing one dominant class I MHC molecule with differing levels of gene expression (39, 193). Birds with heterozygous MHC expression from two different alleles have an advantage over homozygous birds as they are able to express two MHC molecules. This highly selective nature of the MHC expression in the chicken is thought to be the reason for definitive cases of resistance and susceptibility to disease (191). The MHC has extensive regulation on many immune responses thus further implicating its role in the susceptibility and resistance to disease. MHC has been shown to be involved in antibody responses (98, 236), cytokine production (137), activity of cytotoxic and natural-killer cells (340), differential T cell proliferation (21, 380), chemotactic activity of mononuclear leukocytes and macrophages (145, 310). Linked to the MHC is the T cell receptors (TcR) which are the molecules present on T cells which recognise the antigen presented by the MHC. The region of the TcR which binds to the MHC is polymorphic resulting in each circulating T cell having a unique TcR. The variability of the TcR comes from the random reassortment of the genes that encode for the variable (V), diversity (D) and joining (J) segments of the TcR gene (78, 206). It has been demonstrated that the presence of certain polymorphisms and reduced diversity of TcR molecules have a link with disease susceptibility (297, 298, 327). The resultant TcR molecules expressed following V-D-J recombination influence the proportion and quantity of CD4⁺ and CD8⁺ T cells in the blood and peripheral tissues which has implications for immunocompetence (302, 430). Different genetic lines of chickens have been found to have different levels of antibody responses and are categorised as low antibody responders and high antibody responders (307). There is also differential resistance and susceptibility to various diseases relating to whether the bird exhibits as low or high antibody response (353). A bird exhibiting a high antibody response has a higher level of

CD4+ T cells whereas the low antibody responders exhibit a higher level of CD8+ T cells, with resistance depending on whether the pathogen in question is intra- or extracellular (302). The close relationship between the immune system and the intestinal microbiota is likely to result in differences in the intestinal microbiota of certain genotypes being due to polymorphisms in the host immune system. An unrelated study from within our laboratory by Dr Karyn Ridgway, demonstrated changes in the composition of the intestinal microbiota of between control and bursectomised chicks of the same breed. The Bursa of Fabricius, a primary immune organ of the chicken, is colonised by progenitor B cells during the development of the chick (279, 381). The immature B cells undergo gene recombination, differentiation and proliferation to result in a large B-cell repertoire which is important for the adaptive immune response (88, 318). Bursectomised birds have been found to have a much reduced B-cell repertoire but unaffected antibody levels in the serum (249). The mucus layer covering the intestinal epithelia contains a pool of IgA which is produced by the plasma cells in the mucosal lamina propria (358). The IgA opsonises both pathogens and members of the resident microbiota (168) therefore in the event of reduction in the diversity of the B cell repertoire it would not be unsurprising to see a change in the intestinal microbiota. In a further unpublished and unrelated experiment (Richard A. Bailey, unpublished results) it was found that the intestinal microbiota changed between wild type and transgenic mice (obtained from Prof. Simon Carding, IFR/UEA), where there was a reduction in T cell diversity and then further changes in the microbiota in the absence of $\gamma\delta$ T cell receptors (Figure 4.9). These experiments give an indication of the ability of the host mediated factors to control the composition of intestinal microbiota.

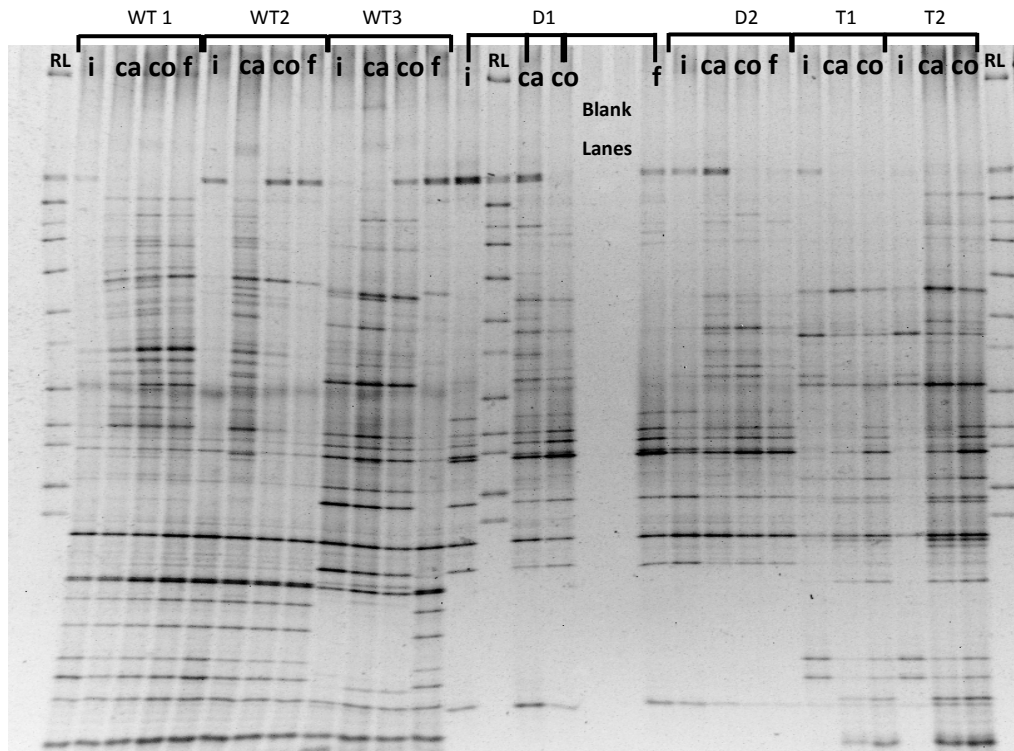


Figure 4.9 DGGE image showing the ileal (i), caecal (ca), colonic (co) and faecal (f) microbial fingerprints from three wildtype mice (WT), two $\gamma\delta$ T cell receptor knock out mice (D) and two OTII knock out mice (T) which have only one type of T cell. There are obvious visual differences in the DGGE profiles of the different mouse genotypes. The results indicate how altering the immune system of an animal can alter the microbial profiles. (RL = reference ladder).

A change in intestinal microbiota of individuals in relation to glycosphingolipid degradation has been demonstrated in humans. Bacteria isolated from individuals with type A histo-blood group would degrade the A antigen but not the B, and similarly bacteria from individuals with the type B histo-blood group would degrade B antigen and not A antigen indicating that there is selection of bacteria based on the nutrient environment provided by the host (170, 309).

The PCA plot from the ilea of the four groups of chicken (Figure 4.4), despite there being no specific segregation, showed a greater degree of clustering for the data points of breeds A and X – the more susceptible breeds. The greater degree of clustering of the data points from one particular breed is indicative of a low variation in band absence and presence from bird to bird, i.e. the microbial composition of that particular sub-population of bird is more alike. It could be speculated that the birds of breeds A and X have low diversity of immune cells dictating the gut microbiota leading to a reduced bacterial diversity between the

birds leading to the breed characteristic of susceptibility. Interestingly breed A is one of the pure breed lines used in the breeding program for breed X. It is possible that certain genetic traits of breed A relating to immunity and general gut health have been inherited by breed X resulting in the shared susceptibility to enteric disease. With the advent of single nucleotide polymorphism (SNP) analyses it is possible to map genetic variations associated with genes controlling immune function within a given population (414). SNPs are point mutations in an organism's genome (331) which can identify candidate genes in a case-control study (440). A case-control study is where you control a population of animals which differ in the factor being tested (437), such as gut health. A case-control study in which SNP patterns of broiler parent and offspring populations with either good or bad intestinal health could be performed to target candidate genes that are involved in intestinal health. The intestinal microbiota of the birds could also be profiled and the presence and absence of certain bacterial species could be correlated to the SNP patterns observed. Furthermore the innate immune system and intestinal microbiota exist in a complex relationship (52) and it has been suggested that over stimulation of the innate immune system could be involved in the onset of IBS in humans (69, 215, 246). The activity of the innate immune system in broilers has been profiled using gene expression levels of cytokines and chemokines (374). This approach could be used in the birds in the above case-control study which would enable the effects of genotype, intestinal microbiota composition and innate immune function on intestinal health. The parent and progeny innate immune profiles, SNP patterns and microbiota composition could be compared in order to investigate whether or not there is vertical transmission of genes involved in the establishment and maintenance of good intestinal health and a favourable immune system.

The ability of the susceptible birds to induce enteric disease in the more resistant birds could be due to a host derived factor or factors that are secreted into the gut lumen and then passed in the faeces into the litter. As chickens ingest litter it is highly likely that the resistant birds ingest the faecal material of the susceptible birds when placed in the same pen. Thus it is possible that this could cause an

upset to the enteric environment resulting in a loss of homeostasis and subsequent disease. There is a need for this work to be taken further by combining information from the microbiota, immune system and genetic make up of the birds to understand why there is a difference in susceptibility and resistance to enteric disease.

5. Investigations into the effects of diet on the intestinal microbiota of broiler chickens

5.1. Introduction

As outlined in section 1.3.4, dietary intake has a major influence on the composition of the intestinal microbiota of an animal. The microbial composition of the intestinal tract has been shown to be modified in response to changes in the carbohydrate source (6, 131, 139), fat source (76, 202) and protein source (93, 241). The shift in microbiota in response to diet is due to individual bacterial species having preferred dietary substrates (84, 244, 265) and also due to changes in the physiological conditions of the GI tract such as intestinal transit time and pH (131, 255). The investigations in this chapter demonstrate the affects of different diets on the composition of the intestinal microbiota in relation to gut health and DB.

5.2. Comparison of the GI tract microbiota of broiler chickens fed on diets differing in protein and energy density.

5.2.1. Background

There is anecdotal evidence that diets higher in protein density can increase the incidence of wet litter and DB, this investigation aimed to explore this hypothesis. In order to test the hypothesis two different diets were formulated. Diet A was a high protein diet with 265g crude protein/kg of feed (equiv of 120% standard diet) and Diet B was a low protein diet with 176g crude protein/kg of feed (equiv of 85% standard diet). As the diversity and composition of microbial communities change in response to dietary composition it is possible that a high protein environment promotes the growth of bacterial species which are detrimental the the gut health of the bird. Proteolytic bacteria such as *Clostridium perfringens*, a well recognised pathogen in poultry known for its role in causing necrotic enteritis (291), have demonstrated increased proliferation in birds fed a higher protein diet (93). A study by Lubbs *et al.* (241) in cats demonstrated that a diet high in protein resulted in an increase in *Clostridium perfringens* and a reduction in *Bifidobacterium sp.* In humans it has been demonstrated that a diet high in animal protein can result in

elevated *Bacteroides spp.* levels (248); it is possible that this phenomena could also be relevant in the case of broiler chickens. This investigation was aimed to look at the effects of protein density rather than protein source, therefore all the diets contained the same base substrates, namely wheat and oil for energy and a mixture of soybean meal and fishmeal for protein; the relative quantities of these products were altered to control the protein density but maintain the energy balance of the diet.

As this investigation was aimed to identify possible bacterial species involved with the onset of DB, the birds sampled in this trial were healthy and not showing any sign of intestinal disease.

5.2.2. Materials and Method

5.2.2.1. Sample Selection

Twelve male birds were selected at random from the groups of broilers fed on diets differing in protein density. All the birds were all housed in the same broiler shed with each group in individual pens with pen mates being fed on the same diet. The birds were all the same age (21 days), the same sex and the same genotype (Ross 308) to remove these factors as variables for changes in the intestinal microbiota. The birds had been fed either the high or low protein diet from hatch. Following selection, the birds were euthanised via cervical dislocation and the GI tract extracted and frozen immediately on dry ice. In line with the results from chapter three, sections were then taken from the ileum (5 cm section 10 cm caudal of Meckel's Diverticulum) and caecum and then stored at -20 °C.

5.2.2.2. DNA Extraction, PCR-DGGE, DGGE gel analysis and band identification

These were all carried out as described in sections 2.3, 2.4 and 2.5

5.2.3. Results

DGGE analysis of ileal microbiota

The ileal DGGE profiles of birds fed on diets A and B revealed very limited differences in banding patterns. Additionally further mean band number calculation, dendrogram construction and PCA analysis revealed no significant differences in the microbial composition or diversity in birds fed on the different diets. Figure 5.1 shows the DGGE profiles obtained from the comparison of the ileal microbiota of the birds fed on diets A and B. The results indicate that nutrient density does not affect the ileal microbiota of birds in this trial when DGGE is used for analysis.

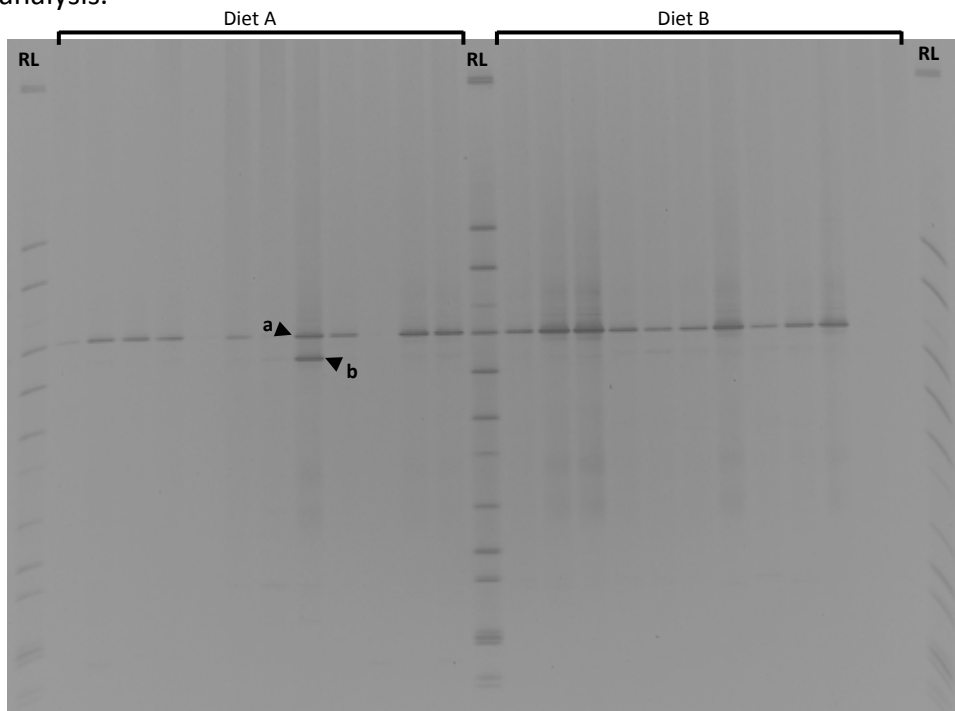


Figure 5.1 DGGE gel visualising DNA obtained from the ilea of the birds fed on diets A and B following PCR amplification of the V3 hypervariable region of the 16S rRNA gene. There are limited differences in the band patterning between birds. The bands present on the gel were sequenced and identified with the 16SrRNA database as being a) *Lactobacillus acidophilus*, *Lactobacillus crispatus* or *Lactobacillus fornicalis* (100%) b) *Lactobacillus salivarius* (100%) (RL = reference ladder). (See appendix II for a table linking all the bacteria identified in this thesis).

DGGE analysis of caecal microbiota

There were no significant differences in mean band number ($p>0.05$) between any of the diet groups indicating that changing the protein and energy density of the diet does not alter the species richness of the caecal microbiota when assessed with DGGE.

DGGE gel analysis

The image obtain from DGGE analysis of the caecal samples from diets A and B can be seen in Figure 5.2, where there are fluctuations in the appearance of specific bands.

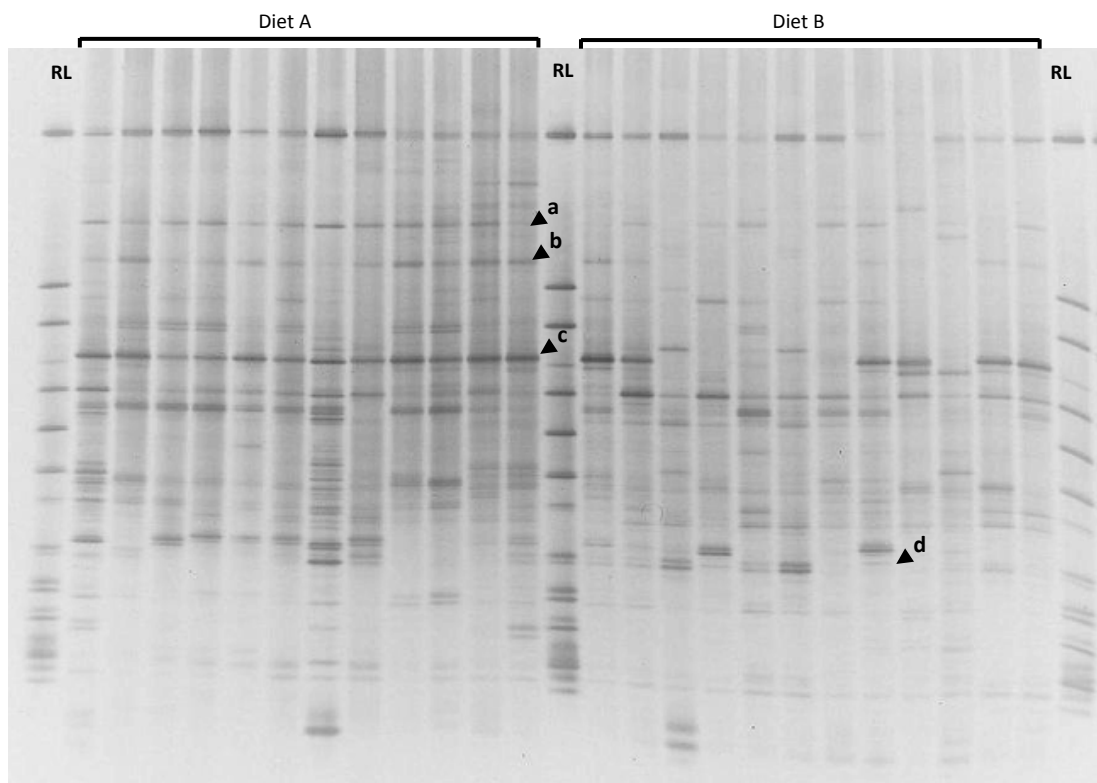


Figure 5.2 DGGE gel visualising DNA obtained from the caeca of the birds fed on diets A and B following PCR amplification of the V3 hypervariable region of the 16S rRNA gene. There are visual differences in the banding patterns and the bands of interest were identified with band sequencing and comparison to the 16S database a) *Bacteroides vulgatus* (100%) b) *Bacteroides ovatus* (100%) c) *Parabacteroides distatonis* (100%) and d) *Escherichia coli* (100%) (RL = reference ladder). (See appendix II for a table linking all the bacteria identified in this thesis).

Apart from a few outliers there was separation of the DGGE profiles for diets A and B when Dice similarity coefficient was constructed as a dendrogram (Figure 5.3).

This indicates that within the dietary groups there is a high degree of similarity between the DGGE profiles but there are distinct differences between the profiles from the birds fed on diet A and B. Further analysis with PCA (Figure 5.4) demonstrates separation which can be related to dietary treatment. PCA analysis of these data did reveal separation of the data points in relation to diets A and B (figure 5.3) thus indicating that altering the protein density of the diet does alter the caecal microbiota.

