The effect of different dietary and management interventions on aspects of foot health, gut function and litter microbiome composition in growing poultry

By

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A thesis submitted to The University of Reading for the Degree of Master of Philosophy

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United Kingdom

January 2020

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

The poultry studies involved undergraduate students who were using some of the data for their own dissertation projects. Some of the sample collection and analysis was therefore undertaken by them under supervision, but all data analysis was undertaken by myself independently.

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Abstract

Foot pad dermatitis (FPD) in poultry is a contact dermatitis that can be painful and has a negative impact on bird welfare and performance. The primary cause is wet litter and so any factors which may cause the litter to become wet (eg poor choice of bedding material, dietary imbalances and poor gut health) may increase the risk of the birds developing FPD . In this thesis, various nutritional and management interventions were investigated to determine their impact on bird performance, aspects of gut and foot health and function, and measures of litter quality. When turkeys were fed whole grain wheat (WGW), either as free choice or mixed with the diet at inclusion rates up to 200 g/kg diet, it was observed that intake of WGW was much more variable in birds offered free choice WGW, although mean intake of WGW was greater. Bird performance was better if WGW was not fed, but if WGW was included in the diet, performance was better when WGW was mixed into the diet rather than offered free choice. WGW reduced gizzard pH, which might inhibit the growth of pathogens in the gut, but at a molecular level it had no effect on the presence of *Clostridium perfringens, Campylobacter jejuni*, *Salmonella* spp or *Brachyspira pilosicoli*. It also had no effect on litter moisture content or incidence of foot pad dermatitis. When broilers were reared on different bedding materials (Envirobed or wood shavings), bird weight was greater if broilers were kept on wood shavings, but birds consumed more Envirobed bedding and this was associated with drier digesta so that the risk of FPD might be lower with Envirobed. Broilers were then fed either a wheat or maize based diet and reared on wood shavings which were either clean or had excreta from mature laying hens added. There was effect on bird performance, but feeding maize may reduce the risk of FPD as it was associated with reduced litter ammonia content, increased litter pH and decreased prevalence of ampicillin resistant *E. coli* in both the birds' gut and the litter. Litter quality is improved (and FPD reduced) if birds are encouraged to consume bedding (or a high fibre feed) and fed maize rather than wheat. The finding of this thesis are that, to reduce the risk of foot pad dermatitis in broilers and turkeys, the birds' litter must remain dry and friable.

This will be achieved by adequate ventilation and good management of drinkers, but also by maintaining good gut health in the birds. In this thesis, drier excreta and litter were associated with higher bedding (or fibre) consumption, and by feeding birds maize rather than wheat.

Acknowledgements

I would like to express a special warmest and appreciation thanks to both my supervisors Dr Caroline Rymer and Dr Darren Juniper for advice, support, encouragement, guidance and for providing me with knowledge of poultry production and management throughout my PhD; all of these are important to me. I would not have this achievement of my research, thesis, or to grow as a research scientist without their encouragement, advice, knowledge and guidance.

I am grateful to all staff at CEDAR research farm, especially Mr Colin Green, Dr Anna Thomson, and Ms Debbie Cockman. I would like to offer my most affectionate thanks to all my postgraduate and undergraduate friends in the University of Reading. My sincere thanks also go to my sponsor, the Ministry of Higher Education and Scientific Research - Kurdistan region-Iraq for funding this project.

I wish to convey my warmest appreciation to my parents, family members, especially to both my brothers (Baxtear and Aram) for their encouragement, guidance and unconditional support, which have helped me to continue my study, and to my wife (Sozan) and kids (Aland, Lare and Rand). Without my family, I know I would never get this far in my education and life. I am sure the success of this work would make them delighted.

Great thanks are also due to Mr Chris Drake for his support and advice during my work at the Chemistry and Biochemistry Laboratory, and to Dr George Gibbings and PhD student Alex Tonks during my work in the Molecular Genetics Laboratory in SAPD, and to Mr Paul Kirton for his work, especially in the quantification of ammonia in poultry litter.

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Chapter 1 Introduction

1.1 General Introduction

A number of issues have arisen with the rise in modern poultry production, and one such issue is the incidence of wet litter (Francesch and Brufau, 2004). This is an issue because of the increased ammonia content associated with wet litter, as well as the increased incidence of foot pad dermatitis (Youssef et al., 2010; Tran et al., 2015) (which is the focus of this thesis). FPD is a widespread problem in commercially grown turkeys and broilers as mentioned in Chapter 2; Section 2.6.1 which causes necrotic lesions on the plantar surface of the foot pad (Shepherd and Fairchild, 2010) and may eventually reduce bird performance resulting in a loss of carcass yield as well as negative effects on bird health and welfare (de Jong et al., 2014; Lister, 2009; Ritz et al., 2009). For this reason, especially in modern poultry production systems, the control of gut health to reduce wet faeces and FPD is a priority so as to increase production and to prevent environmental stress that may impact upon bird welfare.

Litter moisture concentrations can range from 200 to 550 g/kg and litter can differ considerably in moisture content in poultry houses (Miles et al., 2011). Litter becomes wet when the amount of water added to the litter (excreta and spillage of water from drinkers) exceeds the amount of water removed by evaporation, thus leading to increased litter moisture content. Generally, litter water holding capacity is compromised when the litter moisture content exceeds 250 g/kg (Collett, 2012). Hermans et al. (2006) reported that the incidence of wet litter inside poultry houses in the UK was 561 g/kg especially during winter months when ventilation rates are reduced to avoid excessive heat loss.

In poultry houses, wet litter is caused by a variety of factors such as management and housing, diet and disease. Management aspects that affect litter quality include

temperature, ventilation, litter type (Francesch and Brufau, 2004), and drinker design (Lynn and Elson, 1990). Nutritional aspects that influence the incidence of wet litter would include dietary protein concentrations, lipid, minerals and cereals as mentioned in Chapter 2. Section 2.7.3.1; 2.7.3.4; 2.7.3.3 and 2.9.3 respectively (Francesch and Brufau, 2004; McIlroy et al., 1987; Collett, 2012). Wet litter may also develop as a consequence of diarrhoeal disease, which itself may be a consequence of infections within the intestinal tract (Abd El-Wahab et al., 2012). A study in the UK identified that management factors such as temperature, ventilation, litter type and leakage from the water system accounted for 48.4% of the incidence of wet litter and that in 35.3% of cases more than one factor was involved. Problems with ventilation accounted for 28.9% of cases, leaking drinkers 23.5%, adverse weather 3.7%, leaking roofs 2.1%, but poultry disease (diarrhea) was the single most important factor accounting for 61.0% of cases of wet litter (Hermans et al., 2006). For this reason, this thesis focussed on interventions that would improve gut health by reducing caecal dysfunction (imbalance of the caecal fermentation and inflammation of the caecum). Approaches that were investigated included the inclusion of whole wheat in the diet, changing the bedding material, comparing different cereal sources (maize or wheat), and exposing the birds to excreta from healthy, adult birds.

One intervention that can be used to promote gut health is the inclusion of whole cereal grains in the birds' diet (Singh et al., 2014a; Zdunczyk et al., 2013; Amerah and Ravindran, 2008). The main impact that whole cereals appear to have is in encouraging gizzard development and activity, which prevents potentially pathogenic bacteria from entering the intestine by reducing the pH of gizzard digesta. This in turn enhances intestinal function (by increasing hydrochloric acid secretion in the proventriculus), which consequently reduces the growth of pathogenic bacteria and thereby improves gut health (Engberg et al., 2004; Gabriel et al., 2003a; Zdunczyk et al., 2013), Whole

cereals also increase peristaltic movement (Taylor and Jones, 2004), which may further help to improve gut health. Increased gut motility and greater digestion of nutrients are also related to increased grinding activity within the gizzard (Amerah et al., 2007a). The impact of including whole cereals in the diet of growing turkeys on measures of their gut and foot health, and on the litter moisture content, was investigated in the experiment reported in Chapter 3.

There are several types of bedding material using as a litter in poultry production. There is some concern that sawdust or wood shavings may become scarce because of their increased use as fuel (Worley et al., 1999). The broiler industry must look for other readily available bedding materials that meet hygienic requirements, decrease ammonia level throughout the productive cycle, improve bird performance and litter characteristics (Worley et al., 1999). One potential bedding material is Envirobed (Hulet and Cravener, 2007), which was investigated in the experiment reported in Chapter 4.

In modern poultry production, cereals are an important component of the birds' diet, but the protein and non-starch polysaccharide fractions of different cereals might affect the birds' gut health. Compared with maize, wheat increases digesta viscosity and the bacterial population of the gut, reducing the transmission of hydrolysed products to the enterocyte cells and thereby nutrient absorption (Kalantar et al., 2016). The effect of cereal source in the diet of broilers on measures of bird performance, foot health, litter quality, presence of ampicillin resistant *E.coli* in the digesta and the litter, and the composition of the litter microbiome was investigated in the experiment reported in Chapter 5. In this experiment, the effect of exposing birds to the excreta of healthy, adult birds as a possible means of advancing the development of their caecal microbiome (Cressman et al., 2010) was also investigated.

The aim of this thesis was to evaluate different nutritional and management interventions aimed at improving the gut health of turkey poults and broilers, thereby reducing the litter moisture content and incidence of foot pad dermatitis in these birds.

Chapter 2 Literature Review

2.1 Scale and importance of the meat poultry industry

The world demand for poultry meat is increasing (Ruff, 1999). Between 1995 and 2005, poultry production increased globally by 53% for broilers and 13% for turkeys according to the UN Food and Agricultural Organization (Scanes, 2007) and consumption of poultry meat increased by 5 kg per capita between 1975 and 1995 from 23 to 28 kg/person/year. Chicken meat production in the top producing countries (United States, China, Brazil, Mexico and India) in 1995 was, 11.5, 6.1, 4.1, 1.3, 0.6 million tonnes respectively. This increased, in 2005, to 15.9, 10.2, 8.7, 2.4 and 1.9 million tonnes respectively (Scanes, 2007). In 2006 the United States was the largest consumer of poultry meat at 54 kg/capita/year. Brazil was second in terms of individual consumption (33.3 kg/capita/year) and Mexico third (26.1 kg/capita/year). China was the second largest consumer in terms of volume but had lower per capita consumption (10.3 kg/capita/year). In the European Union, consumption was 22.7, 22.9 and 22.2 kg/capita/year in 2004, 2005 and 2006 respectively. The most rapid increase in consumption has been in South America and Asia at +8% a year (Magdelaine et al., 2008). Offsetting this increase in poultry production is the increased efficiency of bird performance. In 1950, it took 16 weeks to reach the marketable body weight of 2 kg for broilers. By 1990, this had decreased to 6-7 weeks (Schmidt et al., 2009). Broiler growth rate has increased in the last 50 years and is expected continue to increase at a rate of 3.3% per year (Zuidhof et al., 2014). In 1957 a broiler weighed 586 g at 42 days with a feed conversion ratio of 2.8, while now a broiler of the same age weighs 2.90 kg with a feed conversion ratio less than 1.70 (Zuidhof et al., 2014). Broilers reaching a weight of 2.34 kg in less than 29 days by the year 2034 is expected as their genetics continue to improve. These improvements in weight gain and feed conversion ratio

mean that changes also need to be made to the birds' nutrition and feeding, and to management practices, to reflect the changing needs of the bird (Tavárez and Solis de los Santos, 2016).

2.2 Production systems

Commercial broiler chickens are usually kept on the litter bedded floor system. A caged system for broiler chickens has not been adopted because of the constraints these have on production (Reece et al., 1971) including leg deformities (Wideman Jr et al., 2012), breast blisters (Reece et al., 1971), skin imperfections and enlarged feather follicles (Andrews et al., 1975) arising from the erosion of the skin against the wire cage floor (Reece et al., 1971; Fu-rong et al., 2007). These problems have negatively influenced meat quality and the increased labour requirements of this system, related to moving broilers in and out of cages, cannot be justified (Reece et al., 1971). However, as the poultry industry continues to expand, it may be that the use of appropriate cages does become more common especially in countries where land is scarce and expensive bedding materials need to be imported. Cages allow a larger number of birds to be reared together inside one building as cages are stacked vertically and decrease costs related to the purchase, removal and disposal of litter. In the Gulf region and Saudi Arabia, chickens are usually reared for five weeks to a weight of just 1.4 to 1.5 kg (Al-Ankari et al., 2004). At this age or weight, the incidence of leg deformities and breast blisters is negligible. Another system is organic broiler production, which has a greater focus on bird welfare requiring the birds have access to free range areas and perches. ′Stocking density is lower than in commercial systems′ to ′Stocking density is lower than in conventional systems′. Organic systems are commercial, but not conventional. In the UK this is set as a maximum of 21 kg/m^2 with fixed housing and 30 kg/m^2 if the house is mobile, whereas conventionally kept birds have a maximum stocking density of 39 kg/m^2. The use of antibiotic growth promoters is banned by the EU for any

system, but other additives are also banned in organic systems. The feed supplied to the chicken must also correspond to organic standards without the use of any artificial fertilisers and 90% of the feed should be 'home produced'. An increasing number of consumers demanding 'healthy' and 'natural' foods have a preference for organic farming (Sundrum, 2001), although the market share of organic poultry meat is still very small. Turkeys can be raised successfully almost anywhere in the world because they are adaptable to a wide variety of climatic conditions if their nutritional requirements are met and protection is provided against diseases, weather conditions and predatory animals (Stanley, 1971). Commercial turkeys are kept in enclosed houses in conventional housing with some side curtains, with environmental control such as temperature, lighting and ventilation. In another system turkeys are housed on deep litter in naturally ventilated sheds with natural light and have access to forage and shelter belts (Hartung et al., 2009). Turkeys are reared to a variety of ages from 3-5 months depending on the strain used. The free range system is beneficial to bird welfare (Kijowski et al., 2005). However, carcass yield decreased in both male and female birds housed in the extensive system (Herendy et al. (2004). Sarica and Yamak, (2010) noted that birds kept in a free range system have a lower incidence of foot pad dermatitis compared with intensive system, this may be because birds have greater exposure to damp litter in intensive systems. In the outdoor system an increase in mortality was observed in the last 2 weeks of growth (20 to 22 weeks) because of ground frost at night (Burs and Faruga, 2006). In turkey welfare stocking density is an important issue. Turkey welfare was poorer at higher stocking densities with negative effects on behaviour, reduced body weight and poorer health compared to lower stocking density (Abdel Rahman 2005).

2.3 Issues facing the industry

Many issues are facing the poultry industry, such as the cost and availability of feed (and competition between feed and food), diffuse pollution, ammonia, stocking density, leg and foot health, breast and hock blisters, cannibalism, food safety and quality, litter management, and the use of antimicrobials in poultry production and the rising incidence of antimicrobial resistance. These issues will be discussed briefly in this section.

2.3.1 Cost and availability of feed (and competition between feed and food)

The high cost and non-availability of feed ingredients are major issues facing broiler producers. The cost of feed is about 60-70% of the total cost of broiler production (Milanovic, 2017). Accessibility of feed quality and quantity at a sensible cost is a key to successful poultry production (Milanovic, 2017). Therefore, it is imperative for broiler producers to source cheap alternative feedstuffs without affecting the quality of the feed and bird performance (Hooge and Rowland, 1978). On the other hand, the global population will continue to grow to 9 billion people by the middle of the century. This will result in higher demand for food and competition for the use of cereals for feed, food and fuel. There will also be increased competition for land, water and energy (Godfray et al., 2010). This will inevitably increase the feed cost pressures on the broiler industry.

2.3.2 Ammonia

A major air quality concern in poultry houses is the emission of ammonia (NH_3). Excess dietary amino acids and non-protein nitrogen are converted to urea and excreted mostly as uric acid. In the poultry litter it is converted back to urea by the enzyme uricase in the presence of oxygen and water. Thereafter, urea is subsequently hydrolyzed to ammonia by the enzyme urease in the presence of water (Liang et al., 2014; Becker and Graves,

2004; Maliselo and Nkonde, 2005). As water is required for both reactions, this conversion is accelerated in conditions of high humidity. Other favourable conditions include a pH of 8-13 and high temperature (Maliselo and Nkonde, 2005). Ammonia is a colourless, irritant gas with a sharp and penetrating odour (Moum et al., 1969). Higher concentrations of ammonia in the poultry house are generally related to higher litter moisture and nitrogen contents; the higher moisture content encourages ammonia volatilization and the higher N (uric acid) content increases the supply of ammonia precursors (Liu et al., 2007). According to Carlile (1984) ammonia is a harmful gas in poultry houses. It can not only affect the environment, but also the health and performance of the birds (Kristensen and Wathes, 2000; Homidan et al., 2003; Shah et al., 2007). In poultry production, ammonia and high litter moisture contents are also correlated with foot pad dermatitis (FPD) as well as hock burn lesions (Haslam et al., 2006). In addition, ammonia causes irritation of the respiratory tract and mucous membranes of the eyes. This can increase the incidence of respiratory disease which in turn may impact on growth rates, feed intake and subsequent feed conversion efficiency (Kristensen and Wathes, 2000). A number of different strategies have been investigated to either reduce the amount of ammonia produced by poultry production, or to mitigate its effect on the environment once released. Reduction and careful control of dietary protein content and amino acid balance can be used to reduce the amount of N that ends up in excreta as a source of ammonia emissions (Becker and Graves, 2004) as an increase in the emissions of ammonia are observed when the protein content of broiler diets increases (Elwinger and Svensson, 1996). Alternatively, urease activity in the litter may be inhibited by the use of feed additives such as yucca plant extracts, and this may reduce ammonia emissions. The use of acidifying agents such as ferrous sulphate, phosphoric acid and aluminium sulphate may reduce poultry litter pH and therefore

urease activity, leading to a reduction in the emission of ammonia (Becker and Graves, 2004).

2.3.3 Litter management

In many places in the world, there is a major concern associated with the disposal of poultry litter because of risks to public health and the environment (Bolan et al., 2010; Waziri and Kaltungo, 2017). Poultry litter may be a harbour for zoonotic pathogens such as coliforms, *Clostridia* and potentially *Salmonella* and *Campylobacter* (Schefferle, 1965; Terzich et al., 2000; Lu et al., 2003b; Macklin et al., 2005) It is also a major source of diffuse pollution and ammonia (Xiaoyan, 2005; Sharpley, 1999; Kaiser et al., 2009). Mean poultry litter production per bird day is approximately 0.11 kg so that a flock of 10 000 birds would produce over 46 t in one 42 d growing period (Waziri and Kaltungo, 2017). In Australia, the scarcity of available litter material and growing production costs has led broiler producers to practice litter reuse in broiler houses (Cressman, 2014). Reusing poultry litter for a second or third batch reduces the amount of litter requiring disposal (Waziri and Kaltungo, 2017) as well as reducing the amount of fresh bedding material required.

2.4 Nutrition physiology and metabolism

The digestive tract of any bird is important in digesting feed into nutrients for absorption for use by the bird for maintenance and growth. Therefore it is necessary to understand the avian digestive tract, not only to enable the formulation of economical diets which will meet the birds' requirements for health and growth, but also to identify when something is wrong so that corrective actions may be taken. The hypothesis on which this thesis is based is that an improvement in the functioning of the digestive system will reduce the risk of the bird developing FPD. It is an indirect risk, but that if the litter on which the bird is kept will be drier. This would then (indirectly) reduce the

risk of the bird developing FPD. The structure and function of the bird's digestive tract is therefore briefly reviewed here. The digestive tract of birds begins at the beak/mouth and ends at the cloaca (Zoetendal et al., 2004). The whole digestive tract of the bird is illustrated in Figure 2.1.

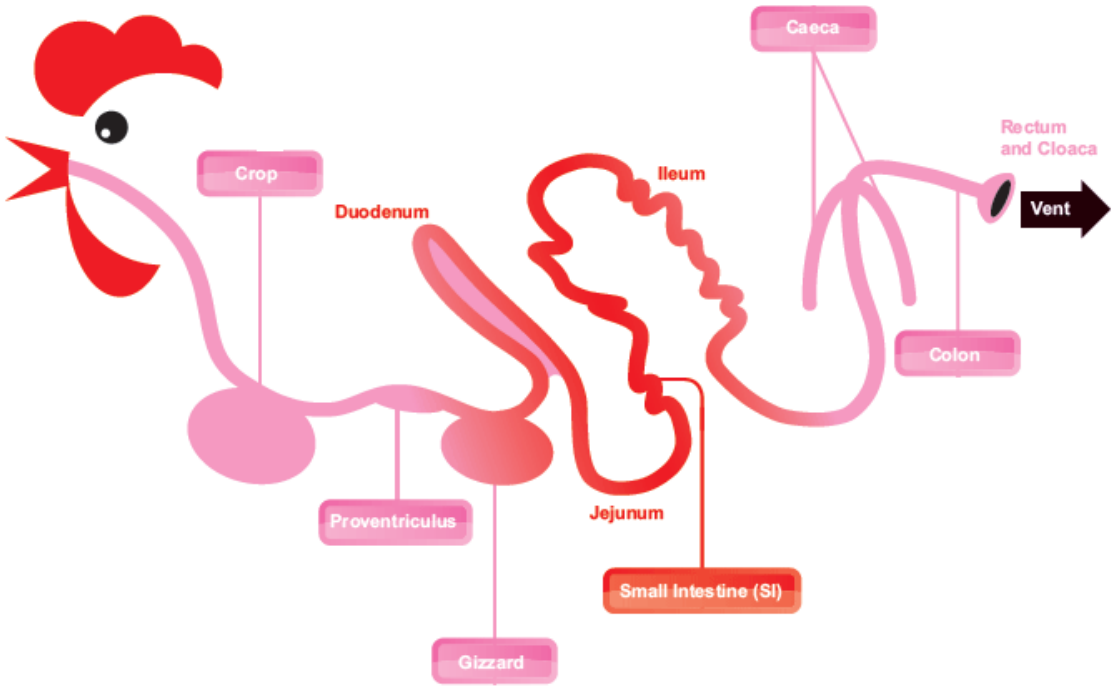


Figure 2.1: Digestive tract of a broiler chicken, modified from Baily (2013)

The beak obtains feed which then enters the mouth. As they do not have teeth, the chicken uses its tongue to push the feed to the back of the mouth (Classen et al., 2016).

The crop is an out-pocketing of the oesophagus and the main function of the crop is not only to store the feed before it passes into the stomach, but also to soften the digesta, which facilitates the beginning of enzyme (endogenous, exogenous and microbial) hydrolysis of the feed. It is the first main defence against poultry pathogens and zoonotic organisms (Classen et al., 2016). Salivary amylase is secreted in the mouth (Leasure and Link, 1940; Duke, 1986) and the action of this enzyme on starch continues in the crop. The crop wall does not have mucus secreting glands. The feed remains in the crop for up to 6 hours during which time the digestion process begins by bacterial

fermentation mainly by members of the *Lactobacillus* genus (Mead, 1997; Barnes et al., 1980). The main products of fermentation are lactic and acetic acids (McDonald et al., 2002). *Lactobacilli* predominate adhering to the crop wall which are able to hydrolyse starch to sugar and much of the sugar thus formed can be lost from the crop by absorption (Bolton, 1965; Hagen et al., 2003). *Streptococci*, coliforms and *Bifidobacteria* have also been found in the crop (Hilmi et al., 2007; Peinado et al., 2013; Petr and Rada, 2001) . The role of the crop in digestion of the diet will be influenced by the amount of time the feed spends in the crop and the proportion of the feed that enters the crop. The absorptive capacity of the crop is much lower than the small intestine because of the nature of the crop epithelium compared to the small intestine, but it does allow absorption by diffusion of organic acids such as lactic acids (Cutler et al., 2005). The type of feed that enters the crop and its residence time in the crop is variable and highly dependent on the nature of feeding behaviour, feed presentation and bird management (Shires et al., 1987). The withdrawal of feed (to induce moulting in layer hens, or prior to slaughter) results in a shift in the bacterial community of the crop and susceptibility of the crop to *Campylobacter spp* and *Salmonella* colonisation. An absence of feed in the crop increases the risk of pathogen colonisation as a result of reduced *Lactobacilli* colonisation, as *Lactobacilli* produce lactic and acetic acid by the fermentation of feed, which decreases the crop digesta pH and strengthens the entry barrier to pathogens (Hinton Jr et al., 2000a; Hammes and Vogel, 1995). The presence of *Campylobacter spp* and *Salmonella* in the crop of broilers at slaughter represents a human disease risk due to the higher chance of carcass contamination at slaughter from the crop compared with caecal rupture (Van Gerwe et al., 2010; Corrier et al., 1999). Inclusion of lactic acid in the drinking water (Byrd et al., 2001), acidified drinking water (Chaveerach et al., 2004) and the administration of glucose (associated with increased growth of lactic acid bacteria) can decrease the rise in the population of

Campylobacter spp and *Salmonella* during the period of feed withdrawal (Hinton Jr et al., 2000b). The crop forms part of the acidic barrier formed by the crop and gizzard which decreases the colonisation and passage of bacteria such as *Clostridium spp, Campylobacter spp* and *Salmonella* to the gut (Sekelja et al., 2012). The crop pH ranges from below 5 to greater than 6 (Józefiak et al., 2006; Hinton Jr et al., 2000a) and the acidity of it can vary with the degree of crop fermentation (essentially by *Lactobacilli*) and the pattern of fermentation (for example a change in the production of lactic acid to the weaker acetic acid will increase pH) (Cutler et al., 2005).

Feed passes through from the crop to the proventriculus; the oesophagus ends at the proventriculus. The proventriculus contains glands which secrete pepsinogen and hydrochloric acid (HCl) which reduces the pH in both the proventriculus and gizzard. The pH of proventricular contents from broiler chickens varies between 3 and 5 (Nkukwana et al., 2015; Mabelebele et al., 2017). The purpose of the low pH is to activate the pepsionogen to pepsin (Auer and Glick, 1984), but it also has a direct inhibitory effect against a variety of pathogens. Pepsin is the first enzyme responsible for protein digestion in the intestinal tract, cleaving the N terminal of aromatic amino acids such as phenylalanine, tryptophan and tyrosine. The acid denaturation and pepsin hydrolysis of proteins produce smaller molecular weight peptides which then enter the small intestine (Chen, 2017). The first step of protein digestion therefore occurs in the proventriculus by exposing ingested proteins to HCl, which denatures the protein and exposes peptide bonds for enzyme hydrolysis. When feed is eaten, distension of the proventriculus occurs enhancing the release of acetylcholine. This binds to G cells which enhances the release of gastrin. Other stimulants of gastrin release are hypercalcaemia and the presence of amino acids and gastrin releasing peptide, which is a neurocrine agent (Hersey and Sachs, 1995). The presence of gastrin encourages the release of histamine from enterchromaffin-like cells in the proventriculus. These

stimulants (acetylcholine, gastrin and histamine) bind to the parietal cells. This stimulates the secretion of HCl from these cells leading to the decrease in pH of both the proventriculus and gizzard (Khan and James, 1998; Bohak, 1973). As a result of the oesophageal contractions, feed passes through the proventriculus to the gizzard (McDonald et al., 2002)

Feed is subjected to mechanical grinding by the contractions of the thick, muscular gizzard wall (McDonald et al., 2002; Svihus, 2014). One of the important functions of the gizzard is assisting digestion by reducing the particle size of the feed (Svihus, 2011); before feed particles leave the gizzard, they need to be ground to a certain critical size (Moore, 1999). The threshold size for being constrained from leaving the gizzard in chickens is between 0.5 and 1.5 mm (Ferrando et al., 1987). In broiler chickens the gizzard pH varies between 1.9 to 4.5 with an average value of 3.5 (Bjerrum et al., 2005; Huang et al., 2006), although it can be as high as between 4.2 and 5.7 (Senkoylu et al., 2009; Boros et al., 1998). Whether the pH is low or high depends on the feed which is eaten by the birds, with large particle feeds such as whole grain wheat leading to a reduction in gizzard pH (Engberg et al., 2004). The contents of the crop, gizzard and proventriculus have a relatively low microbial diversity compared with the small and large intestine (Rehman et al., 2007). When leaving the gizzard the digesta passes into the small intestine, which has three segments, namely the duodenum, jejunum and ileum (Soltan, 2009). In broiler chickens the small intestine is dominated by lactic acid producing bacteria, mostly *Lactobacillus spp, Enterococcus spp*. and *Streptococcus spp*. These bacteria have a purely fermentative metabolism (Bjerrum et al., 2006; Hilmi et al., 2007). In the small intestine the digesta is mixed with bile salts from the liver via the gall bladder. The function of the bile salts is to emulsify the lipids and lipid soluble vitamins to assist in their digestion and absorption (Ridlon et al., 2016). Digestive enzymes produced by the pancreas consisting of proteinases, amylases and lipases are

secreted into the lumen of the small intestine and hydrolyse proteins, carbohydrates and lipids respectively. The pH of the small intestine varies from 6 to 6.5 (Mabelebele et al., 2017). Peptides from the proventriculus and gizzard are digested to amino acids by proteases secreted by the pancreas and small intestine. The pancreatic proteases (trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B) hydrolyse specific peptide bonds (Lipscomb, 1970) and are stored in their inactive form (zymogens) in the pancreas (Puigserver and Desnuelle, 1975). When secreted into the intestine, enteropeptidase activates trypsinogen into trypsin (Light and Fonseca, 1984; Lu et al., 1997) and trypsin activates chymotrypsinogen to chymotrypsin (Freer et al., 1970). Proelastase is converted to elastase (Grant and Robbins, 1957), and procarboxypeptidase to carboxypeptidase (Puigserver and Desnuelle, 1975).

The digesta then passes through the caecal junction into the large intestine, which consists of the caecum, colon and rectum. The caecum consists of two separate blind pouches located where the small and large intestines join. The roles of the caeca are the maintenance of gut health, the microbial fermentation of undigested nutrients and the absorption of water, glucose and volatile fatty acids. The caeca empty every 24-48 hours at which point they are refilled (Clench and Mathias, 1995). The caeca are filled in two ways, firstly by small particles of ingesta from the ileum (Bj Rnhag, 1977; Clemens et al., 1975), and the second is by reverse peristalsis of urinary and digestive fluids from the cloaca (Fenna and Boag, 1974; Frei et al., 2017). Undigested nutrients including starch, protein and fibre that escape digestion in the small intestine reach the caeca, where they may be fermented to produce indole, skatole, phenol, hydrogen sulphide, amines, ammonia and the volatile fatty acids (acetic, propionic and butyric) (Chaplin, 1989; Son et al., 2002; McDonald et al., 2002). Bj Rnhag (1977) observed that 20-34% of urine enters the caeca which is an opportunity for the birds to recycle nitrogen. Movement of material in the lower digestive tract and in the caeca by reverse

peristaltic movements move material from the proximal towards the distal end of the caeca and participate in filling the caeca as well as mixing the contents, however, peristaltic movements also empty (and further mix) the contents of the caeca (Duke, 1989; Clench, 1999). Björnhag (1989) reported that water, salt, monosaccharides and nutrients such as amino acids, B group vitamins and volatile fatty acids may be absorbed from the liquid refluxed in the caeca. The structure and activity of the caeca are affected by diet. The length of the caeca increased 30% and the weight with contents increased at least 3 fold when diets containing 20% glucose were replaced with diets containing the same quantities of pentoses as well as uronic acid (Longstaff et al., 1988). It was also observed that if a large proportion of the monosaccharides passed to the caeca having not been absorbed in the small intestine, the weight and length of the caeca increased (Longstaff et al., 1988). The transfer of materials to the caeca is determined by their physical characteristics. In cockerels about 17% of the excreted water and 18% of the excreted dry matter entered the caeca (Son et al., 2002). It is postulated that smaller particles move more readily into the caeca than larger particles (Hetland et al., 2002). Thus good gizzard function is required for diets containing coarse seed particles or fibrous components to ensure that they are reduced to small enough particles to aid downstream digestion in the small intestine (Svihus, 2011; Amerah et al., 2008a; Hetland and Svihus, 2007) and to be selectively transferred to the caecum. In addition to digestion by the bird's own digestive enzymes, there is extensive microbial fermentation of lactose and oligosaccharides (Carré et al., 1995), and (Duke et al., 1984) observed that there is caecal fermentation of cellulose in turkeys, although fermentation of large fibre particles appears to be very low (JøRgensen et al., 1996). Soluble and insoluble arabinoxylans and beta-glucans from barley passed into the caeca in broiler chickens (Jamroz et al., 2002), and (Denstadli et al., 2010) observed that broiler diets containing coarse or finely ground brewer's spent barley grains, when pre-treated with a

xylanase, resulted in a higher concentration of arabinose and xylanase in the caeca. Non-starch polysaccharides (NSP) such as arabinoxylans, pectins, and β- glucans are not digested by endogenous enzymes of the gastrointestinal tract, but they may be fermented in the caeca and colon to short chain fatty acids and may then be absorbed and utilised in the body (Choct et al., 1999; Jamroz et al., 2002). The fermentation in the caeca may lead to caecal hypertrophy (Redig, 1989). Soluble non starch polysaccharides can increase the viscosity of the gastrointestinal contents in the pre caecal part of the gastrointestinal tract, which may inhibit the secretion of bile acids and endogenous enzymes, which in turn causes morphological changes in the gut as well as reducing the digestibility of nutrients (Bedford and Classen, 1992; Choct and Annison, 1990). Thereafter the gut microflora and water balance of the body may be changed, increasing the incidence of wet litter, sticky droppings and reducing liveweight gain (Teitge et al., 1991; Chickens et al., 1989; Smits and Annison, 1996). Soluble pectin or β- glucans even in small amounts can increase the viscosity of intestinal digesta (Bedford and Morgan, 1996), but insoluble polysaccharides such as cellulose and xylans can hold water and their viscosities are relatively low. The amount of energy that can be extracted from caecal fermentation is only around 3-5% of the total energy requirements of the chicken (Jamroz et al., 2002; JøRgensen et al., 1996). (Józefiak et al., 2011) found a large amount of lactic acid in the crop and ileum, but none was detected in the caeca whereas significant amounts of volatile fatty acids were detected in the caecal contents. The caeca pH is normally slightly acidic at below 6.5 (Jamroz et al., 2002; Józefiak et al., 2006; Nkukwana et al., 2015). (Marounek et al., 1999) observed the caecal fermentation in broiler chickens fed different carbohydrates and reported that the disaccharides lactose and raffinose produced more VFA then inulin, starch, pectin and xylans, and no VFA were produced from carboxymethylcellulose. Small changes in fermentable dietary fibre content may have significant effects on the

extent of caecal fermentation (Jozefiak et al., 2004). Changes in the fibre content of the diet can cause changes in the microflora composition of the caeca. The number of *E. coli* and *Lactobacilli* increased when wheat and barley replaced maize in the diet, whereas the addition of inulin decreased the number of *E.coli* and increased the number of *Bifidobacteria* in the caeca (Rodríguez et al., 2012). When fructooligosaccharides were added to the broiler chicken diet, there was an increase in the total number of *Bifidobacteria* and *Lactobacilli* as well as a decrease in the number of *E.coli* in the caeca (Xu et al., 2003). An increase in the number of *Bifidobacteria* in the caeca was also observed when mannanoligosaccharides were added to the broiler diets (Baurhoo et al., 2007). Apart from diet, the health status and age of the bird also affect their caecal microflora. The predominant caecal bacteria in the first day of life in the healthy chicken are *Enterobacteriacae* spp., *Enterococus* spp., and *Lactobacillus* spp (van der Wielen et al., 2001). *Lactobacillus acidophilus*, *Lactobacillus salivarius*, and *Lactobacillus fermentum* are the most abundant *Lactobacillus* spp. in chickens (Mead, 1989). As the bird matures, the majority of bacteria in the caeca are strictly anaerobic and are dominated by *Ruminococcus, Clostridium, Faecalibacterium, Bacteroides,* and other strict anaerobes (Yeoman et al., 2012). From the ileocaecal junction, digesta then enters the colon. Very little digestion and absorption occurs in the colon apart from the last of the water reabsorption, while urine and excreta collect in the rectum or cloaca (Rose, 1997).

2.5 Anatomy and the composition of the chicken foot

The avian foot contains only part of the ankle bones; the hock in the bird is anatomically equivalent to the human ankle. Unlike humans, the bird does not have a well-developed calcaneum, or heel. Most poultry have four toes, although some breeds, including the Dorking, Faverolle, Houden, Sulatan and Non bearded Silkie Bantams have five toes. In these birds the extra toe arises above the base of the hallux and

projects upward, never touching the ground. The chicken's claw is relatively short and straight and is used for scratching when foraging. The chicken metatarsus has four surfaces, but these are not equal in size and some have an irregular shape. The two or three scutes on the forward surface of the metatarsus demarcate the anterior surface. The marginal boundaries can be seen fully only in medial and lateral views, but the encircling proximal and distal boundaries can be seen in several views (University of Illinois Extension). The proximal boundary is common with the ankle region, the distal boundary is the caudal end of the tarsometatarsus at the junction with the basal end of toes 2, 3 and 4 (the front toes). The metatarsal spur, like the beak has two parts: the underlying osseous structure and the covering of heavily keratinized epidermis. The spur in the chicken projects from the axis of the metatarsus at an angle of about 90 degrees and is pointed posteromedial at about a 45 degree angle. They are placed between the middle and distal thirds of the metatarsus. The phalangeal formula for the chicken is 2, 3, 4, 5. The first toe is the shortest, the third is the longest, the fourth toe has five phalanges and is only slightly longer then the second with three phalanges. In the chicken all flanges are relatively long except the terminals and those of the fourth toe. Although round in cross section, the toes seem to be divided into both a dorsal and ventral surface. The differentiation of these two zones is based chiefly on scale structure and placement of the interdigital webs (University of Illinois Extension). Chicken feet consist of 85% protein, mainly collagen. Other components are fat (3%), ash (2%) and moisture (10%) (Almeida and Lannes, 2013; Gómez-Guillén et al., 2011). Collagen consists of 30% glycine, 11.7% proline, 10-12.7% alanine and glutamic acid (Liu et al., 2001). Collagen is a fibrous protein with a triple helix structure and it is insoluble (Gómez-Guillén et al., 2011). A diagram of the chicken foot and toe is presented in Figure 2.1.

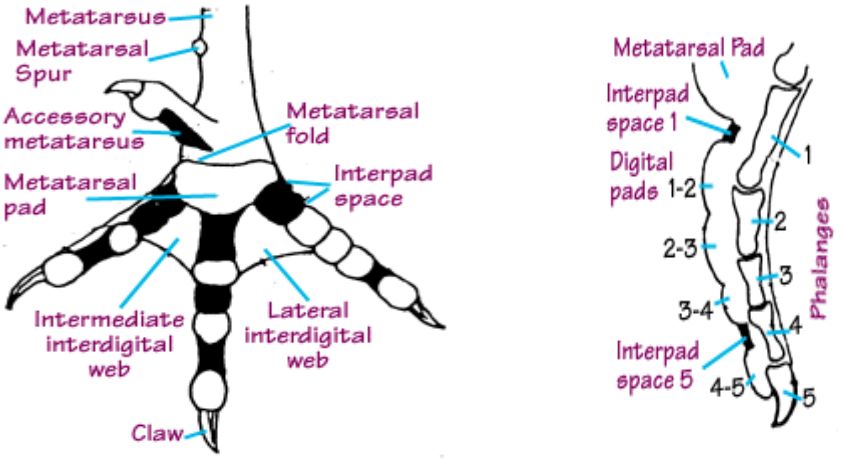


Figure 2.2: Chicken foot on the left and chicken toe on the right (Univerity-of-Illinois, 2018).

2.6 Foot-pad dermatitis

Foot pad dermatitis (FPD) or pododermatitis is a type of contact dermatitis affecting the plantar surface of the feet in poultry, the skin on the hock joint and in severe cases accompanying lesions may affect the breast area as well (Jacob et al., 2016a). At an early stage, lesions begin as small scaly brown scabs on the plantar surface and digital pads of the foot, becoming cracked, eroded and progressively larger in the first few weeks and in severe cases these changes are followed by acute inflammation, swelling, ulcers, hyperplasia and necrosis of the epidermis (Ekstrand et al., 1997; Greene et al., 1985). In turkey poults, the development of foot lesions are succeeded by hyperkeratosis and separation of keratin layers of the footpads by six weeks of age (Platt et al., 2001). Sometimes ulceration spreads into the dermis, although lesions are mainly superficial. The number of granulocytes, lymphocytes and lymph follicles increase within the dermis adjacent to the lesions. The prevalence of superficial lesions decrease whereas more severe ulceration increases after 14 days of age in turkeys (Platt et al., 2001). Martland (1984) observed that the mildest lesions showed an infiltration of heterophils into the stratum germinativum and this is sometimes accompanied by defects in keratin formation. Heterophils in the dermis, sub epidermis and epidermis, as

well as basophilic debris (necrotic cells) in the stratum corneum and small vacuoles often containing heterophils were seen in the epidermis and inside blood vessels in birds affected with these lesions (Martland, 1984). There was complete demolition of the keratin and epidermal layer in the centre of the lesion, exposing necrotic tissue and a mass of inflammatory cells, mostly heterophils (Greene et al., 1985). In more severe, ulcerated lesions the major finding was acute inflammation. In these cases, more dense cellular infiltration occurred and more obvious defects in the stratum corneum were observed (Martland, 1984; Greene et al., 1985). The epidermis was more eroded and fluid filled the dermis, while blood vessels were congested and necrotic and the epidermis was split (Mayne et al., 2006).

2.6.1 Prevalence of foot pad dermatitis in poultry

In turkeys the prevalence of FPD is enormously high, affecting about 98% of turkey poults. The prevalence of foot pad dermatitis in Swedish turkeys was observed to be 20% for severe lesions and 78% for mild lesions (Ekstrand and Algers, 1997). The prevalence of FPD in 60 turkey flocks in western France was observed to be 40.7% for severe lesions, with swelling of the feet observed in 60.0% of cases (Allain et al., 2013). (Krautwald-Junghanns et al., 2013) reported that the prevalence of FPD was nearly 100% in 24 farms in Germany and in the first week of age FPD was detected, with prevalence of lesions increasing up to 22-35 days of age. In Swedish broilers the prevalence of FPD was evaluated to be 5-10% for severe lesions and 10-35% for mild lesions (Berg, 1998). The prevalence of foot pad dermatitis in 101 commercial broiler flocks in Sweden was observed to be 6% for severe lesions, 32% mild lesions while 62% of birds were without lesions (Ekstrand et al., 1997). In France the prevalence of FPD lesions in broilers from 15 farms was 20% of birds (Martrenchar et al., 2002) .A total of 8 985 broiler chickens from 45 flocks were used to determine the prevalence of foot pad dermatitis in Japan. All birds had FPD in three of the flocks, but in the other 42

flocks the lesions ranged from 31.9% to 99.5%. The severity of the lesions were divided into four scores; score 0 none or normal, 1 small scabs < 5% pad area, 2 larger scabs < 25% pad area, 3 severe, large scab (filled ulcers) and the incidence of FPD for the four scores was 13.1, 33.3, 33.4 and 20.2% respectively (Hashimoto et al., 2011). With such a high incidence of this condition, especially in turkeys, it is clear that research and management effort needs to focus on addressing this condition to improve both the birds' welfare and their performance (de Jong et al., 2014; Benevides et al., 2016).

2.6.2 Risk of foot pad dermatitis

The inflammation of foot pads affected with FPD is probably accompanied by pain and suffering, especially in severe cases (Mayne, 2005). This constitutes a welfare problem and affects the final specification of the carcass (Ekstrand et al., 1997; Berg, 1998; de Jong et al., 2014). A high incidence of FPD in birds is often associated with a high prevalence of other types of contact dermatitis such as hock burns and breast blisters (Martland, 1985; Greene et al., 1985). In addition to lower body weight and growth rates of birds suffering from FPD, this may adversely affect the profitability of these flocks (De Jong et al., 2014). In addition, some markets place high economic value on chicken feet and in such cases, FPD would reduce the quality of chicken feet (Shepherd and Fairchild, 2010), and therefore the value of the carcass (Kaukonen et al., 2016).

2.6.3 Foot quality

Foot quality can be determined in the field and in poultry slaughterhouses (Sirri et al., 2010 ; Ask, 2010). There are a number of scales that are used to assess foot quality, these include an eight point scale, (Ekstrand et al., 1997), a three point scale (Bilgili et al., 2006), and a four point scale (Martland, 1984). Grading is based on the size of the lesion on the foot and as a consequence FPD may have a role in the classification of the

carcass in addition to the unintentional mutilations from processing, discoloration, and trauma injuries that may occur during catching and transport. Only 1% of foot downgrades come from catching, live haul injuries or processing mutilations. The remaining 99% are a consequence of FPD lesions (Shepherd and Fairchild, 2010).

2.6.4 Economic value of poultry feet

The economic value of chicken feet in 2008 was worth $280 million in USA (Shepherd and Fairchild, 2010). Therefore reducing the incidence of foot pad dermatitis would enhance both economic production and bird welfare. Increased demand for high quality chicken feet in export markets resulted in increased prices of chicken feet (US Poultry & Egg Export Council, 2009). In the US, prior to the mid-1980s, chicken feet were rendered with blood, feathers and other unsaleable portions of the chicken because chicken feet had little economic value. Little research was done on foot pad dermatitis and at that time companies did not consider FPD a serious economic problem (Shepherd and Fairchild, 2010). However, since chicken feet are cooked and consumed in some Asian countries (Lee et al., 2015) their economic value has begun to be realised in more recent years.

2.7 Aetiology of foot pad dermatitis

The aetiology of FPD is complex, and it seems to be multifactorial. Many contributing factors have been suggested including the composition of the diet, bird weight, sex, litter moisture and litter type (Mayne, 2005). The potential causes of foot pad dermatitis can be divided into three main factors, relating to the bird, its management and its diet. These will be reviewed in the following section.

2.7.1 Factors related to the bird

2.7.1.1 Gender and body weight

It is necessary to understand the effects of gender on the incidence of FPD in poultry. A number of papers have reported that males had a higher incidence and severity of FPD than females (Nagaraj et al., 2007b; Bilgili et al., 2006; McIlroy et al., 1987; Bruce et al., 1990; Buffington et al., 1975). This could be due to body weight, males typically being heavier than females and consequently additional weight is placed on the bird's foot (Bilgili et al., 2006). Buffington et al. (1975) found that body weight had a significant role in increasing the incidence of hock burns and FPD, while (Da Costa et al., 2014) observed that when body weight increased, FPD and gait score worsened. However, some studies reported no effect of body weight on the prevalence of FPD in broilers and turkeys (Martland, 1984; Kjaer et al., 2006). Conversely, other studies found a higher prevalence of FPD lesions in females compared with males (Nagaraj et al., 2007d; Kjaer et al., 2006; Kapell et al., 2012; Martins et al., 2016). This could be related to female skin which contains more fat and less collagen than males and is considered to be more susceptible to skin injury than males (Smith et al., 1977; Shuster et al., 1975) as collagen is the main protein of connective tissue including skin (Miyata, 1981), and also females had significantly weaker skin than males (Christensen et al., 1994). These differences in responses between studies reflect the multifactorial nature of the condition. In those instances where FPD was more prevalent in females, this was presumably because the interaction between litter moisture content and the physical characteristics of the litter were the principal factors predisposing the bird to FPD, and the poorer integrity of the female's skin made them more susceptible to developing FPD. In instances where the interaction between litter moisture and other litter characteristics were perhaps less challenging, then it was only when the greater pressure

on the foot was applied (arising from the greater weight of male birds compared with females) that male birds were more susceptible to FPD.

2.7.1.2 Breed

There is a clear difference in the susceptibility of foot pad dermatitis between different commercial breeds of the same age (Ekstrand et al., 1998; Sanotra and Berg, 2003). suggesting that the susceptibility of FPD may be affected by genotype (Bilgili et al., 2006). Similarly (Kestin et al., 1999) observed that FPD was not just a consequence of poor management, but that there may also be a difference between different strains in their susceptibility to FPD. (Allain et al., 2009) reported that slow growing strains had a lower incidence of FPD and (Kjaer et al., 2006) observed that slower growing, dual purpose breeds had a lower incidence of FPD when compared with the fast growing Ross 308 breed. This is most likely because fast growing birds, by definition, are heavier at earlier ages compared with slow growing birds, and so will exert more pressure on their feet, thereby predisposing them to a higher incidence of FPD. These authors suggested that it should be possible to reduce the severity of FPD by genetic selection. Amongst fast growing broiler breeds, a higher incidence of FPD was observed in Swedish and Danish Ross chicks when compared with Swedish Cobb chicks (Sanotra et al., 2003). With regard to turkeys, large turkey poults had a higher incidence of FPD than Broad Breast Bronze poults when reared in the same conditions on wire floors (Chavez and Kratzer, 1972). More recently, a difference in incidence of FPD was observed between two medium heavy turkey hybrids at 28 d of age (Veldkamp et al., 2017). These findings would suggest that genetic selection may be used as a tool to prevent foot pad dermatitis (Allain et al., 2009; Kestin et al., 1999; Ask, 2010), but the interaction between genotype and bird management and diet also need to be investigated.

2.7.2 Factors related to management

2.7.2.1 Litter materials

Management of litter material is an important component of rearing broilers and turkeys throughout their life. Bedding material fulfils a number of functions, such as moisture absorption, thermal insulation, the ability to maintain birds in floor pens, and allowing natural scratching behaviour. Litter materials must not only be able to absorb moisture, but should also have a reasonable drying time to get rid of that moisture via evaporation (Bilgili *et al*., 2009). The structure of the bedding materials (soft, coarse, sharp and hard) is also key (Bilgili *et al*., 2009). Therefore, the type of litter which is used in the poultry house must also not be too coarse, as a higher incidence of FPD has been observed when turkeys were reared on coarse particle board when compared with other litter sources such as fine particle board and hardwood shavings. This is because the coarse particle board has jagged edges and a coarse texture which can more easily puncture the skin of the foot (Hester *et al*., 1997).

Different bedding materials such as pine bark, pine shavings, mortar sand, chipped pine, chopped wheat straw, ground door filler, cotton gin trash and ground hardwood pellets were evaluated by (Bilgili *et al*., 2009). The researchers found that birds which were reared on soft materials such as mortar sand and ground door filler had a significantly lower prevalence of FPD than birds which were reared on the other types of bedding materials (which had sharp edges) as these caused small puncture wounds on the foot skin. Mortar sand also has the ability to release moisture quickly whereas ground door filler has superior moisture absorbance (Bilgili *et al*., 2009). According to Villagrá et al. (2014), birds reared on different bedding materials (wood shavings, straw, rice hulls and sand) showed a preference for sand to any other bedding materials, but the behaviours performed on the four bedding materials mainly differed for resting which was mostly performed on wood shavings and straw, because when sand was available

broilers dust bathed more, and they were more likely to peck and scratch when bedded on rice hulls. (Garcês et al., 2017) reported that there was no difference between sand, coconut husk, rice hulls, newspaper, corncob and guinea grass on the incidence of foot pad dermatitis.

(Ekstrand and Algers, 1997) observed that turkey poults reared on straw in commercial conditions had a higher incidence of FPD than those reared on wood shavings. This may be due to poor absorbing capacity in straw. Also, Mayne et al. (2007) found that turkeys reared on long barley straw showed high FPD scores regardless of whether the bedding was wet or dry. Similar results were also observed in broilers, where chopped straw was associated with the highest severity of FPD compared to wood shavings (Bilgili et al., 2009) . Straw is associated with a greater incidence of foot pad lesions compared to wood shavings, because of its higher moisture content, and a great propensity toward caking (Bilgili et al., 2009). Birds reared on pelleted wheat straw had lower incidences of foot pad dermatitis when compared to chopped straw in broilers at day 28 and 29 of age because the moisture content of pelleted wheat straw was lower than chopped straw (Kheravii et al., 2017). The water holding capacity of wheat straw was higher than wood shavings and rice hulls, but the evaporation rate in wheat straw was lower (Farhadi, 2014). Common litter materials include either white wood shavings or wheat straw. Normally-wood shavings are preferred to wheat straw as they are more porous and absorbent and birds can turn wood shavings more easily (Meluzzi et al., 2008a).

(Petek et al., 2014) reported that wood shavings were a better solution than rice hulls for foot pad lesions because wood shavings absorb water better than rice hulls. These studies show that the selection of wood shavings as a bedding material is to be recommended as it can reduce the severity of FPD. This is because of its absorptive capacity and ready release of moisture when ventilated, associated with the

lignocellulose in wood shavings. However, there is another litter material derived from timber production that can be used as bedding material which is superior even to wood shavings. This is produced by chopping the wood into fine particles then pressing them into pellet form using steam and high temperature. The incidence of FPD was significantly reduced with these lignocellulose pellets compared with other types of bedding materials including wood shavings, chopped straw and dried maize silage because of its higher absorbing capacity and ability to release water quickly (Youssef et al., 2011a). Kaukonen et al. (2017) observed that peat proved to be more useful for foot pad dermatitis compared to wood shavings and ground straw. Foot pad scores were better with either fresh shavings or peat moss compared to used shavings at day 21 and 42 of age due to peat moss higher ability to absorb moisture than used shavings (Shepherd et al., 2017). There are a number of choices when it comes to bedding for poultry. It is difficult to recommend using only one type of bedding materials because of differences in availability and cost. The bedding material associated with the lowest incidence of FPD is peat moss (Kaukonen et al., 2017) and lignocellulose pellets (Youssef et al., 2011a), but in reality the lower cost and greater availability of white wood shavings make this one of the most popular bedding materials for birds.

2.7.2.2 Wet litter

Wet litter is the most important factor associated with the development of FPD (Kaukonen., 2016). The severity of FPD was much higher when turkeys were reared on litter with a moisture content of 730 g/kg for 8 h/d compared with those reared on litter with a moisture content of 250 g/kg (Youssef et al., 2011a). A number of studies have shown that by itself, wet litter is sufficient to result in a deterioration of foot health and the development of FPD in turkeys and broilers (Tran et al., 2015; Taira et al., 2014). FPD lesions in turkeys can be reduced by maintaining the litter moisture content below 300 g/kg (Youssef et al., 2010). Martland (1984) demonstrated that litter (wood

shavings) which were sprayed with water to produce wet crusty litter with a moisture content of about 650 g/kg resulted in more FPD lesions in white turkey poults reared on untreated wood shavings with a moisture content of approximately 200 g/kg (birds were examined at 6, 8,10, 11,12 and 20 weeks of age). According to Mayne (2005), moving birds from wet litter to dry litter resulted in a reduction in FPD as well. Taira et al., (2014) reared chicks on wood shavings, and litter was either sprayed with water 1-3 times weekly as necessary until 35 days of age to maintain a high moisture content (litter moisture content varied from 309 to 565 g/kg). Litter was turned as necessary 1–3 times weekly until 28 days of age. In the control treatment, chicks were reared on the same dry litter (litter moisture content varied from 151 to 400 g/kg) without being sprayed with water. Litter was turned 3-4 times weekly until 49 days of age. The same bedding material maintained at the same depth of about 10 cm was used in both treatments and the same compound feed (starter, pre-grower, grower and finisher) was used in equal amounts. Water was provided by nipple drinkers. FPD in birds reared on dry litter was not observed until 28 d, whereas birds reared on wet litter showed signs of FPD at 14 d. It was also observed that when birds were moved from the wet litter to the dry litter the incidence of FPD reduced. The evidence from these studies clearly points to the importance of maintaining litter at a moisture content below approximately 300 g/kg to preserve foot health in poultry, particularly turkeys (Youssef et al., 2010; Youssef et al., 2011a).

2.7.2.3 Drinker design and management

Poor drinker design can result in increased moisture content of the litter which then eventually leads to FPD. Flocks receiving water from nipple drinkers were shown to have a lower incidence of FPD than those receiving water from drinker cups (Ekstrand et al., 1997). Flocks which were reared with cup drinkers were in turn shown to have a lower prevalence of FPD when compared with bell drinkers (Ekstrand and Algers, 1997). Bell drinkers increase litter moisture content, and the incidence of hock conditions in broilers compared with small cup and nipple drinkers (Lynn and Elson, 1990). This is likely to be a result of bell drinkers allowing more splashing of water onto the litter compared with the nipple system. In addition, all drinker systems, regardless of their design, can cause wet litter if they are poorly maintained, or badly managed, for example if the drinker is set at the wrong height for the growing birds, or the water pressure setting is set wrongly, leading to wastage of water from drinkers onto the litter (Lister, 2009).

2.7.2.4 Stocking density

In general stocking density is an important factor in bird performance and welfare (Hafez et al., 2016; Farhadi and Hosseini, 2016). A number of studies have observed that higher stocking densities were associated with a greater prevalence of FPD (Haslam et al., 2007; Bessei, 2006). This is probably due to the increase in the amounts of excreta when more birds are kept, which results in increased litter moisture content and poorer litter quality at high population densities (Dozier et al., 2006; Bessei, 2006). Higher stocking densities also increase the relative humidity of the air (through respiration losses), and so increase the need for greater ventilation to dry the litter to prevent FPD.

In broilers, hock and breast lesions also increased as stocking density increased when compared with flocks at a lower stocking density (Bruce et al., 1990; Dozier et al., 2005). This is possibly related to decreased litter quality because of increased litter moisture, and in several studies, decreased litter quality resulted in higher FPD lesions, hock and breast burns and these were associated with higher stocking densities (Meluzzi *et al*., 2008). (Buijs et al., 2009) observed that when broiler chickens were reared at 6, 15, 23, 33, 35, 41, 47 and 56 kg body weight/m^2 the incidence of foot pad dermatitis increased linearly with stocking density. (Farhadi and Hosseini, 2016) reported that when broilers were reared at different densities of 16, 18, 20 and 22 birds/ m^2, the incidence of foot pad dermatitis significantly increased at the highest density of 22 birds/m^2 , but there was no significant difference between the lower densities. (Dozier et al., 2005) also observed that stocking density had a negative impact on live performance especially when densities were over 30 kg/m^2. In addition to maintaining a low litter moisture content, therefore, FPD may also be controlled by maintaining lower stocking densities. The negative effect of high stocking density, however, may be partly ameliorated by increasing the ventilation rate in the poultry house, and this will be reviewed in the next subsection.

2.7.2.5 Ventilation

Ventilation has an important role in poultry houses in maintaining the correct environment for birds (Wang et al., 2014). Ventilation has a significant effect on the prevalence of FPD, hock and breast lesions with the highest incidence of these conditions being observed in the winter months (Ekstrand and Carpenter, 1997; Kyvsgaard et al., 2013). This is because ventilation rates decrease in winter to avoid excessive heat loss in poultry houses and reduce heating costs; this results in poor ambient air quality with high levels of humidity, which in turn prevents evaporation and removal of volatile compounds from the litter (Meluzzi et al., 2008b). (Weaver and

Meijerhof, 1991) reported a significant increase in litter moisture, caking, FPD and ammonia concentrations as relative humidity increased. However, if internal air circulation increased both caking and litter moisture were reduced. Good ventilation and air control such as the type of litter, type of drinker and type of ventilation are the key management factors affecting bird welfare, with maintenance of correct temperature, humidity, air and litter quality being crucial to birds' welfare (Jones et al., 2005).

2.7.3 Factors related to diet

Diet is considered to have an important role in the development of FPD having an impact on water intake, which in turn affects excreta moisture content and quality, and thereby litter moisture and quality (Francesch and Brufau, 2004; Collett, 2012). The modern poultry diet is predominantly composed of cereal grains mixed with protein supplements such as oilseed cakes and meals. This review will focus on dietary factors that affect water consumption and water excretion in excreta because of the effect this will have on litter quality, and the interaction this has with foot health.

2.7.3.1 Protein content and source

Foot pad dermatitis can be caused by high concentrations of protein in the diet (Veldkamp et al., 2016). An excess of dietary protein cannot be stored, thus it is catabolized, used as an energy source and the amino fraction excreted as uric acid (Francesch and Brufau, 2004; Veldkamp et al., 2016). To excrete this uric acid requires water, and so excess protein may lead to increased water consumption by birds, which can then result in additional water excretion in excreta. The wetter litter that this then produces can result in a higher incidence of FPD (Francesch and Brufau, 2004; Veldkamp et al., 2016). (Kamran et al., 2010) observed that the moisture content of litter decreased significantly when low protein diets were fed. (Veldkamp et al., 2017) observed turkeys fed low crude protein diets had lower litter moisture and foot pad

dermatitis compared with those fed diets with a high crude protein content. Broilers fed a diet with a low protein content (but meeting requirements for all essential amino acids) decreased their excretion of nitrogen and thereby improved litter quality and foot health without adversely affecting performance, carcass or meat quality (Shao et al., 2017). A number of studies have demonstrated that the source of protein in the diet can also influence litter quality in poultry houses (Marks and Pesti, 1984; Francesch and Brufau, 2004). For instance (Vieira and Lima, 2005) compared vegetable protein sources (soyabean and maize) with animal products (30 g/kg pork by product, 25 g/kg poultry by product and 15 g/kg feather meal). Birds fed soybean and maize (the vegetable diet) had a higher water intake and higher excreta moisture contents compared with birds fed the diet containing animal products. These conditions would result in a deterioration of litter quality and may have a direct effect on the development of FPD. The incidence of foot pad dermatitis was also higher in chickens fed a diet with all vegetable protein source compared with those fed mixed vegetable and animal protein dietary sources (Nagaraj et al., 2007b). Similar results were reported by (Cengiz et al., 2013) when broilers fed with all vegetable protein had a higher incidence of FPD than those fed mixed vegetable and animal protein. An increased incidence and severity of foot pad dermatitis, total excreta production and litter moisture content was also observed in broilers fed all vegetable diets based only on corn and soybean meal compared with those fed diets with poultry by product (Eichner et al., 2007). This may be because the non-starch polysaccharide in vegetable diets is poorly digested by chickens leading to watery and sticky droppings. This could increase the probability of faeces and litter adhering to the birds' feet, predisposing them to foot pad dermatitis, hock burn and breast blisters (Jensen et al., 1970; Mayne, 2005). The higher protein quality of animal proteins would also satisfy the birds' requirements for essential amino acids with a lower intake of non-essential amino acids that would need to be excreted.

(Nagaraj et al., 2007a) observed that decreased concentrations of protein in broiler diets resulted in reduced concentrations of ammonia in litter at 28 and 42 d of age. (Ferguson et al., 1998a) also observed that decreased protein contents in broiler diets resulted in reduced concentrations of litter ammonia and excretion of nitrogen in litter. A combination of wet litter and high ammonia content in the litter was reported to cause FPD. Ammonia is produced as a result of microbial activity in the excreta as well as litter on uric acid (Martland, 1985; Nairn and Watson, 1972). High levels of ammonia released from the litter may result in severe irritation of the skin and respiratory tract of birds which can in turn led to breast blisters, hock burns and FPD (Alchalabi, 2002). However, to contradict these findings, (Mayne et al., 2007) observed that there was little association between the ammonia released from the litter and the incidence of foot pad dermatitis. This would suggest that it is not the ammonia that is causing the FPD, but rather the excess litter moisture (associated with increased ammonia production) that arises from feeding an excess of protein or an imbalance of amino acids in the diet.