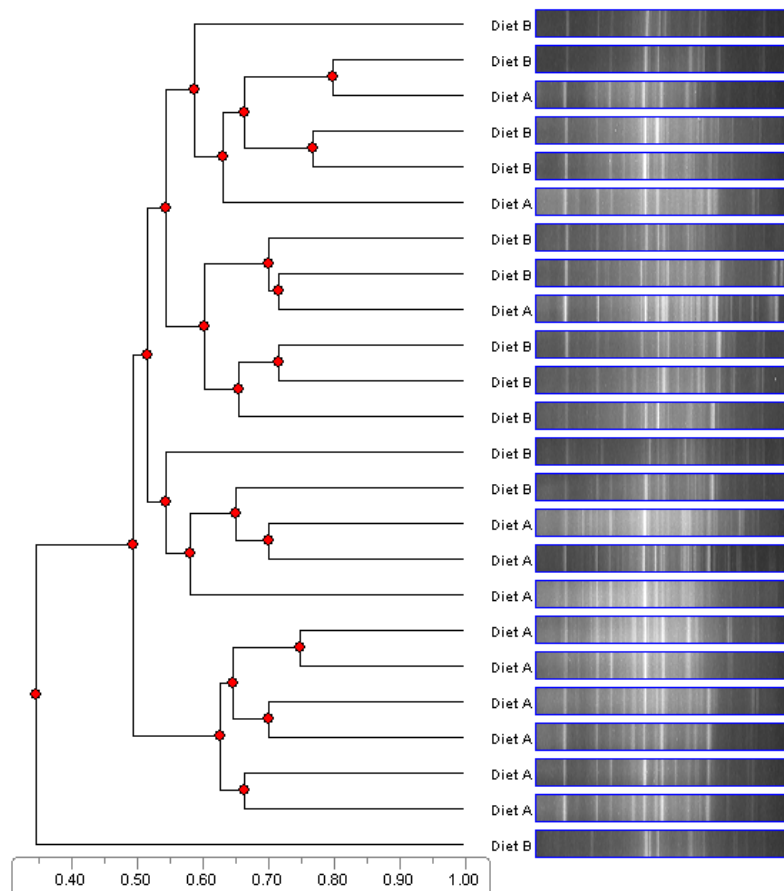


Figure 5.3 Dendrogram of the DGGE lane images in Figure 5.2; relative similarity of band patterns indicated by 0-1 coefficient bar (1=100% similar). There is separation of the DGGE profiles for from the caeca of the birds on diet A and B; indicating a change in microbial communities based on protein level.

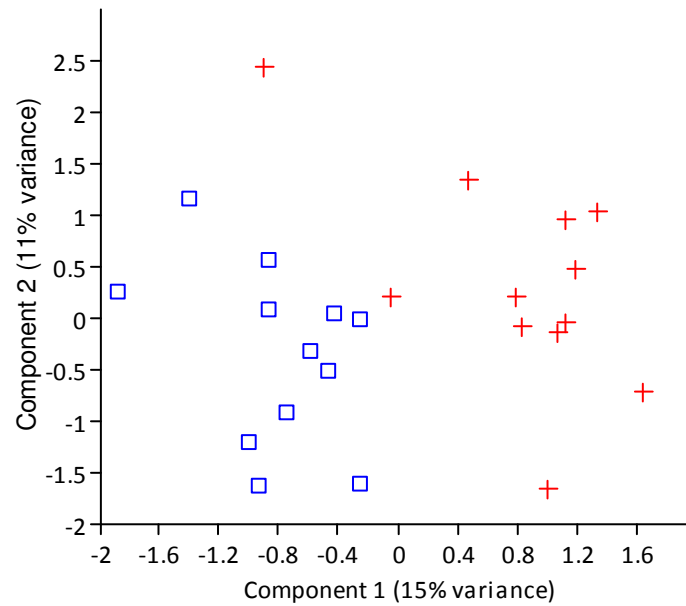


Figure 5.4 Scatter plot following PCA analysis of the data sets from caecal samples of birds on diet A (+) and B (□). In line with the dendrogram there is again separation of the two groups indicating a diet dependant shift in microbiota.

The major bands of interest were excised, reamplified, rerun on DGGE and then sequenced for bacterial identification. The results indicate more of *Bacteroides vulgatus*, *Bacteroides ovatus* and *Parabacteroides distatonis* in the caeca of birds fed on the higher protein diet. There is a minor increase in the presence of *Escherichia coli* in the caeca of the birds fed on diet B, the lower protein density diet. Overall the results indicate that altering protein density in the diet does not alter the composition of the ileal microbiota but does alter the composition of the caecal microbiota

5.2.4. Conclusions

This investigation was aimed to assess how changing the protein density alters the composition of intestinal microbiota of broiler chickens. As mentioned in previous parts of this thesis dietary compounds play an important role in driving the ecology of the intestinal microbiota. The mechanisms behind dietary influence are based on either individual bacterial species having a preferred substrate on a possibly restricted substrate diet (84, 244, 265) or altering the physiological conditions of

the gut making it more or less favourable to specific groups of bacteria (131, 255). The results indicated that there is no change in the composition of the ileal microbiota in relation to changing the protein density. Interestingly the diversity of bacterial species within the ilea of these birds was much reduced compared to that seen in the earlier studies within this thesis. The lack of species richness could be explained by the sensitivity of DGGE; in that it is only sensitive enough to detect bacterial groups which contribute to 1% of the total microbial population (284, 448). Therefore, it is possible that the lactobacillus population over shadows the more minor residents of the ileal microbiota. The minor bacterial groups may be undetectable by DGGE, thus differences between them in the high and low protein diet would not be detected in this study. The living environment is another factor which could have influenced the ileal microbiota in comparison to the analysis of birds used in the previous studies. The birds in this trial were housed in a high hygiene environment on a trial farm with increased biosecurity, whereas the birds in Chapters 3 and 4 were housed in less hygienic commercial environments. In a commercial environment the birds are exposed to a greater array of bacterial species which combined with standard biosecurity and higher stocking densities could leave the birds more open to the colonisation of less favourable bacterial species in the small intestine. Therefore it would be interesting to repeat this study in birds housed in an environment which bears more similarity to commercial production and analyse the composition of the ileal microbiota in a similar fashion.

Analysis of the caecal microbiota revealed that the differences in composition between birds fed on a high protein diet compared to those fed on a low protein diet. In the caeca of the birds fed on a high protein diet there is an increase in members of the bacteroidetes which is similar to the findings in Chapter 3 where an increase in members of bacteroidetes were found in the caeca of DB birds. The caeca is the recipient of excess nutrient which the ileum fails to absorb (188), therefore in a diet containing excess protein there will be an increased concentration of amino acids and proteins being filtered into the caeca available for use by the resident microbiota (187). Furthermore, excess protein which is absorbed from the small intestine is converted to uric acid which is excreted into

the cloaca and then taken up into the caeca via the colon by retroperistalsis (35, 95). Therefore it may be deduced that bacterial species which have proteolytic and ureolytic activity are likely to have increased growth when present in birds fed on a protein rich diet. The bacteroides group of bacteria typically represent the major component of the caecal microbiota of a broiler (431) and members of this group are known to have proteolytic (242, 319) and ureolytic (121, 359) capacity; this could therefore explain the increase in number of the bacteroides seen in the birds fed on the higher protein diet. Proteolytic and ureolytic bacterial species produce potentially harmful substances such as ammonia, amines, phenols and indoles which can be potentially harmful to the enteric environment (131, 141, 229). In addition to a change in substrate concentration in the intestinal lumen it has also been demonstrated that protein content of the feed influences the retroperistaltic waves which occur in the avian digestive tract (35). Waldenstedt and Björnhag (413) revealed that a low protein diet resulted in more intense retroperistaltic transport of urine and thus colonic contents into the caeca. The consequence of this would be an increase in the throughflow of caecal contents of birds fed a lower protein diet making the conditions more favourable for the faster growing bacterial species such as *Escherichia coli* which appeared more frequently in the DGGE profiles of the birds fed on a low protein diet.

It can be concluded from this investigation that diets differing in protein density do result in a differential composition of the intestinal microbiota. The ileal microbiota remained constant in response to dietary protein density. As discussed previously, this could be due in part to the environment in which the birds were kept or that the lactobacillus population in the ileum was well established enough that perturbations in the less dominant bacterial species in the ilea were kept under tight control via a competitive exclusion type mechanism. It is worth bearing in mind that these birds were fed on the low or high protein density diets from hatch so this investigation essentially explores the effect of continual feeding of a high or low density protein diet. Perhaps a future investigation in which the effects of changing from a high protein to lower protein diet and *vice-a-versa* in a more challenging environment would reveal a different picture in terms of gut health,

intestinal microbiota composition and the onset of DB. Perhaps if there is an unstable ileal microbiota or disruption to the ileal mucosa, possibly due to a low level coccidiosis or viral infection, opportunistic pathogens such as *Bacteroides sp.* can establish resulting in DB. Broiler chickens are fed a high protein diet in order to maintain good muscle growth so perhaps there is a need to investigate methods of controlling the growth of less favourable species of bacteria associated with high protein diets and implicated with the mechanism of DB by use of in feed additives such as prebiotics and probiotics.

5.3. Comparison of the intestinal microbiota of broiler chickens fed on either a vegetarian diet or a standard broiler diet

5.3.1. Introduction

During routine industrial surveying by Aviagen Ltd., ten flocks of Ross 308 broiler were examined from seven different broiler farms across the UK. It was observed that the birds examined from two of the flocks had much better gut health in terms of intestinal tissue and digesta consistency following visual inspection during *post mortem* examination. The intestinal tracts of the birds from these two flocks mentioned had much better intestinal integrity based on the muscle tone and strength of the gut wall combined with a healthy pink colour on the intestinal pink surface. The intestinal integrity of the birds from the remaining eight flocks was varied with poor smooth muscle tone, increased vascularisation (such as that seen in birds with DB as described in Chapter 3) and a more reddened unhealthy looking mucosa was also apparent. The Bursa of Fabricius was also examined in the birds from all ten flocks and was found to be visually much larger in the birds from the two flocks with better gut health. The size of the Bursa of Fabricius is a good indicator of overall bird health; stressed or sick birds will have a smaller Bursa in comparison to healthier birds. In addition to this, the litter quality of these two flocks was much improved in comparison to the remaining eight flocks. The question then arose as to why there was such a dramatic difference in the gut health of these birds. One key feature of the two flocks with healthier intestinal

health is that they were both on a farm where, due to contractual obligations, there was a requirement to feed the birds on a completely vegetarian diet. This meant that there was no fishmeal (a standard protein source in broiler diets) present; instead the main protein source for the diet was from the inclusion of soybean meal. The previous investigation in this chapter demonstrated how protein density can alter the caecal microbiota and it is known that protein source can alter the composition of the microbiota. For example, Drew *et al.* (93) demonstrated that *Clostridium perfringens* populations were higher in the ilea and caeca of broilers fed a fishmeal based diet compared to birds fed a soybean based diet. It is therefore possible that the intestinal microbiota in the birds fed on a totally vegetarian diet have a different microbiota resulting in better overall gut health. This investigation aims to characterise the composition of the intestinal microbiota of birds from all ten flocks using PCR-DGGE and relate the findings to the different diets administered.

5.3.2. Materials and Methods

5.3.2.1. Sample Selection

Ten female birds were selected at random from the ten different flocks of broilers (Table 5.1). Following selection the birds were euthanised via cervical dislocation and 5 ml contents (including mucosal scrapings) from the ileum were collected and frozen immediately on dry ice.

Flock	Farm Location	Diet type
1	Suffolk	Standard
2	Suffolk	Standard
3	Cambridgshire	Standard
4	Nottinghamshire	Standard
5	Wiltshire	Standard
6	Wiltshire	Standard
7	Herefordshire	Vegetarian
8	Herefordshire	Vegetarian
9	Lincolnshire	Standard
10	Lincolnshire	Standard

Table 5.1 Description of farm location and diet type of flocks used in this study

In addition 2ml of faecal matter was collected from each bird and frozen immediately for metabolite analysis (discussed in chapter 6)

5.3.2.2. DNA Extraction, PCR-DGGE, DGGE gel analysis and band identification

These were all carried out as described in sections 2.3, 2.4 and 2.5

5.3.3. Results

DGGE analysis of ileal microbiota

The ileal samples were loaded onto five different DGGE gels to reveal differences between the banding patterns and thus microbial profiles. Mean band number revealed differences in species richness in some of the groups of birds as seen in figure 5.5. There doesn't appear to be any correlation between the mean band number and the dietary type.

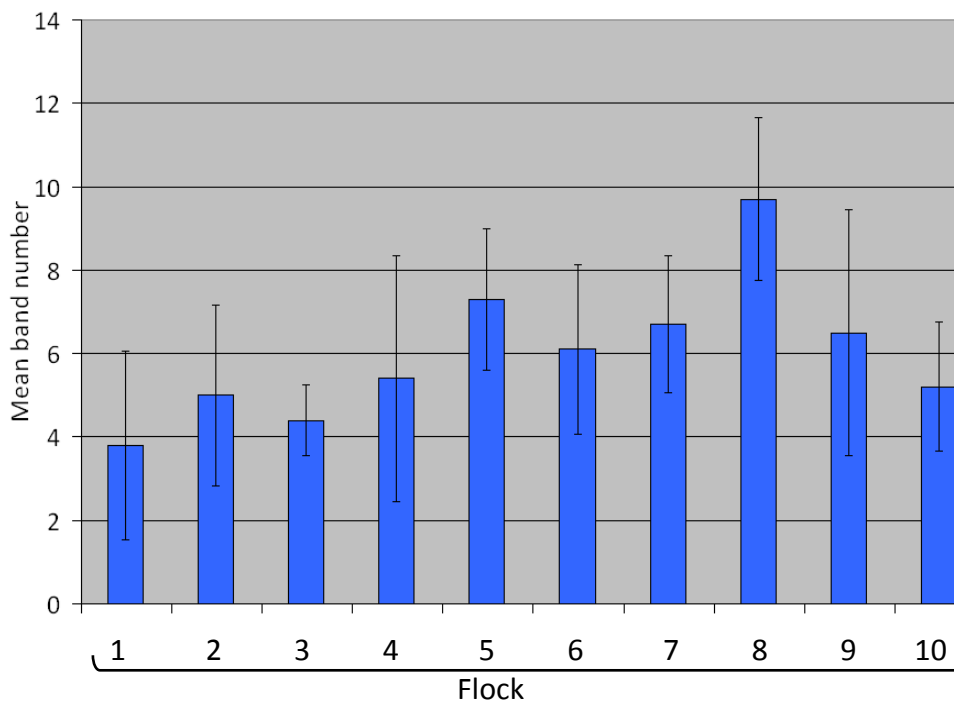
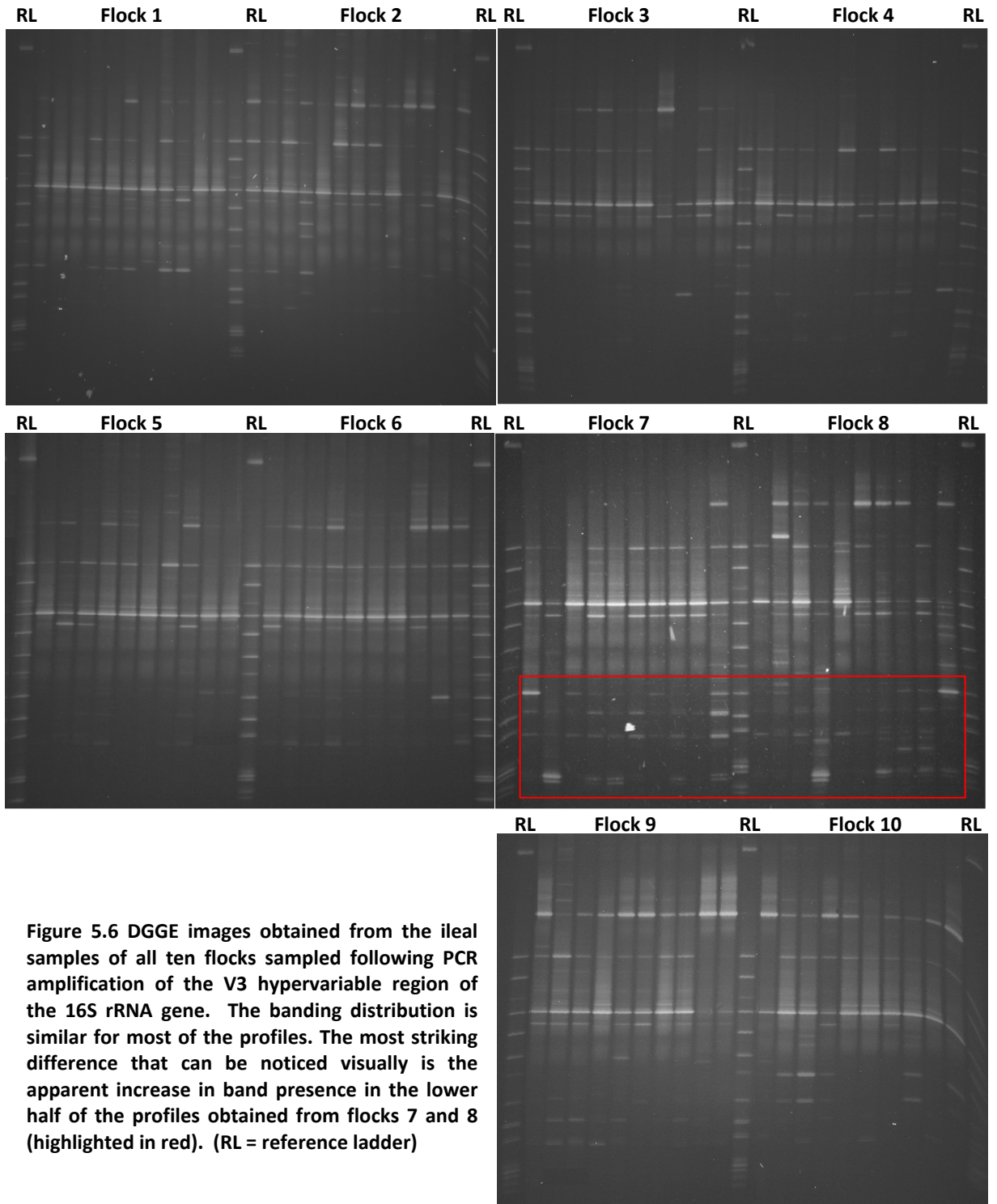


Figure 5.5 Mean DGGE gel band number for the ileal samples from each flock of birds. There doesn't appear to be any correlation between mean band number and dietary type. Bars represent standard deviation.

Figure 5.6 represents the DGGE images obtained from DGGE analysis of the ileal samples. The most striking difference seen is the increased band presence in the lower part of the DGGE profiles for the samples from flocks 7 and 8.



In order to compare the position of the common bands across all the profiles the samples were pooled from each flock and then run side by side for comparison

(Figure 5.7). This revealed that the majority of bands in all these samples aligned, thus indicating common bacterial species across all the gels. Following this the DGGE data was aligned to allow a PCA plot to be constructed containing band presence and absence matrices from all 100 birds in the study (Figure 5.8)

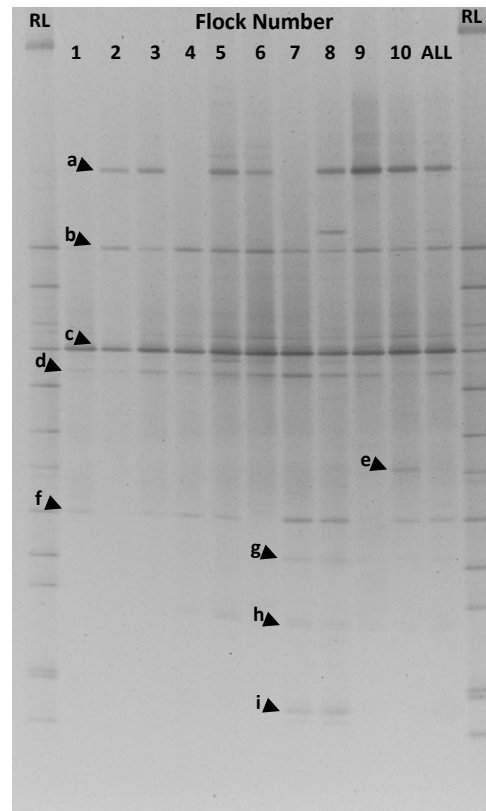


Figure 5.7 DGGE gel visualising pools of DNA obtained from the ilea of the ten flocks (1-10) of birds and a pool of all flocks together (ALL) following PCR amplification of the V3 hypervariable region of the 16S rRNA gene. The bands present are common in the majority of profiles there are some bands present in one a small number of pools. All common bands were identified via band sequencing and the 16S database a) *Lactobacillus aviarius* (100%), b) *Lactobacillus johnsonii* (100%) c) *Lactobacillus crispatus* or *Lactobacillus fornicalis* or *Lactobacillus helveticus* (100%) d) *Lactobacillus salivarius* (100%) e) *Campylobacter jejuni* (96%) f) *Escherchia coli* (100%), g) *Lactobacillus sp.* (100%) h) *Clostridium sp.* (100%) and i) *Bifidobacterium sp.* (100%) (RL = reference ladder). (See appendix II for a table linking all the bacteria identified in this thesis).