2.7.3.2 Nutritional deficiencies

Deficiencies of amino acids such as methionine (Murillo and Jensen, 1976; Clark et al., 2002), vitamins such as biotin (Clark et al., 2002) and riboflavin (Lepkovsky and Jukes, 1936) and trace elements such as zinc (Hess et al., 2001) have all been reported to adversely affect the prevalence of foot pad dermatitis because of their role in the synthesis and maintenance of skin. (Chavez and Kratzer, 1972) observed that supplementation of the diet with methionine decreased the incidence and severity of FPD in turkey poults and in another experiment (Chavez and Kratzer, 1974) found that the incidence of FPD was caused primarily by a deficiency of methionine in the diet of turkey poults. (Youssef et al., 2012) reported an experiment in which turkeys were fed a control (300 μg biotin and 50 mg Zn/kg), high biotin (2000 μg/kg), high Zn (150 mg/kg) and mannan –oligosaccharides MOS), 10 g/kg containing diet. In each group, half the

birds were exposed to wet litter for 8 hour daily while the other half were maintained on litter with a moisture content of 270 g/kg. The wet litter was maintained at an approximate moisture content of 730 g/kg by spraying water on the surface of the litter. They found a high biotin or zinc content in the diets of growing turkeys reduced the incidence of FPD in birds kept on dry litter, but had no effect in birds exposed to wet litter. The interaction between zinc, methionine and biotin requirement was investigated by (Abd El-Wahab et al., 2013), who observed that broilers had substantial foot pad lesions when fed diets containing zinc oxide (150mg/kg) and the recommended requirement of biotin (300 µg/kg), but that no lesions were observed with broilers fed Zn as zinc-methionine 150 mg/kg and a much higher biotin content (2,000 µg/kg). In this experiment wet litter was maintained in all groups by adding water to achieve approximately 35% moisture. As the incidence of foot pad dermatitis increased in all cases when birds were exposed to wet litter, it is clearly essential to reduce exposure to wet litter. However, deficiencies in biotin, zinc and methionine are secondary causes of FPD if litter quality is maintained. The actual concentration of dietary biotin required to prevent FPD is unclear, but a recent study by (Sun et al., 2017) reported that the incidence of foot pad dermatitis in broilers kept in high stocking density (16 broilers/m^2) was reduced when a biotin content of 1521 µg/kg diet compared to a normal biotin content of 155 µg/kg diet was fed, when the litter moisture content was approximately 290 g/kg. The incidence of foot pad dermatitis and the severity of skin lesions were also reduced when broiler diets contained 40000 µg/kg of Zn (Saenmahayak et al., 2010).

2.7.3.3 Electrolyte balance

The effects of dietary electrolytes (Na^+, K^+ and Cl^- balance) have been found to be an important factor that affects litter moisture and the incidence of foot pad dermatitis and several studies with broilers and turkeys have observed that cationic diets (those with

high concentrations of Na and K) are associated with increased water intake and higher excreta moisture contents. This in turn increases litter moisture content and the incidence of foot pad dermatitis (Abd El-Wahab et al., 2011; Cengiz et al., 2012). When turkeys were fed diets containing high concentrations of Na (3.1 g/kg) and K (15.3 g/kg), increased litter moisture content and foot pad dermatitis was observed compared with turkeys fed diets containing the recommended amount of Na (1.6 g/kg) and K (7.8 g/kg) (Abd El-Wahab et al., 2011). In broilers, high dietary Na contents (3.0 g/kg) resulted in increased water intake, litter moisture content and incidence of foot pad dermatitis (Cengiz et al., 2012), while increased incidence of foot pad dermatitis was observed in turkeys fed diets with Na contents of 2.5g/kg (Lichtorowicz et al., 2012). High K concentrations (12.7g/kg) in the diet of broilers adversely affected the moisture content of excreta (Koreleski et al., 2010), while a K content of 14.5 g/kg in the broiler diet also increased litter moisture content and FPD (Koreleski et al., 2010). Intakes of high concentrations of sodium, potassium, and phosphorus in laying hen diets increased water intake, excreta moisture content, litter moisture content and predisposed birds to foot pad dermatitis (Smith et al., 2000). The higher intake of these electrolytes causes significant osmotic changes in the intestinal lumen of the bird, increasing water retention in the digesta (Appleby et al., 1992; Murakami et al., 2000).

2.7.3.4 Fat

Dietary fat can also affect litter quality and the incidence of foot pad dermatitis. An increased incidence of foot pad dermatitis when broiler diets contained high fat contents was observed by (Bilgili et al., 2006). The impact of dietary fat content on foot health is probably mediated through the fat content of the digesta, and the impact this has on the gut wall, and this was in part investigated in a recent study by (Fuhrmann and Kamphues, 2016), who evaluated the effect of fat content in the broiler diet on excreta and litter moisture content, and the development of foot pad dermatitis. Birds were fed

diets containing two different concentrations of fat (55 or 110 g/kg DM mixed fat, consisting of palm oil as well as palm fatty acid distillate) and two different concentrations of calcium (7.5 or 6.6 g/kg DM) and potassium (14.5 or 15.0 g/kg DM). The results indicated that high fat excretion can affect foot pad dermatitis. The excretion of fat may be because of the formation of potassium soaps with the fat. Soap formation decreases the absorption of some fatty acids leading to an increased lipid content in the excreta as well as reduced retention time and water recovery as a result of irritation of the lining of the intestinal tract, thus increasing the moisture content of the excreta (Atteh and Leeson, 1984).

2.8 Prevention and control of foot pad dermatitis

To conclude, management strategies to prevent and control of foot pad dermatitis are summarized below:

2.8.1 Litter management

When the litter becomes wet or if flooding occurs from drinkers, or if there is caking by excreta, the old, wet litter should be removed immediately and replaced with a layer of fresh litter. Litter should be maintained in a dry and friable condition, using litter materials that are soft with an absence of sharp edges. Therefore, it is important to use good bedding materials which are able to absorb and release water quickly, and are soft on the birds' feet.

2.8.2 Ventilation

When the litter moisture content is high, rapid evaporation of the water is required from the bedding materials. Therefore, the litter will be wet especially in humid areas and in cold seasons. In these circumstances, to maintain low litter moisture contents, good ventilation is also required (which can reduce wet litter as well as FPD.

2.8.3 Water (drinker) management

It is important to ensure that drinkers are maintained and properly managed to prevent leakage from drinkers onto the litter. Design of drinker system, water pressure and waterline height all play an important role in the prevention of wet litter and foot pad dermatitis; for example if the pressure is set too high or the line height is too low, wet litter will result. However, if the pressure is set too low or line height is too high, water intake will be restricted which will affect growth rate. A diagram of the nipple drinker height adjustment is presented in Figure 2.2.

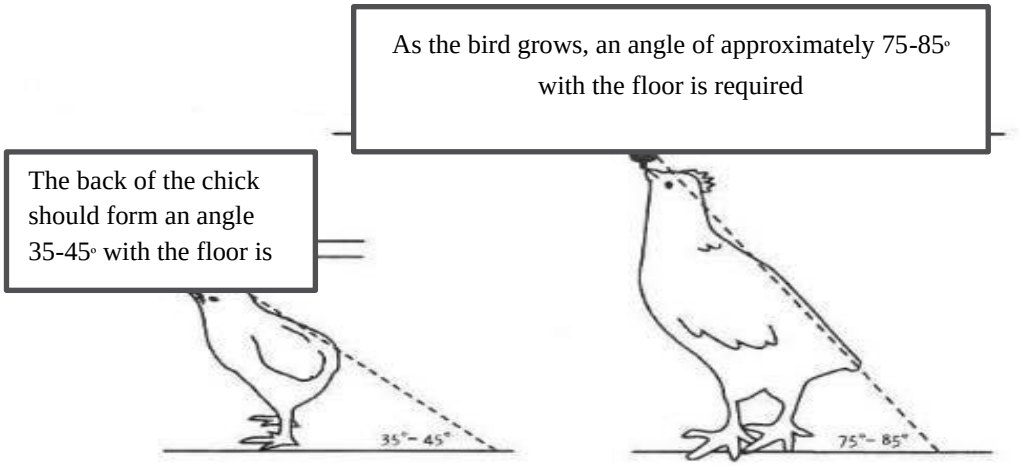


Figure 2.3: Nipple drinker height adjustment

Source of diagram: (Arbor, 2009)

2.8.4 Diet

Nutrition and diet composition are an important factor for the prevention of wet litter and foot pad dermatitis. It is important to make sure all the nutrient requirements of the birds are met, without supplying any to excess. Key micronutrients implicated in the maintenance of foot health are methionine, biotin and zinc.

2.9 Maintenance of gut health (prevention of diarrhoea)

Generally, healthy birds are characterized as having a well-functioning gastrointestinal tract (balance of bacterial population) as this is essential for the efficient conversion of

feed for maintenance, growth and production. When the bird is subjected to stressful conditions including bacterial infections, change of feed, high temperature and humidity then the balance of bacterial population within the gastrointestinal tract is upset (Jin et al., 1997). Any intestinal tract harm caused by proliferation of pathogens leading to poor gut health will reduce the efficiency of nutrient utilisation such as in necrotic enteritis. Thereafter, the health of the gut may affect the way nutrients are partitioned, mobilized and utilized for organ development, tissue growth and immune system maturation (Kelly and Conway, 2001). Intestinal bacteria may be divided into species that are potentially pathogenic (harmful) or beneficial to the host. Pathogenic bacteria, through their production of toxins, may cause harmful effects such as diarrhoea, localized or systemic infections, intestinal putrefaction, liver damage and carcinogenesis. Commensal bacteria, however, may inhibit the growth and establishment of harmful bacteria, reduce the production of fermentation gases and the intestinal distension this can be associated with, and improve the digestion and absorption of essential nutrients, stimulate the immune system and synthesise vitamins which can be utilised by the bird (Jeurissen et al., 2002).

2.9.1 Coccidiosis

Coccidiosis is one of the most common and important diseases caused by protozoan parasites of the genus *Eimeria* that affect poultry and it results in a great economic loss worldwide due to reduced weight gain, poorer feed conversion rates, and increased mortality rates (Williams, 2005). The use of vaccines or anticoccidial drugs for the prevention and treatment of coccidiosis lead to increased production costs. In turkeys the clinical signs of coccidiosis are huddling, ruffled feathers, appetite loss, drooping wings, listlessness and diarrhoea (Chapman, 2008; Hafez, 2011; Hafez, 2008). Coccidiosis is also one of the most important infectious agents contributing to wet litter (Lister, 2009), thus predisposing birds to foot pad dermatitis (Mayne, 2005; Abd El-

Wahab et al., 2012). (Hafez, 2008) observed seven species of *Eimeria* in turkeys, and seven species of *Eimeria* in chickens, but *Eimeria adenoeides and Eimeria tenella* are considered the most pathogenic infecting the caeca of birds.

2.9.2 Necrotic enteritis

Necrotic enteritis (NE) is a worldwide poultry disease responsible for high mortality and reduced bird welfare resulting in production losses which have been estimated to cost the global poultry industry about US$5-6 million annually (Wade and Keyburn, 2015; McDevitt et al., 2006). In recent years the risk of NE has increased due to the removal of antibiotic growth promoters (McDevitt et al., 2006). Necrotic enteritis is caused by *Clostridium perfringens* (Moore, 2016; Parreira et al., 2017). *Clostridium Perfringens* is a Gram positive anaerobic bacterium that lives in the litter and in the intestinal tract of both healthy and diseased birds (Branton et al., 1997) and is found in soil, dust, faeces and intestinal contents. After hatching the disease usually occurs in chickens aged 2-6 weeks and is differentiated by mucosal necrosis and diarrhoea caused by the rapid proliferation of *Cl. perfrigens* in the small intestine (Fukata et al., 1991) and the production of α-toxin. The α-toxin is a zinc-metalloenzyme which has both phospholipase C and sphingomyelinase activity (Awad et al., 2001; Songer, 1997). The number of *Cl. perfringens* in the small intestinal digesta is normally about 10^4 cfu/g, but may increase to 10^7 -10^9 cfu/g of digesta when disturbances occur predisposing the bird to the development of clinical NE (Kondo, 1988). In poultry the infections are mainly caused by *Cl. perfringens* type A, and to a lesser extent by type C (Engström et al., 2003). *Cl. perfringens* type A is very prevalent in the intestinal tract of healthy animals (Smedley Iii et al., 2004). If necrotic enteritis occurs in birds (and this is one of the most common enteric diseases of poultry, (Wade et al., 2015; McDevitt et al., 2006), then birds' enteric health and nutrient digestibility will be compromised, leading

to wetter droppings, and increased litter moisture content, and this in turn will lead to increased foot pad dermatitis.

2.9.3 Cereal source

In commercial poultry production, cereal grains are the main source of energy and comprise 600-700 g/kg of the diet. The metabolisable energy content of cereal grains for poultry varies widely between as well as within cereal species (Hughes and Chockt, 1999). In many countries wheat rather than maize, is the main cereal in poultry diets (Pran et al., 1991). In broiler chickens, diets containing whole wheat have been reported to reduce numbers of *Salmonella typhimurium* in the gizzard and ileum, as well as decrease numbers of *Clostridium perfringens* compared with those fed a pelleted diet (Bjerrum et al., 2005). Annett et al. (2002) reported a significantly lower proliferation of NE bacteria in birds fed a digested maize diet compared with those fed digested wheat and barley. They suggested that the lower incidence of necrotic enteritis in broilers fed the maize diet may be due to reduced clostridial proliferation related to the maize diet compared to those fed wheat and barley diets. In addition necrotic enteritis was related with feeding increased amounts of wheat and barley in broiler diets (Kaldhusdal and Skjerve, 1996). When a barley based diet was fed to broiler chickens instead of maize, decreased numbers of coliforms, *Lactobacilli* and *Streptococci* and increased numbers of *Clostridium perfringens* were observed in intestinal contents (Kaldhusdal and Hofshagen, 1992). In a recent study by (Umar et al., 2016), the objective was to determine the influence of a wheat based diet on the pathology of necrotic enteritis in turkeys, and it was observed that the wheat based diet led to a significant increase in necrotic enteritis compared to those fed a normal commercial (maize based) diet. Yan et al. (2016) observed that dietary carbohydrase containing xylanase, galactosidase and glucanase improved nutrient absorption, dysbacteriosis and growth performance, but a rye wheat based diet containing a mild mixed species *Eimeria* challenge predisposed

birds to subclinical enteritis characterized by inefficiencies of digestion and dysbacteriosis.

These studies above have observed that wheat and barley based diets increase the severity and the incidence of necrotic enteritis compared to a maize based diet. This would also predispose the birds to FPD because of the wet litter that would result from the birds' diarrhoea. However, the actual mechanism of the effect of cereal on necrotic enteritis is unknown, but researchers have established that the population of bacteria in the intestinal tract and bird performance may be affected by complex carbohydrates (non starch polysaccharides). Wheat, barley, oat and rye contain different types and concentrations of non-starch polysaccharides such as arabinoxylans and β- glucans which may result in increased viscosity of digesta (as poultry do not have enzymes to digest NSP), which would lead to increased transit time of intestinal contents while limiting contact between digestive enzymes and digesta, and blocking absorption of digested nutrients (Choct et al., 2010; Yan et al., 2016) .

2.9.4 Physical form (particle size)

In poultry, the physical form of cereal components of feed can affect the morphological structure and physiological function of the gastrointestinal tract (Brunsgaard, 1998; Engberg et al., 2004). An increase in villus height and in the villus height to crypt depth ratio was observed in the small intestinal mucosa of broilers fed pelleted diets compared with those fed mash diets, but there was no impact on crypt depth (Zang et al., 2009). Birds fed pelleted diets had greater villus height and crypt depth in both the duodenum and jejunum compared to those fed mash (Amerah et al., 2007b). Duodenal villus height increased linearly as the dietary particle size increased (Nir et al., 1995; Nir et al., 1994). Greater villus height can increase the absorption of nutrients and consequently the transport of nutrients at the villus surface (Cera et al., 1988). Greater crypt depth is

an indicator of increased turnover rate of intestinal mucosa, which in turn increases the maintenance requirement of the gut (Zang et al., 2009). In recent years, feeding larger particle size diets and whole grain to poultry has gained increasing popularity because of reports of improved gizzard function and bird health (Amerah et al., 2008b; Biggs and Parsons, 2009). The use of wheat ground with a roller mill (coarsely ground) reduced mortality to 18.1%, but a hammer mill ground wheat diet (finely ground) resulted in mortality rate of 28.9%. Mortalities in this experiment were related to a combination of necrotic enteritis and coccidiosis (Branton et al., 1987). (Bennett et al., 2002b) observed that the inclusion of whole barley in the diet of turkeys reduced total mortality rates. Whole wheat can improve gut health in broilers through the development of the gastrointestinal tract, particularly the gizzard, and result in increased absorption of dietary nutrients in the digestive tract (Yasar, 2003; Taylor and Jones, 2004; Engberg et al., 2004). (Huang et al., 2006) conducted an experiment to investigate the effects of feed form (mash and pellet) and feed particle size (coarse and fine) on the prevalence of *Salmonella typhimurium* (ST). They found ST concentrations in the caeca were lower in broilers fed mash diets than those fed pellet diets, and that the pelleted diet also increased ST in the gizzard. When broilers were fed either ground corn-soybean meal, coarsely ground corn and soybean meal, ground triticale-soybean meal or whole triticale and soybean meal, lower *Salmonella* populations were observed in the caeca at 42 d in broilers fed whole or coarsely ground grains (Santos et al., 2008). Broilers fed a finely ground corn, compared with a whole triticale based diet from 0 to 42 days, had lower microbial diversity and a higher prevalence of *Salmonella* in their intestinal tract. It was concluded that the increased coarseness and combination of high dietary fibre content in the whole triticale diets was responsible for the observed beneficial effects (Santos et al., 2007).

Feeding broilers diets with different levels of coarse maize 0,150,300, 450 and 600 g/kg in mash diets resulted in counts of *Clostridium spp*., *Campylobacter spp*. and *Bacteroides spp*. decreasing and those of *Lactobacillus spp*. and *Bifidobacteria spp* increasing with increased levels of inclusion of coarse maize (Singh et al., 2014b). Broiler chickens fed pelleted diets had lower counts of *Lactobacilli spp*. and *Cl. perfringens* and higher counts of coliforms and *Enterococci spp*. in their digestive tract compared with broilers fed a mash diet (Engberg et al., 2002). The results of these studies clearly indicate the beneficial effects of feeding birds diets with larger particle sizes in terms of their gut health (and so, indirectly, their foot health). The mechanism for these changes in the number of pathogenic bacteria may be because of the increased secretion of HCl (by encouraging gizzard development and its grinding activity). This decreases digesta pH which has an antimicrobial effect on pathogenic bacteria entering the gastrointestinal tract (Engberg et al., 2002). Alternatively, the colonisation of commensal bacteria is encouraged, which reduce pathogen numbers by competitive exclusion (Bjerrum et al., 2005).

As this literature review has shown, FPD is a multifactorial condition, and small changes in the health and performance of the bird may have indirect and unintended negative effects on the incidence of FPD. In this thesis, a number of nutritional and management interventions that might improve aspects of bird health and performance were investigated to determine the impact they had on foot health. The interventions that were investigated included the inclusion of whole grain wheat in the diet, the effect of cereal source, the selection of bedding material and the reuse of litter, and the inclusion of probiotics in the diet. These investigations were undertaken to address the objective of this thesis, which is reiterated below.

2.10 Objective of this thesis

The objective of this thesis was to investigate the effect of different interventions aimed at improving bird gut health and performance on the birds' foot health and gait. The hypotheses on which the interventions were based are presented below.

a. Whole cereal (wheat)

Feeding whole grain wheat to turkeys will increase the grinding activity and therefore the musculature of the gizzard. This will increase the gizzard weight and encourage greater acid production by the proventriculus. The result of this will be a decrease in gizzard pH, and potentially caecal, digesta pH which will inhibit the proliferation of pathogens in the intestinal tract, improving gut health and thereby reducing the incidence of wet droppings which will indirectly reduce litter moisture content and thereby reduce the incidence of FPD.

b. Bedding material

Softer bedding materials reduce the irritation of the foot and directly reduce the incidence of FPD. Bedding materials are also consumed by the birds as they forage and the intake of fibre from this will encourage the muscular activity of the gizzard and the production of acid from the proventriculus. This might improve gut health, reduce the incidence of wet droppings and thereby reduce the litter moisture content, indirectly reducing the incidence of FPD.

c. Litter microbiome

Although FPD is a contact dermatitis, the litter microbiome that the foot is in contact with may affect the progression of the condition. There is little information on the composition of the litter microbiome, and this thesis investigated the effect of reused litter on the composition of the litter microbiome, and the impact this had on foot health

d. Cereal type

Different cereals vary in the composition of their non-starch polysaccharides, protein and starch composition. This affects the viscosity of digesta in birds that consume these cereals, and thereby the moisture content of their droppings. This would indirectly affect the litter moisture content and thereby the incidence of FPD in birds fed these cereals.

Chapter 3 Effect of whole grain wheat (WGW) on turkey performance, digestion and health

3.1 Introduction

The relationship between caecal dysfunction (characterised by caecal distension and abnormal caecal droppings) and foot pad dermatitis is poorly understood in turkeys, but is of growing concern in the turkey industry because of negative impacts on bird welfare and economic performance (Zdunczyk et al., 2013). If such a relationship exists, then an improvement in gut health would reduce the incidence of both wet litter and FPD in turkeys. One means of improving gut health that has been investigated is the inclusion of whole (unground) cereal grains in the birds' diet, and this has become common practice in many countries (Svihus et al., 2004). The birds are offered whole grain wheat, but because of its lower protein content compared with the complete diet, the protein supply of the diet is diluted (Forbes and Covasa, 1995). This may explain the poorer feed conversion ratio and diminished weight gain observed by (Amerah and Ravindran, 2008) when they offered birds such a diet. In this feeding system, birds are free to select between the whole grain and another feed (which may be a complete diet or 'balancer' feed, formulated to provide all the nutrients required by the bird if it consumes an 'expected' amount of the whole grain). This method of feeding has some practical limitations especially in the intensive poultry industry, because it does require extra equipment so that the whole grain cereal can be offered in one feeder and the complete diet or balancer feed can be offered in a separate feeder. In addition, the proportion of whole grain cereal consumed by the birds cannot be controlled (Singh et al., 2014a). As discussed in Chapter 1 (Section 1.1), the other ways in which whole cereals may be added to the diet include the mixing of the cereal with the pelleted diet or with a diet premix that has been formulated, as with the free choice system, based on the amount of whole cereal that has been added. Alternatively, whole cereals can be fed

as the only feed for fixed amounts of time before being replaced with the balanced diet or protein/energy supplement.

The gut microbial content has co-evolved with the birds' gastrointestinal tract (Rinttilä and Apajalahti, 2013) and its composition should be monitored for both animal welfare and food safety reasons (Apajalahti et al., 1998). The composition of the microbial population in the gut may have a great influence on the birds' metabolism as well as its health status (Svihus et al., 2013), but there are few data on what constitutes a healthy microbiome (especially in turkeys) and most work focuses on the effect of different dietary interventions on the prevalence of key pathogens.

It has been observed that the population of *Clostridium perfringens* and *Salmonella* were decreased when whole wheat was fed (Bjerrum et al., 2005), as mentioned in Chapter two Section 2.9.3. This may be of benefit as the *α*-toxin producing *Cl. perfringens* type A causes necrotic enteritis (NE) in chickens, which can lead to increased mortality, impaired feed conversion, and retarded growth rate (Petit et al., 1999; Kaldhusdal et al., 2001). Another reported effect of feeding whole wheat and oat hulls to birds was a significantly reduced *Campylobacter jejuni* colonization in the caeca (Gracia et al., 2016). *C. jejuni* is a leading cause of diarrhoeal disease and foodborne gastroenteritis in humans, and poultry have been found to be one of the most important sources for transmission to humans (Solomon and Hoover, 1999). *Brachyspira* species, including *B. pilosicoli* can cause infection in broilers (Muniappa et al., 1996; Prapasarakul et al., 2011) and other poultry species such as turkeys (Shivaprasad and Duhamel, 2005). In many regions of the world colonization and disease have been reported (McLaren et al., 1996; Phillips et al., 2005; Medhanie et al., 2013; Amin et al., 2014; Illanes et al., 2016). Increased mortality rates depend on infections and can vary from being asymptomatic to severe. However, infections are

usually mild or moderate and generally are characterized by diarrhoea with excreta that are caramel in colour. Loss of egg production in laying hens and an increased water content of excreta which results in wet litter was reported by (Trampel et al., 1994), and decreased egg quality and growth rate was reported by (Stephens and Hampson, 2001; Smit et al., 1998). Dwars et al. (1993) reported that infection by an avian *B. pilosicoli* strain in adult breeder chickens resulted in an increased excreta moisture content. The caeca of the infected birds were gassy and the contents of the caeca were frothy, fluid and pale (Dwars et al., 1993). Another important pathogen to consider is *Salmonella.* One of the most important sources of *Salmonella* in human infection is poultry meat (Anumolu and Lakkineni, 2012; Saravanan et al., 2015), which can make poultry meat unsafe for humans (Manoj and Singh, 2015). It is estimated that gastroenteritis caused by *Salmonella spp* amounts to 93.8 million cases and 155,000 deaths in the world/year (Majowicz et al., 2010). In this study of potential challenges to the caecal health (and thereby indirectly foot health) of turkeys should therefore consider *Cl. perfringens, C. jejuni. B. pilosicoli* and *Salmonella.* The objective of the two experiments reported in this chapter was therefore to determine the effect of inclusion of whole grain wheat in the diet of turkey poults (both by a free choice method and by mixing the wheat in the diet) on bird performance, gut and foot health, and the presence of *Cl. perfringens, C. jejuni, B. pilosicoli* and *Salmonella* in the caecal digesta of the birds.

3.2 Material and methods

The second of the two experiments reported in this chapter has been published in: Ahmed, R., Juniper, D., Tonks, A. and Rymer, C., 2018. *The effect of incremental inclusion of whole grain wheat in the diet of growing turkeys on growth performance, feed conversion ratio, cecal health, and digesta characteristics.* *Livestock Science*, 214, 36-41

3.2.1 The experimental area

Housing, feeding and management of turkey poults, euthanasia of turkeys and collection of feed samples and digesta collection were conducted at CEDAR, Hall Place Farm, Arborfield. The experiments were subject to local review and conducted in accordance with the University of Reading's current animal research policy and conformed to the United Kingdom's Animal (Scientific Procedures) Act 1986.

3.2.2 Experimental Design

Experiment 1 was powered based on the growth of birds, assuming (from previous studies at the unit) a coefficient of variation of 6%, to detect a difference ($P<0.05$) between treatments of 7.5%, thereby requiring six replicates. A total of 192 four week old, as hatched, commercial line turkeys (Meleagris gallopavo var. domesticus) were provided by Aviagen. Birds were individually tagged, weighed, blocked by live weight and then randomly allocated to one of two dietary treatments (six pens/treatment, 16 birds/pen, Table 3.1). The experiment was conducted between October and December 2014. Birds were offered their experimental diet following allocation to pens (on arrival) and turkeys received their experimental diets throughout the experiment. The control (CON) treatment received a proprietary starter pellet (F66502 GP Starter pellets, GLW Feeds Leicestershire, UK) from 28 to 48 d of age, a proprietary grower 1 diet (F66503, GLW-Feeds) from 49-69 d of age and a proprietary grower 2 diet (F66504,

GLW-Feeds) from 70-84 d of age. The whole grain wheat with starter pellet (WGW+SP) treatment received whole grain wheat plus the starter diet (F66502) for the entirety of the experimental period (from 28-84 d) (Table 3.2). All feed added and removed from pens were weighed and recorded weekly. A running sample (100 g) of each diet was taken each week throughout the experiment, bulked and submitted for analysis of crude protein, starch, sucrose, oil, calcium, phosphorus and magnesium to Sciantec Services (Cawood, North Yorkshire). The chemical composition (for details of analysis, see section 3.3) and calculated apparent metabolisable energy content for both studies of the starter, grower1 and grower 2 diets and of the wheat are shown in (Table 3.3). The diets used were proprietary compounds; the vitamin and mineral contents of the diets was not determined. Diet changes were conducted abruptly and at the same time for all pens.

Table 3.1: Experimental design (Experiment 1)

Treatment	**Total No. of birds/pen**	**Total No. of pens/treatment**	**Total No. of birds/treatment**
*CON	16	6	96
**WGW+SP	16	6	96
Total		12	192

*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

Table 3.2: Treatment diets (Experiment 1)

Treatment group	Feeder 1	Feeder 2
28-48 d		
*CON	Starter pellets	Starter pellets
**WGW+SP	Starter pellets	Whole grain wheat
49-69 d		
CON	Grower 1 pellets	Grower 1 pellets
WGW+SP	Starter pellets	Whole grain wheat
70-84d		
CON	Grower 2 pellets	Grower 2 pellets
WGW+SP	Starter pellets	Whole grain wheat

* Birds were fed a proprietary, pelleted diet appropriate to their age.

** Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

Table 3.3: Chemical composition (g/kg as fed) of wheat and pelleted diets used in Experiments 1 and 2

Parameter	Starter	Grower 1	Grower 2	Wheat
Crude protein	246	257	237	124
Starch	265	343	386	607
Sugar (sucrose)	47	67	42	21
Oil A (Ether Extract)	67	85	90	19
Ca	12.4	15	9.9	0.7
Mg	2.2	2.2	2.1	1.1
P	8.7	8.8	6.6	3.2
†Metabolisable energy, MJ/kg DM	11.1	13.5	13.7	13.4

Source: Sciantec Analytical Services. Stockbridge Technology Centre, Cawood, North Yorkshire Y08 3SD Tel: 01757242400 Fax 01757242401 www.sciantec.uk.com

† The metabolisable energy was calculated using the equation:

ME / MJ/ kg as fed = (0.1551× CP) + (0.3431× Oil) + (0.1669 × STA) + (0.1301× SGR)

Where:

CP = Crude Protein %

Oil = Oil %

STA = Starch %

SGR = Total Sugars % (as Sucrose)

Source: (McDonald et al., 2002)

In Experiment 2 a total of 72 six week old commercial line turkeys (*Meleagris gallopavo* var. *domesticus*) were used, again provided by Aviagen. Upon arrival the turkeys, which were already tagged, were weighed, blocked by live weight and then randomly allocated to one of three dietary treatments (n= 24 turkeys/treatment). There were four replicate pens per treatment with six birds in each pen. The experiment was powered based on growth rate, with earlier studies indicating a minimum replication of n=4 being required to detect a difference of 9%. All birds were initially fed grower 1 (F66503) diet from weeks 1-3 of the study (bird age 6-8 weeks), and grower 2 (F66504) diets from weeks 4-6 of the study (bird age 9-11 weeks). Birds were changed on to grower 1 and grower 2 diets one week younger in this study compared with the previous study in consultation with the commercial producers who were supporting this work because for logistical reasons the study could only last six weeks and making the diet changes at six and nine weeks of age minimised the number of diet changes in the experiment. All feed added and removed from pens were weighed and recorded. Diet changes were conducted abruptly at the same time for all pens. WGW was mixed with the compound (pelleted) to WGW was mixed with the compound (pelleted) feed so that the birds were offered a mixture of pellets and WGW (WGW was not included in the pellet) at a rate (g/kg, as fed) of 0 (0WGW), 100 (LWGW) or 200 (HWGW), depending on the dietary treatment allocated (Table 3.4).

Table 3.4: Experimental design (Experiment 2)

Treatment	Total No. of Birds/pen	Total No. of pens/treatment	Total No. of birds/treatment
*0 WGW	6	4	24
**LWGW	6	4	24
***HWGW	6	4	24
Total		12	72

*Birds were fed a proprietary, pelleted diet appropriate to their age without WGW.

**Whole grain wheat was mixed at a rate 100 g/kg with a proprietary pelleted diet appropriate to their age

***Whole grain wheat was mixed at a rate 200 g/kg with a proprietary pelleted diet appropriate to their age.

3.3 Determination of chemical composition

3.3.1 Determination of crude protein

Samples were weighed in duplicate into nitrogen free foil parcels and dropped into a hot furnace (Leco FP528, Leco Inc., St Joseph, MI). They were then flushed with pure oxygen to produce rapid combustion. The combustion products were passed through filters and a thermoelectric cooler to remove water and then collected in a ballast tank and allowed to equilibrate. An aliquot of the gaseous mixture was swept through hot copper to remove O_2 and reduce NO_X to N_2. Carbon dioxide and water were removed by chemical absorption and the remaining nitrogen was measured by a thermal conductivity cell. The crude protein content of the sample was calculated from its nitrogen content by multiplying by 6.25.

3.3.2 Determination of Starch

The method comprises two determinations. In the first, the sample was treated whilst warm, with dilute hydrochloric acid. After clarification and filtering, the optical rotation of the solution was measured by polarimetry. In the second, the sample was extracted with 40 % denatured ethanol. After acidifying the filtrate with hydrochloric acid,

clarifying and filtering, the optical rotation was measured under the same conditions as the first determination.

3.3.3 Determination of total sugars

Sugars were extracted from the sample by shaking with water. The solution was clarified with Carrez reagent, filtered and an aliquot of the extract was heated with dilute hydrochloric acid to convert any disaccharides to reducing sugars. These sugars were then determined by the Luff Schoorl Copper reduction (titration) method whereby the sample extract was refluxed with Luff Schoorl reagent and remaining excess copper (II) ions were titrated with sodium thiosulphate solution. A blank titration using only the Luff Schoorl reagent was also carried out. A table relating the difference between the blank and sample titration values was consulted to give the equivalent concentration of the glucose in the solution. A factor of x 0.95 was applied to this value to give the equivalent result as sucrose.

3.3.4 Determination of Oil B

Samples were boiled in hydrochloric acid to release the bound fat and the digest is filtered with a filter aid and washed until neutral. The fat is retained by the filter paper and filter aid. After drying the residue was extracted with light petroleum ether in an extraction tube, the solvent was removed by evaporation and the dry oil weighed and added to the weight of oil retained by the filter paper and filter aid.

3.4 Birds and management

In Experiment 1, birds were kept on white wood shavings at a depth of approximately 12 cm. Feeders and water drinkers were maintained at a height equivalent to the birds' backs. The total floor area for each pen was 4.07 m^2 which was divided into 1/3 drinking space and 2/3 feeding areas. Lighting pattern was 16 hours of continuous light/d at 40 lux followed by an 8 h period of darkness. Bright lights were occasionally

used when people were working in the room. Litter was removed when flooding occurred because of spillage from the drinkers, which would have been observed through daily observations and in this circumstance, litter material was replaced. Environmental conditions of floor space, temperature, lighting, bird density, feeder and water space were similar for all treatment groups. For birds fed the control (CON) diet, both feeders contained the appropriate, pelleted diet. For birds fed the diet containing WGW, one feeder contained a pelleted, starter diet while the other feeder contained WGW. In both studies water was provided *ad libitum* from a bell type drinker in each pen and was filled with fresh water each day.

In Experiment 2 the turkeys were kept in an open fronted shed with Yorkshire boarding on the sides to provide natural ventilation. Halogen heat lamps were suspended over each pen and left on continuously for the first three weeks of the experiment. Natural sunlight dictated the light pattern with no extra artificial lighting (other than from heat lamps). Each pen provided approximately 0.5 m^2/bird, and was bedded with white wood shavings to a depth of approximately 12 cm. There was one feeder placed in each pen, containing the appropriate, pelleted diet in which WGW was mixed in at the appropriate rate. One bell type drinker containing fresh water (replenished each day) was also suspended in each pen.

In both studies the test facility, pens, and birds were observed at least twice daily for general flock condition, lighting, water, feed, ventilation and unanticipated events. If abnormal conditions or abnormal behaviour was noted at any of the daily observations they were documented in writing in the study records.

In Experiment 1 the minimum-maximum temperature and humidity of the test facility were recorded once daily with a digital temperature and humidity recorder that was situated at one end of the room (Figure 3.1). After the birds were seven weeks old, it

became necessary to open the external door during the day to increase the ventilation in the room because of the accumulation of ammonia, which is why the temperature then began to fluctuate more.

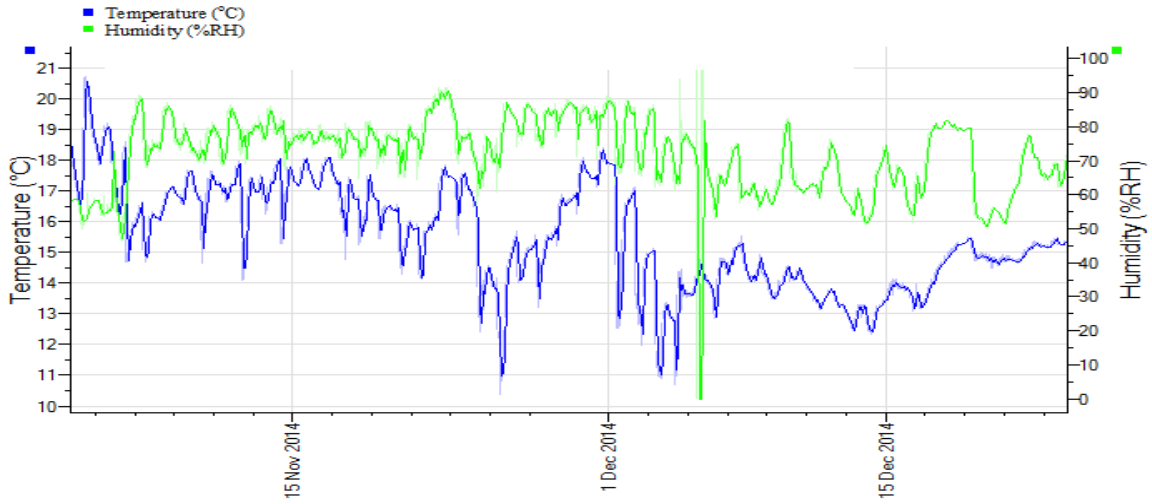


Figure 3.1: Temperature and humidity experiment 1

In Experiment 2 the temperature and humidity was different from experiment 1 as in this experiment birds were kept in an open fronted shed making it more difficult to control temperature and humidity. The minimum-maximum temperature and humidity of the test facility were recorded once daily (Figure 3.2).

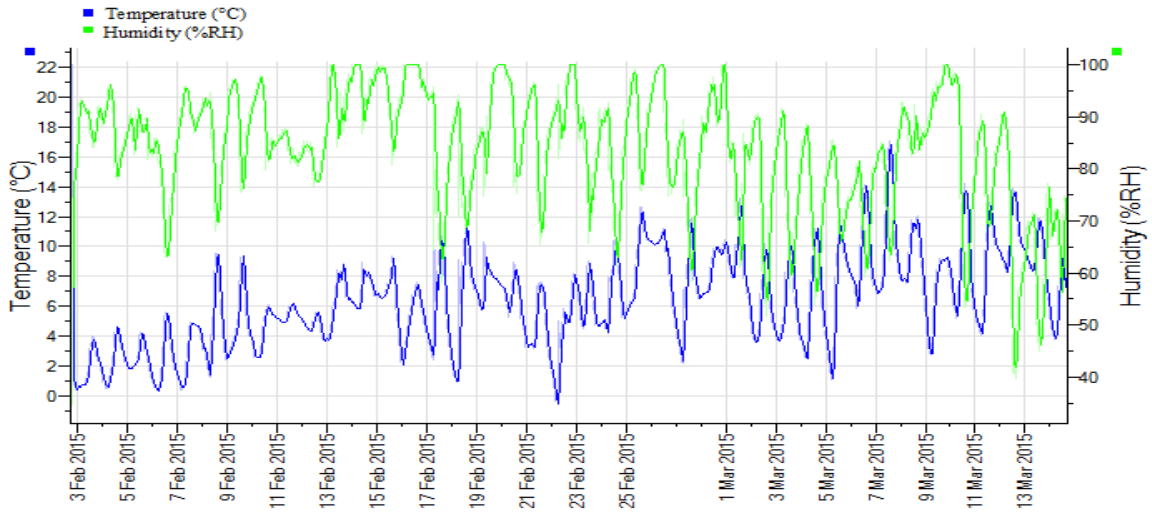


Figure 3.2: Temperature and humidity experiment 2

In both studies, feed was provided *ad libitum* throughout the experiment via feed hoppers. Bags of feed were prepared for each pen, and the amount of feed added to the bag was recorded on a weekly basis. Feed was dispensed from these bags to the hoppers in the pen, and the amount of feed remaining in the hopper and the bag was recorded each week. By difference, the amount of feed consumed by the birds in the pen was calculated. Daily feed consumption per bird was then calculated according to the following equation:

$$\text{Feed intake g/bird/day} = \frac{\text{Feed consumption in pen during week (kg)}}{\text{Number of birds in pen during week}} / 7 \text{ X}1000$$

3.5 Bird performance

In both studies birds were weighed at the beginning of the study and at weekly intervals, Growth rate was calculated for each bird as follows:

Growth rate g/bird/day = Body weight (g) at the end of week – Body weight (g) at the onset of week /7.

The feed conversion ratio was calculated for each pen as follows:

$$\text{Feed conversion ratio} = \frac{\text{Feed consumption (kg/pen)}}{\text{Weight gain (kg/pen)}}$$

In Experiment 1, the effect of treatment (diet) on feed intake, growth rate, feed conversion ratio and bird weight was determined between the start of the experiment (birds 28 d old) until they were 48 d old; again between 49 and 69 d of age, and between 70 and 84 d (coinciding with when the diets were changed from starter, grower 1 and grower 2). In Experiment 2, the effect of treatment (diet) on feed intake, growth rate, feed conversion ratio and bird weight was determined between the ages of 42- 56 d and between 57-77 d (coinciding with when the diets were changed from grower 1 and grower 2).

3.6 Processing and preparation of samples for analysis

3.6.1 Sampling of birds

In Experiment 1 on the Tuesday following the birds' arrival and on each subsequent Tuesday for the duration of the study (eight weeks), either one bird was randomly removed from each pen (when birds were aged 5 to 9 weeks) or two birds were taken from each pen (when birds were aged 10 to 12 weeks); the number of birds taken each week was increased to reduce the atmospheric load because of an accumulation of ammonia in the birds' house. Turkeys were weighed prior to slaughter and euthanased either by cervical dislocation (<5 kg body weight) or by stunning (CASH Poultry Killer, Accles and Shelvoke, Sutton Coldfield, UK) followed by rapid exsanguination (>5kg body weight). One bird per pen was then sampled. The body cavity was instantly opened and the segments of digestive tract (crop, gizzard, caeca, duodenum, ileum, jejunum and colon) were removed. The length and empty weight of the duodenum (gizzard to pancreatic loop), jejunum (pancreatic loop to Merckel's diverticulum), and ileum (Merckel's diverticulum to ileo-caeca junction) were recorded. The weight of the emptied crop, gizzard, pancreas and liver were recorded. A sample of crop, ileum and excreta contents were taken for the proportion of wheat in crop as mentioned in this Chapter; Section 3.7.1 and for the acid insoluble ash as mentioned in this Chapter; Section 3.7.2. The pH of gizzard and caecal digesta was recorded. The caecal contents and appearances were scored, and a sample of caecal digesta placed in an Eppendorf tube and stored in dry ice before being stored (-80^{0}C) pending molecular analysis for the presence of *Cl. Perfringens, C. jejuni, B. pilosicoli* and *Salmonella.* . A sample of caecal contents was taken when the birds were ten weeks old and stored in a sterile tube (in an anaerobic environment for *Campylobacter*) for the determination of population size of C. perfringens and *C. jejuni*. Following sacrifice both legs were removed at the hock joint by knife and subsequently washed for the determination of foot pad score.

In Experiment 2 at the end of the grower 1 phase (when the birds were approximately eight weeks old), and grower 2 phase (when the birds were approximately 11 weeks old), three birds were randomly removed from each pen. Birds were weighed prior to slaughter and euthanased by turkey captive bolt followed by rapid exsanguination. The body cavity was instantly opened as in experiment one. The pH of gizzard and caecal digesta was also recorded. The caecal contents and appearances were also scored. A sample of crop and ileum contents were taken for the proportion of wheat in the crop as mentioned in this Chapter; Section 3.7.1 and for acid insoluble ash content as mentioned in this Chapter; Section 3.7.2. The bids' feet were taken for the determination of foot pad score.

3.7 Preparation of samples

3.7.1 Crop digesta

In both studies, the crop was excised from the bird, emptied, and weighed. Crop digesta was placed in a polythene bag labelled with the pen number, bird identification and date. The bag was sealed and kept frozen (-20 ^{o}C) pending analysis of acid insoluble ash for an estimate of overall diet dry matter digestibility. This was done because it was evident from an early stage of the experiment that (for birds offered WGW), the intake of WGW was highly variable and also that all birds were consuming variable amounts of bedding. As the indirect estimation of digestibility relies on an accurate measure of the marker in the ingested material, it was decided to use the crop contents as the best estimate of what was actually consumed by the bird although it was recognised that a limitation to this was that different materials may reside for different amounts of time in the crop.

3.7.2 Caeca score

In both studies the caeca were flushed with water, blotted on tissue, weighed and given a score for the degree of distension observed (Table 3.5) and also given a score for the appearance of the caecal contents (Table 3.6).

Table 3.5**: Scoring system used for the assessment of caecal appearance of turkeys**

Appearance Scoring
0 = No pathological changes.
1 = Mild distension with no colour change.
2 = Moderate distension with pale colour change.
3 = Complete distension with blood present in the wall.
4 = Complete distension with severe cell necrosis.

Source: Adapted from (Raman et al., 2011).

Table 3.6**: Scoring system used for the assessment of caecal contents of turkeys**

Content Scoring
0 = No pathological changes (light brown, smooth consistency).
1 = Thick and viscous, brown/dark brown in colour.
2 = Foamy/liquid content, pale yellow in colour.
3 = Foamy/liquid content, pale yellow in colour with blood present.
4 = Thick coagulated blood present.

Source: Adapted from (Saif, 2011)**.**

3.7.3 Gizzard and Caecal digesta pH

Gizzard and caecal contents were transferred to a plastic container and distilled water (approximately 50 ml per 5 g digesta) was added to cover the probe. The contents were mixed and the pH determined with a pH Meter (HI 2210, Hannah Instruments, Leighton Buzzard, UK). The probe was cleaned with distilled water and calibration checked between samples.

3.7.4 Ileal digesta

The digesta from the last 10 cm of the ileum was gently expressed into a polythene bag. This bag was labelled with the pen number, bird identification and date, sealed and kept frozen (-20^{o}C) pending analysis of acid insoluble ash for estimation of small intestinal diet dry matter digestibility. The amount of ileal digesta harvested from each bird was too small for such analysis until the birds were ten weeks old.

3.7.5 Excreta

The colon was excised from the bird then emptied as soon as possible. The colon content was gently expressed into a polythene bag. This bag was labelled with the pen number, bird identification and date, sealed and kept frozen (-20^{o}C) pending analysis of acid insoluble ash for estimation of whole tract diet dry matter digestibility.

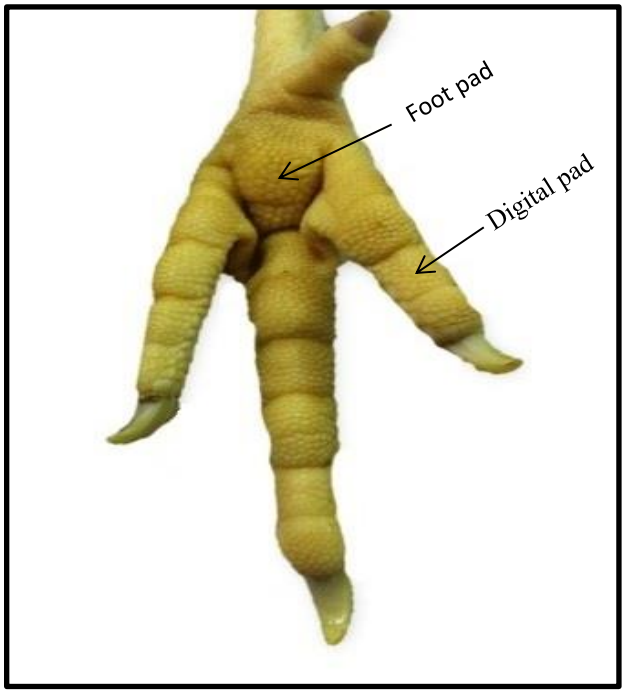
3.7.6 Foot pad score

Foot pad score was divided into eight categories by (Mayne et al., 2007) from completely normal (score 0) to over half of the foot covered by lesions (score 7). Feet were photographed and subsequently scored for the extent of foot pad lesions (Table 3.7). Examples (from this study) of each of these scores are illustrated in Figure 3.3.

Table 3.7: Foot pad scoring system

Score	Description of foot pad
0	Normal foot pad and digital pads.
1	Slight swelling or redness of the skin of the foot pad.
2	The foot pad feels harder and denser than unaffected pad.
3	Small black necrotic areas on the foot pad.
4	The area of necrosis is less one-eighth of the foot pad.
5	The necrotic area extends to a quarter of the foot pad.
6	Half of the foot pad covered by necrotic cells.
7	Over half of the foot pad covered in necrotic scales.

Source: (Mayne et al., 2007



A: FPD score 0

B: FPD score 1

C: FPD score 2

D: FPD score 3

E: FPD: score 4

F: FPD**:** score 5

G: FPD**:** score 6

H: FPD: score 7

Figure 3.3: foot pad score 0-7 from (A to H)

Source: R Ahmed

3.8 Analysis of samples

3.8.1 Wheat proportion in crop

To estimate how much WGW was consumed by an individual bird relative to the amount of pelleted diet, the samples of crop contents were thawed, and then whole wheat was separated from the pelleted contents by hand. Wheat and pelleted contents were then placed in labelled, aluminium trays, dried (65^0C, overnight) and weighed. The proportion of crop contents that comprised whole wheat was then calculated:

$$\text{Proportion of whole wheat in the crop (g/kg DM)} = \frac{\text{Weight of whole wheat}}{\text{Weight of whole wheat + weight of pellet}} \text{X1000}$$

3.8.2 Determination of acid insoluble ash

Samples of crop contents, ileal digesta and excreta were thawed at room temperature. Ileal contents were pooled by treatment to provide n = 6 replicates. Crop, ileal contents and excreta were then analysed for acid insoluble ash as follows:

An empty crucible was weighed, and a sample of mixed digesta was then added, and the crucible and its contents were weighed again. The crucibles were transferred to a muffle furnace and heated (450^oC) overnight. The crucibles were then cooled in a desiccator and weighed. Hydrochloric acid (20 ml approximately, 6 M, prepared by mixing equal volumes of concentrated HCl, 36% v/v, with water) was added and the crucible was then placed on a hot plate until its contents were boiling. The contents of the crucible (residue) were moistened with HCl (4 ml, approximately 36% v/v) and the crucible was then covered with a watch glass and boiled gently for 2 min and the crucible was then removed from the heat. The watch glass was then removed and rinsed, the washings being retained in the crucible. Distilled water (20 ml) was added, then covered with a watch glass and heated until boiling. The crucible was then removed from the hot plate,

the watch glass was rinsed and the washings collected in the crucible. The contents of the crucible were then filtered through a 110 mm Whatman No.541 filter paper. The filter paper containing the insoluble residue was returned to the crucible and dried in an oven at 102^{o}C. Samples were then transferred to a muffle furnace, heated to 600^{o}C overnight, cooled in a desiccator and weighed.

The acid insoluble ash content (of the contents of the crop, ileum or excreta) was calculated according to the following equation:

$$\text{Acid insoluble ash g/kg} = \frac{(\text{Weight of crucible} + \text{A.I.A}) - \text{Weight of crucible}}{\text{Weight of original sample}} \times 1000$$

Nutrient availability (digestibility) was calculated as follows:

Digestibilty = 1-([AIA in diet]/[AIA in digesta])

3.8.3 Bedding material sample

In Experiment 1, two core samples of bedding material were taken from each pen weekly. Core samples were taken from random points around the pen, one close to the feeder, and another from near the gate. Samples were placed in labelled, polythene bags and sealed. Each sample was thoroughly mixed by hand and then a sample (approximately 20 g) from each bag was taken. The samples were analysed for dry matter using a forced draft oven set at 100^{o}C. Drying was done on the day of collection and samples were dried in the oven overnight. Litter dry matter was calculated as follows:

$$\text{Litter dry matter (g/kg fresh weight)} = \frac{\text{Dry sample weight (g)}}{\text{Fresh sample weight (g)}} \times 1000$$

3.9 Microbiological analysis

Microbiological analysis was conducted on samples taken in Experiment 1. The DNA from caecal samples (collected when birds were six, eight, ten and 12 weeks old) was extracted. Molecular analysis of the DNA was undertaken to determine the presence of *Clostridium perfringen* type A, *Brachyspira pilosicoli, Salmonella* and *Campylobacter jejuni.* Caecal contents (pooled from the two birds sacrificed in each pen) were cultured to enumerate the population of *Clostridium perfringens* and *Campylobacter spp* when the birds were ten weeks old.

3.9.1 Retrieval of caecal contents

The caeca were removed using a sharp scalpel, flush with the ileo -caecal junction. In Experiment 1 the caecal contents were then squeezed into 2 ml Eppendorf tubes. These tubes were then 'snap frozen' in liquid nitrogen and then placed in dry ice before being transported to the laboratory where they were placed in a labelled bag and stored at -80°C pending molecular analysis for the presence of *Clostridium perfringens, Brachyspira pilosicoli, Salmonella* spp and *Campylobacter jejuni* .

3.9.1.1 Culturing of caecal contents for microbial analysis

The sample of caecal contents for *Campylobacter* enumeration were placed in a Campy pouch (BD Diagnostics, Sparks, MD) before being transported to the laboratory. For both *Cl. perfringens* and *Campylobacter*, serial dilution (with sterile Brain Heart Infusion, BHI, broth) and plating of samples onto selective media was done in an anaerobic cabinet. Plates were incubated (37^0C) for 24 h in anaerobic conditions. The selective media (Oxoid, Basingstoke, UK) used were Campylobacter Selective Agar for *Campylobacter* spp, and Perfringens Agar Base with egg yolk emulsion and Tryptose Sulphite Cycloserine (TSC) supplement for *Cl. perfringens.* Isolated colonies were counted on a colony counter.

3.9.1.2 DNA Extraction

Samples (0.15 g) of caecal contents were extracted with a Powersoil® DNA Isolation Kit (Qiagen, Manchester, UK). The caecal sample was defrosted and processed according to the Powersoil® protocol, although approximately 0.15 g sample was weighed out rather than 0.25 g as suggested by the manufacturer as the caecal contents were a richer source of DNA than would be the case for soil. The resulting DNA was then transferred to an Eppendorf tube and its quality determined to ensure that it was within the correct range to be able to undergo effective PCR. 1µl of C6 solution from the Powersoil® kit was used to calibrate the NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA) used to detect quality. A sample of DNA (1 µl) was added to the NanoDrop and the concentration of DNA and the A260/280 nm recorded. Any DNA concentration less than 5-10ng/µl with A260/280 <1.65 was discounted and not analysed by PCR.

3.9.1.3 Polymerase Chain Reaction (PCR) analysis

The aim of the PCR is to amplify the DNA in the sample. This is done by denaturing the DNA, splitting its double helix structure, before allowing the primers to anneal to the complementary ends. From this DNA polymerase (the Taq that is added in the Mastermix) provides the nucleotides to allow combination, and therefore extension, of the DNA strand from the 3' end to the 5' end of each of the primers. The amplification of the target sequence is necessary to allow the ethidium bromide to sufficiently stain the final product so it is clear on the gel at the final stage (Schneeberger *et al.*, 1993). This amplification doubles the identical DNA double helix structures after each cycle. This is completed by heating and cooling the sample under controlled conditions, which are specific for each sequence.

PCR Mastermix (ThermoFisher Scientific, Manchester, UK) was made up on ice, at a concentration of 0.25 µmol/40 µl, using the following components: Taq 20.0 µl, forward primer 1.6 µl, reverse primer 1.6 µl, nuclease free water 14.8 µl. Once the Mastermix was prepared, 2 µl of DNA from each sample was added to individual PCR tubes followed by 38 µl of the corresponding Mastermix. The tubes were then mixed on a vortex.

Two primer pairs were used for the detection of each species investigated (Table 3.8). The 16S targeted a region of the 16S rDNA of *B.pilosicoli* and Cpa targeted a region of the Cpa gene encoding the α enterotoxin of *Cl. perfringens.* The hippicurase (Hip) gene is unique to *C. jejuni*. V4 and V5 targeted a region of the 16S rRNA gene of *Salmonella.*

Table 3.8: Primer sequences for PCR amplification of species-specific genes

Species	Target gene	Primer sequence
B.pilosicoli[1]	16S	F: 5'- AGAGGAAAGTTTTTTCGCTTC-3'
		R: 5'-GCACCTATGTTAAACGTCTTG-3'
C.perfringens[2]	Cpa	F: 5'-TGCTAATGTTACTGCCGTTGATAG-3'
		R: 5'-ATAATCCCAATCATCCCAACTATG-3'
Salmonella[3]	V4-V5	U515F (5'-GTGYCAGCMGCCGCGGTA)
		U927R (5'-CCCGYCAATTCMTTTRAGT)
Campylobacter[4]	Hip	F: GTACTGCAAAATTAGTGGCG
		R: GCAAAGGCAAAGGATCCATA

1- (La et al., 2003)
2- (Greco et al., 2005)
3- (Ellis et al., 2013)
4- (Keramas et al., 2004)

The cycle conditions for the polymerase chain reaction for each species investigated are summarised in Table 3.9.

Table 3.9: PCR cycle condition

	Denaturation	Annealing	Extension	Final Extension
Campylobacter	94°C 15s	60°C 15s	72°C 15s	-72°C 600s
Salmonella	95°C 30s	55°C 35s	72°C 1min	72°C 8min
Cl.perfringens	95 5 min	60 30s	68 60s	4 Infinite
B.pilosicoli	95 5 min	60 30s	68 60s	4 Infinite

PCR products were then visualised by ethidium bromide stained gel electrophoresis.

3.9.1.4 Gel electrophoresis

1.5% agarose gels were prepared by adding 0.45 g agarose to 30 ml 1 x Tris-acetate-EDTA (TAE) buffer (per gel). TAE buffer was prepared by diluting 20 ml TAE with 980 ml distilled water (such that the solution contained 40 mM Tris, 20 mM acetic acid and 1 mM EDTA). The mixture was heated in a microwave until the agarose dissolved before the addition of 8 µl ethidium bromide (EtBr). The mixture was poured into a gel caster with a comb set near the top to form wells and left to set for 20 minutes at room temperature. When set, the comb was removed and the gel was placed into a gel tray which was then filled with 1 x TAE buffer. For reference, 4 µl of a DNA ladder mixed with 1 µl of a loading dye was loaded into the first well; 5 µl PCR products were then loaded into subsequent wells. The gel tray was connected to a power supply and run at 75 V for 40 minutes. Gels were then removed from the gel bed and bands were illuminated under UV light. The presence of the bacterial species of interest was confirmed by the presence of an obvious band at the relevant ‘height’ with respect to the DNA ladder.

3.10 Statistical analysis

Data relating to bird performance, nutrient availability, gut morphology, digesta pH and the comparison between studies were subjected to analysis of variance (ANOVA) as repeated measures using a general linear model (GLM). Factors included in the model were treatment and time and first order interaction between these terms. Means were separated using the Tukey simultaneous pairs test. To compare the performance of birds between the two experiments, the feed intake, growth rate and feed conversion ratio of birds was determined when they were eight and eleven weeks old (times when birds were of the same age and being fed the same diet; these ages were selected so that both the grower 1 and grower 2 diets would be selected). The effect of study, bird age and whether or not WGW was included in the diet, and the interactions between these terms, was determined by ANOVA.

Results are presented as least square means with the standard error of the mean and associated P-value. Differences were considered significant at $P<0.05$. In both studies, categorical data pertaining to post-mortem measures for foot health and caecal health were analysed using a non–parametric test (Chi-Square). Frequency counts were reported against categories for each measure. Treatment was used as the only factor. Results are presented as either tables or graphs showing the Chi Square, degrees of freedom and associated P-value. Differences were considered significant at $P<0.05$. In Experiment 1, the number of samples from each pen that tested positive (by molecular analysis) for each bacterial species was determined. The association between dietary treatment and these variables was determined by Chi square analysis. For birds offered WGW, the relationship between the proportion of the diet consisting of WGW (determined on a pen basis) and the size of the *Cl. perfringens* and *Campylobacter spp* populations were determined by regression analysis. To investigate possible relationships between bacterial infection and the development of caecal dysfunction in

Experiment 1, the number of birds infected with each bacterial species was calculated for each caecal score (analysing appearance, contents and lesions separately). The association between caecal score and presence of infection was determined by Chi square analysis. The association between caecal score and combinations of infections was also assessed by one way Chi square analysis.

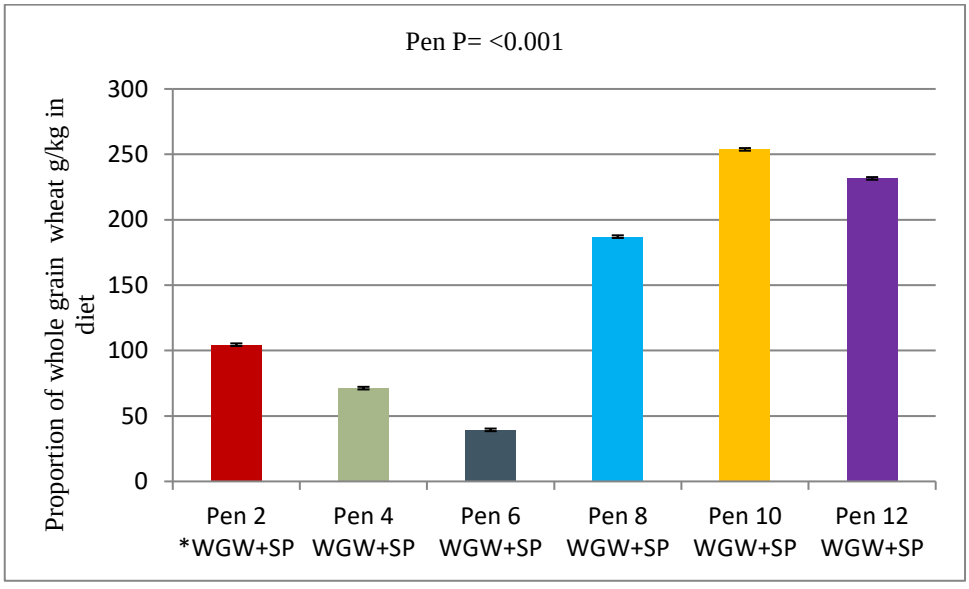
Simple linear regression analysis was used to determine relationships between a predictive variable and response variable. Simple linear regression analysis was used to determine relationships between foot health and bird performance. Foot health was the predictor and aspects of bird performance (feed intake, daily live weight gain and feed conversion ratio) served as the response variable. In addition, simple linear regression analysis was used to determine the relationship between the proportion of wheat in the diet and bird performance, foot pad score and bird performance. All analyses were conducted using the Minitab v 17 software package (Minitab Inc., PA).