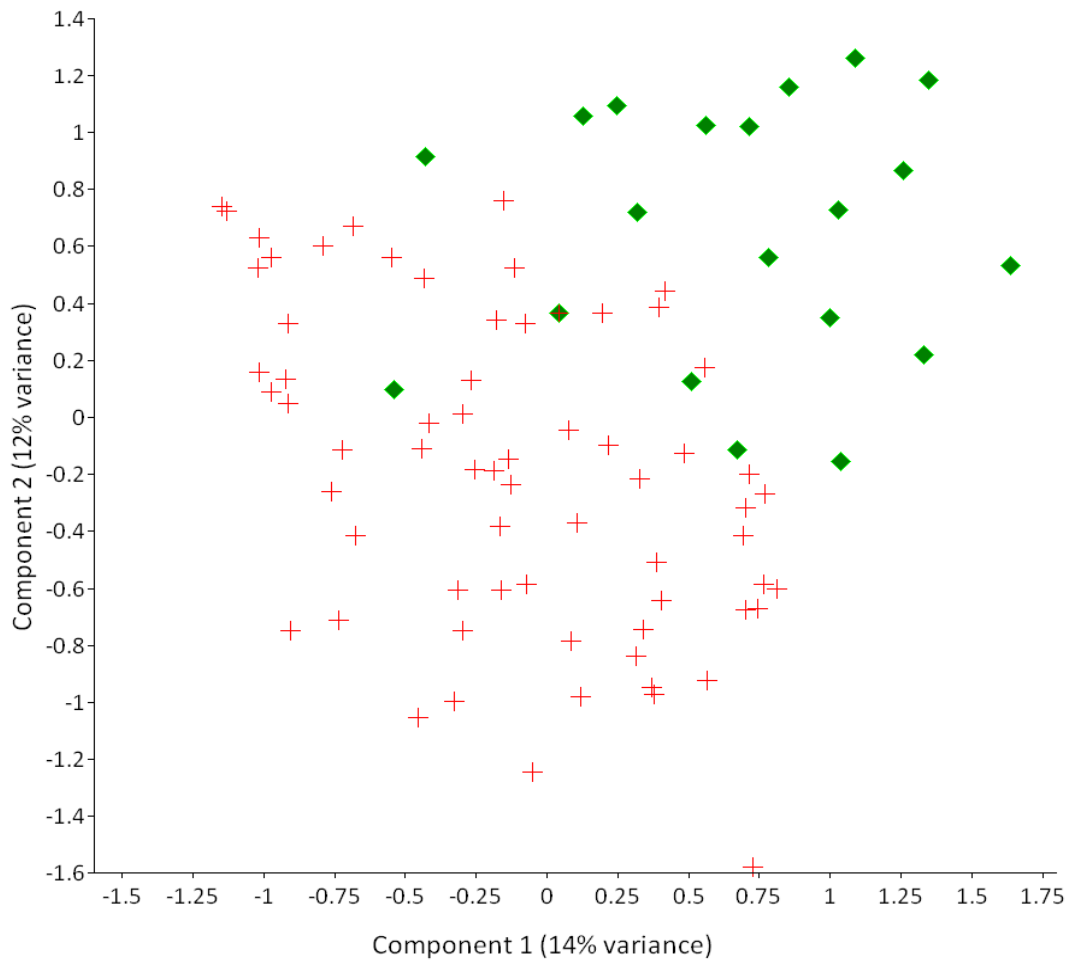


Figure 5.8 Scatter plot following PCA analysis of the combined data from all ten flocks (◆ VG, + SD). There is segregation of the data points indicating variance between the data points based on which diet type the birds were fed.

5.3.4. Conclusions

This study was aimed to further investigate the effects of diet on the intestinal microbiota. As stated previously it is known that dietary components can shape the composition of the intestinal microbiota. Visual differences in the health of the gut between the two groups of birds, those fed on either a vegetarian diet or a standard broiler diet containing fishmeal during an industrial survey instigated this investigation. The hypothesis put forward was that feeding birds a completely vegetarian diet resulted in more favourable intestinal microbiota resulting in improved intestinal health. Mean band analysis didn't reveal any correlation between mean band number and dietary type, thus indicating that dietary type doesn't necessarily increase species richness in the ileal microbiota.. Visual

inspection of the all the DGGE gel images revealed that the birds in flocks 7 and 8 (the vegetarian diet flocks) had a greater presence of bands in the lower part of the gel in comparison to the other profiles and PCA analysis indicated separation of the VG diet birds away from the SD diet birds. The common bands of interest were identified as a *Lactobacillus sp.*, a *Clostridium sp.* and *Bifidobacterium sp.*, the first two species were found in the DGGE profiles of some of the birds in the other flocks however the latter species was only present in flocks 7 and 8. Lactobacilli and bifidobacteria are well documented to have beneficial effects on intestinal health and many are included in probiotic supplements in human and animal nutrition (305, 365). Vegetable and cereal crops such as soybean meal contain a high proportion of indigestible matter in the form of plant oligosaccharides, hemicelluloses and pectins (166, 406) which act as prebiotics. Prebiotics are defined as 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health' (140). The increased presence of lactobacilli and bifidobacteria in the birds fed on the vegetarian diet suggests that the VG diet provides a prebiotic substance preferentially utilised by the lactobacilli and bifidobacteria. Therefore, due to the better intestinal health of the birds fed the VG diet one could deduce that a diet that results in an increased presence of these bacterial species in the intestinal microbiota of the birds is beneficial to intestinal health.

Alternatively, the differences in intestinal health seen in these birds could be related to the effect of fish meal on the physiology of the intestinal tract itself. There is evidence that fishmeal can be detrimental to intestinal health in poultry. Histamine and gizzerosine (a derivative of histidine and histamine) are found in varying levels in fishmeal and have been implicated in the onset of gizzard erosion in broilers (156, 180, 254, 384, 390). Excessive stimulation of histamine receptors in the proventriculus can result in excessive acid secretion and the intestine becoming dilated and filled with watery contents (350). The reduction in pH due to the acid can lead to a reduction in digestive enzyme activity and malabsorption (164). Gizzard erosion has been found to correlate with increased cell counts of

Clostridium perfringens in the intestinal tract of broilers (294). A study in laying hens found that gizzerosine induced inflammation and necrosis in the duodenum in birds fed experimental diets containing gizzerosine (8). It is therefore possible that high levels of fishmeal in the diets of broilers causes a disturbance in intestinal homeostasis allowing other opportunistic pathogens such as members of the bacteroides group within the resident microbiota to proliferate and cause enteric disease. It has been demonstrated that polyunsaturated fatty acids (PUFAs) affecting the growth and adhesion of lactobacilli (186, 190). However a study by Geier *et al.* (138) found that addition of dietary omega-3 PUFAs did not alter the intestinal microbiota of broilers. Work carried out at the IFR (Dr Kerry Bentley-Hewitt, unpublished results) demonstrated a reduction in growth of an isolate of *Lactobacillus johnsonii* from chicken caeca in the presence of n-3 PUFAs derived from fish oils. It is therefore possible that inclusion of fishmeal in the diet of broiler chickens is detrimental to the lactobacillus population along the GI tract, resulting in a reduced potential for competitive exclusion by lactobacilli. There is also the possibility that fishmeal is not compatible with the GI tract of chickens and the associated microbiota meaning that more suitable dietary substrates are needed or feed additives that counteract the negative effects of fish meal may be required.

5.4. Discussion

Diet has for many years been implicated as an important factor for enteric health in broilers. High protein diets have been shown to be associated with greater incidence of intestinal disease in poultry such as necrotic enteritis (233) and coccidiosis (348). Many bacterial species such as the clostridia and bacteroides have proteolytic capability, thus in a diet high in protein these bacterial species will utilise the protein which may result in the formation of compounds which are detrimental to intestinal health (131, 141, 229). A study by Lhost *et al.* (221) showed that feeding rats a diet higher in protein resulted in higher levels of isobutyrate, valerate and isovalerate in the colonic contents when compared to rats fed a lower protein diet. These compounds are indicators of proteolytic activity by bacteria. Furthermore it was shown that higher levels of these compounds correlated with elevated pancreatic enzyme activity. Vieira and Lima (406)

examined the effect on water intake, live performance and excreta characteristics of broilers when fed vegetarian or fishmeal based diets. They found that birds fed a vegetarian diet grew at a similar rate to the birds fed on the fishmeal based diet but they produced greater amounts of faeces and drank larger volumes of water. These two studies along with the work performed in this thesis highlight that changing nutrient density and source alter a variety of processes in the intestinal tract such as host enzyme production and feed passage times as well as the intestinal microbiota. Despite both the investigations in this chapter revealing that changing the density and source of nutrients in a broiler diet alters the intestinal microbiota, neither the different diets induced the onset of DB. This indicates that there are other factors involved with the onset of the disease. During the life of a chicken, the diet it will receive will vary in nutrient quality and density due to seasonal variation in the source and quality of feed raw materials. For example, there is anecdotal evidence from veterinarians and poultry farmers that the incidence of DB increases when the feed changes from the inclusion of winter wheat cultivars to spring wheat cultivars (Philip Hammond, Crowshall Veterinary Services, personal communication). Typically spring wheats have a higher protein content than winter wheats due to the higher yields of winter wheats which in effect reduces the protein relative to the amount of starch (Prof Julian Snape, John Innes Centre, personal communication). With this mind it is possible that changing the wheat source in the broiler diet alters the intestinal physiology and associated microbiota in response to increased protein density and decreased fibre.

The birds suffering from DB housed a different microbiota in both the small intestine and the caeca where as the birds fed on diets of differing protein densities had only different caecal microbiota. During periods of fasting (such as in periods of darkness) it has been found that the intestinal tract of chickens had enhanced retroperistaltic activity; known as a rhythmic oscillating complex (ROC) (61, 183, 323). It has been suggested that ROCs re-establish the motility patterns in the GI tract associated with satiety whilst also enabling the birds to recycle food from the caeca or distal intestine maximizing their nutrient resources during fasted periods (61). Therefore during periods where the birds are not actively feeding the caecal

bacteria can enter the proximal intestine, this could give an opportunity for the opportunistic bacteria to colonise the small intestine. This study indicated that omitting fishmeal from the diet of broilers appeared to improve intestinal health when compared to birds fed only soybean meal; this could imply that fishmeal diets (or those higher in amines such as gizzerosine) predispose broilers to poor enteric health in the small intestine making them more sensitive to perturbations in the composition of the intestinal microbiota. It could be proposed that the mechanism of DB involves firstly a disruption to the small intestinal homeostasis which either perturbs the resident microbiota making it unstable or there is a disruption to the intestinal mucosa. Secondary to this an event occurring such as a change in dietary composition may lead to the opportunistic members of the resident microbiota in the caeca being able to proliferate and enter the small intestine thus setting off a cascade which results in the onset of increased microbial growth and DB.

The birds in the studies in this chapter were fed one diet only, so the effect of changing the diet of a bird was not fully explored. Further studies investigating how the intestinal microbiota of a bird changes when it is fed a diet of one type (e.g. high protein or fishmeal based) and then another diet (e.g. a low protein diet or vegetarian diet) may reveal more information on dietary effects on the intestinal microbiota. To take this hypothesis further, in addition to the change in intestinal microbiota, the effect of changing diets on the intestinal physiology, gut mucosa and intestinal metabolites should be investigated. This would give a clearer picture on the mechanisms involved.

6. Metabolic profiling of intestinal contents of broiler chickens using proton nuclear magnetic resonance

6.1. Introduction

The intestinal microbiota of an animal plays an important role in the health and wellbeing of the host and is a great focus of interest due to the role it plays in digestion, protection from pathogens and development of the immune system (150, 167, 169, 402, 452). With the advent of molecular based tools microbiologists have been able to gain much more accurate insight into the composition of the intestinal microbiota and characterise the species of bacteria present (5, 12, 451, 452). It is well documented that the composition of the intestinal microbiota changes in relation to age, dietary intake and the health status of an animal (5, 131, 240). However, the biochemical mechanisms that result from the changes in the microbiota composition remain undefined (423). Which therefore begs the question of what these bacterial species are doing and are they good or bad for the health of the host (3). It is therefore imperative that in addition to profiling the complex microbiota we assess the metabolic activity of the intestinal microbiota in parallel. Metabolite profiling is such a tool that can start to answer these questions; as characterising the metabolic profile in a biological sample provides a snapshot of ongoing biological processes, which via the presence of a particular metabolite, panel of metabolites, or a certain ratio of metabolites can indicate normal homeostasis, a response to biological stress, or even a specific disease state (347). Wang *et al.* (417) demonstrated differences in the metabolite profile of different regions of the humans GI tract, where the differences seen could be attributed to functional alteration in energy metabolism, osmoregulation, microbial activity and oxidative protection. Martin *et al.* (252) took this idea further by comparing the metabolite profiles of the duodenum, jejunum, ileum, proximal and distal colon of conventional mice with germ free mice which were either inoculated with the microbiota of conventional mouse or of human baby intestine. Each intestinal region exhibited a specific metabolite profile altered by the microbiota present indicating the influence of the microbiota on the metabolome. Wikoff *et al.* (423) explored the effect of the intestinal microbiota on the level of blood

metabolites by comparing plasma metabolite concentrations in conventional and germfree mice. The results they obtained indicated that the presence of bacteria in the intestinal tract resulted in a vast increase in metabolites in the blood plasma. These studies together demonstrate the influence of the presence and composition of the intestinal microbiota on host biochemistry and metabolism. The microbiota of the GI tract derive most of their energy from the host diet via fermentation (5, 73). The resultant metabolites, such as SCFAs, can be utilised by the host and have a positive effect on the physiology and biochemistry of the intestinal tract (243, 314, 328, 436). However, the intestinal microbiota has been linked to the onset of both systemic (363, 392) and intestinal (15, 250, 299, 392) disease. By applying a metabolic approach to the study of disease there are implications that changes in metabolites are associated with intestinal disease, such as a decrease in urinary hippurate in sufferers of Crohn's disease (426). A study by Martin *et al.* (251) provided evidence of altered energy metabolism, fat mobilisation and amino acid metabolism as a result of hyper contractility and hypertrophy of intestinal smooth muscle related to post infective irritable bowel syndrome (IBS). Furthermore these shifts in metabolites and symptoms of IBS were reduced by the administration of a probiotic in the form of *Lactobacillus paracasei* which had previously demonstrated an ability to normalise the abnormal muscle characteristics seen in IBS (404). A study by van Nuenen *et al.* (400) implied that microbial metabolites could be involved in the induction of local immune responses in the event of a loss of epithelial integrity in the intestinal mucosa. These studies all highlight the influence of the intestinal microbiota and their metabolites on host health and give insight into the possible mechanisms involved in the pathogenesis of gut disorders.

In Chapter 3 a difference in the composition of microbiota was described in the ilea and caeca of birds affected with DB compared to the microbiota of healthy birds. Similarly in Chapter 5, an improvement in intestinal health combined with an alteration in intestinal microbiota composition was noticed in birds fed a vegetarian diet compared to a standard diet. By adopting a metabolomic approach, this chapter focuses on characterising the metabolic profiles of the intestinal samples of the birds from the studies in Chapters 3 and 5. Where possible this chapter aims to

link changes in the metabolite profiles obtained to the changes in intestinal health and fluctuations in the composition of the intestinal microbiota. Furthermore common shifts in the metabolite profiles in relation to intestinal health status and DB could identify possible biomarkers that can be used for monitoring gut health and identifying periods of enteric upset so that intervention strategies can be implemented in a timely manner

6.1.1. Methodology

6.1.1.1. Principles of NMR

The most common methods used in metabolomics are outlined in a review by Nicholson and Lindon (290), where they state that in disease studies, gas chromatography coupled with mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) are the most commonly used methods in metabolomic studies. The former usually requires separation of the metabolites from biological fluids with HPLC (high performance liquid chromatography) and is more sensitive, however the sample preparation can result in the disruption of the structures and interactions of molecular structures. NMR on the other hand is sensitive enough to detect metabolites unmodified in bodily fluids, whilst at the same time providing a greater level of accuracy when quantifying metabolites. By comparing the resultant NMR spectra from control and test individual (such as healthy and DB birds) key metabolites can be identified. Each individual molecule has a specific NMR spectra related to its behaviour in the presence of electromagnetic radiation, which is dependant on its molecular structure. The nucleus of an atom can be considered as a charge in rotational motion. Atoms of hydrogen (^1H) are considered to behave like bar magnets when placed within a strong electromagnetic field (43). The nuclear spin of an atom will align with or against the field and are said to be in a high energy state or a low energy state respectively (43). There is equilibrium between the nuclei in the high or low energy states but this equilibrium can be altered in the presence of increasing magnetic radiation. By increasing the magnetic radiation it is possible to excite the nucleus so that it switches energy state from low to high. Following excitation, as the magnetic field returns to its baseline the equilibrium between high and low energy states returns to the original

level at which point the peak for that individual atom is measured. The position of the peak, or chemical shift as it is termed, is influenced by the chemical environment of the atoms (such as ^1H) being measured which is defined by the neighbouring atoms and associated chemical bonds. Thus each ^1H atom residing in a different compound will have a different chemical shift resulting in multiple peaks in a sample containing many compounds. Additionally as many compounds have more than one chemical environment, multiple peaks may come from the same compound which must be addressed when analysing the sample. In order to have a baseline so that many samples can be compared, a reference compound with a chemical shift value of 0 is used and the chemical shift of signals in the spectrum in relation to the reference compound is calculated and measured in parts per million (ppm). This then enables multiple spectra to be compared and contrasted in order to identify peaks which differ from spectrum to spectrum. This technique is quantitative as the strength of the NMR signal correlates to the concentration of a compound. Therefore variations in the presence and relative concentration of compounds can be measured and related to the samples being examined. One of the major benefits of this technique is that no prior knowledge of the chemical composition of the sample is required as the chemical shift of all compounds in the sample will be measured and also requires very little sample preparation time (290).

6.1.1.2. Analysis of NMR data

The resultant NMR spectra from these investigations are complex due to the large number of compounds found within the intestinal tract therefore multivariate statistical analysis must be used (290). Chemo metrics is the name that has been given to the application of a range of statistical techniques favoured for use in analysing highly dimensional data such as NMR spectra (197). The most common statistical method applied to NMR spectra is Principal Component Analysis (PCA) which as mentioned previously in this thesis takes multidimensional data and converts it into two dimensional data in order to easily identify patterns and relationships in the data set (196). Another method used to compress the multivariate data is partial least squared (PLS) analysis which classifies data into sets

by combining the features of the data in a linear fashion often with a discriminant analysis (197). Both PCA and PLS have their limitations. PCA displays the information related to the data matrix that contains the largest amount of variability. Sometimes, the variance between two groups of samples (for example healthy and DB birds) does not necessarily show up due to the way the PCA factors are calculated. An alternative to this is to provide a second matrix of information which places the samples into classes and performs the multivariate calculations using both the data matrix and the second matrix. This means that the PLS factors produced are associated with the variance between the sample groups, however this approach can result in over fitting of the PLS model. However, validation of the model with cross-validation overcomes this problem. This approach leaves a sample or a subset of samples aside to test the PLS model independently. Another way is to use two third of the samples to build the PLS model and use the remaining third to test it. Variations include a sampling set (used to build the model), a tuning set (to evaluate the robustness of the model) and a test set (to truly test the model). Iterations are often used (the test set consisting of one or several samples and is iteratively reshuffled). For example, it is possible to construct ten PLS models and obtain an average percentage of success of sample reclassification based on ten different test sets.