3.11 Results

3.11.1 Intake of WGW

Overall, birds that were offered WGW free choice consumed sufficient WGW that it constituted 138 g/kg diet when they were 28-48 d, 176 g/kg when they were 49-69 d and 154 g/kg when they were 70-84 d old. There was, however, a significant difference between the six pens that were offered WGW (using the estimates of intake in each week of the experiment) in the proportion of whole grain actually consumed in the diet (Figure 3.4; P=0.001) with some pens consuming approximately 250 g/kg and others <50 g/kg. There was no effect of bird age on the proportion of whole grain wheat in the diet (Figure 3.5; p=0.762). In week 8 the proportion of WGW was numerically higher than in other weeks which may be because birds consumed more WGW in week 8 compared with other weeks (Figure 3.5). Or because there was greater spillage in this

week. When protein intake was expressed as a proportion of metabolisable energy (g crude protein/MJ ME), it remained relatively constant (20.4 g/MJ when birds were 28-48 d, 19.9 g/MJ when 49-69 d, and 20.2 g /MJ when 70-84 d in Experiment 1). The protein contents in the starter, grower 1, grower 2 and wheat feeds were 22.2, 19.0, 17.2 and 9.3 g/MJ respectively. In Experiment 2 (when the wheat was mixed with the compound feed) the protein content (g/MJ) when grower 1 was fed was 19.0, 15.0 and 17.1 respectively for the inclusion rates of wheat of 0, 100 and 200 g/kg. When grower 2 was fed, the protein contents were 17.2, 16.4 and 15.5 g/MJ respectively. The crude protein content of the diets were 257, 13 and 49 g/kg for the wheat inclusion rates of 0, 100 and 200 g/kg respectively when grower 1 was fed. The corresponding values when grower 2 was fed were 237, 22 and 45 g/kg in LWGW and HWGW respectively.



*WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period

Figure 3.4: Proportion of diet composed of whole grain wheat (g/kg diet) in Experiment 1 observed in pens of birds that had free choice access to WGW.

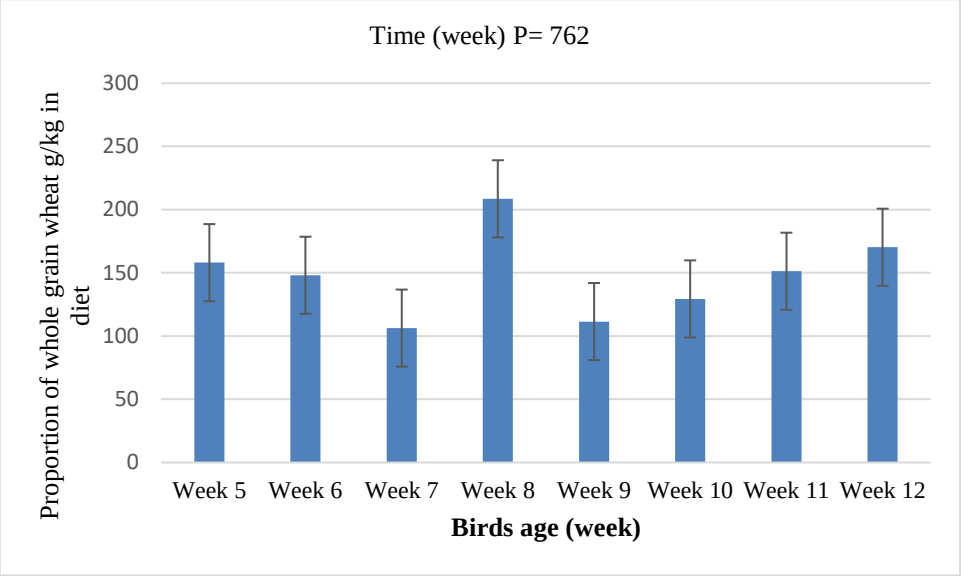


Figure 3.5: Proportion of whole grain wheat in diet g/kg in different times in Experiment 1

3.11.2 Effect of experiments on bird performance

The effects of treatment in free choice whole grain wheat on feed intake, growth rate, feed conversion ratio and turkey weight in the starter (28-48) d, grower 1 (49-69 d) and grower 2 (70-84 d) phases are shown in (Table 3.10). There were significant effects of treatment on feed intake and FCR at both 28-48 d and 70-84 d old, with birds offered WGW eating more feed but without growing more (indeed, having a lower growth rate (P=0.018) when they were 28-48 d old) so that their FCR was greater than birds fed CON. This difference in feed intake and FCR was not observed when birds were fed grower 1 and were 49-69 d old. There was no significant interaction between treatment and time on turkey live weight (P =0.802). Overall, there was no difference between treatments in Experiment 1 in the intake of either ME or crude protein (Table 3.11). However, birds offered WGW had a higher intake of ME (P=0.017) in the starter phase but a lower intake (P=0.022) in the grower 1 phase, and a higher (P=0.044) intake of crude protein in the grower 2 phase. The effect of mixing WGW into the diet (Experiment 2) on bird performance is summarised in Table 3.12. Treatment did not affect total feed intake at grower 1(42-56 d old) and at grower 2 (57-77 d old). ME

intake was not affected in either phase, but there was a tendency (P=0.092) for crude protein intake to be greater if WGW was not mixed in the diet. This was associated with reduced growth rates (P=0.027) and poorer feed conversion ratio (P=0.002) when WGW was included in the diet of younger birds (42-56d old). When the birds were older (63-84 d), there was no significant effect of treatment on bird performance. This reduction in feed efficiency with the inclusion of WGW in the diet is in line with what was observed in Experiment 1.

Table 3.10: Effect of free choice WGW on bird performance experiment one

	*CON	WGW intake	SP intake	**WGW+SP	SEM	P- value
Starter (28-48 d)						
Feed intake (g/b/d)	181[a]	33	207	240[b]	9.81	< 0.001
Growth rate (g/b/d)	125			120	1.21	0.018
FCR (g feed/g gain)[†]	1.099			1.530	0.081	0.004
Turkey weight (Kg)	1.715			1.706	0.196	0.976
Grower 1 (49-69 d)						
Feed intake (g/b/d)	419[a]	81	380	461[a]	35.1	0.414
Growth rate (g/b/d)	178			180	2.44	0.619
FCR(g feed/g gain)[†]	2.723			3.051	0.218	0.314
Turkey weight (Kg)	4.621			4.666	0.235	0.894
Grower 2 (70-84 d)						
Feed intake (g/b/d)	545[a]	101	554	655[b]	25.4	0.004
Growth rate (g/b/d)	223			229	4.95	0.387
FCR(g feed/g gain)[†]	2.466[b]			2.869[a]	0.126	0.032
Turkey weight (Kg)	8.723			9.033	0.344	0.528
Overall						
Feed intake (g/b/d)	373[a]	75	380	455[b]	13.1	< 0.001
Growth rate (g/b/d)	182			184	13.6	0.875
FCR(g feed/g gain)[†]	2.149[a]			2.538[b]	0.08	0.003
Turkey weight (Kg)	5.433			5.564	0.446	0.528

*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

†Feed conversion ratio

Table 3.11: Effect of free choice WGW on the intake of ME and crude protein.

	Diet[1]		SEM	P
	CON	WGW+SP		
ME intake (MJ/bird/d)				
Starter (28-48 d)	6.04	8.21	0.537	0.017
Grower 1 (49-69 d)	18.8	16.8	0.51	0.022
Grower 2 (70-84 d)	12.6	13.9	0.91	0.336
Overall	12.4	12.8	0.46	0.549
Crude protein intake (g/bird/d)				
Starter (28-48 d)	134	165	11.0	0.072
Grower 1 (49-69 d)	357	335	8.2	0.090
Grower 2 (70-84 d)	217	273	17.1	0.044
Overall	238	256	8.3	0.167

Table 3.12: Effect of 0W GW, LWGW and HWGW on bird performance experiment two

	*0WGW	**LWGW	***HWGW	SEM	P-value
Grower 1 (42-56 d)					
Feed Intake (g/b/d)	360	346	365	9.0	0.352
ME intake (MJ/bird/d)	4.85	4.66	4.93	0.141	0.435
CP intake (g/bird/d)	92	84	84	2.7	0.092
Growth rate (g/b/d)	140[a]	123[b]	131[ab]	3.1	0.027
†FCR (g feed/g gain)	2.635[a]	2.928[b]	3.036[b]	0.0448	0.002
Grower 2 (57-77 d)					
Feed Intake (g/b/d)	536	530	550	17.2	0.705
ME intake (MJ/bird/d)	7.34	7.25	7.51	0.320	0.851
CP intake (g/bird/d)	127	120	118	5.2	0.458
Growth rate (g/b/d)	227	221	233	5.61	0.064
†FCR (g feed/g gain)	2.294	2.290	2.316	0.0762	0.968

*0WGW: Birds were fed a proprietary, pelleted diet appropriate to their age without WGW.

**LWGW: Whole grain wheat was mixed at a rate 100 g/kg with a proprietary pelleted diet appropriate to their age

***HWGW: Whole grain wheat was mixed at a rate 200 g/kg with a proprietary pelleted diet appropriate to their age

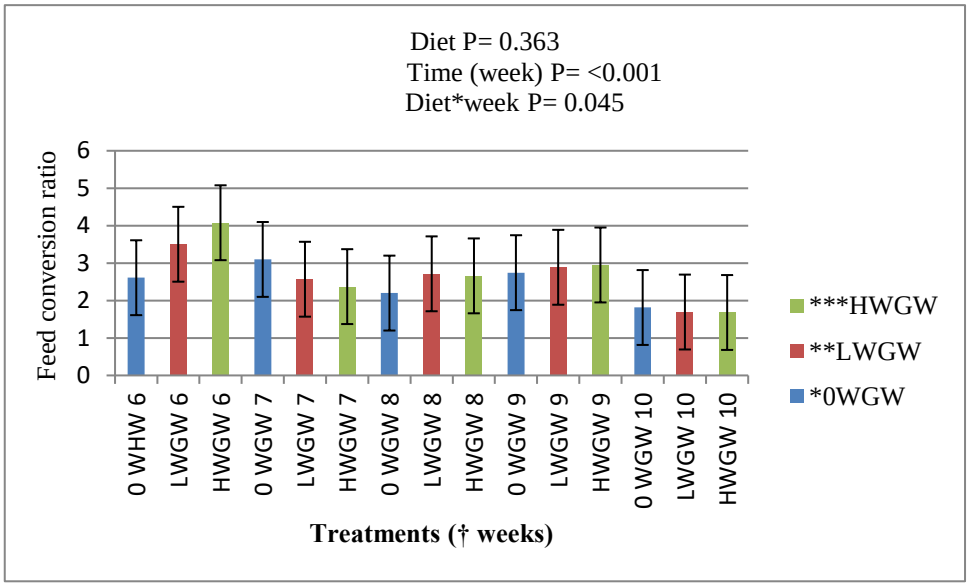
†Feed conversion ratio

Different superscripts within row differ significantly($P < 0.05$)

Note: These values were different in this thesis compared with the values in the Livestock Science paper because different time periods were used to determine bird performance so that the results from the two experiments could be compared.

Surprisingly, feed conversion ratio decreased as the birds got older ($P<0.001$), and there was a significant interaction ($P=0.045$) between treatment and time on feed conversion ratio with

young birds fed WGW having a much higher FCR compared with older birds, regardless of what they were fed (Figure 3.6).



*0WGW: Birds were fed a proprietary, pelleted diet appropriate to their age without WGW.

**LWGW: Whole grain wheat was mixed at a rate 100 g/kg with a proprietary pelleted diet appropriate to their age

***HWGW: Whole grain wheat was mixed at a rate 200 g/kg with a proprietary pelleted diet appropriate to their age

†Weeks: Age of birds (6-10 weeks)

Figure 3.6: Effect of treatment and time on FCR Experiment 2

The data from the two experiments were then combined, and are summarized in Table 3.13. There was no effect of study on the feed intake and FCR although birds grew more slowly in Experiment 2. Older birds (11 weeks old) ate more and grew more quickly than younger birds (eight weeks old) but there was no significant difference (P=0.159) in FCR between these two ages. Across these two experiments, the inclusion of WGW in the diet had no effect on bird performance. Feed intake increased more in Experiment 1 as birds got older compared with Experiment 2. Any reduction in feed intake with the inclusion of WGW when the birds were eight weeks old was not observed in the older birds (P=0.010). There was a significant interaction between study and age on feed intake and between age and diet on feed intake, growth rate and FCR.

Table 3.13: Effect of study, bird age and the inclusion of whole grain wheat in the diet on bird performance

	Study								SEM	P						
	1 (Free choice)				2 (Mixed in diet)					Study	Age	Diet	SxA	SxD	AxD	SxAxD
	Bird age (weeks)				Bird age (weeks)					(S)	(A)	(D)				
	8		11		8		11									
	CON	WGW	CON	WGW	CON	WGW	CON	WGW								
Performance (g/bird/d):																
Feed intake	399	326	543	638	456	353	509	504	33.8	0.404	<0.001	0.393	0.0144	0.192	0.010	0.477
Growth rate	180	172	212	238	152	144	203	198	7.33	<0.001	<0.001	0.798	0.774	0.157	0.095	0.155
FCR	3.15	2.64	2.59	2.69	3.10	2.45	2.51	2.55	0.239	0.523	0.159	0.152	0.977	0.782	0.072	0.905

CON: Birds fed control diet (no whole grain wheat); WGW: Birds offered whole grain wheat free choice (Experiment 1) or mixed in the diet (Experiment 2); FCR: Feed conversion ratio

3.11.3 Relationship between whole wheat in the diet and bird performance (Experiment 1)

There was no relationship between the amount of WGW consumed and the birds' growth rate, and there was a weak relationship between feed intake and FCR. (Table 3.14)

Table 3.14: Relationship between whole wheat intake of birds offered whole grain wheat and starter pellet and their growth rate, feed intake and feed conversion ratio.

	Regression		Constant			Coefficient		
	R^2	P	Value	SE	P	Value	SE	P
Feed intake	0.183	0.000	-10.3	11.2	0.359	0.104	0.023	0.000
Growth rate	0.068	0.017	-17.9	23.7	0.453	0.325	0.125	0.017
FCR	0.174	0.000	-26.7	16.6	0.111	27.63	6.63	0.000

3.11.4 Effect of treatment on weight of gastrointestinal organs

There was no significant effect of treatment on the weight of the different parts of the gastrointestinal tract (crop, gizzard, liver, pancreas and caecum) of turkeys fed different diets at starter (28-48 d), grower 1 (49-69 d) and grower 2 (70-84 d old), but there was a significant effect on caecum weight (P= 0.038) at grower 2 stage (70-84 d old, Table 3.15). The weight of the duodenum, jejunum, ileum and caecum increased with bird age as expected.

Table 3.15: Effect of treatment on crop, gizzard, liver and pancreas weight (g)

	*CON	**WGW+SP	SEM	P- value
Starter (28-48 d)				
Crop	9.0	8.6	0.22	0.162
Gizzard	49.3	53.0	1.95	0.192
Liver	54.7	56.5	1.73	0.479
Pancreas	5.8	6.2	0.20	0.209
Caecum	14.8	14.4	0.69	0.736
Grower 1 (49-69 d)				
Crop	16.0	15.5	1.17	0.770
Gizzard	92.3	90.8	3.63	0.778
Liver	88.5	92.9	4.25	0.485
Pancreas	9.7	9.0	0.62	0.515
Caecum	24.8	27.7	1.30	0.153
Grower 2 (70-84 d)				
Crop	23.0	23.3	0.85	0.784
Gizzard	116.1	125.8	3.73	0.072
Liver	150.5	146.3	3.93	0.442
Pancreas	11.1	12.7	0.39	0.010
Caecum	45.8 [b]	49.5 [a]	1.21	0.038

*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

3.11.5 Effect of treatment on length of digestive tract

Measurements of lengths of the four segments of small intestine (duodenum, jejunum, ileum and caecum) were not significantly affected by treatment. However, there was a tendency (P=0.092) for caecal length at starter (28-48 d) to be greater in birds fed WGW+SP (Table 3.16). The length of the duodenum, jejunum, ileum and caecum increased with bird age as expected. Generally these data in Table 3.15 and 3.16 suggest that the inclusion of WGW in the diet did not affect the morphology of the birds' gut.

Table 3.16: Effect of treatment on lengths of duodenum, jejunum, ileum and caecca (cm)

	*CON	**WGW+SP	SEM	P- value
Starter (28-48 d)				
Duodenum	30.99	30.64	0.66	0.709
Jejunum	73.56	73.03	1.22	0.763
Ileum	73.89	73.50	1.19	0.820
Caecum	23.03	24.81	0.71	0.092
Grower 1 (49-69 d)				
Duodenum	32.33	32.42	1.09	0.958
Jejunum	88.67	89.42	1.93	0.790
Ileum	85.92	87.75	1.10	0.266
Caecum	30.00	32.13	1.31	0.278
Grower 2 (70-84 d)				
Duodenum	36.00	35.38	0.85	0.608
Jejunum	101.73	105.50	2.24	0.247
Ileum	101.68	103.54	2.01	0.521
Caecum	35.05	37.50	1.27	0.186

*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

3.11.6 Effect of treatment on digesta pH and proportion of wheat in the crop

In Experiment 1, the proportion of WGW in the crop was approximately 35 g/kg when birds were 28-48 d old, 25 g/kg when birds were 49-69 d and 171 g/kg when they were 70-84 d old (Table 3.17). WGW intake was therefore generally lower when offered free choice compared with the intake of WGW when it was mixed into the diet (Experiment 2, Table 3.18). When mixed with the pelleted diet, the proportion of WGW in crop contents broadly reflected the inclusion rate of WGW in the mixed diet. In Experiment 1, digesta pH was affected by treatment (P=0.045) gizzard digesta pH being lower when birds were offered WGW at 28-48 d old (Table 3.17). There was considerable

individual variation between birds in the amount of WGW found in the crop, but no relationship (R^2=0.059, P=0.019) was observed between the proportion of wheat in the crop and either gizzard or caecal pH (R^2<0.001, P=0.713). It was also observed when samples were being taken from the birds that many of them had consumed bedding along with their feed, and in some cases this consumption of bedding appeared to be substantial.

Table 3.17: Effect of treatment on digesta pH and the proportion of wheat observed in crop contents experiment one.

	*CON	**WGW+SP	SEM	P- value
Starter (28-48 d)				
Proportion (g/kg DM) of wheat in crop contents	0.00	35.00	0.0135	0.096
Gizzard (pH)	3.83[a]	3.60[b]	0.0742	0.045
Caeca (pH)	6.36	6.22	0.188	0.613
Grower 1 (49-69 d)				
Proportion (g/kg DM) of wheat in crop contents	0.00 [b]	24.97 [a]	0.00605	0.014
Gizzard (pH)	3.45	3.59	0.0862	0.270
Caeca (pH)	5.88	5.98	0.172	0.710
Grower 2 (70-84 d)				
Proportion (g/kg DM) of wheat in crop contents	0.00 [b]	170.80 [a]	0.0380	0.003
Gizzard (pH)	3.54	3.47	0.0851	0.549
Caeca (pH)	5.79	5.84	0.0951	0.744

*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

As in Experiment 1, the inclusion of WGW in the diet in Experiment 2 did not affect caecal pH (P=0.535) when birds were younger, P=0.145, when birds were older). In Experiment 1,

gizzard pH was reduced when WGW was offered to birds, and this was observed in Experiment 2 (P=0.006) when the birds were younger but not when they were older (P=0.211). Interestingly, it was younger birds fed LWGW that had the lowest gizzard pH, but there was no significant difference between birds fed 0WGW and HWGW (Table 3.18).

Table 3.18: Effects of treatment on digesta pH and the proportion of wheat observed in crop contents experiment two.

	*0WGW	** LWGW	***HWGW	SEM	P- value
Age of birds (56 d)					
Proportion (g/kg DM) of wheat in crop contents	0 [a]	124 [ab]	232 [b]	0.048	<0.001
Gizzard	3.45 [a]	2.60 [b]	3.05 [ab]	0.175	0.006
Caeca	5.78	5.78	5.49	0.210	0.535
Age of birds (77 d)					
Proportion (g/kg DM) of wheat in crop contents	0 [a]	102 [b]	225[C]	0.018	<0.001
Gizzard	3.43	3.38	3.06	0.135	0.211
Caeca	5.87	5.81	5.49	0.140	0.145

*0WGW: Birds were fed a proprietary, pelleted diet appropriate to their age without WGW.

**LWGW: Whole grain wheat was mixed at a rate 100 g/kg with a proprietary pelleted diet appropriate to their age

***HWGW: Whole grain wheat was mixed at a rate 200 g/kg with a proprietary pelleted diet appropriate to their age

Different superscripts within row differ significantly (P<0.05)

3.11.7 Effect of treatment on intestinal and whole tract nutrient availability

The estimates of nutrient availability were low, especially when the birds were young (28-48 d and 49-69 d old). The estimates were more credible at 70-84 d old, with no effect of treatment on nutrient availability when birds were fed WGW+SP compared with CON (Table 3.19).

Table 3.19: Effect of treatment on intestinal and whole tract nutrient availability experiment one

	*CON	**WGW+SP	SEM	P- value
Starter (28-48 d)				
Nutrient availability Crop/excreta	0.30	0.36	0.0475	0.421
Nutrient availability Crop/ileum	0.25	0.30	0.0405	0.400
Grower 1 (49-69 d)				
Nutrient availability Crop/excreta	0.45	0.44	0.0278	0.907
Nutrient availability Crop/ileum	0.27	0.32	0.0990	0.748
Grower 2 (70-84 d)				
Nutrient availability Crop/excreta	0.68	0.71	0.017	0.136
Nutrient availability Crop/ileum	0.62	0.65	0.030	0.473

*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

In Experiment 2, the estimates of ileal nutrient availability were similar to those observed for the control diet in Experiment 1. As in Experiment 1, there was no effect of treatment on

intestinal nutrient availability in Experiment 2 (Table 3.20).

Table 3. 20: Effect of treatment on intestinal nutrient availability experiment two

	*0WGW	**LWGW	***HWGW	SEM	P-value
Age of birds 77 d					
Nutrient availability Crop/ileum	0.68	0.65	0.67	0.0860	0.960

*0WGW: Birds were fed a proprietary, pelleted diet appropriate to their age without WGW.

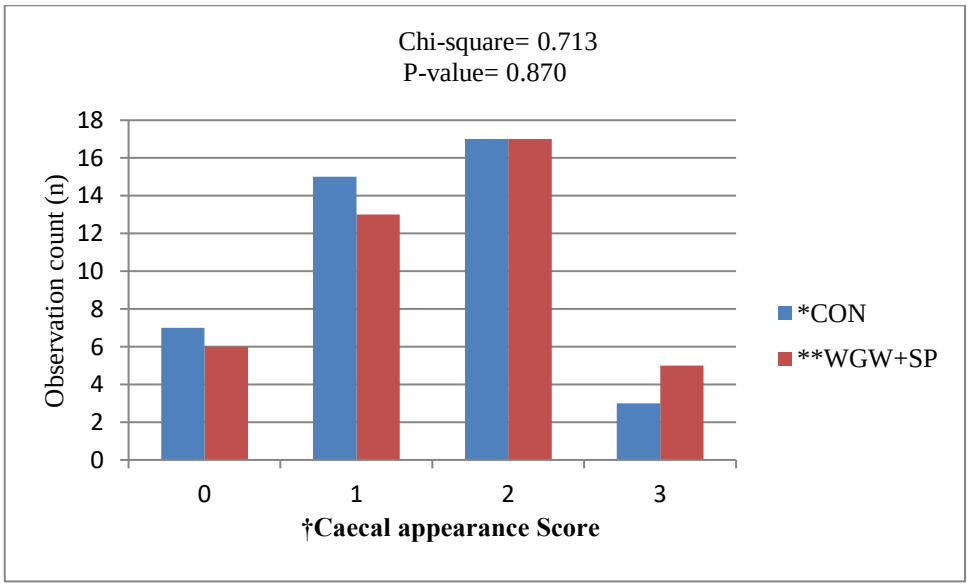
**LWGW: Whole grain wheat was mixed at a rate 100 g/kg with a proprietary pelleted diet appropriate to their age

***HWGW: Whole grain wheat was mixed at a rate 200 g/kg with a proprietary pelleted diet appropriate to their age

Different superscripts within row differ significantly (P<0.05)

3.11.8 Effect of treatment on measures of caecal health

In Experiment 1, the caeca of most birds, regardless of dietary treatment (P=0.870) appeared mildly or moderately distended (Figure 3.7), while most caecal contents were described as either thick and viscous or foamy and liquid but without blood (Figure 3.8), and dietary treatment had no effect on the appearance of the caecal contents (P = 0.250).

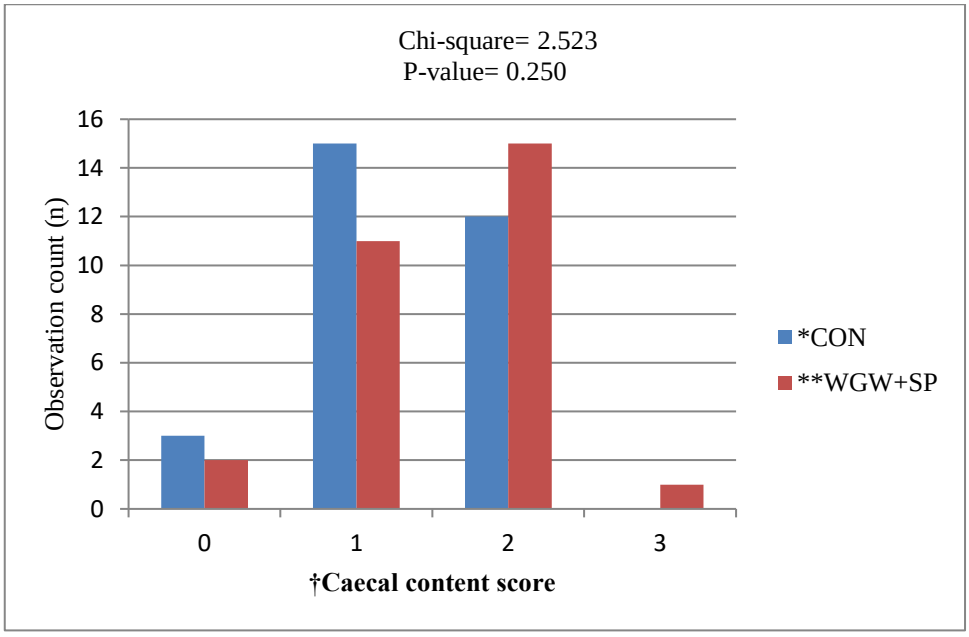


*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

†Caecal appearance score: 0= no pathological changes, 1 = mild distension with no colour change, 2= moderate distension with pale colour change, 3= complete distension with blood present in the wall.

Figure 3.7: The effect of treatment on caecal appearance score Experiment 1



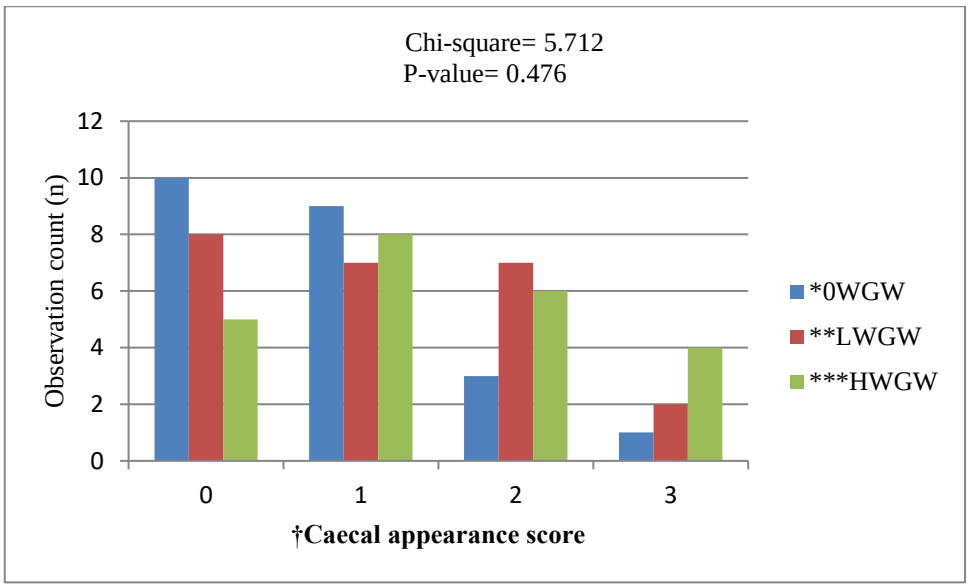
*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

†Caecal content score: 0= no pathological changes (light brown, smooth consistency), 1= thick and viscous content, brown/dark brown in colour 2= foamy/liquid content, pale yellow in colour, 3= foamy/liquid content, pale yellow in colour with blood present

Figure 3.8: The effect of treatment on caecal content score Experiment 1

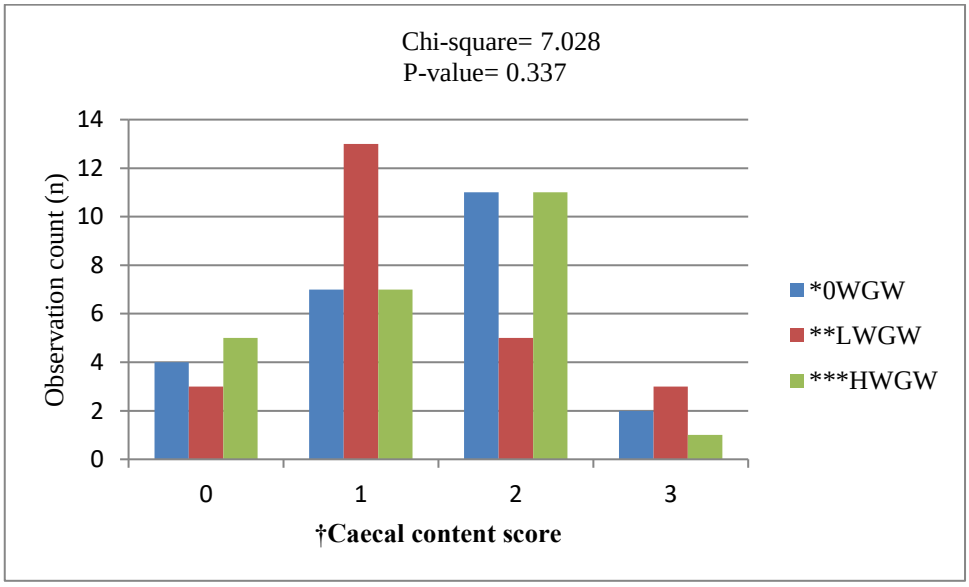
In contrast to Experiment 1, most birds in Experiment 2 showed either no pathological change or only mild distension of their caeca. As before, there was no effect of treatment on caecal appearance scores (Figure 3.9; P-value = 0.476). The description of caecal contents were similar to Experiment 1 (thick and viscous or foamy but with no blood present), and were not affected by treatment (Figure 3.10; P value = 0.337).



*0WGW: Birds were fed a proprietary, pelleted diet appropriate to their age without WGW. **LWGW: Whole grain wheat was mixed at a rate 100 g/kg with a proprietary pelleted diet appropriate to their age. ***HWGW: Whole grain wheat was mixed at a rate 200 g/kg with a proprietary pelleted diet appropriate to their age

† Caecal appearance score: 0= no pathological changes, 1 = mild distension with no colour change, 2= moderate distension with pale colour change, 3= complete distension with blood present in the wall.

Figure 3.9: Effect of treatment on caecal appearance score Experiment 2



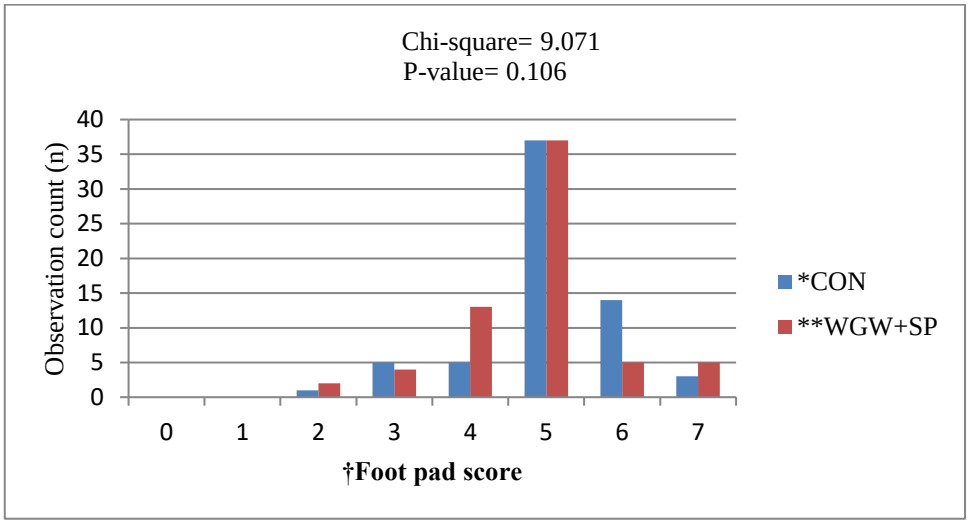
*0WGW: Birds were fed a proprietary, pelleted diet appropriate to their age without WGW. **LWGW: Whole grain wheat was mixed at a rate 100 g/kg with a proprietary pelleted diet appropriate to their age. ***HWGW: Whole grain wheat was mixed at a rate 200 g/kg with a propri etary pelleted diet appropriate to their age.

† Caecal content score: 0= no pathological changes (light brown, smooth consistency), 1= thick and viscous content, brown/dark brown in colour 2= foamy/liquid content, pale yellow in colour, 3= foamy/liquid content, pale yellow in colour with blood present

Figure 3.10: Effect of treatment on caecal content score Experiment 2

3.11.9 Effect of treatment on foot pad score

In Experiment 1, most birds had a necrotic area covering a quarter of the foot pad (Figure 3.11) and this was not affected by dietary treatment (P = 0.106). There was a much more even spread of scores in this experiment compared with Experiment 2, but again there was no significant effect of treatment (P= 0.869) on foot health (Figure 3.12).

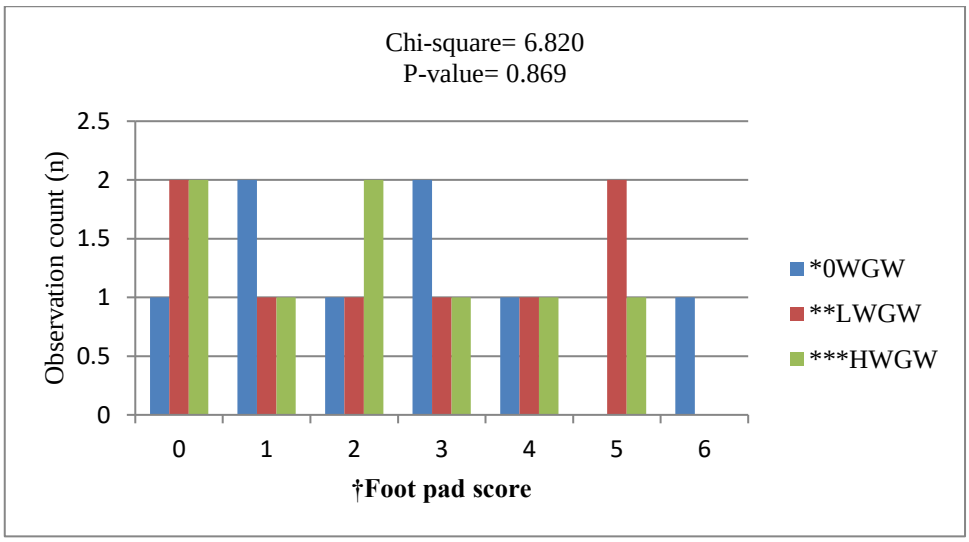


*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

†Foot pad score: 0= normal foot pad and digital pads, 1= redness skin of foot pad, 2= the foot pad feels harger and denser, 3= small black necrotic on the foot pad, 4= the area of necrosisis less than one- eighth of the foot pad, 5= the necrotic area extendsto a quarter of the foot pad, 6= half of the foot pad covered by necrotic cells, 7= over half of the foot pad covered by necrotic cells.

Figure 3.11: Effect of treatment on foot pad score Experiment 1



*0WGW：Birds were fed a proprietary, pelleted diet appropriate to their age without WGW.

**LWGW: Whole grain wheat was mixed at a rate 100 g/kg with a proprietary pelleted diet appropriate to their age

***HWGW: Whole grain wheat was mixed at a rate 200 g/kg with a proprietary pelleted diet appropriate to their age

†Foot pad score: 0= normal foot pad and digital pads, 1= redness skin of foot pad, 2= the foot pad feels harger and denser, 3= small black necrotic on the foot pad, 4= the area of necrosisis less than one- eighth of the foot pad, 5= the necrotic area extendsto a quarter of the foot pad, 6= half of the foot pad covered by necrotic cells, 7= over half of the foot pad covered by necrotic cells.

Figure 3.12: Effect of treatment on foot pad score Experiment 2

There was no relationship (R^2<0.1) between foot pad score and either bird performance (feed intake, growth rate, feed conversion ratio) or caecal scores in Experiment 1. However, in Experiment 2, foot pad score explained some of the variation observed in bird performance. (Table 3.21).

Table 3.21: Relationship between foot pad score and bird performance in Experiment 2

	Regression		Constant			Coefficient		
	R^2	P	Value	SE	P	Value	SE	P
Feed intake	0.358	0.003	7.22	1.47	0.000	-0.0108	0.003	0.003
Growth rate	0.607	0.000	7.23	0.87	0.000	-0.021	0.003	0.000
FCR	0.181	0.038	0.22	1.04	0.838	0.980	0.444	0.038
Turkey weight	0.664	0.000	7.05	0.74	0.000	-0.0007	0.0001	0.000

3.11.10. Effect of treatment on litter dry matter content (Experiment 1)

Litter dry matter content declined (P<0.001) during the experiment from approximately 642 g/kg at the beginning to 444g/kg at the end (Table 3.22). There was no effect of dietary treatment (P=0.737) or interaction between treatment and time (P= 0.361).

Table 3.22: Effect of treatment on litter dry matter

	Litter dry matter content g/kg
* CON	554
**WGW+SP	562
SEM	16.7
P-value	0.737
Age of bird (weeks)	
6	642
7	609
8	640
9	554
10	459
11	444
SEM	26.6
P-value	0.000

*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

There was no relationship between litter dry matter content and the birds' foot pad score (R^2=0.002, P=0.756).

3.11.11 Microbial profile of the caecal contents

The molecular analysis of the samples of caecal contents, using a target gene (hippicurase) that is unique to *Campylobacter jejuni*, indicated that there was no *C. jejuni* in any of the birds that were sampled. Conversely, *Cl. perfringens* were present in all samples, although in most cases this was not associated with any clinical signs of

pathogenesis. The effect of WGW inclusion in the diet and relationship between the presence of particular bacterial species and caecal score is therefore confined to a consideration of *Brachyspira pilosicoli* and *Salmonella.*

Samples were categorised as containing either *Brachyspira* alone, or *Salmonella,* or both, or neither. Chi square analysis indicated that there was no association between diet and the combination of *Brachyspira* and *Salmonella* presence, Chi Square: 0.727, P=0.867 (Table 3.23).

Table 3.23: The frequency with which different diets were associated with the presence of *Brachyspira pilosicoli* and/or *Salmonella* in the caecal contents of turkey poults in experiment 1.

Diet	Neither *Brachyspira* nor *Salmonella*	*Brachyspira* alone	*Salmonella* alone	Both *Brachyspira* and *Salmonella*
*CON	11	2	14	3
**WGW+SP	13	3	11	3

*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

There was also no association between the score for caecal external appearance, Chi Square: 8.326, P>0.5 (Table 3.24) and the presence of these bacteria, nor between the score for caecal contents and these infections (Table 3.25).

Table 3.24: The frequency with which different scores for the appearance of the caecum were associated with the presence of *Brachyspira pilosicoli* and/or *Salmonella* in the caecal contents of turkey poults in experiment 1.

*Score	Neither *Brachyspira* nor *Salmonella*	*Brachyspira* alone	*Salmonella* alone	Both *Brachyspira* and *Salmonella*
0	4	2	8	1
1	11	2	7	1
2	7	1	10	3
3	2	0	0	1

*Caecal appearance score: 0= no pathological changes, 1 = mild distension with no colour change, 2= moderate distension with pale colour change, 3= complete distension with blood present in the wall.

Table 3.25: The frequency with which different scores for the appearance of caecal contents were associated with the presence of *Brachyspira pilosicoli* and/or *Salmonella* in turkey poults in experiment 1.

*Score	Neither *Brachyspira* nor *Salmonella*	*Brachyspira* alone	*Salmonella* alone	Both *Brachyspira* and *Salmonella*
0	2	0	4	1
1	7	1	9	1
2	13	1	7	1
3	1	0	0	0

*Caecal content score: 0= no pathological changes (light brown, smooth consistency), 1= thick and viscous content, brown/dark brown in colour 2= foamy/liquid content, pale yellow in colour, 3= foamy/liquid content, pale yellow in colour with blood present

Although the molecular analysis of the samples indicated that *Campylobacter jejuni* was absent, when caecal contents were cultured (when the birds were eight weeks old) a colony was identified as being *Campylobacter spp.* Although neither of these bacterial populations were affected by diet as determined by analysis of variance (Table 3.26), a positive relationship was observed in birds offered WGW in terms of the dietary proportion of WGW consumed (on a pen basis) and the population size of *Cl.*

perfringens (Figure 3.13, R^2=0.788, P=0.045). No such relationship was observed with the *Campylobacter* spp.

Table 3.26: The effect of diet on the size (log CFU/g) of the *Campylobacter spp* and the *Clostridium perfringens* population of caecal contents taken from poultry in Study 1.

Bacterial species	Diet		SEM	P
	*CON	**WGW+SP		
Campylobacter spp	4.98	4.93	0.222	0.885
Clostridium perfringens	3.98	4.33	0.298	0.457

*CON: Birds were fed a proprietary, pelleted diet appropriate to their age.

**WGW+SP: Birds had free access to a proprietary, pelleted starter diet in one hopper and whole grain wheat in a second hopper throughout the experimental period.

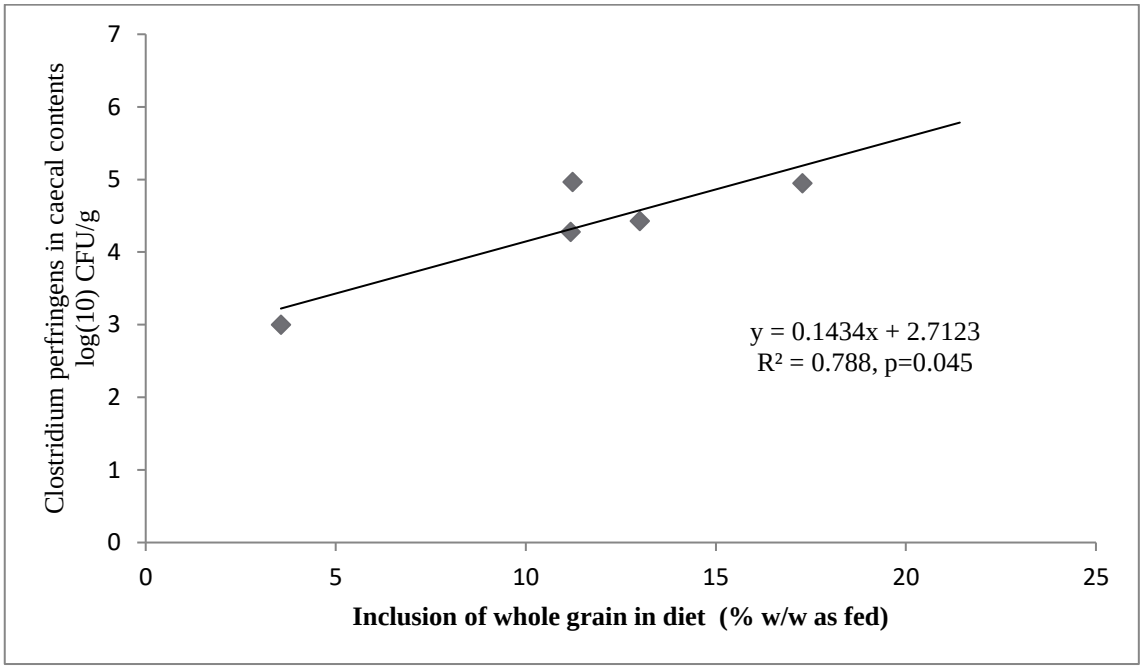


Figure 3.13: The relationship between the proportion of the diet that was comprised of whole grain wheat (in birds offered free choice whole grain wheat) and the size of the population of Clostridium perfringens observed in caecal contents taken from those birds.

- To examine the relationship between (pen) WGW intake (% of total intake represented by WGW) and Cl. perfringens population (log CFU/g), only the six pens that were offered WGW were used, and there was one missing value (failed to culture any CFU), leaving five estimates of dietary WGW content and Cl. perfringens population.

3.12 Discussion

These two experiments investigated the effects of supplementing turkey diets with whole grain wheat in both a free choice feeding (FCF) system and mixing the whole grain wheat in the turkey diet on bird performance and maintenance of gut health. Generally, turkeys fed whole grain wheat in a free choice feeding system or mixing with 20/g/kg WGW increased feed intake especially in free choice feeding. This might be because the consumption of whole grain wheat resulted in higher grinding pressure and abrasive action within the gizzard which in turn led to improved efficiency of digestion of whole grain wheat as observed by (Rose et al., 1995; Preston et al., 2000). These workers also reported that whole grain wheat was more efficiently digested and absorbed because of grinding in the gizzard and this resulted in increased feed intake by the birds (Preston et al., 2000; Rose et al., 1995). However, the lack of any significant difference between treatments in the estimates of nutrient availability in this experiment would suggest that this was not the case in this study. In these experiments, growth rate, bird weight and feed conversion ratio were not improved by increased WGW consumption, and indeed the consumption of WGW worsened FCR in some cases. This may be a result of a lower availability of net energy with the WGW diets because of the energy required for the grinding of the wheat (Hetland *et al*., 2002). Alternatively, it may reflect a lower supply of crude protein, oil, sugar and minerals when WGW was consumed, although the reduction in oil and sugar supply would be partly offset by the much higher starch content of WGW compared with the pelleted diet. However, a lower intake of crude protein by birds fed WGW was only observed in younger birds in Study 2, and in Study 1 there were some phases when crude protein intake tended to be higher with birds fed WGW. The high starch content of whole wheat may affect digestion as rapid starch digestion has a negative effect of bird performance compared with more slowly digestible starch as the lower rate of digestion leads to improved efficiency of

digestion of protein and amino acids, consequently leading to improved bird performance (Weurding et al., 2001). That said, the rate of starch digestion in untreated, whole wheat is likely to be rather low. The findings of this experiment is in agreement with the study of (Erener et al., 2003) who observed that free choice whole wheat in broiler chicken diets negatively affected bird performance parameters with the highest total feed intake being recorded in the FCF group.

In Experiment 2, whole grain wheat was mixed with the control diet (0WGW). The findings of Experiment 2 are similar to those of (Bennett and Classen, 2003); Experiment 2 found that although there were no effects of treatment on intake, there were effects with respect to rates of weight gain and subsequent feed conversion efficiency with rates of gain and efficiency of feed use being poorer in younger birds offered diets containing WGW (Table 3.12). Bennett and Classen (2003) reported that the inclusion of WGW reduced both weight gain and feed conversion efficiency as WGW inclusion increased (150-350 g/kg). In contrast, (Zdunczyk et al., 2013) reported that the feeding of WGW at low, medium and high inclusion rates (82,133, and 182 g/kg) did not adversely affect feed intake or body weight gain but improved (lowered) feed conversion ratio when compared with the inclusion of the same amounts of ground and pelleted wheat. The changes in bird performance observed in Experiment 2 and that of Bennett and Classen (2003) may be a consequence of the dilution of crude protein. Within studies, the metabolisable energy contents of diets were comparable between contrasting treatments, but crude protein contents were different. In Experiment 2, the crude protein content of the diets, when compared with the control, were reduced by 35 to 70 g/kg with an ME content across diets of 13.5 MJ/kg, whereas in the study reported by Bennett and Classen (2003), crude protein contents were reduced by 70 to 125 g/kg with an ME content across diets of 12.6 MJ/kg. In both

experiments, weight gain of the birds was depressed and FCR poorer when WGW was fed, probably because of an inability of the gizzard to process high levels of whole wheat fast enough to maintain growth (Picard et al., 1999).

In common with the findings of other studies, including WGW in the diet of turkeys did not affect the weight of the crop, gizzard, liver, pancreas or caecum. This was also observed by (Ravindran et al., 2006; Amerah et al., 2011), (Table 3.15). These results of gut weights are also consistent with other studies (Amerah et al., 2011; Gabriel et al., 2003a; Gabriel et al., 2008; Ravindran et al., 2006; Jankowski et al., 2014b). The only exception to this was the small increased weight of the caecum observed in 70-84 d old in birds fed WGW (Table 3.15). Including WGW in the diet had no effect on the length of the duodenum, jejunum ileum, or caecum as also observed by (Ravindran et al., 2006), In younger birds, WGW did appear to increase the length of the caecum slightly (Table 3.15). This result agrees with (Amerah and Ravindran, 2008) who reported increased length of caeca in birds offered free choice whole wheat compared to those fed the ground wheat. This might be due to the caeca being highly adaptive and altering in length according to the form of the diet which in turn led to an increase in the size of caeca when the birds were fed whole grain wheat (Fenna and Boag, 1974; Pulliainen and Tunkkari, 1983). However, most studies have shown no changes in the weight and length of digestive tract when whole wheat was fed (Wu et al., 2004; Jones and Taylor, 2001; Preston et al., 2000; Svihus and Hetland, 2001; Wu and Ravindran, 2004). WGW did not have much effect on bird performance, but did have an effect on gizzard pH as expected and as discussed, whole wheat feeding is generally associated with reduced gizzard pH (and potentially increased counts of beneficial microflora) and it has been hypothesised that this is a consequence of increased grinding activity by the gizzard. The consumption of whole wheat may in part be an attempt by the bird to influence

gizzard pH and microflora in the gut (Gabriel et al., 2003b; Bjerrum et al., 2005; Santos et al., 2008). However, any such benefit was not observed in this experiment in terms of measures of caecal health and the presence of potential pathogens.

There are a number of studies reporting that an increase in gizzard weight is an indication of greater gizzard development and that this is associated with improved digestive health in poultry fed whole grains; increases in gizzard weights in both turkeys (Zdunczyk et al., 2013; Jankowski et al., 2012; Jankowski et al., 2013) and broilers (Bennett et al., 2002a; Gabriel et al., 2003a; Williams et al., 2008; Preston et al., 2000; Ravindran et al., 2006; Abdollahi et al., 2016; Gracia et al., 2016) have been reported when fed diets containing whole grains. In Experiment 2, gizzard weight was not recorded but gizzard digesta pH was. The reduction in pH of gizzard contents has been reported in both turkeys (Zdunczyk et al., 2013) and broilers (Gabriel et al., 2003a; Engberg et al., 2004; Gracia et al., 2016). (Svihus, 2011) proposed that this reduction in pH was most likely due to whole cereals causing an increase in gizzard volume leading to increased digesta retention time resulting in a stimulatory effect on gizzard activity and hydrochloric acid secretion. Benefits of this acidic environment may include reduced pathogenic bacteria (Engberg et al., 2004) and improved gastric digestion (Gabriel et al., 2003; Zdunczyk et al., 2013), but again, such benefits were not observed in this experiment.

Zdunczyk et al., (2013) reported that ceacal content pH was lower in turkeys fed diets containing high concentrations of whole wheat (225 g/kg) and this was associated with increases in the concentrations of acetic and butyric acids in caecal digesta. The findings of Experiments 1 and 2 were unable to establish any significant effects of treatment on caecal pH nor were short chain fatty acids (SCFA) quantified in caecal digesta. However, it is interesting to note that caecal pH was numerically lower (P

=0.181; p =0.145) in the HWGW group compared with the LWGW and 0WGW groups, which might suggest some alterations in SCFA production. Birds remained healthy throughout Experiment 2 although there was evidence of mildly distended caeca (caecal scores 2 and above). These were not treatment related, suggesting that the provision of whole wheat in the diet did little to reduce incidence. Similarly, caecal content scores indicated that the majority of caeca contained foamy/liquid content but this too was not related to treatment.

In both studies there was no effect of dietary treatment on scores of caecal health (appearance and content, Figures 3.8, 3.9, 3.10 and 3.11), and while very few had score 1 the scores recorded indicated that the birds were generally in a good state of caecal health, since no birds had a score of 4 and very few birds had a score of 3. There was therefore no evidence to suggest that WGW affected caecal health. If stress is a necessary predisposing factor in the development of caecal distension, then the birds in Experiment 2 would be expected to be more prone to the condition. In Experiment 2, the birds were taken from their home flock when less than seven weeks old, and transported to the research facilities at Reading. This was done in January, at the coldest time of year, and they were then kept in an outside barn with limited shelter and artificial heat. However, no clinical signs of stress were observed, and dietary treatment in this experiment had no effect on foot pad score either (Figure 3.12). In Experiment 1, scores of FPD were generally high in both groups. The foot pad lesions that were observed might be a consequence of water from the bell drinker being spilled on to the bedding material increasing the moisture content of bedding material rather than a response to diet. Lynn and Elson (1990) found that bell drinkers increased litter moisture content compared with small cup as well as nipple drinkers, and as has already been noted, litter moisture content alone is a primary cause of foot pad dermatitis in

turkeys (Mayne et al., 2007). In Experiment 2, despite the perceived greater stress that the birds were exposed to, most birds had a foot pad score of 0, which is the score for normal/healthy foot pads. This appears to be related to better litter conditions in Experiment 2 compared with Experiment 1 as the turkeys were kept in an open fronted shed, with natural ventilation and heat lamps were left on overnight which might result in dryer litter which in turn led to a lower foot pad score, but it would have been beneficial to determine the litter moisture content in this experiment.

The absence of any real clinical cases of distended caeca and caecal dysfunction in this experiment largely explains why there was no effect of diet, or association with caecal scores. In Experiment 1 the few cases where birds' caecal contents indicated the presence (at a molecular level) of both *Brahcyspira* and *Salmonella*, it was observed that this was not associated with any change in caecal distension and inflammation. This might indicate either that the level of infection was very low (and there were certainly no clinical signs of infection), or that neither of these species promote a strong inflammatory response. The effect of wheat encouraging proliferation of *Cl. perfringens* was also observed by (Annett et al., 2002) *in vitro* and by (Jia et al., 2009) in broilers. Digesta viscosity was not measured in this experiment, but it is possible that the inclusion of WGW increased the flow of some nutrients to the caecum, encouraging the proliferation of some bacterial species. Four of the ten successfully cultured samples indicated *Cl. perfringens* populations in excess of the commensal population level of 10^4 CFU/g observed by (Kondo et al., 1988) and three of these four samples were from birds fed WGW. However, none of the birds showed any clinical signs of necrotic enteritis, and so even though the WGW may have encouraged the growth of *Cl. perfringens*, there are clearly other predisposing factors required for pathogenesis (such as stress) that were not encountered by these birds. Wheat encourages the proliferation

of *Cl. perfringens* and this is attributed to the NSP content of wheat. Most *Cl. perfringens* strains are non-pathogenic and an increase in their number (perhaps associated with the reduction of other pathogens, including pathogenic strain of *Cl. perfringens)* in response to WGW may be expected. There was no evidence from this study that the feeding of WGW affected the quality of the bedding or the incidence of FPD' before 'It has also been noted.

3.13 Conclusion

Free choice feeding of whole wheat with a more concentrated protein source (the starter pellet) increased gizzard weight and the inclusion of whole wheat in the diet reduced gizzard digesta pH. This may be beneficial for the development of a healthy intestinal flora and nutrient digestion, although no evidence of either of these outcomes was observed in this experiment. The severity of FPD was higher in Experiment 1 compared with Experiment 2 but it is likely that this is a consequence of differences in the housing of the birds rather than the diets they were fed. Throughout both experiments, the severity of caecal dysfunction was low, and no clinical signs of major digestive dysfunction were observed. There were no clear indications that any of the potential pathogens investigated were associated with signs of caecal distension, and it seems likely that other predisposing factors (such as stress) are needed to precipitate these clinical signs. In the absence of caecal distension and dysfunction, there were no health or performance benefits associated with including whole wheat in the diet of these poults. It has also been noted that there was evidence that many of the birds had consumed at least some bedding material in addition to the feed they were offered. In the next experiment, therefore, it was decided that two different sources of bedding would be compared to determine what effect bedding source had on the consumption of bedding by broilers and on the characteristics of their digesta.

Chapter 4 Effect of bedding type on bird performance

4.1 Introduction

Natural behaviours of birds such as pecking and scratching at their litter suggests some ingestion of litter (Van Hierden et al., 2002; Malone et al., 1983). Indeed (Malone et al., 1983) reported that around 4% of poultry consumption consists of litter. Certainly in the previous experiment (Chapter 3), some litter was observed in the crop of birds in addition to the pelleted diet (and whole wheat if that was offered as well), but the amount of litter consumed was not quantified. The estimates of nutrient availability (approximately 0.65) were also low which may be partly because of the errors associated with determining acid insoluble ash but also because the ileal digesta or excreta were diluted with undigested bedding material. In a separate experiment, using titanium dioxide as an inert marker, negative coefficients were calculated for fibre digestion and this was attributed to the consumption by birds of unknown amounts of wood shaving bedding (C. Poulos, pers. comm.). The consumption of bedding may in part be an attempt to meet a nutrient or structural fibre requirement of the bird (Hetland et al., 2005). It is hypothesised that the consumption of litter may have a beneficial effect on digesta quality and gut health, and that different beddings may have different effects on these parameters. One alternative to wood shavings, although little used by poultry, is recycled paper waste marketed in the UK as Envirobed (Enviro Systems UK Ltd, Preston, UK). Like wood shavings, Envirobed is easy to handle and has good absorbent qualities (Hulet and Cravener, 2007). The objective of this experiment was to compare wood shavings with Envirobed (when birds were fed the same diet) to determine the effect of bedding source on estimates of the amount of bedding consumed, and on measures of gut health (digesta dry matter content and the incidence of *Campylobacter jejuni* and *Clostridium perfringens* in the caecum of birds) and diet

dry matter availability (assessed using titanium dioxide as a marker). These were selected as indicators of possible increased moisture excretion, which would thus result in an increased risk of foot pad dermatitis. In chickens the gastrointestinal tract harbours a complex microbiota that plays an important role in digestion, absorption, immune system development and pathogen exclusion (Pan and Yu, 2014). Chickens continuously take up microorganisms from the surrounding environment during their growth cycle. The bedding material used in chicken houses is usually mixed with excreta and thus harbours a complex microbial community and is a potential influence on microbiome of chicken gut (Coufal et al., 2006). The prevalence of *Campylobacter jejuni* and *Clostridium perfringens* pathogens and the risk of disease can be lowered by a healthy gastrointestinal tract microbiota through colonization resistance and competitive exclusion (Kerr et al., 2013). The two bacterial species investigated were selected as the former is a potential zoonosis and the latter a risk factor for necrotic enteritis and therefore poor welfare and economic performance (McDevitt et al., 2006; Hermans et al., 2012b), particularly since it was the gene for the α toxin that was being identified.

4.2 Material and methods

4.2.1 The study area

This experiment was carried out at the same area as in the previous experiment (CEDAR, Hall Place Farm, Arborfield).

4.2.2 Experimental design

This experiment was powered based on growth rate, with earlier studies indicating a minimum replication of n=4 been required to detect a difference of 9%. A total of 144 Ross 308 broiler chicks were collected on the day of hatching. The wings of the chicks

were individually tagged with numbered identification tags and their weight recorded. Tagged chicks were then randomly allocated to one of two bedding material treatments (Envirobed or Wood shavings). There were four replicate pens per treatment with 18 chicks in each pen (Table 4.1).

Table 4.1: Experimental design

Treatment	**Total No. of Birds/pen**	**Total No. of pens/treatment**	**Total No. of birds/treatment**
Envirobed	18	4	72
Wood shavings	18	4	72
Total		8	144

The four treatment pens were bedded with a layer of around 5 cm of either Envirobed or wood shavings. The size of each pen was 1x1.25 m and they were lined with cardboard (approximately 60 cm height) to prevent draughts and reduce contact and transfer of feed and litter material between pens. A proprietary starter diet (Countrywide Chick Crumbs, Countrywide Farmers, Evesham, UK) was fed for 9 d before being abruptly changed to a grower diet from 10-21 days and a finisher diet was then fed until the completion of the experiment (at 38 d). It was a proprietary vitamin/mineral mix produced by Target Feeds. The formulation of the grower and finisher diets are presented in Table 4.2. All feed added and removed from pens was weighed and recorded and diet changes were conducted at the same time for all pens.

Table 4.2: Formulation (g/kg as fed) of the grower and finisher diets.

Ingredient	**Grower**	**Finisher**
Corn	640	658.6
Soya Hi Pro	310	280
L-Lysine HCl	23	17
DL-methionine	29	26
L-threonine	13	11
Soya Oil	4	18
Limestone	12.5	12
Monocalcium phosphate	13.5	12.5
Salt	3	3
Sodium bicarbonate	1.5	1.5
Vitamin and Trace mineral premix	4	4
Titanium dioxide	5	5
Calculated nutrient composition (g/kg as fed)		
Crude Protein	209.2	195.6
ME (MJ/kg)	12.29	12.71
Calcium	9	8.6
Phosphorus	6.5	6.1
Lysine	13.1	11.7
Methionine	6.1	5.7
Total sulphur amino acids	9.5	8.8

4.2.3 Birds and management

Lighting was via incandescent lights with 23 h continuous light per 24 h period for the first seven days, followed by 18 h continuous light (6 h darkness in each 24 h period). Feeders were maintained at a height equivalent to the birds' backs. Feed was supplied *ad libitum* via hoppers and water was supplied *ad libitum* via nipple drinkers. The target temperature for the whole room was 30^0C for the first three days, dropping to 28^0C on

day 3, and then reducing by 1^0C every three days until a temperature of 20^0C was attained, although this was adjusted in response to observed bird behaviour.

4.2.4 Bird performance

Birds were weighed at the beginning of the study and at weekly intervals. The amount of feed added to hoppers and remaining in the hopper was determined on a pen basis at weekly intervals to calculate daily feed consumption in the same manner as described in Chapter three, Section 3.4 Growth rate and feed conversion ratio were calculated as in Chapter three (Section 3.5).

4.3 Sample collection

4.3.1 Selection of birds

At the end of the starter period (day 9), grower period (day 22) and finisher period (day 38), two birds were randomly selected and removed from each pen. Disposable overshoes and gloves were worn at all times and changed between pens to reduce bacterial transfer between pens. Birds removed from the pen were taken to a separate room for slaughter. Birds were weighed prior to slaughter by cervical dislocation (all birds were <3 kg body weight).

4.3.2 Determination of dry matter content in digesta

The crop, gizzard, ileum and colon of each individual bird was opened and the contents were collected, placed in a labelled plastic tube and stored frozen (-20^0C pending analysis. A sample of fresh digesta (approximately 1 g) was taken and placed in a weighed, labelled aluminium tray and placed in an oven at 105^0C overnight. Drying was done on the day of collection.

4.3.3 Estimation of bedding consumption by bird

It is assumed that the proportions of bedding and feed in the crop contents reflected the relative amounts of bedding and feed that the birds ate. It was therefore assumed that a dynamic steady state was achieved, and that the residence time of both feed and bedding in the crop was the same. If the bird ate no bedding, then the concentration of Ti in the feed and the crop contents should be the same. The lower the concentration of Ti was in crop contents compared with the feed, the more bedding it was assumed that the bird ate. The estimate of the proportion of bedding that made up the bird's diet (feed plus bedding) was calculated from:

Estimate of the proportion of bedding in the bird's diet = 1-([Ti] in crop contents/[Ti] in feed).