6.1.1.3. Materials and method

Sample preparation

50 mg of frozen intestinal sample was mixed with 1 ml of NMR buffer which contained internal reference markers TSP (sodium 3-(Trimethylsilyl)-propionate-d₄) 0.5 mM and DFTMP (difluorotrimethylsilylphosphonic acid) 0.5 mM in phosphate buffered saline (8.1 mM NaH₂PO₄, 1.9 mM Na₂HPO₄, 150 mM NaCl) made up with 100% D₂O as described by Saric *et al.* (338). Following mixing the sample tubes were centrifuged at full speed (14000 g) for 1 min to separate the debris. The resulting supernatant in each tube was removed and filtered via 0.45 μm membrane (Sartorius, UK) into clean 1.5 ml tubes. 650 μl of the filtrate was transferred to a 5 mm glass NMR tube for ^1H NMR analysis.

NMR recording and data processing

The ^1H NMR spectra were recorded at 27°C on a 600 MHz BRUKER spectrometer fitted with an auto-sampler at IFR. The D_2O present in the buffer acted as the internal lock. Each spectrum consisted of 64 scans of 32768 complex data points with a spectral width of 8389.262 Hz. The *noesypr1d* pre-saturation sequence was implemented to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay (2 sec). Spectra were Fourier transformed with 1 Hz line broadening, automatically phased and manually baseline corrected using the TOPSPIN software. Spectra were converted to ASCII file for statistical analysis using Matlab®, version 6.1 (The MathWorks Inc, Natick, Massachusetts, USA).

Data acquisition and analysis

In order to analyse the NMR data, the chemical shifts of the spectra were allocated into variable width buckets (or bins) along the horizontal axis and the intensities within each bucket were calculated using the Bruker AMIX software v3.9. Background noise and water resonance was removed from the spectra to normalise it, this was performed using the AMIX Underground removal tool present in the software. Following normalisation the intensities of the buckets were assembled individually as rows using Microsoft Excel as a table. This allowed multivariate statistical analysis using PCA and PLS-DA) to be performed via the PLS Toolbox (Eigenvector Research Inc., Wenatchee, Washington, USA) and scripts written in house at the IFR (Dr Kate Kemsley) which ran in Matlab®, version 7.6 (The MathWorks Inc, Massachusetts, USA). The significance of the differences between variables (chemical shifts or bins) was calculated in Excel which produced p values for all the variables. The most significant differences could be found and then the metabolites responsible for those bins could be identified.

Identification of metabolites

Following the allocation of the chemical shifts into bins and the sorting of the bins in terms of significant differences the metabolites of interest could be characterised

based on their chemical shifts. Due to the complex nature of some of the compounds present some of the NMR peaks were subject to coupling where peaks can form multiplets rather than a single peak. This occurs when neighbouring nuclei mutually influence the spin of each other. By combining the chemical shift and coupling constants, metabolites can be identified using published values in papers (32, 182, 338) and on the web (Human Metabolome Database, <http://www.hmdb.ca/>).

6.2. Comparison of the metabolites in faecal water of broiler chickens fed on either a standard diet or a vegetarian diet using ^1H NMR.

6.2.1. Background

This investigation was a continuation of the study performed in section 5.3 in which the composition of the small intestinal microbiota of birds from ten different broiler flocks (ten birds from each flock) was compared. Improved intestinal health was observed in two out of the ten flocks in comparison to the other eight flocks. These two flocks had been given a completely vegetarian diet containing soybean meal as opposed to the standard broiler diet containing fishmeal on which the remaining eight flocks were fed. PCR-DGGE revealed a shift in the composition of the ileal microbiota in the birds fed a vegetarian diet where an increased presence of a *Lactobacillus sp.* and a *Bifidobacterium sp.* was observed. Members of these bacterial genera are known for their positive influence on intestinal health and are commonly used as probiotics (128, 365) and could therefore be contributing to the improvement in intestinal health observed. Furthermore the potential deleterious effects of fishmeal on intestinal health were discussed and the possibilities of subsequent damage to the mucosal epithelia which could be highlighted by a change in the metabolite profile. Different microbial species produce a range of metabolites (174, 306) thus change in composition of the intestinal microbiota is likely to be reflected in a change in metabolite profile. By adopting a metabolomic approach with ^1H NMR, this investigation aimed to characterise and compare the metabolite profiles of faecal samples of the birds from all ten flocks in order to unravel the mechanisms behind the apparent difference in intestinal health and

microbiota composition. Furthermore this approach aimed to identify metabolites that could be used as potential biomarkers for indication of gut health in the live bird could therefore be used as a monitoring tool.

6.2.2. Materials and Method

Sample Selection

As described in section 5.3.2.1, faecal matter was collected from ten female broiler chickens from ten different flocks around the UK. During euthanasia some of the birds expelled their faeces, thus they weren't available thus some groups $n < 10$.

Flock	Number of faecal samples
1	9
2	9
3	8
4	10
5	10
6	9
7	8
8	8
9	8
10	8

Table 6.1 Faecal sample number per flock (Standard diet (SD) $n = 71$, vegetarian diet (VG) $n = 16$)

6.2.3. Results and discussion

In order to identify any potential metabolites of interest and identify any differences in the metabolite profiles in relation to diet it is necessary to align all the spectra. Following alignment the spectra obtained from the NMR recording were combined (figure 6.1) and divided into 204 variable bins. The matrix obtained from the combination of spectra was then analysed using PCA to reveal possible separation (figure 6.2) however with the uneven sample groups (SD diet $n = 71$, VG diet $n = 16$) the more supervised method of PLS-DA was performed to further highlight the separation of the data (figure 6.3). In order to ensure the PLS-DA analysis had not 'overfit' the data a cross validation model was created (figure 6.4). This model highlights on a scale of 1% (worst) to 100% (best), the sensitivity (false negatives) and specificity (false positives) of the allocation of the data points into the PLS classes.

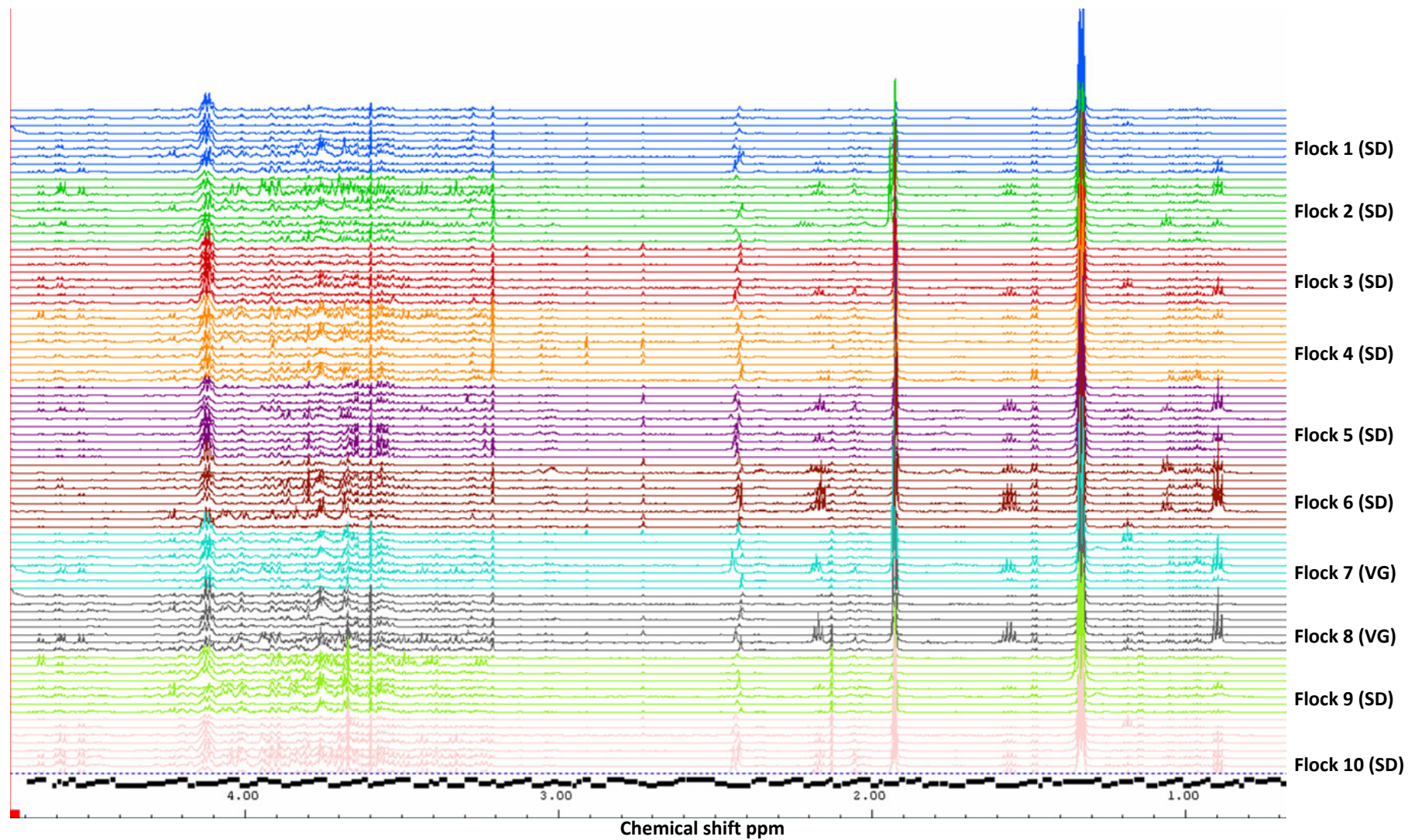


Figure 6.1 Combined spectra of all 87 samples. Colours refer to the flock origin as labelled 1-10. The horizontal axis highlights the variable bins used for analysis. (SD standard diet, VG vegetarian diet)

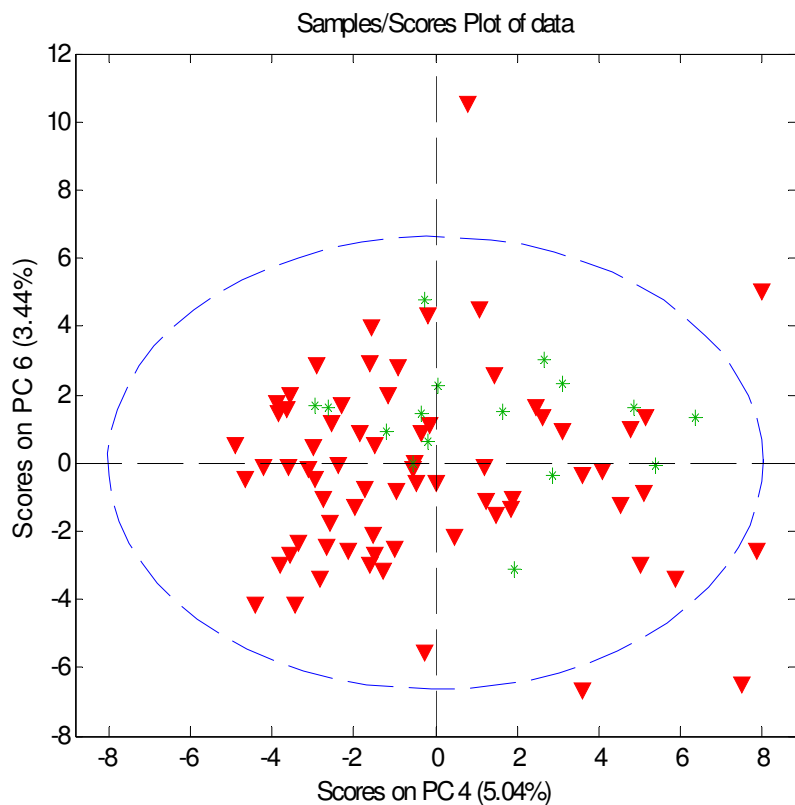


Figure 6.2 PCA plot of the variable bins obtained from the NMR spectra. The separation of the spectra from birds fed the SD diet (\blacktriangledown) and birds fed the VG diet (\ast) is small using the unsupervised method.

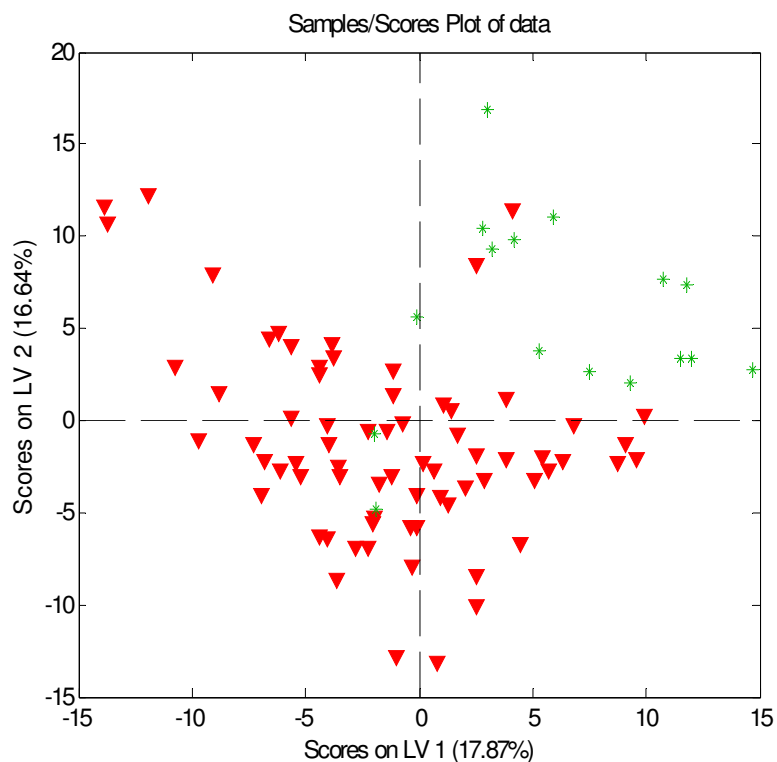


Figure 6.3 PLS-DA plot of the variable bins obtained from the NMR spectra. The separation of the spectra from birds fed the SD diet (\blacktriangledown) and birds fed the VG diet (\ast) is much more pronounced than the PCA plot highlighting a difference in metabolite profile between the two groups

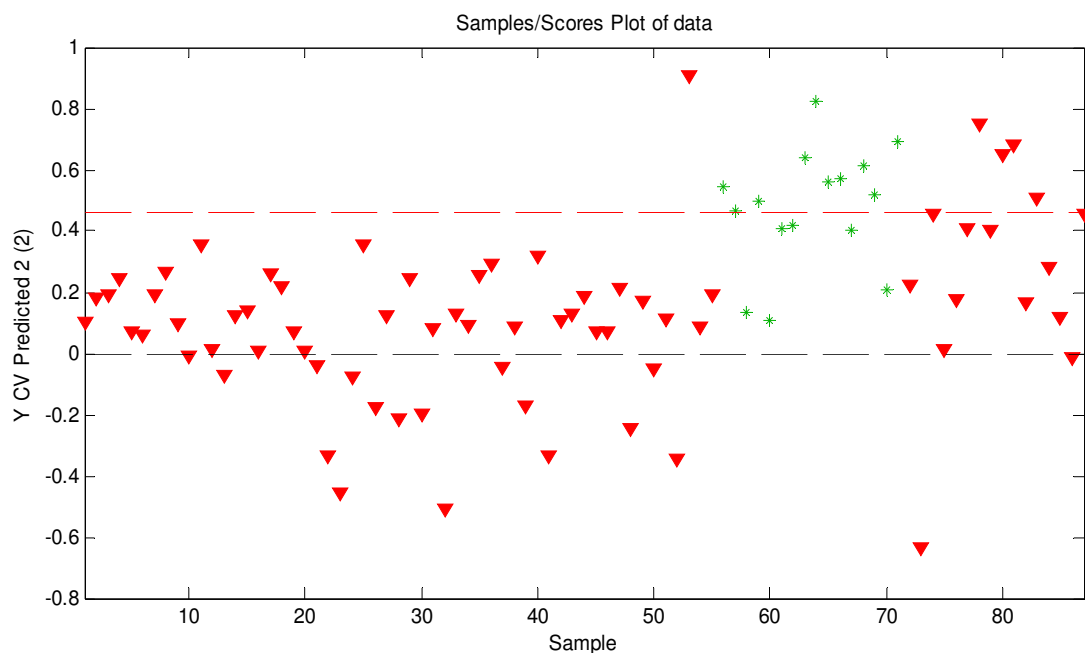


Figure 6.4 The data points for the SD diet birds (\blacktriangledown) should lay within the black and red dotted lines and data points for the VG diet birds (\star) should lay above the red dotted line. This cross validation of the PLS-DA shows that the separation of birds fed on the SD diet have a better than chance separation.

The cross validation model revealed that the classification of the data points for the SD diet birds (\blacktriangledown) had a sensitivity of 91% and a specificity of 64% and the data points for the VG diet birds (\star) had a sensitivity of 64% and a specificity of 91%. This indicates that there is a separation in the data points relating to the diet however, due to the small number of samples in the VG group in comparison to the SD group the differences seen are not that large.

Figure 6.5 shows the identification of the variable bins which differ the most between the SD and VG diet groups, i.e. the metabolite markers. By calculating the selectivity ratio (explained variance/residual variance) for each of the bins, the power of that particular bin as a marker of differences between diet groups can be calculated (312). The bins found to be the most powerful markers are displayed in table 6.2, where possible the metabolite responsible is mentioned. Figure 6.7 shows the dot plots for the marker metabolites for the birds fed either the standard or vegetarian diet.

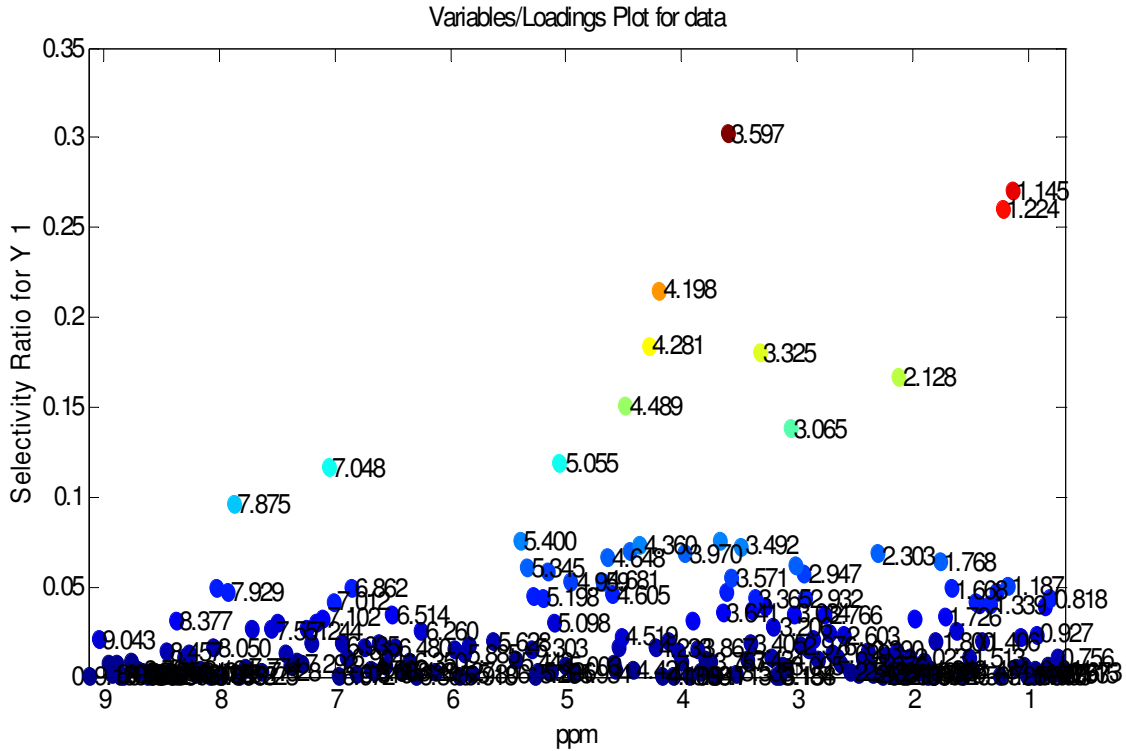


Figure 6.5 Identification of the variable bins responsible for the differences between the SD diet spectra and the VG diet spectra. The horizontal axis represents the bin number (chemical shift) and the vertical axis represents the selectivity ratio – the higher the ratio the more influential the bin on separating the two groups. Bins 1.22, 1.14, 1.23, 1.33, 2.13, 3.60, 3.67, 4.20, 4.28, 4.49, 5.06, 5.40 and 7.88 ppm shown as different colours have the highest selectivity ratio and differed significantly between the two groups, thus are potential markers.

There are several bins that act as possible markers to separate the distinction between the birds fed on a vegetarian diet or a standard diet; table 6.2 outlines these bins and metabolites. The signals at bins 1.23 ppm and 1.33 ppm are from lactate which are significantly higher in the birds fed on a standard broiler diet ($p < 0.0001$ and $p < 0.05$ respectively). Lactate, is produced as part of bacterial fermentation in the intestinal tract and is often utilised by other residents of the microbiota to produce further short chain fatty acids (SCFA) such as acetate, butyrate and propionate (24). Lactate does not normally accumulate in the intestinal tract of healthy individuals due to its utilisation by bacteria and host absorption (24, 97). Elevated levels of lactate have been seen in human colonic metabolite profiles in some cases of gut disorders such as ulcerative colitis (405). However it has been demonstrated that the lactate in the intestinal lumen can be of host origin from inflamed mucosa (172). The intestinal integrity of the birds fed on the standard broiler diet was observed to be worse when compared to the birds fed on the vegetarian diet.

Bin	p value t-test	Metabolite trend	Identification
1.22	<0.00001	S>V	SCFA
1.14	0.0013151	S<V	propylene glycol and 2,3-butanediol*
1.23	<0.00001	S>V	Lactate
1.33	0.0227667	S>V	Lactate
2.13	0.0019988	S<V	Acetyl-propylene glycol.*
3.60	0.0011615	S<V	singlet (62.5-85.5)
3.67	0.0005333	S<V	Ethylene glycol*
4.20	0.0034261	S<V	with 1.22 ppm
4.28	0.0011358	S<V	sugar
4.49	0.0086385	S<V	sugar
5.06	0.0030263	S<V	sugar
5.40	0.0191516	S<V	sugar
7.88	<0.00001	S>V	Phenolic compound

Table 6.2 List of marker metabolites that distinguish the ileal metabolite profiles of the birds fed either a standard or vegetarian broiler diet. (S>V metabolite present in higher levels in the birds fed on standard diet. S<V metabolite present in higher levels in the birds fed on vegetarian diet)
*provisional assignment

Furthermore the mucosa of the birds fed on the standard diet was more reddened (Chapter 5.3.1) suggesting some degree of irritation, this could account for the elevated levels of lactate in the lumen of the standard diet fed birds. As shown in Chapter 5 the microbiota of small intestine of these birds was found to differ depending on the dietary formulation. There was an increased presence of a *Bifidobacterium sp.* and a *Lactobacillus sp.* in the VG diet fed birds. The observed differences in microbiota and subsequent metabolic profiles could be explained by the composition of the vegetarian diet. The soybean meal used in the vegetarian diet in place of fish meal has much lower digestibility than fishmeal due to the higher level of carbohydrates in the form of plant oligosaccharides, hemicelluloses and pectins (406). The consequence of the high level of plant oligosaccharides, hemicelluloses and pectins is a more viscous excreta and increased faecal output (71, 216). The higher level of sugars seen in the metabolic profiles of the birds fed the vegetarian diet, as seen in bins 4.28, 4.49, 5.06 and 5.40 could be due to the

higher level of plant material. Alternatively as vegetarian broiler diets increase gut transit time and faecal output in comparison to a standard diet (406), the decreased sugar levels seen in the birds fed a standard diet could be due to the bacteria having more time to utilise them before they are voided in the faeces. If these sugars could be further characterised then a greater understanding could be gained. There is further variation in the SCFA profiles with one uncharacterised SCFA at bin 1.22 ppm being higher in the birds fed a standard diet this is likely to be the result of the combination of the different diet and different microbiota composition. The signal at 1.14 ppm is a doublet made up of signals at 1.143 and at 1.146 ppm (Figure 6.6). The first signal at 1.143 ppm has a structure CH_3CHOH - and could arise from a SCFA however the second signal at 1.146 indicates the compound is a di-alcohol derivative as the chemical shift is similar to that of butanediol and 1,2 propanediol (propylene glycol). There is also elevated presence of another glycol (possibly ethylene glycol) in bin 3.67 in the birds fed the vegetarian diet. A change in microbiota composition in the intestinal tract of these birds will result in different fermentative pathways which is likely to account for the different metabolites.

The formation of glycols via the fermentation of glycerol by bacteria has been recognised in lactic acid bacteria (135), species of clostridia (151) and members of the enterobacteriaceae (31). Glycerol is released during the digestion of lipids (48), the composition of the lipids found in fish and vegetables differ in their structures (163). Fish oils are high in levels of eicosapentaenoic (C20:5) and docosahexaenoic (C22:6) acids (155, 179), whereas plant oils such as soybean oil are high in linoleic (C18:2) and oleic (C18:1) acids (163, 185). The presence of alcohol derivatives from the fermentation of glycerol in the intestinal tracts of the chickens fed on the vegetarian diet indicated different active metabolic pathways within the intestinal lumen. Unrelated studies investigating the effects of the addition of a variety of fatty acids to the diets of laying hens have shown higher levels of scour in birds fed on diets containing the longer chain fatty acids from fish oil (Dr John Tarlton, University of Bristol, personal communication). Fat source has been shown to affect the intestinal microbiota and digestive processes in poultry (202). It is

possible that with an increased presence of plant lipids the metabolic profiles of the birds fed on the vegetarian diet induce active pathways that result in better overall gut health.

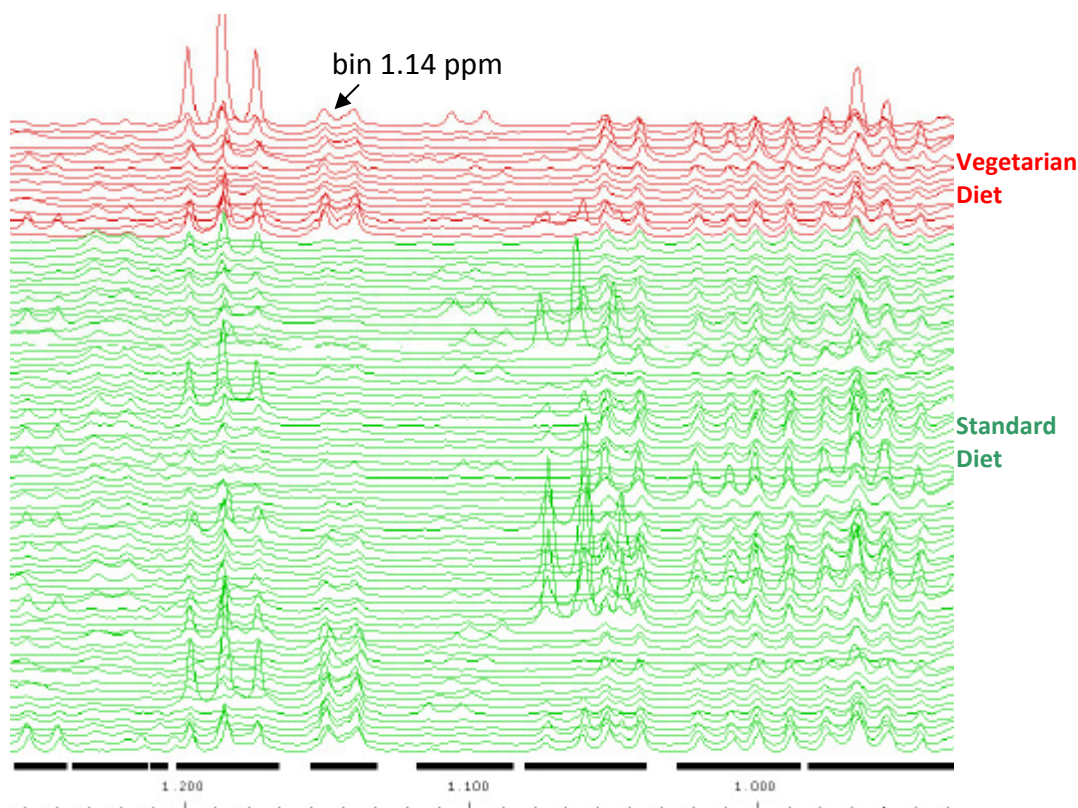


Figure 6.6 Part of ^1H NMR spectra of faecal extracts from broilers fed either a standard diet (green) or vegetarian diet (red). Bin 1.14 ppm is a doublet comprising of two peaks 1.143 and 1.146 which suggest the presence of a SCFA or a di-alcohol derivative similar to butanediol or 1,2 propanediol. These signals are absent from the majority of the standard diet fed birds thus could be an important marker in identifying a more preferable intestinal environment.

A further marker at 7.88 ppm was identified as a phenolic compound and was present at higher levels in the birds fed on the standard diet. Phenols are produced by the addition of one or several hydroxyl group(s) (OH) onto an aromatic carbon group. The gut bacteria have been implicated in the production of phenols via degradation of aromatic amino acids such as tyrosine (37). Phenol production by the intestinal microbiota has been shown to decrease in an environment with a lower pH and increased carbohydrate availability (361). The decrease in phenol production seen in the vegetarian group could be the result of a higher level of carbohydrates being available for the intestinal microbiota in the form of oligosaccharides, hemicellulose and pectins. Phenols have been implicated in the destabilisation of tight junctions in human epithelial cell lines leading to loss of

barrier function of the intestinal epithelia (258). Thus it is possible that bacterial production of phenols in the lumen of the GI tract of broiler chickens is detrimental to gut health by disturbance of the mucosal integrity. There are two further bins which separate the birds fed on the two diets. Firstly the signal in bin 2.13 ppm is higher in the birds fed on a vegetarian diet and has been identified as an acetylated compound. Acetylation is the addition of an acetyl group (COCH_3) to an organic compound. Further investigation revealed similarities of this compound to the chemical structure of acetyl-propylene glycol. This compound was further investigated and the SDBS spectral database (Japanese NMR spectral database) gave chemical shifts that match those seen in this investigation. Thus this compound was putatively assigned to acetyl-propylene glycol, which has not yet been found in intestinal metabolite profiles.

The results in this study indicate that there are definite distinctive differences in the metabolite profiles of the faecal samples of the birds on the two diet types. It would appear that the differences observed are related to the utilisation of the dietary components leading to different bacterial metabolites. To gain more information it would be beneficial to combine the metabolite data with the DGGE data and correlate the presence or absence of specific metabolites with the presence or absence of specific bacterial species. Further work with a bigger sample size of vegetarian fed birds and repeated studies would be needed to confirm the ability to use these markers as an indicator of enteric health.

6.3. Comparison of the metabolite profiles in the ilea and caeca of birds affected with dysbacteriosis using ^1H NMR

6.3.1. Background

This study follows on from the investigations in Chapter 3 where the composition of the intestinal microbiota was compared between healthy birds and birds suffering from DB. The primary symptoms of dysbacteriosis include the production of wet, sticky and mucoid faeces resulting in wet litter. The small intestinal and caecal microbiota of the DB affected birds was found to have an altered composition when analysed with PCR-DGGE in comparison to that of healthy birds. Two farms were included in the investigation and the results indicated increased presence of members of the bacteroidetes, species of clostridia, *Escherichia coli* and *Lactobacillus aviarius* within the intestinal microbiota of DB affected birds. As different bacterial species produce different metabolites (174, 306, 376) it is likely that an alteration in the metabolite profile within the intestinal lumen will occur in the birds suffering from DB. In the DB there is an increased presence of bacteria with proteolytic capacity seen birds which produce amines and ammonia, in contrast to the saccharolytic bacteria such as the lactobacilli and bifidobacteria which produce SCFA (204, 375, 376). Therefore the aim of this study was to characterise the differences in metabolic profile in birds suffering from DB compared to healthy birds and relate that to the change in the composition of the intestinal microbiota observed earlier. In addition to this there is sometimes a specific odour associated with DB; farmers and veterinarians often describe a distinct sour smell present in houses of birds suffering from DB (Philip Hammond, Crowshall Veterinary Services, personal communication). This observation suggests a compound being expelled in the faecal or caecal droppings of DB birds which if detected early enough could be an indicator of the deterioration of intestinal health and used as a tool for directing early intervention in the event of enteric upset.

6.3.2. Materials and Method

Sample selection

As described in section 3.2, dysbacteriosis was identified on two separate broiler farms. Ten healthy male and ten DB affected male broilers were selected and molecular profiling of the intestinal microbiota was performed using PCR-DGGE. This analysis revealed changes in the microbial composition of the small intestine as well as in the caeca. In line with this metabolite profiling of the ilea and caeca of the DB and healthy birds was performed using ^1H NMR. As the intestinal tracts of the birds had been retained and frozen at -20°C , it was possible to obtain gut contents from the same area of gut from which the microbial profiles were obtained.

Flock origin	Group code	Status	Number of ileal samples	Number of caecal samples
Farm 1	H	Healthy	10	9
	D	Dysbacteriosis	10	9
Farm 2	UH	Healthy	10	10
	UD	Dysbacteriosis	10	10

Table 6.3 Groups and sample numbers used in this study. N.B. n=9 in farm 1 caecal samples due to lack of quantity of caecal contents.

NMR recording and analysis

This was performed as described in section 6.1.1.3.

6.3.3. Results and discussion

The spectra obtained from the NMR recording were combined (figure 6.7) and divided into 194 variable bins. Analysis of the spectra was then performed to look for variations in metabolite profiles in the caeca and ilea of the healthy and DB birds.

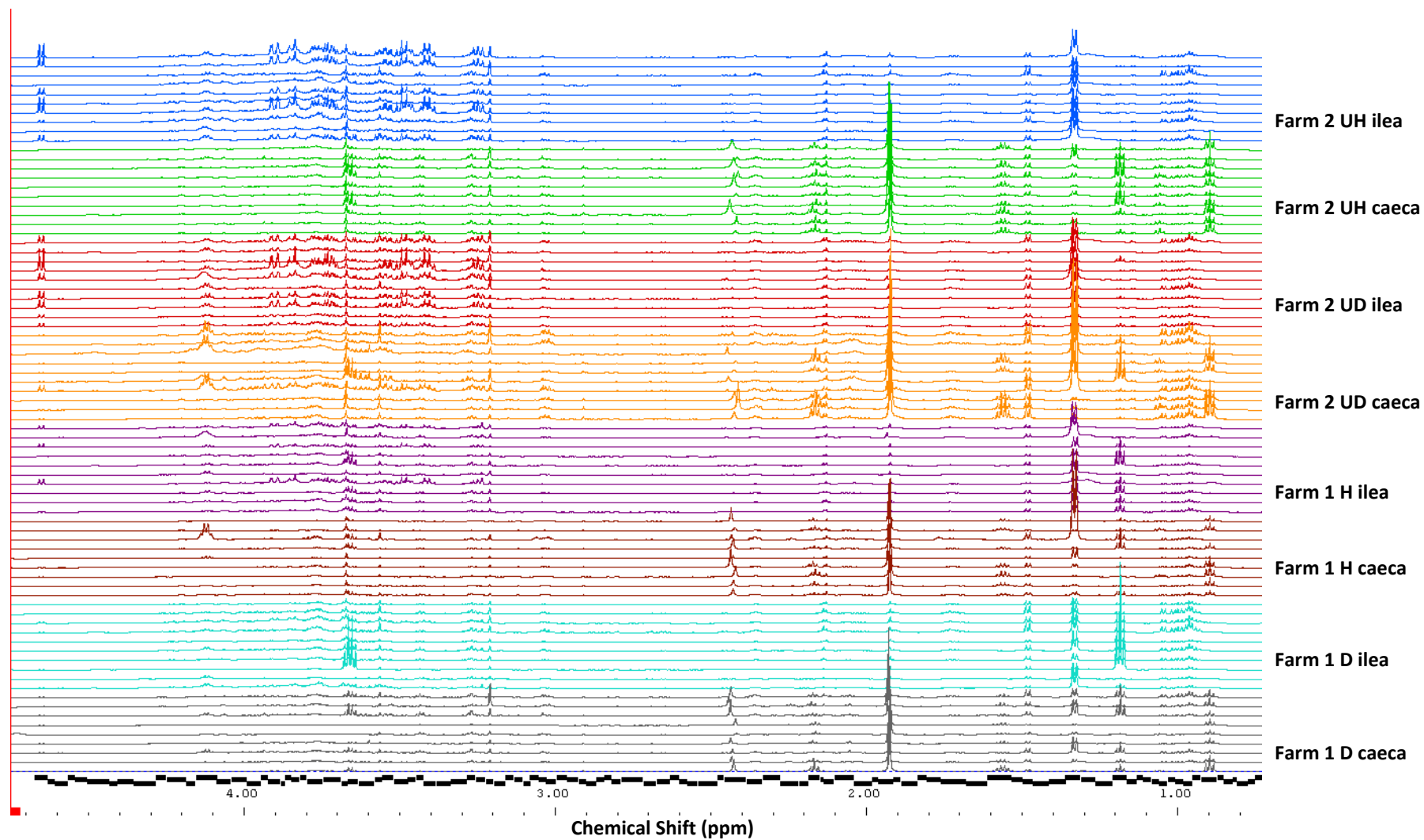


Figure 6.7 Combined spectra of all 78 samples. Colours refer to the different groups farm 1 or 2 and ileum or caecum as labelled. The horizontal axis highlights the variable bins used for analysis.

Metabolite profiles of ilea

PCA analysis (not shown) revealed no separation of the data points however PLS-DA (figure 6.8) demonstrated separation in relation to healthy and DB birds.

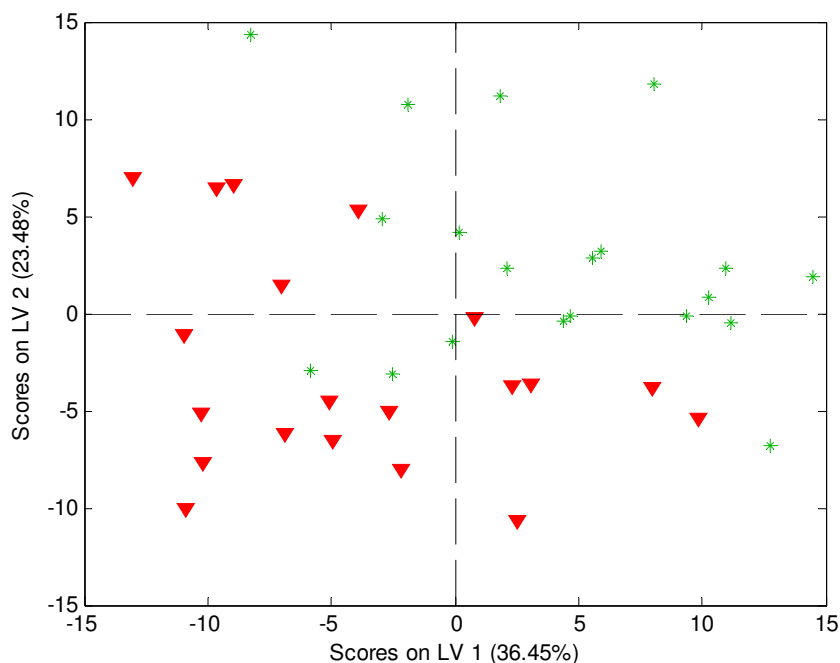


Figure 6.8 PLS-DA plot of the variable bins obtained from the NMR spectra from ileal contents. There is separation of the metabolite spectra from healthy birds (▼) and DB birds (★) ilea.