4.3.4 Dry matter digestibility

Feed and digesta samples of (0.1 g) were weighed into porcelain crucibles and ashed at 580°C for 13 h. The crucibles were then cooled and H_2SO_4 (10 ml 7.4 M) was added to each crucible. To completely dissolve the crucible contents, all samples were boiled gently for 60 min. After cooling, the solutions from each crucible were poured quantitatively into a small beaker containing 25 ml distilled water. The contents of the beakers were poured into 100 ml volumetric flasks through filter paper (Whatman 541). 20 ml H_2O_2 was added to each flask and the contents made up to volume with distilled water. Aliquots of these solutions were then transferred to a cuvette and their absorption at 410 nm determined with a spectrophotometer; a scan revealed that 410 nm was the wavelength which gave the optimum absorbance.

To prepare the calibration curve 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of the standard titanium dioxide solution (0.5 mg ml^{-1} TiO2) was pipetted into individual 100 ml volumetric flasks, 7.4 M sulphuric acid was added so that the combined volume was 10

ml. 20 ml H_2O_2 solution was added and the contents of the flasks made up to 100 ml using distilled water. The sample without titanium was used as the blank for the spectrophotometer readings. The samples were measured at 410 nm and a calibration curve was derived from the readings. The relationship between absorbance and concentration was linear up to the highest concentration (0.05 mg ml^{-1}).

4.4 Bacterial identification

Bacterial identification of *Campylobacter jejuni* and *Clostridium perfringens* were determined in the same manner as described in (Chapter 3, Section 3.9.1.1).

4.4.1 DNA Extraction

DNA extraction was determined as in (Chapter 3, Section 3.9.1.2)

4.4.2 Polymerase Chain Reaction (PCR) analysis

PCR Mastermix (ThermoFisher Scientific, Manchester, UK) was made up on ice, at a concentration of 0.25 µmol/40 µl, using the following components: Taq 20.0 µl, forward primer 1.6 µl, reverse primer 1.6 µl, nuclease free water 14.8 µl. The primers used for each bacterium are presented in Table 4.3, and were produced by Eurofins Genomics (Ebersberg, Germany).

Table 4.3: The genetic code of the required forward and reverse primers needed in the Mastermix for each corresponding bacterium.

Primer	***Campylobacter jejuni*** **(coding for hippuricase, Hip)**	***Clostridium perfringens*** **(coding for α-toxin, Cpa)**
Forward	GTACTGCAAAATTAGTGGCG	AGTCTACGCTTGGGATGGAA
Reverse	GCAAAGGCAAAGGATCCATA	TTTCCTGGGTTGTCCATTTC

Once the Mastermix was prepared, 2 µl of DNA from each sample was added to individual PCR tubes followed by 38 µl of the corresponding Mastermix. The tubes were then mixed on a vortex. The conditions used for the two bacteria are presented in Table 4.4.

Table 4.4: Conditions of the PCR cycles for each bacterial species investigated.

	No. of cycles	Initial heating	Stage 1: Denaturation	Stage 2: Annealing	Stage 3: Extension	Final Extension
Campylobacter jejuni[1]	28	-95°C 600s	94°C 15s	60°C 15s	72°C 15s	-72°C 600s
Clostridium perfringens[2]	35	-95°C 600s	95°C 60s	55°C 60s	72°C 60s	-72°C 600s

1= (Keramas et al., 2004)

[2] = (Baums et al., 2004)

Samples that had been amplified by PCR were stored refrigerated (5^{o}C) while gels were being made. 70 ml of Tris-Acetate-EDTA buffer was added to a 100 ml Erlenmeyer flask containing 0.70 g of Agarose powder. After swirling to mix, an upturned glass beaker was placed over the mouth of the flask before being placed into the microwave for 30 s. After 30 s, the solution was removed and swirled again, before being put back into the microwave for 10 s intervals, being careful not to boil the solution. Clarity at each interval was checked until all the precipitate had dissolved. Once fully dissolved, 4 µl of the nucleic acid stain, ethidium bromide was added. Casts were made up using masking tape around each end, ensuring no leaks of the gel could occur. Once set up, the agarose gel was poured gently into the cast and two well casts were put in. Bubbles were pulled down by a pipette tip, toward the bottom of the gel to avoid altered or unclear images at the end. This was then left to set for 15-20 minutes. Once set, the masking tape was removed and the cast was lowered carefully into the electrophoresis

machine containing TAE x 1 buffer that fully covered the gel. Samples were collected from the fridge. In a tray of small wells, 2 µl of blue dye was added to each followed by 5 µl of each of the PCR products which were mixed with the blue dye using the pipette tip. Loading of the wells started with the corresponding ladder on the furthest left well on each row. A lambda ladder was used for *Cl. perfringens* electrophoresis to be able to measure the 900bp band. A 100bp ladder was used for *C. jejuni* to allow detection of the 149bp length. These base pair lengths corresponded to the DNA helix length. The sample DNA was then added to wells. A positive control (if available) and a negative was included, to allow a visual comparison of what an absence or presence of the particular bacterial DNA would look like. The lid was then firmly put on and wires plugged in to the corresponding points. The machine was then turned on and ran at 110V for 25 minutes.

The gel was then slid off onto the tray of a UV Transilluminator machine. Care was taken not to expose the gel to the UV light for too long to prevent further degradation of the DNA. Images were then printed and labelled. The presence of a band in line with the correct bp length relative to the ladder was interpreted as evidence of the presence of the particular bacterial species.

4.5 Statistical analysis

Data relating to bird performance, digesta dry matter content and nutrient availability were subjected to analysis of variance (ANOVA) as repeated measures using a general linear model (GLM). Factors included in the model were treatment (d.f. =1), time (d.f. =6) and the interaction between treatment and time. Results are presented as least square means with the standard error of the mean and associated P-value. All analyses were conducted using the MINITAB Vs. 17 software.

4.6 Results

4.6.1 Effect of litter type on bird performance

The effect of bedding source on bird performance is summarised in Table 4.5. Birds bedded on wood shavings rather than Envirobed tended to eat more (P=0.083), grew more quickly (P=0.040).

4.6.2 Effect of treatment on dry matter content g/kg in digestive tract

Digesta dry matter content was significantly higher in the crop and gizzard than in the ileum and colon (P<0.001, Figure 4.1). Digesta was drier in birds at 15 d of age compared with birds that were 36 d old (P=0.014). Although the effect of bedding source was not significant (P=0.707), there was a significant interaction between bird age and bedding source (P=0.004). At 15 d, there was no significant difference between bedding sources, but when the birds were 36 d old, birds kept on Envirobed had drier digesta than those kept on wood shavings.

Table 4.5: Effect of treatment on bird performance

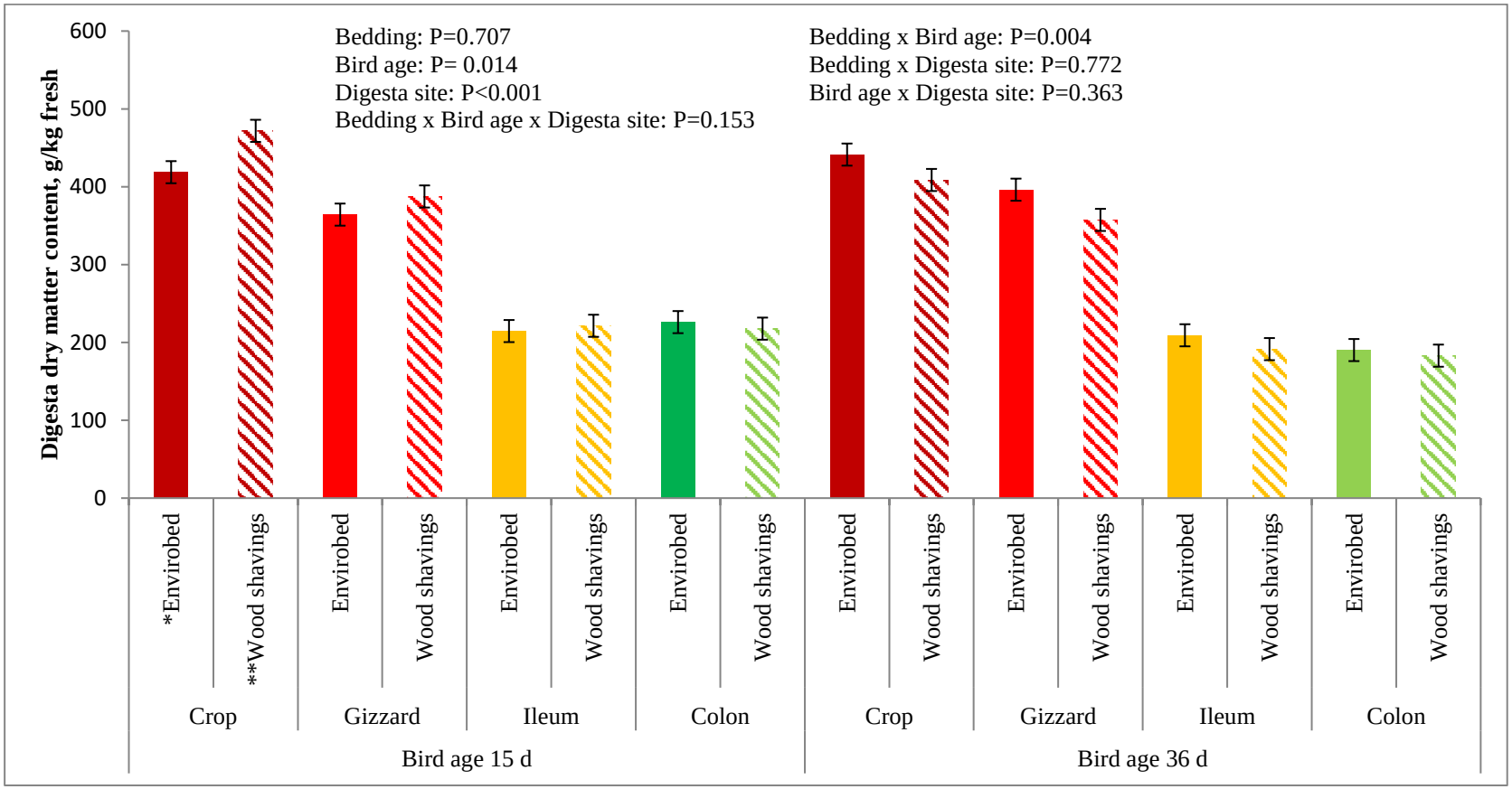
	* Envirobed				** Wood shavings						
	Age of bird (d)				Age of bird (d)					p	
Parameters	15	22	29	36	15	22	29	36	SEM	†Treatment	Time
Feed Intake (g/bird/d)	36.0	89.4	116.8	178.9	35.7	90.0	125.8	192.2	4.42	0.083	<0.001
WeightGain (g/bird/d)	27.0	58.0	72.5	88.5	29.0	62.0	79.4	92.0	2.66	0.040	<0.001
†FCR (g feed/g gain)	1.33	1.54	1.61	2.02	1.24	1.45	1.58	2.08	0.0336	0.133	<0.001

*Envirobed: Birds were bedded on Envirobed throughout the experiment

**Woodshavings: Birds were bedded on woodshavings throughout the experiment

†FCR: Feed conversion ratio

† Treatment: Effect of bedding supplied (Envirobed or wood shavings)



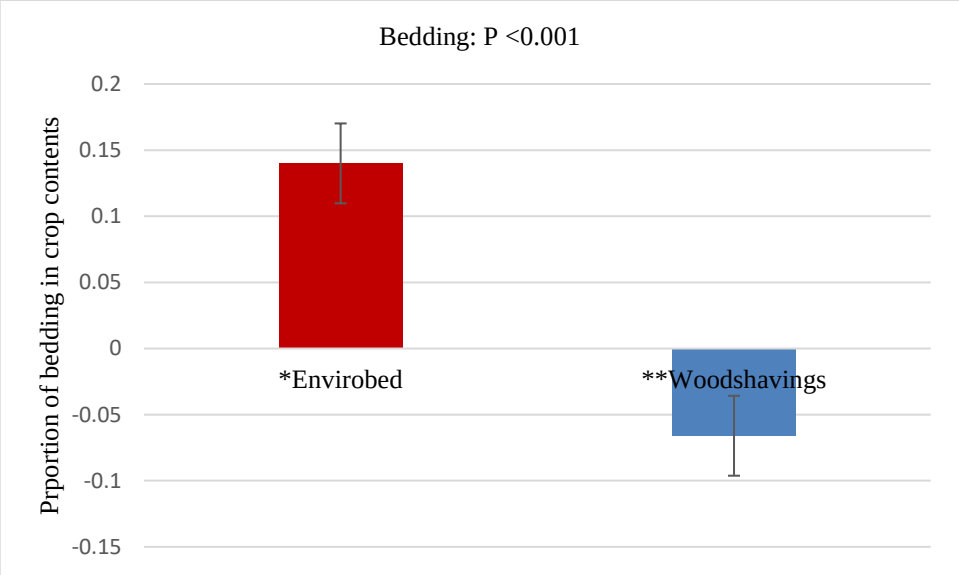
*Envirobed: Birds were bedded on Envirobed throughout the experiment

**Woodshavings: Birds were bedded on woodshavings throughout the experiment

Figure 4.1: Effect of treatment on dry matter content (g/kg fresh digest) in digestive tract

4.6.3 Effect of treatment on proportion of bedding in crop contents (total diet)

The proportion of the bedding in the crop in those birds that were reared on Envirobed was significantly greater than that found in the crop of birds kept on wood shavings (P<0.001, Figure 4.2). Bedding intake therefore appeared to be much lower in birds reared on wood shavings compared with those reared on Envirobed. Obviously, the estimate of bedding intake cannot be <0, but these data would suggest intake of wood shavings was very low, and some of the feed (a part that did not contain Ti) had already escaped from the crop so the concentration of Ti in the crop was in fact higher than it was in the feed and the errors involved in the estimation of Ti and therefore litter content mean that mathematically an estimate <0 was obtained..



*Envirobed: Birds were bedded on Envirobed throughout the experiment

**Woodshavings: Birds were bedded on woodshavings throughout the experiment

Figure 4.2: Effect of treatment on proportion of bedding in crop contents

4.6.4 Effect of treatment on dry matter digestibility

When the birds were 36 days old, the estimates of dry matter digestibility were low, but the estimates were more credible at 15 days old, with no effect of treatment and interaction between treatment and time (Table 4.6).

Table 4.6: Effect of treatment on dry matter digestibilty

	* Envirobed		** Wood shavings					
	Age of bird (d)		Age of bird (d)			p		
Parameters	15	36	15	36	SEM	Treatment	Time	Interaction
Nutrient availability Crop/Ileum	0.660	0.454	0.621	0.398	0.0504	0.369	<0.001	0.861

*Envirobed: Birds were bedded on envirobed throughout the experiment

**Woodshavings: Birds were bedded on woodshavings throughout the experiment

4.6.5 *Campylobacter jejuni*

Campylobacter jejuni was not detected in any of the samples (Figure 4.3), and thus there was no evidence that the choice of bedding affected the establishment of this bacterium in the chickens' caeca.

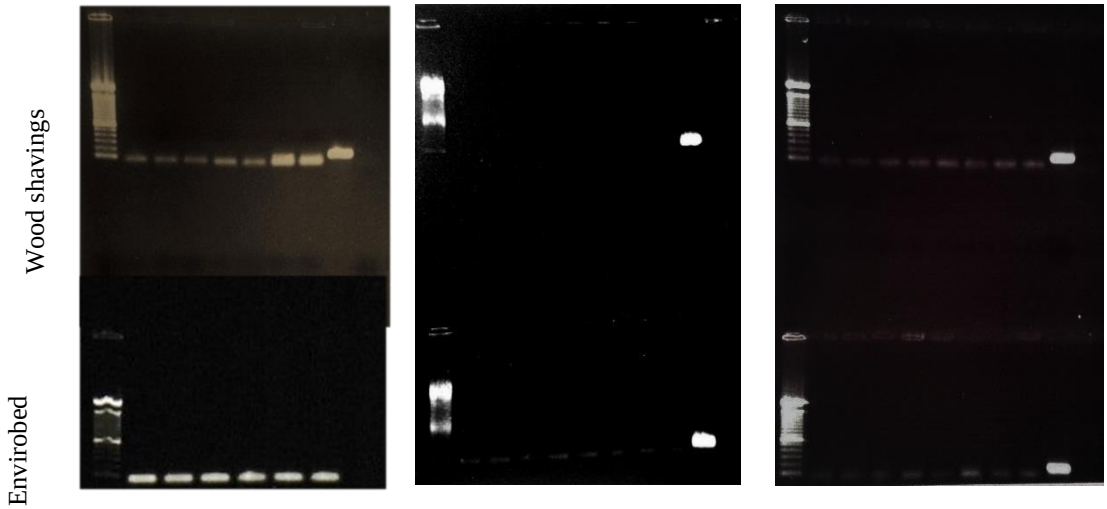


Figure 4.3: Left UV image of day 22 and right of day 38, both bedding type on both days indicated absence of *Campylobacter jejuni*, measured against the 100bp ladder and the positive band, brightly lit on the far right of both images.

4.6.6 *Clostridium perfringens*

The gene coding for the α toxin was present in all samples (Figure 4.4) with no evidence that choice of bedding had any effect on the presence in the chicken caecum of *Clostridium perfringens* type A.

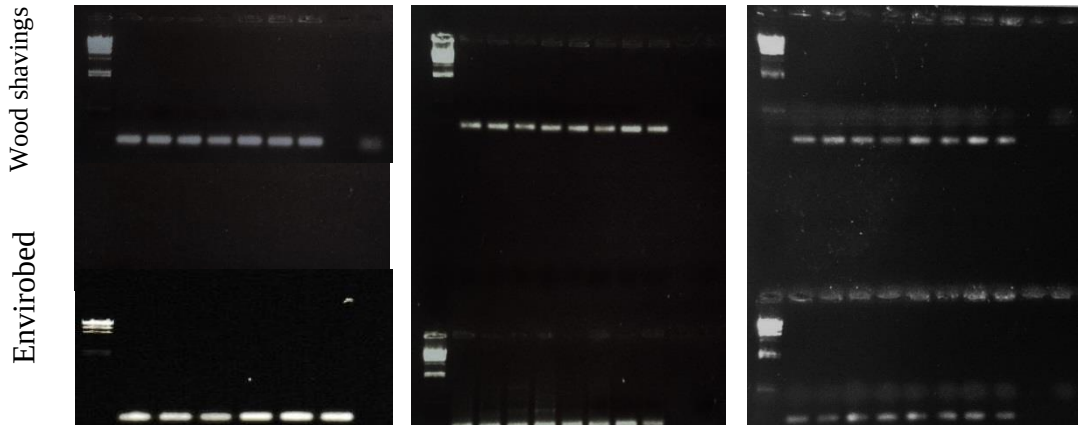


Figure 4.4: Left UV image on day 22 and right on day 38, indicated the presence of Type A alpha enterotoxin *Clostridium perfringens* in all both bedding samples, measured against the lambda ladder (far left of each image).

4.6.7 Foot health and mobility

In all of the observations of the birds, during both weighing and sampling, there was no evidence of any lesions on the foot, or of any impaired mobility by the bird. The choice of bedding had no effect on these observations. Litter remained dry and friable throughout. As the dry matter content of digesta in the digestive tract was determined, litter dry matter content was not assessed since the determination of digesta dry matter content was assumed to be a more direct estimate of gut condition.

4.7 Discussion

This experiment investigated the effects of two different bedding materials (wood shavings and Envirobed) on broiler performance, the presence of *Campylobacter jejuni* and *Clostridium perfringens* in the caecum of the chicken, and the dry matter content of the digesta and the nutrient availability of the diet. Findings suggested wood shavings were beneficial for weight gain and bird liveweight throughout the experiment ($P=0.040$; $P<0.001$) respectively. Birds reared on Envirobed appeared to consume more bedding material than those reared on wood shavings (based on the estimates of bedding material in crop contents) and so the poorer performance of birds reared on Envirobed may reflect dilution of their diet with bedding. These results are in agreement with several studies which have reported that bedding type can influence bird performance (Huang et al., 2009; Youssef et al., 2010; El-Deek et al., 2011; Toghyani et al., 2010; Torok et al., 2009; Garcês et al., 2013), although many others have reported that bedding type had no effect on bird performance (Hafeez et al., 2009; Simsek et al., 2009; Teixeira et al., 2015). Although bird performance may have been compromised by the consumption of bedding, birds reared on wood shavings had wetter digesta in their lower gut at 36 d compared with those on Envirobed. This finding is interesting, as it suggests that gut health was improved when birds consumed more bedding material (in the form of Envirobed). The wetter digesta is likely to be associated with wetter excreta (and thus wetter litter), so that birds reared on wood shavings may be at greater risk of developing foot pad dermatitis than those reared on Envirobed. There was no impact on FPD, but it is assumed that the wetter digesta increased the risk of FPD. With hindsight, litter DM content should have been determined, but it was not. It is not clear whether Envirobed itself promotes greater gut health, or whether it is primarily a consequence of more Envirobed being consumed relative to the wood shavings. If

consuming bedding does decrease excreta (and therefore litter) moisture content, it raises the question as to whether birds consume bedding material to increase their fibre intake and potentially improve their gut health (and potentially improve their foot health as well). However, if it was simply an unmet requirement for structural fibre that the bird was trying to satisfy, it does not explain why birds reared on wood shavings did not consume as much bedding material as those reared on Envirobed. The smaller particle size, and perhaps the softer nature of the particles in Envirobed may make it a more attractive substrate for birds than the harsher wood shavings. When the diet does not provide the minimal amount of fibre required by birds, they may show abnormal behaviour such as consumption of litter and feather pecking (Hetland et al., 2005). In addition fibre in chicken diets results in drier bedding material and lower mortality (Hetland et al., 2005). Mateos, (2012) reported that under commercial conditions the mortality is lower when birds are fed diets that contain a high level of fibre such as inclusion of sunflower meal and barley than birds fed diets based on corn and high protein soybean meal.

Absence of *Campylobacter jejuni*

There was no evidence of the presence (at a molecular level) of *C. jejuni* in any of the birds, which is good from a public and possibly bird health perspective. The chicks were from a commercial hatchery suggesting the hatchery is not the source of C. *jejuni* in birds entering the food chain. The paradigm of *C. jejuni* being often considered to be a harmless commensal bacterium of the chicken gut (Humphrey et al., 2014; Hermans et al., 2012a), is beginning to be questioned for fast growing broilers such as the Ross 308 used in this experiment. Faster growing breeds have been observed to have a stronger immune inflammatory response to *C. jejuni* infection compared with slower growing breeds, and this can lead to gut lining damage and subsequent diarrhoea (Humphrey et

al., 2014). *C. jejuni* infection in fast growing broilers may therefore, in addition to the public health risk, also increase the risk of reduced litter quality and increased incidence of hock burns and foot pad dermatitis (Humphrey et al., 2014).

This experiment was carried out in autumn/winter, perhaps accounting for the absence in this experiment. According to Altekruse et al. (1999); (Denis et al., 2001) *C. jejuni* colonisation is greater in the summer months. (Jorgensen et al., 2011) reported a significant difference in *C. jejuni* colonisation in different geographical locations of the UK and within regions there was seasonal variation with the highest prevalence in June and in flocks reared in Northern Britain compared with central and South Britain $P<0.001$).

Presence of *Clostridium perfringens* type A

The Cpa gene coding for the α-toxin of *Clostridium perfringens* was observed in all the caecal contents, confirming the ubiquitous nature of this bacterial species. There was no evidence that the bedding on which the birds were kept (wood shavings or Envirobed) affected this finding. These findings agree with the previous study in turkeys, and in studies by (Dahiya et al., 2006; Gholamiandekhordi et al., 2006) who also detected the presence of the α-toxin in both flocks suffering with Necrotic Enteritis (NE) and a healthy flock without NE. More recent data indicate that the NetB toxin, rather than the α toxin, is the main virulent factor of Necrotic Enteritis (Keyburn et al., 2008; Keyburn et al., 2010). However, the presence of either the α-toxin or the NetB toxin (and certainly of the *Cl. perfringens* strains that produce the toxins) is not considered to be the sole cause of Necrotic Enteritis (Timbermont et al., 2011). Other predisposing factors (such as the presence of *Eimeria* infection and the feeding of wheat rather than maize) (Akhtar et al., 2012; Kim et al., 2017) need to be present to

encourage the over-proliferation of the *Cl. perfringens* type A and the production of the α and NetB toxins.

In this experiment, focus has been on *Cl. perfringens* and *C. jejuni*. However, studies have suggested that it is the composition of the whole microbiome and perhaps dysbacteriosis (over growth of certain microorganisms) that is the major cause for disease and consequent poor welfare (Teirlynck et al., 2011; De Gussem, 2007) rather than the presence or absence of particular bacterial species. The source and sink for these bacteria for the broiler is the litter, but there has been little if any investigation of the composition of the litter microbiome. The aim of the next study in this project was therefore to determine the effect of litter quality (fresh, or deliberately contaminated with poultry excreta) on the composition of the litter microbiome, and whether this was altered by the cereal source that the birds were fed.

4.8 Conclusion

This is the first report that has attempted to associate Envirobed and wood shavings consumption by poultry with measures of gut health. The caecal presence of either *Campylobacter jejuni* or *Clostridium perfringens* was not affected by the source of bedding used. Rearing birds on wood shavings did appear to be beneficial to broiler performance (although this may be an artefact of measuring liveweight), but birds reared on wood shavings consumed less litter and had wetter digesta than those reared on Envirobed.

Chapter 5 investigating the effect of diet and bedding on the performance and gut microflora of broiler chickens

5.1 Introduction

The accumulation of massive quantities of wastes such as excreta and litter is one of the main problems facing the poultry industry, with the pollution risk associated with this waste. Poultry litter is a mixture of excreta, bedding material, feather and waste feed removed from poultry houses (Kelleher et al., 2002a; Terzich et al., 2000). It contains many unwanted pathogens which may develop inside litter. Therefore, it is necessary to know the composition of bacteria in litter to minimise poultry disease, protect consumer and bird health, and to reduce the effect of litter on the environment (Lu et al., 2003b; Terzich et al., 2000; Roll et al., 2011).

The reuse of poultry litter is a common practice, particularly in modern poultry production, in the United States and Brazil because of the availability and pricing of fresh bedding materials, and the requirement to decrease the environmental impact of poultry production (Roll et al., 2011). However, reused litter may increase the risk of FPD when rearing birds on it, because of its increased moisture content (low litter quality). Yamak et al. (2016) observed that the incidence of foot pad dermatitis and its associated lesions were significantly lower when birds were reared on new litter rather than reused litter. Similarly Paz et al. (2013) observed that the incidence of foot pad dermatitis was lower with new litter than reused litter. However, Xavier et al. (2010) reported that foot pad dermatitis for the first litter use, 4^{th} reuse and 5^{th} reuse were 68, 20 and 35% respectively, indicating that the incidence of FPD decreased with reused litter. In addition the lowest incidence of FPD was observed with reused litter by (Jacob et al., 2016b). Ruiz et al. (2008) reported there were no differences between new and reused litter on FPD. These contrasting data would suggest that it is not the reuse of

litter per se that increases the risk of FPD, but rather the quality (specifically the dry matter content) of the litter, be it fresh or reused. It is well known that moisture increases the risk of FPD, and if litter has been used then it may be moister than fresh litter. However, if the litter is dried before it is used then it should not pose a risk to birds, and whether litter is fresh or reused, it is essential to control the environment in the poultry house to maintain litter quality and bird performance. In addition to the birds' diet, the litter is an important source of material that is consumed by the bird, and it has been noted in previous experiments in this thesis that birds do consume significant (if unknown) amounts of litter. Cloacal drinking by the young bird will also mean that bacteria in the litter will enter the bird's hindgut (Wang et al., 2016; Kers et al., 2018). The litter microbiome is an important source of commensal and pathogenic bacteria for the bird that has not been studied in detail before, but likely plays an important role in the establishment of the gut microbiome in the bird. In this regard, it is noteworthy that the caecal microbiome of birds was advanced, with the microbiome of younger birds appearing to be more similar to those of older birds, when the birds were reared on reused litter (Borda-Molina et al., 2018). This evidence would suggest a beneficial effect of reusing litter, and would suggest that this practice would help reduce the risk of FPD, by improving the gut health of the bird.

Cereals form the basis of the commercial poultry diet and are the main source of energy and protein (Steenfeldt, 2001; Cowieson, 2005; Hellin and Erenstein, 2009). The lower fibre and more readily digestible starch content of maize means that bird performance is usually better when birds are fed maize rather than wheat (Widyaratne and Zijlstra, 2007). The higher digestibility and lower digesta viscosity associated with feeding maize (Qiu et al., 2016) would also improve litter quality (and therefore reduce the risk of FPD) compared with birds fed wheat. However, the interaction between cereal source

and litter quality (fresh or reused litter) on the establishment of a healthy gut is unclear. The objective of this experiment was therefore to investigate the effects of cereal source and litter quality (fresh, or bedding to which poultry excreta were added), and the interaction between these two variables, on bird performance, foot health, litter quality, presence of ampicillin resistant *E.coli* in the digesta and the litter, and the composition of the litter microbiome.

5.2 Materials and methods

5.2.1 The Study area

This experiment was conducted in the same place as before at CEDAR, Hall Place Farm Arborfield.

5.2.2 Experimental design

A total of 168 commercial one day old male broiler chicks (Ross 308) were obtained from PD Hook Hatchery (Cote, Bampton). Birds were reared on either clean litter (wood shavings), or litter to which poultry excreta had been added. Birds were also fed either a wheat based or maize based diet, in a 2x2 factorial design. From days 1-14, There was one pen for each treatment (36 chicks per pen) to improve brooding conditions. Birds were fed a starter diet (based on either wheat or maize), with pens being bedded with clean litter, or fresh litter to which 500 g poultry excreta was added. Excreta was collected from the house of a free range laying hen flock, because we had access to excreta from laying hens but not broilers, and as adult birds the excreta microbiome might more closely resemble an adult bird's microbiome (and arguably what birds reared by mother hens might be exposed to). On day 15, birds were weighed and allocated to one of six replicate pens per treatment with six birds/pen (Table 5.1).

They were offered a grower/finisher diet from 15-35 days. The study was conducted between September and October 2016. The formulation of the diets is presented in

Table 5.2. A running sample of the diets was taken throughout the experiment and submitted for analysis of chemical composition to Sciantec Analytical Services (Cawood, North Yorkshire). The chemical composition and calculated ME content of each diet is presented in Table 5.3.