The lack of separation using PCA but subsequent separation by PLSA-DA indicates that the separation of the healthy and DB profiles is not associated with a large variance. The cross validation model highlights the sensitivity (false negatives) and specificity (false positives) of the allocation of the data points into the PLS classes healthy and DB birds. When the ileal data set is placed into the validation model there is a difference in the metabolite profiles of the healthy and DB birds with sensitivity of 87% and 76% specificity (figure 6.9).

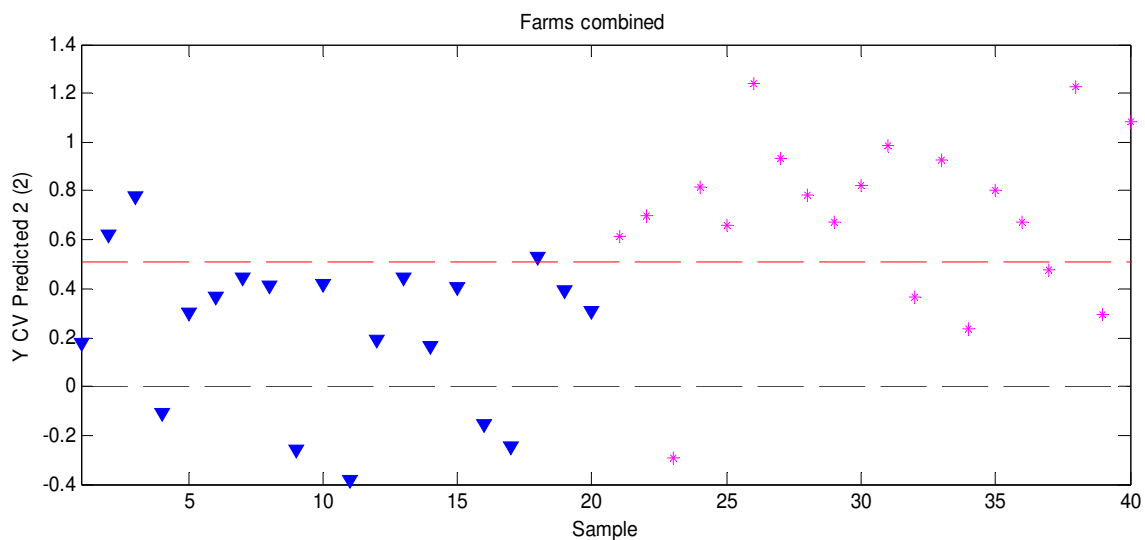


Figure 6.9 The data points for the healthy birds (\blacktriangledown) should lie within the black and red dotted lines and data points for the DB birds ($*$) should lay above the red dotted line. This cross validation model combined with the PLS-DA shows that the separation of healthy birds and DB birds based on ileal metabolite profiles is better than chance alone.

As the healthy and DB groups contained birds from two different farms the PLS-DA cross validation was performed on the spectra from each individual farm for comparison and this was found to improve the sensitivity (figure 6.10). The model showed that for farm 1 the sensitivity was 90% and specificity was 80%, and then for farm 2 the sensitivity was 90% and the specificity was 60%. Both farms demonstrate separation of the healthy bird and DB bird spectra indicating a difference in metabolite profiles.

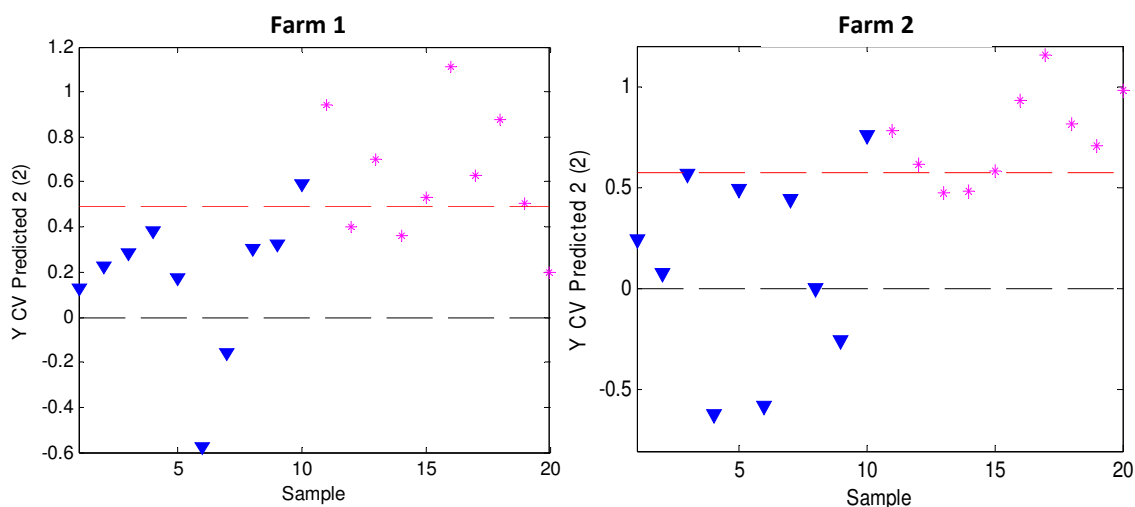


Figure 6.10 Cross validation model. The data points for the healthy birds (\blacktriangledown) should lay within the black and red dotted lines and data points for the DB birds ($*$) should lay above the red dotted line.

Selective ratio plots were then constructed for the birds from each farm (figure 6.11). The results indicate that there are definite markers that are found in the ileal metabolites that allow differentiation between healthy and DB birds' profiles from both farms.

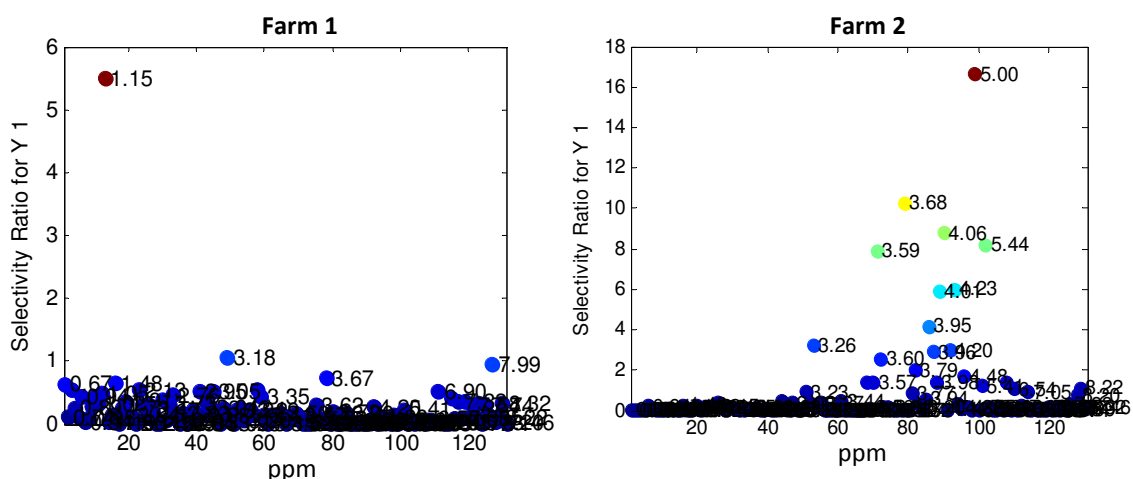


Figure 6.11 Identification of the variable bins responsible for the differences between the healthy and DB bird spectra. The horizontal axis represents the bin number (chemical shift) and the vertical axis represents the selectivity ratio – the higher the ratio the more influential the bin on separating the two groups.

The chemical shifts and identity of the metabolites whose presence is significantly different between the healthy and DB affected birds are shown in table 6.4. These metabolites have been listed as they were highlighted as important variables using the selective ratios therefore they could give some insight into the mechanisms involved in DB. In the ilea of the DB birds there appears to be an increase in the level of nucleosides and amino acids. One of the main functions of the ilea is to absorb nutrients such as amino acids and nucleosides (364, 418) which suggests, owing to the elevated levels of amino acids and nucleosides in the lumen of the DB birds' ilea, that there is malabsorption occurring in these birds. There are increased levels of creatine in the ilea of the DB birds this is likely to be also due to malabsorption of dietary creatine. Furthermore in the spectra of the DB birds there are elevated levels of taurine in combination with decreased bile acid which suggests bile acid deconjugation.

Bin ppm	p value t-test	Trend	Ratio H/D	Identification	Bin ppm	p value t-test	Trend	Ratio H/D	Identification
3.58	0.00024	H>D	1.3	Glycerol	3.04	0.0006	H<D	0.7	Creatine
3.56	0.003	H>D	1.0	Glycine	3.27	0.002	H<D	0.8	Taurine
4.05	0.02	H>D	1.2	Glycerol	3.28	0.008	H<D	0.8	
0.67	0.0008	H>D	1.6	Bile	3.26	0.009	H<D	0.9	
6.87	0.0009	H>D	1.3	p-hydroxyphenylacetic acid	3.44	0.010	H<D	0.8	
7.17	0.01	H>D	1.2		8.22	0.002	H<D	0.6	Nucleoside
1.14	0.006	H>D	1.3	Propylene glycol and 2,3-Butanediol*	8.20	0.02	H<D	0.7	Nucleoside
1.15	0.01	H>D	1.2		3.04	0.003	H<D	0.7	Lysine
1.14	0.01	H>D	1.3	Unknown doublet	3.03	0.008	H<D	0.7	
1.16	0.003	H>D	1.3		1.48	0.004	H<D	0.7	Alanine
4.19	0.005	H>D	1.2	Sugar	2.36	0.007	H<D	0.8	Glutamine
4.21	0.007	H>D	1.2	Sugar	1.05	0.01	H<D	0.7	Valine
4.23	0.02	H>D	1.2	Sugar	1.04	0.01	H<D	0.8	
4.22	0.02	H>D	1.3	Sugar	6.91	0.03	H<D	0.8	Tyrosine
3.84	0.01	H>D	1.2	Glucose	2.14	0.02	H<D	0.8	Methionine
3.90	0.03	H>D	1.2		2.65	0.03	H<D	0.8	
2.74	0.01	H>D	1.4	Unknown singlet					
2.73	0.02	H>D	1.2	Unknown singlet					
2.76	0.02	H>D	1.3	Similar to Sarcosine					
3.67	0.05	H>D	1.2	Ethylene glycol*					

Table 6.4 Identification of metabolite markers of the ilea that distinguish between healthy and DB birds on both farms. On the left are the markers which are lower in the DB birds (H>D) and on the right are the markers which are elevated in the DB birds (H<D). * Provisional assignment

Sodium taurochenodeoxycholate and sodium taurocholate are the most predominant acids found in poultry (103) and are formed by the conjugation of taurine to chenodeoxycholic acid and cholic acid (142, 205). The major role of bile acids in the intestinal tract is to emulsify lipids for digestion which can only occur when the salts are conjugated (48). In parallel to this, glycerol was highlighted as one of the major distinguishing markers between the healthy and DB birds, having a decreased level in the gut lumen of the DB birds. Emulsified lipids reach the ileum in the form of triacylglycerols and phospholipids (92); triacylglycerol is broken down into glycerol and fatty acids (48). Thus the reduction in glycerol in the ilea of the DB birds combined with the possible deconjugation of bile salts could imply compromised lipid metabolism in birds with DB. Birds suffering from DB often have growth depression, this would most certainly be a consequence of malabsorption. A study by Gracey *et al.* (144) showed that feeding rats deconjugated bile salts for a number of days resulted in ultrastructural damage to the microvilli and intracellular compartments of the small intestinal epithelium, these changes were only evident using electron microscopy as light microscopy revealed no change. This could explain the reason why no histopathological changes have been observed when tissue samples from the GI tract of birds with DB have been taken and examined by veterinarians (Philip Hammond, Crowshall Veterinary Services, personal communication). If the intestinal mucosa of chickens reacts in the same manner then it would help to explain the malabsorption seen. A reduction in glucose and other unidentified sugars is seen in the ilea of the DB birds; as there is an overgrowth of the intestinal bacteria it is likely that the bacteria present are utilising the sugars causing the level of sugar to be lower in the ilea. Interestingly bacterial species such as members of the bacteroidetes (64, 194), clostridia (253, 399) and lactobacilli (142, 200, 351) have been recognised for their ability to deconjugate bile acids. Members of these bacterial species were found to have an increased presence in the birds suffering from DB and could be responsible for the bile acid deconjugation, thus an increase in their presence results in increased deconjugation. Further analysis to combine the DGGE data and NMR spectra would be needed to correlate the metabolite profiles to the presence or absence of bacterial species.

There are lower levels of p-hydroxyphenylacetic acid, ethylene glycol, glycine and a compound of similar structure to sarcosine in the ilea of the DB birds. Glycine, sarcosine and ethylene glycol are produced during the degradation of choline (101, 324), which is added to the diets of chickens (94). It is possible that these compounds are lower in the DB birds due to disruption of the microbiota and a reduction in the pathways involved in producing them. Or alternatively the intestinal bacteria that have an increase in growth in cases of DB could be utilising the compounds as there is evidence intestinal bacteria are able to degrade glycine (11), sarcosine (269) and ethylene glycol (99, 425). P-hydroxyphenylacetic acid has been shown to be a urinary metabolite in humans, originating from tyrosine metabolism (38). As urine is drawn into the caecum and ileum of birds via retroperistalsis it is likely that the intestinal tract will contain urinary metabolites. Thus a reduction in p-hydroxyphenylacetic acid in the intestinal lumen of the DB birds could be suggestive of reduced tyrosine metabolism, which could be due to the malabsorption of tyrosine implied by the elevated levels of this amino acid in the DB birds. Alternatively as p-hydroxyphenylacetic acid can be produced by bacterial metabolism (379), there is again the possibility that there is a reduction in the bacterial species responsible for this metabolic pathway in the birds suffering from DB. There are also some unknown doublets and singlets present at higher levels in the healthy birds but without further characterisation it is not possible to conclude anything from them other than suggest that they are just components of normal intestinal metabolism. Thus a variation in these metabolites could indicate that in birds suffering from DB there is a deviation from the normal metabolic processes that are seen in a healthy chicken. Additionally feed transit through the intestinal tract is increased in the birds with DB thus normal metabolic pathways operating in the healthy birds may not have the time required in the birds with DB.

Metabolite profiles of the caeca

Both PCA analysis (figure 6.12) and PLS-DA (figure 6.13) demonstrated separation of the data points in relation to healthy and DB birds, as with the profiles from the ileal contents. The PLS-DA separation was improved when the spectra were separated in the farm 1 and farm 2 (figure 6.14). Cross validation of the individual farm PLS-DA plots (figure 6.15) revealed that separation of the caecal data based on health and DB birds for farm 1 had a sensitivity of 96% and a specificity of 91%. Farm 2 had similar levels with a sensitivity of 93% and specificity of 91%. These results therefore confirm the hypothesis that there was a difference in metabolite profiles in the caeca of healthy and DB birds.

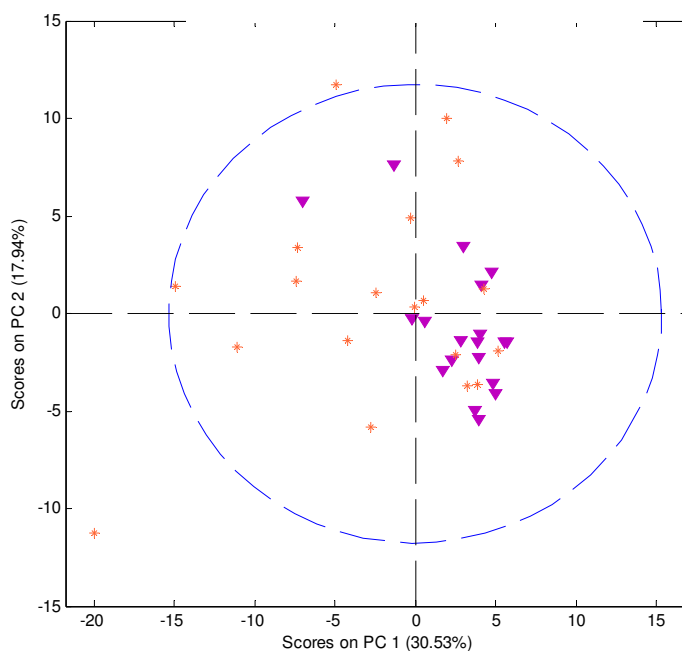


Figure 6.12 PCA analysis plot of data points from the spectra from the caeca of healthy (\blacktriangledown) and DB (\star) birds from farms 1 and 2. There is some separation of the data points indicating differences in metabolite profiles.

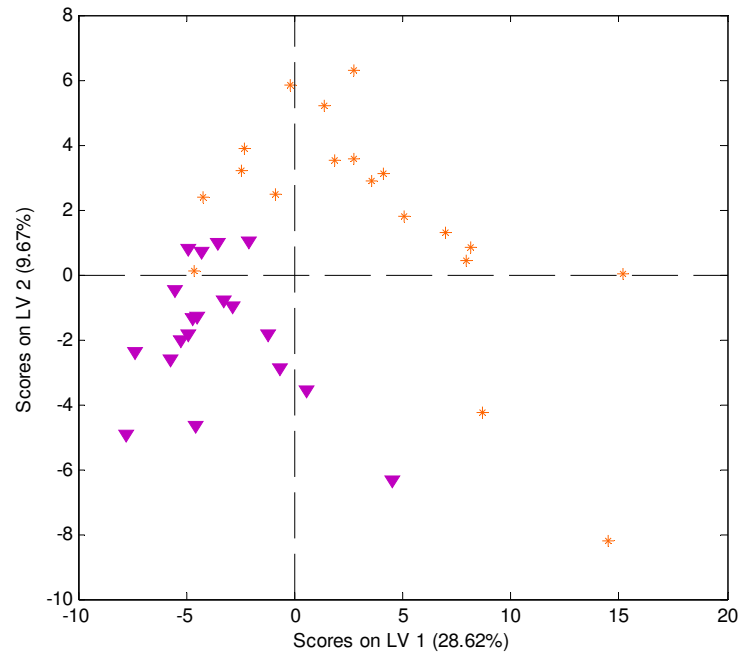


Figure 6.13 PLS-DA plot of data points from the spectra from the caeca of healthy (▼) and DB (★) birds from farms 1 and 2. The separation of the two groups indicates a difference in metabolite profiles in the healthy and DB bird caeca.

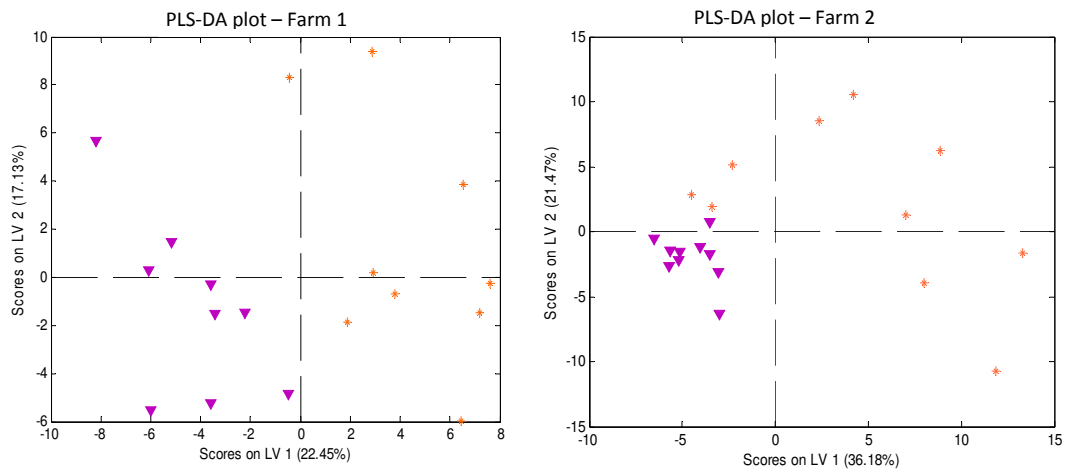


Figure 6.14 Individual farm PLS-DA plots of data points from the spectra from the caeca of healthy (▼) and DB (★) birds. In both cases there is separation of the data points indicating differences in caecal metabolite profiles in cases of dysbacteriosis.

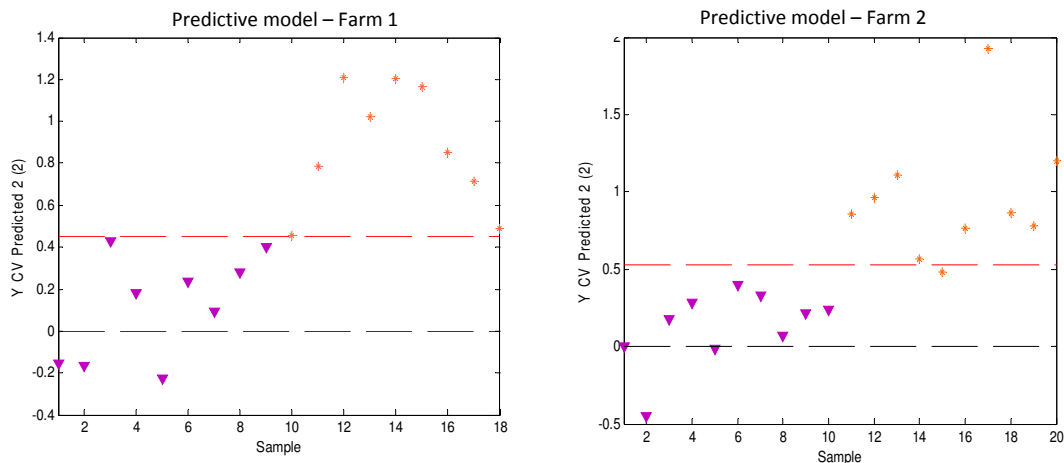


Figure 6.15 The cross validation models for the Individual farm PLS-DA data points from the spectra from the caeca of healthy (\blacktriangledown) and DB (\star) birds. The data points for the healthy birds should lay within the black and red dotted lines and data points for the DB birds should lay above the red dotted line. Both sets of plots indicate that there is a difference in the metabolite profiles in healthy and DB birds.

Selective ratio plots were then constructed for the variable bins from farms 1 and 2 (figure 6.16). The results from these plots indicate that there are definite markers for differences between healthy and DB birds and which are common between farms. The plots reveal that there are several markers that are important in distinguishing between the healthy and DB bird caecal spectra thus are potential marker metabolites, these are detailed in table 6.5.

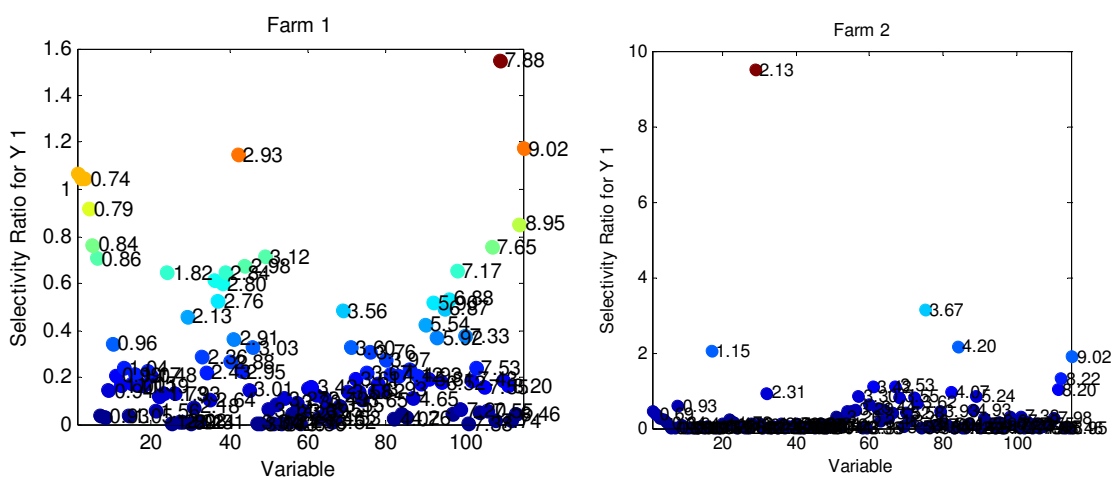


Figure 6.16 Identification of the variable bins responsible for the differences between the healthy and DB bird spectra from both farms. The horizontal axis represents the bin number (chemical shift) and the vertical axis represents the selectivity ratio – the higher the ratio the more influential the bin on separating the healthy and DB birds.

Bin ppm	p value t-test	Trend	Ratio H/D	Identification	Bin ppm	p value t-test	Trend	Ratio H/D	Identification
2.13	<0.0001	H>D	1.6	acetyl-ethylene glycerol*	3.30	0.03	H<D	0.8	Taurine
3.67	0.0001	H>D	1.6	Ethylene glycol*	3.42	0.003	H<D	0.7	
1.14	0.0001	H>D	1.5	Propylene glycol and 2,3-butanediol*	3.56	0.01	H<D	0.8	Glycerol
1.15	0.001	H>D	1.4		3.61	0.003	H<D	0.7	
1.14	0.01	H>D	1.4	Unknown doublet	4.07	0.01	H<D	0.8	
1.16	0.04	H>D	1.2		3.54	0.005	H<D	0.7	
7.17	0.001	H>D	1.6	p-hydroxyphenylacetic acid	3.40	0.01	H<D	0.7	Glucose
6.87	0.003	H>D	1.6		1.48	0.01	H<D	0.8	
4.20	0.001	H>D	1.3	Sugar	5.27	0.03	H<D	0.7	Sugar
3.11	0.003	H>D	1.3	Singlet					
2.68	0.002	H>D	1.3	Aspartic acid					
2.86	0.01	H>D	1.3	Asparagine					
2.91	0.02	H>D	1.3	Trimethylamine					
2.73	0.01	H>D	1.2	Singlet					
2.74	0.01	H>D	1.2	Singlet					
1.82	0.02	H>D	1.2	Acetic acid					
1.93	0.15	H>D	1.2						
2.43	0.03	H>D	1.4	Succinic acid					
8.95	0.02	H>D	1.2	Singlet					

Table 6.5 Identification of metabolites markers in the caeca that distinguish between healthy and DB birds on both farms. On the left are the markers which are lower in the DB birds (H>D) and on the right are the markers whose concentrations are elevated in the DB birds (H<D). Compounds which have the same pattern in the ilea are in **bold green typeface** and those that have an inversed pattern are in **bold violet typeface**. *Provisional assignment

Figure 6.17 shows the area of the NMR spectra relating to metabolite markers that are higher in the caeca of diseased birds.

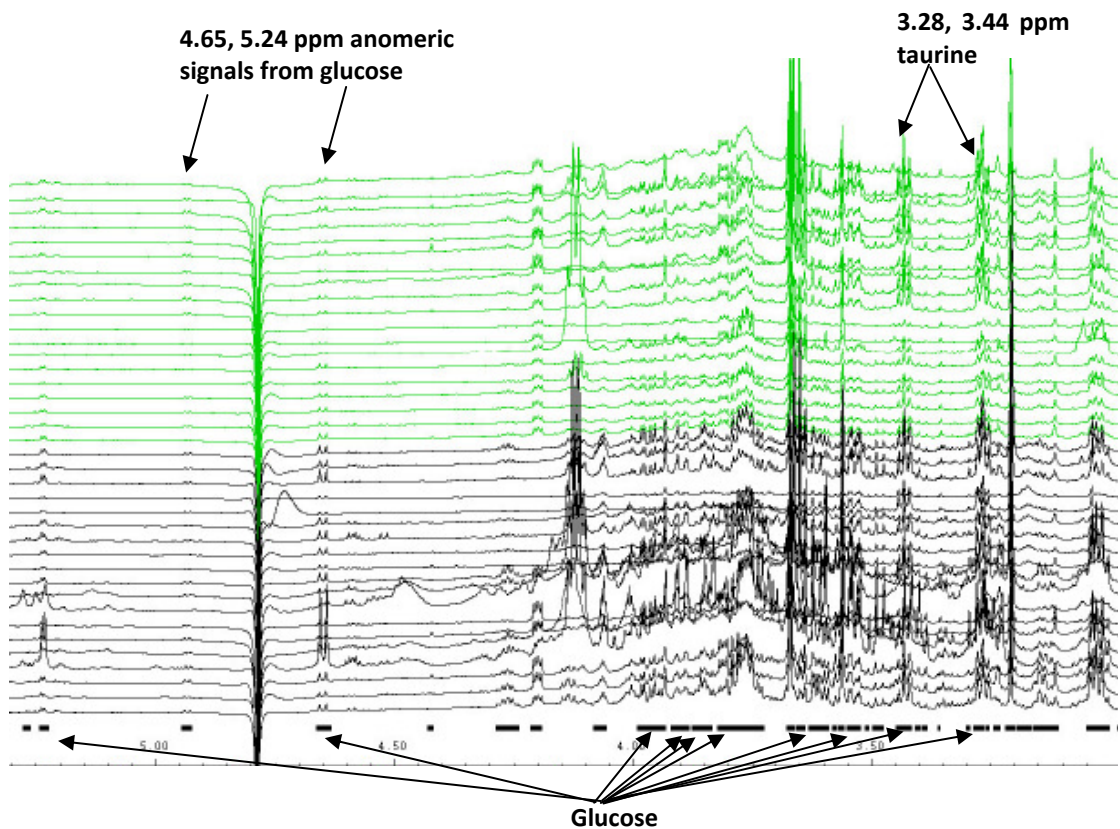


Figure 6.17 Extract of the NMR spectra of the caecal metabolite profiles. The green spectra are from healthy birds and the black spectra are from DB. Glucose and taurine were found to be increased in the caeca compared to the ilea.

There are some similarities in the ileal and caecal metabolite profiles in the healthy and DB birds. Firstly there is a similar increase in the level of taurine in the caeca of birds suffering from DB, the taurine will either originate from the ilea or will be from further deconjugation of bile in the caeca. Alanine was also found to be elevated in the caeca as well as the ilea of the DB affected birds. It is possible that the increased levels of this amino acid in the caeca is due to it being passed from the small intestine into the caeca however it could be expected that other amino acids would also be elevated. It is possible that alanine is not a preferred substrate for intestinal bacteria or it could be that the alanine originated from the urine as an indicator of gluconeogenesis. As the birds are potentially suffering from malabsorption and impaired lipid digestion it is likely that the birds are undergoing gluconeogenesis in order to obtain energy. Gluconeogenesis is the generation of glucose from a non-carbohydrate source such as glycerol or amino acids. As broiler

chickens are selected for their ability to convert their feed into muscle, young broilers have very little fat deposits around their bodies therefore will rely on amino acids for energy in times of low energy availability such as when suffering from malabsorption. Muscle is the major source of protein in an animal's body thus is the target for gluconeogenesis in humans and rats. Alanine is one of the most common amino acids released during gluconeogenesis. It is released into the blood and taken up by liver cells which deaminate it to form pyruvate which is then further converted to glucose (55, 114). It is possible that due to the elevated levels of alanine in the blood that it could be filtered out in the urine which would then be taken up into the caeca. As seen in the ilea there is a reduction in the substance (3.67 ppm) provisionally assigned to ethylene glycol, p-hydroxyphenylacetic acid (6.87 and 7.17 ppm) in the dysbacteriosis affected birds' caeca along with a decrease of the unknown singlets (2.73 and 2.74 ppm), propylene glycol and 2,3-butanediol and an unknown doublet (1.15 ppm). These patterns probably link to the same disruption in the normal metabolic patterns in the birds suffering from DB as mentioned in the ileal metabolite results above. The alteration in the normal metabolic pathways is likely to explain the reduction in the levels of aspartic acid, asparagine and trimethylamine. There is a further reduction in the level of acetic acid and succinic acid in the caeca of DB affected birds. Succinic and acetic acids can be the products of bacterial fermentation of glucose and glycerol (104, 217, 218) thus one could propose that in the caeca of DB affected birds there is a reduction in the number or the activity of the bacterial species that perform these fermentative pathways to create acetic and succinic acids. This theory is further backed-up by a higher level of glucose and glycerol in the caeca of the DB affected birds. The ileal results indicate that there is a change in the metabolic pathways in the small intestine of DB affected birds. It is therefore logical that the digesta from the ilea and colon which inoculates the caeca will differ in the DB affected birds, thus altering the conditions within the caeca resulting in a disruption to the caecal homeostasis. The birds suffering from DB demonstrated an increase in bacterial species in the caecal microbiota with certain species of less dominant bacteria becoming more dominant. It is quite possible that there is a change in the level of bacterial species responsible for producing organic acids such as acetic and succinic

acids, however due to them being less dominant any fluctuation in their presence is undetectable by DGGE. If there is a reduction in the production of organic acids within the caeca it could result in a change in the pH within the GI tract of DB affected birds which would alter the physiological conditions within the gut which could allow the growth of bacterial species which are detrimental to intestinal health.

The results from the ilea and caeca reveal that there are differences in the metabolic profiles of the healthy and DB. It would appear that the DB affected birds have bile acid deconjugation leading to impaired lipid digestion in addition to malabsorption of nutrients. These findings would explain the lack of flock uniformity in the DB affected birds. There also appears to be a change in the metabolic pathways in the DB affected birds which could be an indicator of the differences in bacterial composition of the GI tract. Further work would be needed to correlate the changes in bacterial species and the fluctuations in the presence of metabolites. The metabolite profiling gives a good insight into the mechanisms occurring in the birds suffering from DB and provides more information for understanding the pathogenesis of the condition.

6.4. Conclusion

The aim of these studies was two fold. The primary aim was to profile the metabolites of the GI tract and relate that to the changes in intestinal health and differences in bacterial composition. The second aim was to potentially identify metabolite markers that could be used as biomarkers for monitoring intestinal health over time. The results from both studies have revealed differences in metabolite profiles which can be linked to intestinal health and the composition of the intestinal microbiota. A literature search revealed no previously published papers on metabolite analysis of the intestinal tracts of broilers relating to intestinal health or dysbacteriosis using ^1H NMR analysis. Therefore it is not possible to compare the results in this study to previous work.

In the diet study the birds fed on the vegetarian diet appeared to have much improved intestinal health which was accompanied with an increased presence of a

Lactobacillus sp. and a *Bifidobacterium sp.* The increased presence of these bacterial species could be explained by the nature of the diet as soybean meal has increased fibre which would act as a prebiotic promoting the growth of beneficial bacterial species such as lactobacilli and bifidobacteria which use these as substrates. Subsequently there would be increased fermentation which would explain the increased levels of di-alcohols in the faecal samples. It wasn't possible to identify all the compounds in these samples at this time and so further work is needed to fully understand the active metabolic pathways in the GI tracts of the birds fed the different diets. However, it was clear that the birds fed on the vegetarian diet had improved intestinal health due to the GI tract being a healthy pink colour with good tone and consistency of contents. The litter was of better quality in the house of the birds fed the vegetarian diet, as it was dry and friable. Therefore it is rational to conclude that the bacterial composition and associated metabolic pathways are beneficial to the enteric health of the birds. The presence of lactate in the microbial profiles of the birds fed on the standard diet could be linked with a potential intestinal disorder as lactate has been seen to be elevated in human patients suffering from bowel disease (405). Phenols can destabilise epithelial tight junctions thus impairing the barrier function of the intestinal wall (258), therefore a higher level of a phenol in the spectra from the standard diet fed birds could be compromising intestinal integrity. Potentially both these compounds could be used as faecal markers for a poor enteric environment however further studies would be needed to confirm this. The results suggest that vegetarian diet is more beneficial to the intestinal health of a broiler which could be due to the specific substrates of the vegetarian diet leading to the growth of more favourable bacteria. It is interesting that there are indications that the oils present in fish may not be beneficial to the homeostasis of the enteric environment in chickens. The results from this study are encouraging and indicate that future trials to explore the effects of feeding a vegetarian diet on the intestinal health of broilers.

In the second study the metabolite profiles of the ilea and caeca of birds suffering from DB were compared to those of healthy birds. As there are no published papers on the metabolites found in the GI tract of birds suffering from DB it is

impossible to compare these results to the literature. The resultant profiles from the ilea revealed that in the DB affected birds there is an increase in amino acids, nucleosides and taurine seen along with a decrease in sugars, glycerol, bile and a variety of other bacterial metabolites. These findings suggest that there is bile acid deconjugation which could be explained by the increase in bacterial species with the capacity to deconjugate bile salts. Furthermore there is evidence to suggest malabsorption of nutrients from the small intestine. Deconjugated bile acids have been implicated in disturbing the structure and function of the gut epithelia in rats (144) thus would contribute to the malabsorption seen. The decrease in sugars in the lumen of the ilea of the DB affected birds is likely to be a result of either the increased bacterial load which would utilise the sugars or due to the increased feed passage time resulting in a reduction in the time for the breakdown of complex carbohydrates to simple sugars. The differential levels of the other metabolites that are observed indicate that there is a shift in the active metabolic pathways in the small intestine of the birds suffering from DB. This could be due to a change in the nutrient availability for the resident microbiota or because there is a change in the dominant species present. The caecal metabolites reveal a further change, with there being a selection of differences in the metabolite profiles. There is a reduction in the level of organic acids in the caeca of the DB affected birds which would likely result in a higher pH which could provide a more favourable environment for certain bacterial species. Feeding of exogenous organic acids to poultry has been used as an alternative to antibiotic growth promoters (441). Organic acids have been found to affect the growth of campylobacter (57) and acidification of drinking water with organic acids has been found to reduce campylobacter levels in drinker line so as to reduce colonisation in broiler chickens (56). Feeding a blend of organic acids to broilers has been found to increase the level of lactobacilli in the ileum of broiler chickens (288) which is probably due to their ability to tolerate acidic conditions (259). Thus it could be proposed that feeding of organic acids or providing chickens with probiotic bacteria with the ability to secrete organic acids would be more beneficial for gut health.

By combining the metabolite profiles from both studies there appears to be a number of markers that are commonly in the metabolite profiles of the healthy birds and those fed on the vegetarian diet namely the compound provisionally assigned similar to ethylene glycol (3.67 ppm), the acetylated compound provisionally assigned to acetyl-ethylene glycol (2.13 ppm) and the propylene glycol and 2,3-butanediol at 1.14 ppm. If these compounds are further characterised and verified they could provide a marker for indicating a favourable enteric environment and also lead to the isolation of the bacterial species responsible for the metabolites. This would enable them to be trialled as probiotics in poultry or the metabolites to be produced for addition to poultry feed to promote gut health.

This chapter highlights the valuable information provided by parallel profiling for the microbiota and the intestinal microbiota. The next step with the data obtained would be to combine the data sets and look for correlations between the presence and absence of bacterial species and the level of metabolites.

7. *General discussion and future directions of research*

The intestinal microbiota of an animal plays an important role in the health and well being of an animal. With bacterial cell numbers outnumbering the host's own cell number by ten to one; it is not surprising that animal hosts rely on the vast metabolic entity residing inside us. Bacteria reside in all known habitats therefore animals have had to evolve in a world full of bacteria; part of the evolutionary process has resulted in the symbiotic relationship between an animal and its microbial residents. It has been found that the intestinal microbiota aids digestion, protects against pathogens, produces nutrients and plays a role in the maturation of the immune system.

With the advent of culture independent DNA based technologies, the knowledge of the composition of the intestinal microbiota has improved greatly. Using these techniques it has been suggested that the intestinal microbiota of the chicken is comprised of around 640 species of bacteria from 150 different genera (5). The increased application of molecular methods revealed that culture based methods had vastly underestimated the complex community of bacteria within an animal's intestinal microbiota (1, 403). Culture independent techniques have allowed microbiologists to learn more about the relationship between the host and its resident microbiota. It has been demonstrated that the composition of the intestinal microbiota is affected by factors such the sex, age, dietary intake and health status of the host. The exact relationship between the host and its resident microbiota is still a mystery however it is becoming more apparent that the intestinal microbiota is massively influential in terms of host health.

The primary aim of this thesis was to gain a better understanding of dysbacteriosis (DB), a digestive disorder of concern within the poultry industry. There have been increased reports of the condition however very little is known about it. DB has been associated with an imbalance in the intestinal microbiota leading to poor flock uniformity and the production of wet faeces resulting in wet litter. The condition typically affects birds of 20-30 days of age and it has been suggested that it is triggered by changes in diet, stress and overcrowding. Currently the condition is

treated by administration of antimicrobials however it is not unheard of for DB to return once antibiotic therapy has finished. As enteric health is an important issue, there is increasing pressure from within the poultry industry for poultry breeders and veterinarians to provide solutions on how to tackle conditions such as DB. As there is a lack of literature surrounding DB there is a need to improve the knowledge surrounding the pathogenesis of the condition so that it can correctly managed without the need for antimicrobials.

The first set of investigations in Chapter 3 explored the hypothesis that birds suffering from DB have an imbalance in their intestinal microbiota. By using PCR-DGGE 16S rRNA microbial DNA fingerprints were produced from the contents of the crop, gizzard, jejunum, ileum, caeca and colon from ten healthy and ten DB affected birds from two separate farms. The DGGE results showed that the microbial composition of the jejunum, ileum and caeca of DB birds was different to that of their healthy counterparts. The microbial groups responsible for these changes were members of the bacteroidetes (*Bacteroides dorei* and *Barnesiella viscericola* in the birds from farm 1 and *Bacteroides ovatus* and *Bacteroides vulgatus* in the birds from farm two) uncultured *Clostridia sp.* (different species in farm one and two), *Escherichia coli* and *Lactobacillus aviarius*. Bacteroides, clostridia, lactobacilli and *Escherichia coli* have been all been implicated in small intestinal microbial overgrowth in a variety of mammalian species (153, 154, 246). It has been suggested that *Bacteroides vulgatus* and *Bacteroides ovatus*, along with other bacteroides species have the ability to cause damage to the brush border of the small intestine and compromise brush border sucrase and maltase activity by secreting proteases (319). As a consequence of brush border damage there will be a reduction in the surface area available for nutrient uptake leading to malabsorption (319). Interestingly the metabolite profiling results in Chapter 6 support this suggestion of malabsorption in the ileum of DB affected birds, as there were elevated amino acid and nucleoside levels compared to the ileal contents of healthy birds. The metabolomic analysis also revealed evidence of bile acid deconjugation in the small intestine of the DB birds. Bacteroides, clostridia and lactobacilli have been known to have the ability to deconjugate bile acids, thus

impairing lipid metabolism. The malabsorption and compromised lipid digestion would certainly account for the loss of growth seen in the birds suffering from DB. The ^1H NMR analysis highlighted that there were reduced levels of certain organic compounds such as succinic acid, acetic acid, glycols and di-alcohols in the GI tract of the DB affected birds. These compounds are common microbial metabolites thus indicate a disruption to the normal metabolic pathways operating in the GI tract of a healthy bird. The change in the composition and activity of intestinal microbiota seen is the logical explanation for this change in metabolite levels. However comparative analysis and correlation of the ^1H NMR spectra and DGGE profiles will need to be performed in order to verify the relationship between the variation in metabolites and the presence and absence of bacterial species. It was found through selective plating that some of these bacterial species could be isolated from the intestinal tracts of healthy birds despite the species of bacteria not being detected in the DGGE profiles. This gives an indication that the changes in composition of the intestinal microbiota in cases of DB are due to an imbalance of the resident autochthonous microbiota as opposed to allochthonous species entering the gut. It is not clear what causes the change in the composition of the microbiota or how the changes contribute to the onset and pathogenesis of DB. As mentioned in the discussion in Chapter 3, DB has parallels with dysbiosis, which is a shift in microbiota resulting in overstimulation of the gut immune system and thought to be a precursor to IBS in humans. *Bacteroides spp.*, *Clostridia spp.* and *Escherichia coli* are considered to be opportunistic bacteria and their increased proliferation in the intestinal tract has led to opportunistic enteric diseases (36, 239, 409). The capacity of these species to cause disease is likely down to their ability to enhance mucosal permeability, promote bacterial uptake, cross the epithelial barrier and induce the synthesis of proinflammatory cytokines by interacting with epithelial cells and macrophages (77, 201, 325, 326, 420). Thus it is quite possible that in birds suffering from DB there is an immune component that contributes to the onset of this disease – any future studies should include profiling of the immune system to compare the immune activity of healthy and DB affected birds. . This could involve looking at the innate immune system by cytokine or chemokine analysis or by characterising acute phase proteins to give an indication

of ongoing immune processes. The development of the digestive system and immune system of any animal is heavily influenced by the composition of the resident microbiota and the pioneering species which first colonise. Therefore it is essential that a young animal is exposed to a favourable bacterial community as soon as possible. There is the possibility that due to the high hygiene status of a commercial hatchery that the broiler chicks have delayed exposure to a suitable microbiota and this delay has a detrimental affect on the development on the GI tract and associated immune response.

There is evidence from within the poultry industry that certain breeds of broiler are predisposed to developing DB and that the onset of the condition is linked to dietary change; Chapters 4 and 5 reported the studies that investigated these hypotheses. Comparing the microbial composition of the ilea and caeca of four different genetic lines of birds explored the influence of host genotype on intestinal microbiota. As the interest here was to investigate potential causative bacterial species the birds were healthy and showing no sign of DB. Out of the four genetics lines, two of the genetic lines were susceptible to enteric disease and two were resistant. Furthermore two of the lines were commercial broiler breeds with the remaining two being pure breed lines used in the breeding program to produce commercial broilers. The hypothesis to be tested was that there were common factors in the composition of the intestinal microbiota of the susceptible and resistant lines in order to explain the differential predisposition to enteric disease. The results however did not reveal any differences based on susceptibility or resistance; instead there was separation of the DGGE profiles based on whether the birds were pure line or commercial broiler lines. This suggests that the susceptibility to enteric disease lies in a factor or factors determined by host genetics. The mechanism behind breed disposition might not be due to the composition of the intestinal microbiota in the healthy bird but how the immune system responds to changes in the intestinal microbiota. In the birds more susceptible to enteric disease it is possible that when the microbiota contains more immunostimulatory bacterial species such as bacteroides and clostridia that the resultant immune response is not enough so that the bacteria are able to cause an

upset to the intestinal environment. The other possibility is that the immune response is too strong resulting in a condition similar to irritable bowel disease where a change in intestinal microbiota results in overstimulation of the immune system. For example, in humans there appears to be genetic predisposition and environmental factors that can trigger irritable bowel disease (IBD) via a loss of antigen tolerance and subsequently the intestinal microbiota appear then to play a role in the development and persistence of IBD (382). A similar mechanism may be occurring in broilers and further work would be needed to elucidate the relationship between host genetics, the immune system and the changes in composition of the intestinal microbiota. A case-control study approach to examine the combined effects of host genetics, immune response and the composition of the intestinal microbiota is outlined in the discussion in Chapter 4.

Chapter 5 examined the influences of diet on the intestinal microbiota. The two studies in this chapter examined the changes in intestinal microbiota of broilers fed on diets differing in nutrient density and nutrient source. The results from these studies reveal difference in the microbial composition of the GI tract in relation to dietary type. These findings are in agreement with previous studies which show that dietary changes such as the carbohydrate source (6, 131, 139), fat source (76, 202) and protein source (93, 241) influence the composition of the intestinal microbiota. It has been proposed by veterinarians that diets with higher protein density can lead to the onset of DB (Dr Barry Thorp, Aviagen Ltd, personal communication). Comparing the intestinal microbiota of birds fed on diets with either a high or low protein density revealed an increase in the presence of members of the bacteroidetes in the caeca of birds fed on a high density protein diet. The ileal microbiota remained unchanged, however this could be due to the high hygiene status of the farm where these birds were housed. There is evidence that various members of the intestinal microbiota, such as members of the bacteroidetes, have the ability to obtain energy through proteolytic pathways (242, 265). Proteolytic metabolism by bacteria can result in the formation of ammonia, amines, phenols and indoles which can be potentially harmful and detrimental to the enteric environment (131, 141, 229). Increased levels of ammonia will result in

a higher luminal pH which could make the condition in the intestine more favourable for specific bacterial species (330). Phenols have been shown to destabilise epithelial tight junctions and affect gut barrier function (258). The inclusion of more fermentable carbohydrates and a lower luminal pH results in lower production of phenolic compounds by intestinal bacteria (361), thus could be a potential mechanism to lower the production of phenols. In comparison to fishmeal, soybean meal has a lower concentration of protein and a higher proportion of indigestible material such as oligosaccharides, hemicelluloses and plant pectins (159, 166). In the experiment comparing the two different protein levels in Section 5.2, the low protein diet had a higher ratio of soyabean meal than fishmeal which would result in a higher level of indigestible material in this diet compared to the high protein diet. Some indigestible plant carbohydrates have prebiotic potential and may improve intestinal health by stimulating beneficial bacterial groups such as bifidobacteria and lactobacilli which preferentially utilise the prebiotic compounds (85, 140). The subsequent investigation in Chapter 5 involved the comparison of the small intestinal microbiota of birds fed either a standard broiler diet (which contains fishmeal) or a totally vegetarian diet. *Post mortem* examination of the groups of birds revealed improved gut health in the birds fed the vegetarian diet. Characterisation of the ileal microbiota revealed increased presence in a species of lactobacillus and a species of bifidobacterium in the birds fed on the vegetarian diet. It is possible that the vegetarian diet administered to these birds results in the proliferation of a more favourable microbiota which subsequently improves intestinal health. Lactobacilli and bifidobacteria are considered to be probiotic bacteria due to their beneficial affects on host health (68, 140, 150, 402). Many studies have shown the benefits to the health of broilers administered with either probiotics, prebiotics or a mixture of both (synbiotics) (9, 10, 58, 162, 224).

Metabolomic analysis of the intestinal contents of the birds fed on either the standard or vegetarian diet revealed differences in the metabolic profiles in relation to diet. The metabolites that were found to vary are potentially due to the differences in microbiota seen, although further analysis to correlate the DGGE data

with the ^1H NMR spectra is needed. The soybean based diet is potentially providing nutrients which the lactobacilli and bifidobacteria preferentially utilize and the products of their fermentation are beneficial to intestinal health. There is elevation of a phenolic compound in the GI tract of the birds fed the standard diet and as mentioned previously phenols can destabilise the intestinal epithelia, this could be playing a role in the unhealthy appearance of the GI tract of these birds. It has been demonstrated that some of the compounds found in fishmeal, such as histamine and gizzerosine, have the potential to be detrimental to intestinal health in poultry and have been implicated in the onset of gizzard erosion in broilers due to stimulating excessive acid production by the proventriculus (156, 180, 254, 384, 390). These compounds can also lead to the small intestine becoming inflamed, dilated and filled with watery contents (8, 350). The lowering of pH due to the excessive acid production can lead to a reduction in digestive enzyme activity and malabsorption (164). Damage to the intestinal mucosa can be correlated with an increase in enteric pathogens. For example the presence of *Clostridium perfringens* (the causative agent of necrotic enteritis) has been found to be increased in cases of gizzard erosion (294) and coccidiosis (427) where there is mucosal damage. Dietary change has been implicated in the onset of DB in broiler chickens. Taking all of this into account it is possible that there are dietary factors that are detrimental to the integrity of the intestinal tract of broiler chickens which play a role in the onset of DB in the event of a diet induced change in microbial composition of the resident microbiota.

This thesis provides a greater understanding of the role of intestinal bacteria, genetics and diet in relation to DB and forms a firm foundation for further studies. In order to determine whether the bacteria seen to alter in the DB are the cause of the disease then healthy chickens could be inoculated with intestinal contents of DB affected birds. If the birds then went on to develop the disease it would suggest that the bacterial species are involved with the onset of the condition. If this was found to lead to the onset of DB then a disease model could be created and management strategies could be investigated. To ascertain the involvement of the immune system in the pathogenesis of DB, a further case study of healthy and DB

affected birds would be required and blood samples taken for the analysis of immune parameters such as cytokines and chemokines to give an immunological perspective on the mechanism of the disease. The administration of probiotics to day old chicks immediately post hatch could promote favourable development of the GI tract and the immune system, whilst at the same time providing a favourable pioneering bacterial species to colonise the neonate gut. This could be a means to prevent the onset of DB and act as an alternative to antibiotic growth promoters. Inoculating a group of newly hatched chicks with probiotic bacteria and another group with a placebo as an experimental set up could evaluate this by taking the necessary samples from members of the groups at different time points for comparison. Samples could be taken to analyse the composition of the intestinal microbiota, immune responses (such as cytokine and chemokine expression levels) and the metabolite profiles of intestinal contents. In addition the histological morphology of the GI tract could be compared to look at how the probiotic affects the structural development of the GI tract. Taking this further if probiotic treated group and the placebo group could then be inoculated with the intestinal contents of a DB affected bird and similar comparisons measured. In this scenario a good indicator of the integrity of a bird's intestinal health and immune system would be for the intestinal microbiota to return to what it was before inoculation.

There has been no published work on the characterisation of the intestinal microbiota of broiler chickens suffering from DB using PCR-DGGE or H^1 NMR, therefore by combining microbial and metabolomic profiling this thesis provides the first comprehensive investigation into the condition and related risk factors. A major conclusion of this study is that in birds suffering from DB, there was a shift in the composition of the intestinal microbiota involving the excessive proliferation of members of the resident microbiota including members of the bacteroidetes, clostridia, *Lactobacillus aviarius* and *E. coli*. There is a change in the biochemistry within the gastrointestinal tract of the DB affected birds which suggests bile acid deconjugation and nutrient malabsorption which could account for the depression in growth seen in affected birds. Furthermore increasing dietary protein density (a proposed risk factor) affects the resident microbiota, with the changes in bacterial

composition being similar to that seen in the DB affected birds. Analysis of the intestinal microbiota of different genotypes either susceptible or resistant to enteric disease did not reveal any bacterial related differences, suggesting one possibility is that the susceptibility relates to genotype specific responses to shifts in the intestinal microbiota. The feeding of a vegetarian diet did appear to promote more favourable bacteria in the small intestine, thus could be a possible strategy for maintaining good enteric health.

It is likely that a dietary change or stress causes an overgrowth of the intestinal microbiota which, due to the host response or compromised intestinal integrity, leads to further deterioration of intestinal function. At present intestinal health or robustness to change in the composition of the microbiota is not a phenotype considered when selecting the chickens to be involved in the breeding program of broilers. The removal of pharmaceuticals for the treatment and control of diseases in food producing animals is going to be a continual hurdle in disease control. Thus one could suggest that with increased knowledge and genome sequencing capability, that an intestinal health and robustness phenotype should be included in the selection criteria of the next generation of broiler breeders.

Appendix I – Recipes for media used in culturing of bacteria in this thesis**Brain Heart Infusion (BHI) broth**

Calf Brains, Infusion from 200 g	7.7 g/litre
Beef Heart, Infusion from 250 g	9.8 g/litre
Proteose Peptone	10.0 g/litre
Dextrose	2.0 g/litre
Sodium Chloride	5.0 g/litre
Disodium Phosphate	2.5 g/litre

Brain Heart Infusion (BHI) agar

Calf Brains, Infusion from 200 g	7.7 g/litre
Beef Heart, Infusion from 250 g	9.8 g/litre
Proteose Peptone	10.0 g/litre
Dextrose	2.0 g/litre
Sodium Chloride	5.0 g/litre
Disodium Phosphate	2.5 g/litre
Agar	15.0 g/litre

Bacteroides specific agar

Peptamin	20.0g/litre
Sodium Chloride	5.0g/litre
Yeast Extract	2.0g/litre
Reducing Agents/Peroxide Inhibitors	1.5g/litre
Dextrose	1.0g/litre
Sodium Bisulfite	0.1g/litre
Hemin	5.0mg/litre
Vitamin K	1.0mg/litre
Vancomycin	40ml/litre
Kanamycin	40ml/litre
Laked Horse Blood	50ml/litre
Agar.....	15.0g/litre

Phosphate Buffered Saline (PBS)

NaCl.....	137mM
KCl	2.7mM
Na ₂ HPO ₄	8mM
KH ₂ PO ₄	2mM

Appendix II – Table of bacterial species identified in this thesis.

Species number	Figure	Band letter	Bacterial species*	100% DNA match to species identified **
1	3.6	a	Uncult. <i>Clostridium</i> sp. (AB218299)	4
2		b	<i>Lactobacillus aviarius</i>	8, 14, 29
3		c	<i>Escherichia coli</i>	10, 18, 28, 34
4		d	Uncult. <i>Clostridium</i> sp. (AB218299)	1
5	3.12	a	<i>Bacteroides dorei</i>	-
6		b	<i>Barnesiella viscericola</i>	-
7	3.21	a	<i>Bacteroides vulgatus</i>	9, 12, 25
8		b	<i>L. aviarius</i>	2, 14, 29
9		c	<i>B. vulgatus</i>	7, 12, 25
10		d	<i>E. coli</i>	3, 18, 28, 34
11		e	Uncult. <i>Clostridium</i> sp (EF708193)	-
12	3.27	a	<i>B. vulgatus</i>	7, 9, 25
13		b	<i>Bacteroides ovatus</i>	26
14	4.3	a	<i>L. aviarius</i>	2, 8, 14
15		b	<i>Lactobacillus johnsonii</i>	30
16		c	<i>Lactobacillus crispatus</i> (FR683088), <i>Lactobacillus fornicalis</i> (AB290883) or <i>Lactobacillus helveticus</i> (FR683085)	21, 31
17		d	<i>Enterococcus</i> sp. (AB189703)	-
18		e	<i>E. coli</i>	3, 10, 28, 34
19		f	<i>Clostridium</i> sp. (FJ503326)	22
20	4.6	a	Uncult <i>Bacteroides</i> sp. (XO77B42)	-
21		b	<i>L. crispatus</i> (FR683088), <i>L. fornicalis</i> (AB290883) or <i>L. helveticus</i> (FR683085)	16, 31
22		c	<i>Clostridium</i> sp. (FJ503326)	19
23	5.1	a	<i>L. crispatus</i> (AY335496) or <i>L. fornicalis</i> (AJ621639) or <i>Lactobacillus acidophilus</i> (M99704)	-
24		b	<i>Lactobacillus salivarius</i>	32
25	5.2	a	<i>B. vulgatus</i>	7, 9, 12
26		b	<i>B. ovatus</i>	13
27		c	<i>Parabacteroides distatonis</i>	-
28		d	<i>E. coli</i>	3, 10, 18, 34
29	5.7	a	<i>L. aviarius</i>	2, 8, 14
30		b	<i>L. johnsonii</i>	15
31		c	<i>L. crispatus</i> (FR683088), <i>L. fornicalis</i> (AB290883) or <i>L. helveticus</i> (FR683085)	16, 21
32		d	<i>L. salivarius</i>	24
33		e	<i>Campylobacter jejuni</i>	-
34		f	<i>E. coli</i>	3, 10, 18, 28
35		g	<i>Lactobacillus</i> sp. (FJ268889)	-
36		h	<i>Clostridium</i> sp. (AM933653)	-
37		i	<i>Bifidobacterium</i> sp. (AB064846)	-

* Numbers in brackets refer to accession numbers of unknown or uncultured species

** Numbers refer to the species number in the first column in this table

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