Table 5.1: Experimental design

Treatment	Birds/pen (starter)	Birds/pen (grower/ finisher)	Pens/ treatment (grower/ finisher)	Birds/ treatment (grower/ finisher)
Clean x maize[1]	42	6	6	36
Clean x wheat[2]	42	6	6	36
Dirty x maize[3]	42	6	6	36
Dirty x wheat[4]	42	6	6	36
Total	168		24	144

1: a maize based diet with birds bedded on new wood shavings. 2: a wheat based diet with birds bedded on new wood shavings. 3: a maize based diet with birds bedded on new wood shaving, each pen mixed with 500 g of excreta. 4: a wheat based diet with birds bedded on new wood shaving, each pen mixed with 500 g of excreta

Table 5.2: Formulation of the experimental diets (g/kg as fed)

Ingredient	**Starter (0-14 days)**		**Grower/finisher (15-36 days)**	
	Maize	**Wheat**	**Maize**	**Wheat**
Maize	515	0	560	0
Barley	40	40	40	40
Wheat (12.5% CP)	0	500	0	550
Soybean meal (48% CP)	320	320	270	265
Rapeseed meal	42	42	42	42
Soybean oil	35	50	50	65
L-lysine HCl	4	4	1	1
DL-methionine	3.45	3.45	2	2.42
L-threonine	2.05	2.05	2	2.02
Sodium bicarbonate	2.5	2.5	2.5	2.5
Salt	2	2	2.5	2.5
Limestone	12	12	8	8.56
Poultry vitamins/minerals	2	2	2	2
Dicalcium phosphate (QPRDC)	20	20	18	17

Table 5.3: Chemical composition (g/kg as fed) of the experimental diets

	Starter (0-14 days)		Grower/finisher (15-36 days)	
	Maize	**Wheat**	**Maize**	**Wheat**
ME MJ/kg	12.7	12.8	13.4	13.4
Crude protein	230	244	194	216
Total oil	62.3	64.5	84.5	92.8
Sugar	55.6	46.6	34.8	46.2
Starch	377	369	422	378
Alanine	8.6	8.9	8.7	8.6
Arginine	13.9	14.2	11.7	13.7
Aspartic	20.6	21.5	17.9	19.9
Cystine	3.6	3.5	2.8	3.7
Glutamic	47.1	47.7	32	45.8
Glysine	8.8	9.2	7.8	9
Histidine	5.4	5.5	4.6	5.3
Iso – leucine	9.3	9.6	7.8	9
Leucine	15.9	16.3	15	15.6
Lysine	13.8	15.5	10	11.4
Methionine	7	6.4	4.7	5.6
Phenylalanine	10.7	11.1	8.9	10.6
Proline	13.7	14.1	10.1	12.7
Serine	10.4	10.6	8.5	10.3
Threonine	9.2	9.6	8.9	10.1
Tyrosine	4.6	5.5	4.2	5
Valine	9.9	10.1	8.4	9.6
Tryptophan	1.9	1.9	1.7	2.1

Analysis feed by: Sciantec Analytical Services. Stockbridge Technology Centre, Cawood, North Yorkshire Y08 3SD Tel: 01757242400 Fax 01757242401. Sciantec Analytical Servies is a division of the Cawood Scientific group. Registered Number: 5655711. Registered Office: Coopers Bridge, Braziers Lane, Bracknell, Berkshire RG42 6NS. www.sciantec.uk.com

Table 5.4: Composition of the vitamin/mineral mix added to the broiler

Micronutrient	Unit	iu or mg/g supplement
Vitamin A	iu	0.2
Vitamin D3	iu	0.2
Vitamin E	iu	2
Riboflavin	mg	0.1
Vitamin K	mg	0.1
Nicotinic acid	mg	0.7
Pantothenic acid	mg	0.3
Folic acid	mg	0.03
Thiamine	mg	0.04
Pyridoxine	mg	0.1
Biotin	mg	0.1
Vitamin B12	mg	0.2
Choline chloride	mg	64.7
Iron	mg	1.1
Chloride	g	
Cobalt	g	
Mn	mg	3.9
Cu	mg	1.3
Zn	mg	2.8
Iodine	mg	0.04
Se	mg	0.6
Choline	mg	

The composition of the poultry minerals/vitamins referred in Table 5.2. Diets were formulated to be equal in lysine and methionine content. Diets were not balanced for protein because the aim of the experiment was to compare wheat and maize, and it was decided that if the supply of the first limit amino acids was balanced then other differences would be a consequence of the different cereal sources.

5.3 Birds and management

On arrival, birds were wing tagged, weighed and then randomly allocated to one of four large brooding pens (n=42/pen). Half the pens were bedded with clean shavings and half were bedded with clean shavings to which was added 500 g of excreta collected from the house of a free range laying hen flock. On day 14, birds were weighed again

and randomly allocated to one of 24 smaller pens according to their treatment group. Birds were kept on floor pens with a litter of wood shavings with a depth of approximately 10 cm (to which was added 100 g of excreta from the same batch as before if the birds had previously been in a pen to which excreta had been added). Litter material was replaced or fresh dry litter was added to the pens if the litter became very wet because of leakage from the drinkers. A solid plygene wall was placed around each pen to exclude draughts and reduce transfer of feed and litter material from neighbouring pens. Disposable overshoes were worn when entering the pens, and were changed when moving from one pen to another. Lighting was via incandescent lights with 23 h continuous light per 24 h period for the first seven days, followed by 18 h continuous light in each 24 h period. The birds were brooded according to the breeder's recommendations using infrared lights to provide supplementary heat. Feeders were maintained at a height equivalent to the birds' backs. The total floor area for each pen in the grower/finisher phase was 1.5 m^2; the feeder was round with a diameter of 26cm. Birds were vaccinated against infectious bronchitis prior to arrival.

Feed was provided on an *ad libitum* basis from suspended hoppers. Feed added and removed from pens was weighed and recorded on a weekly basis and diet changes were conducted at the same time for all pens. Water was provided via cup drinkers from a single source.

The daily observations were the same as in the previous experiment. The minimum-maximum temperature and humidity of the test facility were recorded three times daily (0800, 1600 and 2200 h) (Table 5.5). At the beginning of this experiment the temperature was low because of the door being left open when the birds arrived.

Table 5.5: Temperature and humidity of the room in which the birds were kept

Age days	Maximum Temp °C	Minimum Temp °C	Maximum Hum %	Minimum Hum %
2	29	27	32	25
4	31	29	35	31
6	31	30	36	33
8	30	29	39	36
10	31	30	43	40
12	31	30	48	45
14	31	30	55	52
16	28	27	51	47
18	25	23	37	32
20	25	23	35	29
22	25	24	40	37
24	24	24	38	37
26	24	23	35	32
28	24	23	35	31
30	23	20	32	27
32	21	20	36	31
34	21	20	36	34
36	22	21	39	36

During the study, any bird that was removed, found dead or was sacrificed was weighed and removed from the pen.

5.3.1 Bird performance

Daily feed consumption was calculated as described in Chapter three Section 3.4. Birds were weighed on arrival, and on days 14, 21, 28 and 35. Daily live weight gain was calculated for each week. Growth rate was calculated for each pen as described in

Chapter three Sections 3.5. Feed conversion ratio was calculated for each pen as described in Chapter 3 Sections 3.5.

5.3.2 Foot pad score

Scoring of FPD was conducted at weekly intervals for the last three weeks of the experiment as described in Chapter three Section 3.7.6.

5.3.3 Gait score

The gait score (GS) was used to determine the walking ability of the birds. Walking ability was divided into four categories by (Ferket et al., 2009), from completely normal (score 0) to immobile (score 3). Two birds per pen were removed from the pen each week and placed on the floor of the poultry hall and then given a score for their degree of walking ability (Table 5.6).

Table 5.6**:** Gait scoring system

Gait Score	Description of leg abnormalities
0	normal mobility
1	mobile with mild hobble
2	significant hobble with limited mobility
3	unable to move

Source: (Ferket et al., 2009)

5.4 Microbial data

5.4.1 Sample collection

One bird from each of twelve pens (three replicate pens per treatment) was randomly selected and euthanased on days 15, 22 and 36. Samples of digesta (approximately 1g) were taken immediately post mortem from the caecum, small intestine (the midpoint between the proximal duodenum and terminal ileum), and gizzard. These were placed

into sterile McCartney bottles and stored on ice for transport back to the lab for analysis. Samples of litter (a small pinch, taken using a gloved hand) were also taken from each pen at random points on the same day that birds were sampled. These samples were also placed in sterile McCartney bottles and analysed in the same manner as the digesta samples.

5.4.2 Analysis of samples

A sample (1 g) of digesta or litter was transferred to a sterile 15 ml tube, to which was added warm Luria-Bertani (LB) broth (10 ml) and the mixture was vortexed thoroughly. A sample of the suspension (100 µl) was plated in duplicate onto MacConkey agar and MacConkey agar with 20 µg/ml ampicillin. Litter samples were also spread on plates containing MacConkey agar and either 250 µg/ml ampicillin or 150 µg/ml cefotaxime on days 23 and 36. Colony numbers were counted and morphology was noted after 24 hours incubation at 37^0C.

5.4.3 Identification of isolated strains

Single colonies isolated from different points of the digestive tract or from the litter samples were selected and purified. This was done by selecting a single *E. coli* (pink) colony from the original plates and streaking using a four-way streak onto MacConkey's agar. After repeating this again, the same process was done on Eosin Methylene Blue (EMB) agar (taking one colony from the repeated MacConkey plate and streaking it on the EMB agar). Colonies with a green, metallic sheen were selected. Finally this same process was done on a final media of nutrient agar. After incubation, all the colonies were then collected and placed in a cryo-tube and frozen at –80^{o}C. From the frozen samples a small sample was taken and grown on nutrient agar. This was then used to streak colonies onto selective media to isolate *E. coli* strains in terms of their

biochemical properties. The media used were: MacConkey agar, Eosin methylene blue (EMB), M9 minimal media with either sucrose, sorbose or dulcitol.

Plates were then incubated for 48 hours at 37^{o}C and the presence or absence of growth was recorded. Colonies were identified as *E. coli* if they were pink when grown on MacConkey agar, had a green, metallic sheen with EMB, and cream coloured when grown on M9 media.

5.4.4 Bedding material sample for determination of microbial composition in litter

A sample of bedding material was taken from each pen at the end of the experiment. Samples were taken from at least half depth of litter in the left, right and middle of pens. Samples were placed in labelled, polythene bags and sealed and stored frozen (-20^{0}C). The determination of the microbial composition of the litter material in each pen was determined by molecular analysis. The litter samples (0.25 g) were defrosted and processed according to the Powersoil ®protocol as described in Chapter 3; Section 3.8.1.1. The plate was sealed and then submitted for analysis of the microbiome using the Axiom microarray (Oxford Genomics Centre, Wellcome Trust Centre for Human Genetics, Oxford University).

5.5 Bedding material samples for pH, moisture and ammonia

Litter samples were collected from each pen at week 3, 4 and 5. Twenty four samples were collected from the surface of the litter from different locations of the pens (right, middle and left). Samples were placed in labelled, polythene bags and sealed. Each sample was thoroughly mixed by hand.

One sample (ca 30 g) from each pen was blended with 200 ml of distilled water. Litter pH was then measured (pH Meter model HI 2210, Hannah Instrument, Eden Way, Pages Industrial Park, Leighton Buzzard, Bedfordshire). The determination of litter pH

was done on the day of collection. The dry matter content of the litter sample was determined by weighing a single (ca 50 g) sample of litter from each pen into an aluminium tray and then drying in an oven (100^{o}C) for 16 h before weighing the tray and sample again. Dry matter content was determined on the day of sample collection.

The ammonia content of one sample of litter from each pen was determined. Approximately 50 g of fresh litter from each pen was soaked and blended with 500ml of 0.05 M sulphuric acid (to produce ammonium sulphate from the ammonia in the litter). The ammonium sulphate solution was then filtered through filter paper and a subsample taken. The subsample of filtrate (5 ml) was then transferred to a Kjeldahl tube and diluted with ca 100 ml distilled water and steam distilled (Büchi B-324, Büchi, Switzerland) with the distillate trapped in a conical flask containing a receiver solution of boric acid and pH indicator. This solution was then titrated with hydrochloric acid (0.1 M) to determine the ammonia-N content of the sample.

5.6 Statistical analysis

Data relating to bird performance were subjected to analysis of variance (ANOVA) as repeated measures using a general linear model (GLM). Factors included in the model were cereal source (d.f. = 1) and litter type (d.f. =1), time (d.f. =3) and first order interaction between these terms. Means were separated using the Tukey simultaneous pairs test. Results are presented as least square means with the standard error of the mean and associated P-value. Differences were considered significant at $P<0.05$.

Categorical data pertaining to measures for foot health and gait score were analysed using a non–parametric test (Chi-Square). Frequency counts were reported against categories for each measure. Cereal source and litter type were used as the only factor. Results are presented as tables/graphically showing the Chi Square, degrees of freedom

and associated P-value. Differences were considered significant at $P<0.05$. Simple linear regression analysis was used to determine the relationship between variables.

When Gram negative bacteria were enumerated in the microbiological analysis, it was observed that in many instances the growth of bacteria was such that the colonies were too numerous to count (TNTC). Plates were therefore classified as showing abundant (>300 colonies), medium (100-300 colonies) or little (<100 colonies) growth. Chi-square analysis was then used to determine whether there were associations ($P<0.05$) based on the site of digesta sampling (gizzard, small intestine or caecum), the presence of ampicillin, the age of the bird, the cereal source and the bedding quality. The effect of location (site of digesta sampling), cereal source, bedding, and age were also compared against the number of times each different biochemical property was identified in samples that were isolated from plates containing ampicillin. Interactions between age and location were also investigated. The presence and absence of bacteria in litter, and their association with cereal source, litter quality and treatment were determined by chi square analysis. All analyses were conducted using the Minitab v. 17 software package (Minitab Inc., PA).

5.7 Results

5.7.1 Bird performance

The effects of treatment on feed intake, crude protein intake, growth rate and feed conversion ratio are presented in Table 5.7. ME intake was not analysed as the diets were isoenergetic and so differences in feed intake would reflect differences in ME intake. Birds fed wheat had a higher intake of crude protein ($P=0.038$). There was significant effect between treatments on growth rate and FCR, but no significant of bedding (clean or dirty) and interaction between diet and bedding on bird performance during the grower/finisher phase.

Table 5.7: Effect of diet and bedding on bird performance from 15-36 d old

Parameters	*Maize		*Wheat		SEM	P-Values		
	**Clean	** Dirty	Clean	Dirty		Diet	Bedding	Diet*bedding
Feed intake (g/d)	145.3	141.4	141.0	143.9	3.657	0.828	0.969	0.751
Crude protein intake (g/d)	26.4	25.6	28.4	29.4	1.28	0.038	0.998	0.538
Growth rate g/b/d	83.3	77.6	86.2	82.9	1.28	0.000	0.349	0.808
†FCR(g feed/g gain)	1.71	1.80	1.62	1.71	0.04	0.039	0.166	0.968

*Maize:A maize based diet
*Wheat: A wheat based diet
**Clean: Birds bedded on new wood shavings
**Dirty: Birds bedded on new wood shavings each pen mixed with 500 g of excreta
†FCR: Feed conversion ratio

The effect of treatment and bird age on the weight of the birds is illustrated in Figure 5.1. There was a significant difference between treatments p = <0.001 on chickens weight to be greater in clean bedding wheat rather than other 3 treatments. The weight of birds approximately less than ½ kg in 2 weeks of age, but the weight of birds increased more than 2 kg in 5 weeks of age. Obviously, as birds got older, their weight increased (P<0.001). The interaction between treatment and age of birds was significant (p=0.045) during the grower/finisher phase (Figure 5.1).

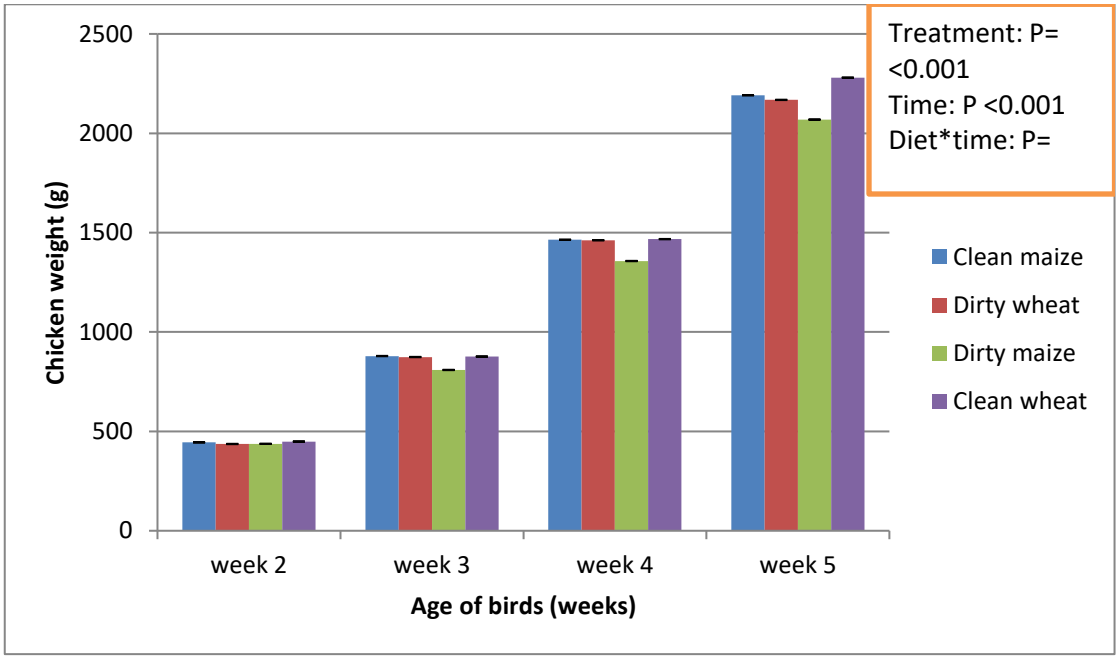


Figure 5.1: Effect of treatment and age of birds on chicken weight (g)

5.7.2 Effect of diet and bedding on litter quality

Litter pH was significantly lower (P=0.019) although litter ammonia content was significantly higher (P = 0.016), but there was no significant effect on litter dry matter content when birds were fed wheat rather than maize. There were no significant effects of bedding on these measures of litter quality (Table 5.8). There was also no significant interaction between diet and bedding on litter dry matter content, litter pH and litter ammonia content during the grower/finisherphase.

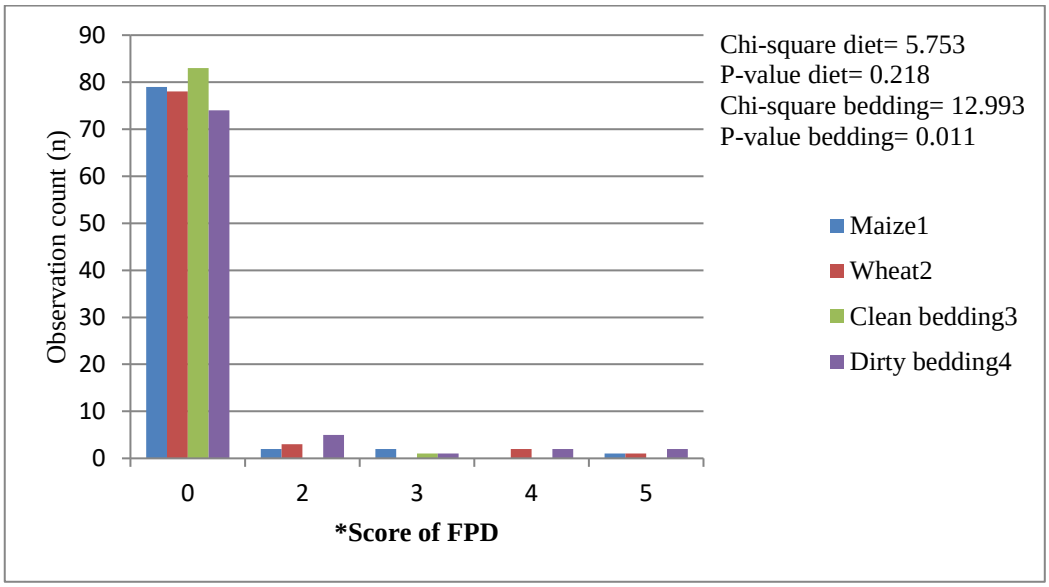
Table 5.8: Effect of diet and bedding on litter dry matter, litter PH and ammonia content in litter from 15-36 d old

Parameters	*Maize		*Wheat		SEM	P-Values		
	**Clean	**Dirty	Clean	Dirty		Diet	Bedding	Diet*bedding
Litter dry matter g/kg	739	751	754	760	11.2	0.582	0.474	0.796
Litter pH	6.44	6.60	6.25	6.29	0.065	0.001	0.357	0.551
Ammonia content in litter N g/kg	0.016	0.021	0.028	0.023	0.0014	0.001	0.904	0.108

*Maize: A maize based diet
*Wheat: A wheat based diet
**Clean: Birds were bedded on new wood shavings
**Dirty: Birds were bedded on new wood shavings each pen mixed with 500 g of excreta

5.7.3 Effect of diet, bedding and age of birds on foot pad score

The incidence of foot pad lesions was very low, with 94, 93, 99 and 88% of observations for maize, wheat, clean and dirty litter respectively being a score of 0 (Figure 5.2). Dietary cereal source did not affect the foot pad score (Chi square =5.753; P = 0.218), but there were more incidences of a foot pad score >0 with birds kept on bedding to which excreta had been added (Chi square =12.993; P = 0.011). The birds' age was associated with changes in foot pad score (Figure 5.3, Chi square = 37.740; P <0.000); although most birds at all times had a low lesion score, more birds had a score of 2 when they were five weeks old.



1 Maize: A maize based diet

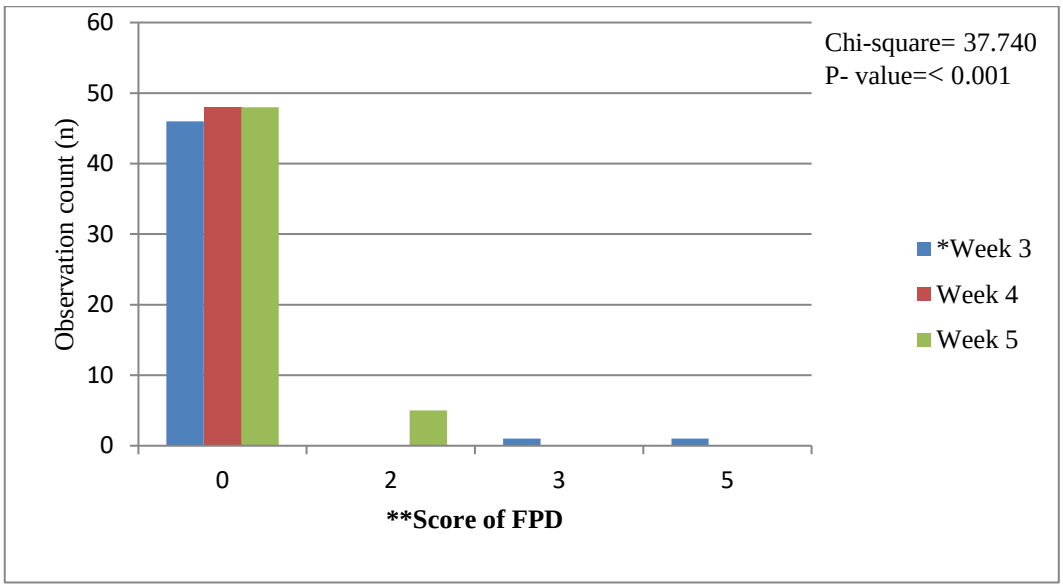
2 Wheat: A wheat based diet

3 Clean bedding: Birds were bedded on new wood shavings

4 Dirty bedding: Birds were bedded on new wood shavings each pen mixed with 500 g of excreta

*Score of FPD: 0= normal foot pad and digital pads, 1= redness skin of foot pad, 2= the foot pad feels harder and denser, 3= small black necrotic on the foot pad, 4= the area of necrosisis less than one- eighth of the foot pad, 5= the necrotic area extends to a quarter of the foot pad, 6= half of the foot pad covered by necrotic cells, 7= over half of the foot pad covered by necrotic cells.

Figure 5.2: Effect of diet and bedding on FPD score



*Age of birds 3, 4 and 5 weeks old

**Score of FPD: 0= normal foot pad and digital pads, 1= redness skin of foot pad, 2= the foot pad feels harder and denser, 3= small black necrotic on the foot pad, 4= the area of necrosisis less than one- eighth of the foot pad, 5= the necrotic area extends to a quarter of the foot pad, 6= half of the foot pad covered by necrotic cells, 7= over half of the foot pad covered by necrotic cells.

Figure 5.3: Effect age of birds (week 3, 4 and 5) on FPD score

5.7.4 Relationship between foot pad score and bird performance

There was a very weak negative relationship between foot pad score and feed intake and growth rate (Table 5.9), and foot pad score was not related to feed conversion ratio (R^2=0.069), litter dry matter content (R^2=0.04) or chicken weight (value of coefficient =0.00).

Table 5.9: Relationship between foot pad score and bird performance

	Regression		Constant			Coefficient		
	R^2	P	Value	SE	P	Value	SE	P
Feed intake	0.194	0.000	1.98	0.40	0.000	-0.01	0.003	0.000
Growth rate	0.182	0.000	2.32	0.50	0.000	-0.02	0.006	0.000

5.7.5 Effect of diet, bedding and age of birds on gait score

Regardless of diet or bedding (Chi square = 1.392; P = 0.238) or bird age (Chi square = 2.521; P = 0.472), gait score was good throughout the experiment with the vast majority of birds staying sound (score of 0) throughout the experiment.

5.7.6 Relationship between litter pH and ammonium-N in litter

Litter pH was positively related to ammonium-N (Table 5.10).

Table 5.10**:** Relationship between litter pH and ammonium-N in litter

	Regression		Constant			Coefficient		
	R^2	P	Value	SE	P	Value	SE	P
Litter pH	0.388	0.000	5.90	0.09	0.000	22.50	3.37	0.000

5.7.7 Relationship between litter dry matter content, litter pH and ammonia in litter

Weak negative relationships between litter dry matter content and litter pH and ammonia content were observed (Table 5. 11).

Table 5.11: Relationship between litter dry matter content, litter pH and ammonia in litter

	Regression		Constant			Coefficient		
	R^2	P	Value	SE	P	Value	SE	P
Litter pH	0.275	0.000	1133	74.3	0.000	-59.7	11.6	0.000
Ammonia in litter N g/kg	0.183	0.000	789.8	11.3	0.000	-17.6	444	0.000

5.7.8 Relationship between ammonia content and bird performance

Litter ammonia content was related to bird performance. Positive relationships (R^2>0.5) were observed between litter ammonia content and bird feed intake, growth rate, and chicken weight, although the value of the coefficients were very low. Only a

weak (positive) relationship between litter ammonia content and feed conversion ratio was observed (Table 5.12).

Table 5.12: Relationship between litter ammonia content and bird performance

	Regression		Constant			Coefficient		
	R^2	P	Value	SE	P	Value	SE	P
Feed intake	0.552	0.000	-0.00	-0.003	0.013	0.0002	0.00002	0.000
Growth rate	0.603	0.000	0.02	0.004	0.000	0.0005	0.00005	0.000
FCR	0.139	0.001	0.009	0.009	0.343	0.08	0.005	0.001
Chicken weight	0.629	0.000	0.005	-0.002	0.079	0.00002	0.000002	0.000

5.7.9 Relationship between litter pH and bird performance

Weak positive relationships were also observed between litter pH and bird feed intake, growth rate, feed conversion ratio and chicken weight (Table 5.13).

Table 5.13: Relationship between litter pH and bird performance

	Regression		Constant			Coefficient		
	R^2	P	Value	SE	P	Value	SE	P
Feed intake	0.469	0.000	5.36	0.14	0.000	0.0002	0.007	0.000
Growth rate	0.346	0.000	5.28	0.19	0.000	0.0135	0.002	0.000
FCR	0.332	0.000	4.66	0.29	0.000	1.010	0.171	0.000
Chicken weight	0.374	0.000	5.65	0.12	0.000	0.0005	0.00007	0.000

There was no relationship between foot pad score or gait score and any measure of litter quality.

5.7.10 Ampicillin resistance

Litter samples taken from pens before the birds were placed in them (on day 14) all showed the presence of Gram negative bacteria (125 CFU/g) with no effect of treatment (P=0.261). None of these samples showed any evidence of ampicillin (20 µg/ml) resistance. However, once birds were introduced to the pens, the growth of coliforms in the litter was such that all samples produced colonies that were too numerous to count (TNTC) when samples were cultured on MacConkey agar alone. All litter samples were also resistant to ampicillin (at both 20 and 250 µg/ml), producing plates that were TNTC on both days 23 and 36. However, no litter samples showed resistance to cefotaxime (150 µg/ml). When single colonies were taken randomly from each plate and characterized (confirmed as *E. coli* if they produced pink colonies on MacConkey agar, a metallic green sheen on EMB agar and a cream colony on M9 agar) it was observed many of them were not *E. coli*. There was no effect of bird age, with half the samples taken when birds were both 22 and 36 d old not being *E. coli*. There was a tendency (χ^2=2.72, P=0.099) for samples taken from pens in which birds were fed wheat to be ampicillin resistant (Amp^R) *E coli* (8/12 samples) whereas only 4/12 samples from birds fed maize were confirmed as Amp^R *E coli*. Similarly, 8/12 samples taken from pens that had the 'clean' bedding were Amp^R *E coli* compared with 4/12 samples taken from pens that had had the poultry excreta added (χ^2=2.72, P=0.099). 5/6 samples taken from 'clean wheat' pens were Amp^R *E coli* compared with 1/6 samples from 'dirty maize' (χ^2=5.82, P=0.121; 3/6 samples from 'dirty wheat' and 'clean maize' were Amp^R *E coli*).

There was a significant effect of intestinal site on the relative numbers of Gram negative coliform bacteria that were cultured, regardless of the birds' age (Table 5.14). In the absence of ampicillin, there was little growth of bacteria from samples taken from the gizzard, whereas half the samples taken from the small intestine and all the

samples taken from the caecum showed abundant growth of Gram negative bacteria. In the presence of ampicillin (20 µg/ml), there was again little or no growth of Gram negative bacteria from samples taken from the gizzard, but the abundant growth that was observed with half the samples taken from the small intestine in the absence of ampicillin was replaced with little or no growth when the birds were 14 or 22 d old. By the time they were 36 d old, half the samples taken still showed little or no growth, but the other half showed medium or abundant growth. Most of the samples taken from the caecum, however, continued to show abundant growth and this increased from 67% of the samples when the birds were 14 d old to 75% of samples when they were 22 and 36 d old.

The growth of ampicillin resistant (20 µg/ml) coliforms was not affected by cereal source (χ^2=2.14, P=0.344) or bedding quality (χ^2=4.11, P=0.128) but birds that were fed maize and reared on 'dirty' bedding produced samples with fewer ampicillin resistant coliforms than those birds reared with the other treatments (Figure 5.4). However, when a single culture was taken randomly from the plate and confirmed as being *E coli*, it was observed that the experimental treatment to which the birds had been assigned had a more significant effect. There was no significant difference (χ^2=0.056, P=0.812) in the number of observations of Amp^R *E coli* between birds (positive observations, n=20, 19; negative observations, n=52, 54) raised on either fresh or 'dirty' bedding respectively. However, significantly more (χ^2=18.1, P<0.001) birds fed wheat produced samples that were positive for Amp^R *E coli* compared with those fed maize (31 positive and 42 negative for wheat, compared with 8 positive and 64 negative for maize). Birds that were reared on 'dirty' bedding (those pens to which poultry excreta was added) had a higher than expected number of positive observations for Amp^R *E coli* if they were fed wheat, but lower than expected if they were fed maize (Figure 5.5, χ^2=25.7, P<0.001)

Table 5.14: The effect of bird age and intestinal site on the number of observations of different counts of Gram negative bacteria in the absence of ampicillin

Bird age (d)	Abundant growth			Medium growth			Little growth			X^2	P
	Gizzard	Small intestine	Caecum	Gizzard	Small intestine	Caecum	Gizzard	Small intestine	Caecum		
No ampicillin added											
14	4	12	24	2	7	0	18	5	0	46.59	<0.001
22	6	14	24	0	2	0	18	8	0	33.86	<0.001
36	9	14	24	1	2	0	14	8	0	22.90	<0.001
Ampicillin added (20 µg/ml)											
14	1	2	16	1	4	7	22	18	1	44.91	<0.001
22	2	2	20	0	2	2	22	20	2	45.55	<0.001
36	0	8	19	0	3	1	24	13	4	38.41	<0.001

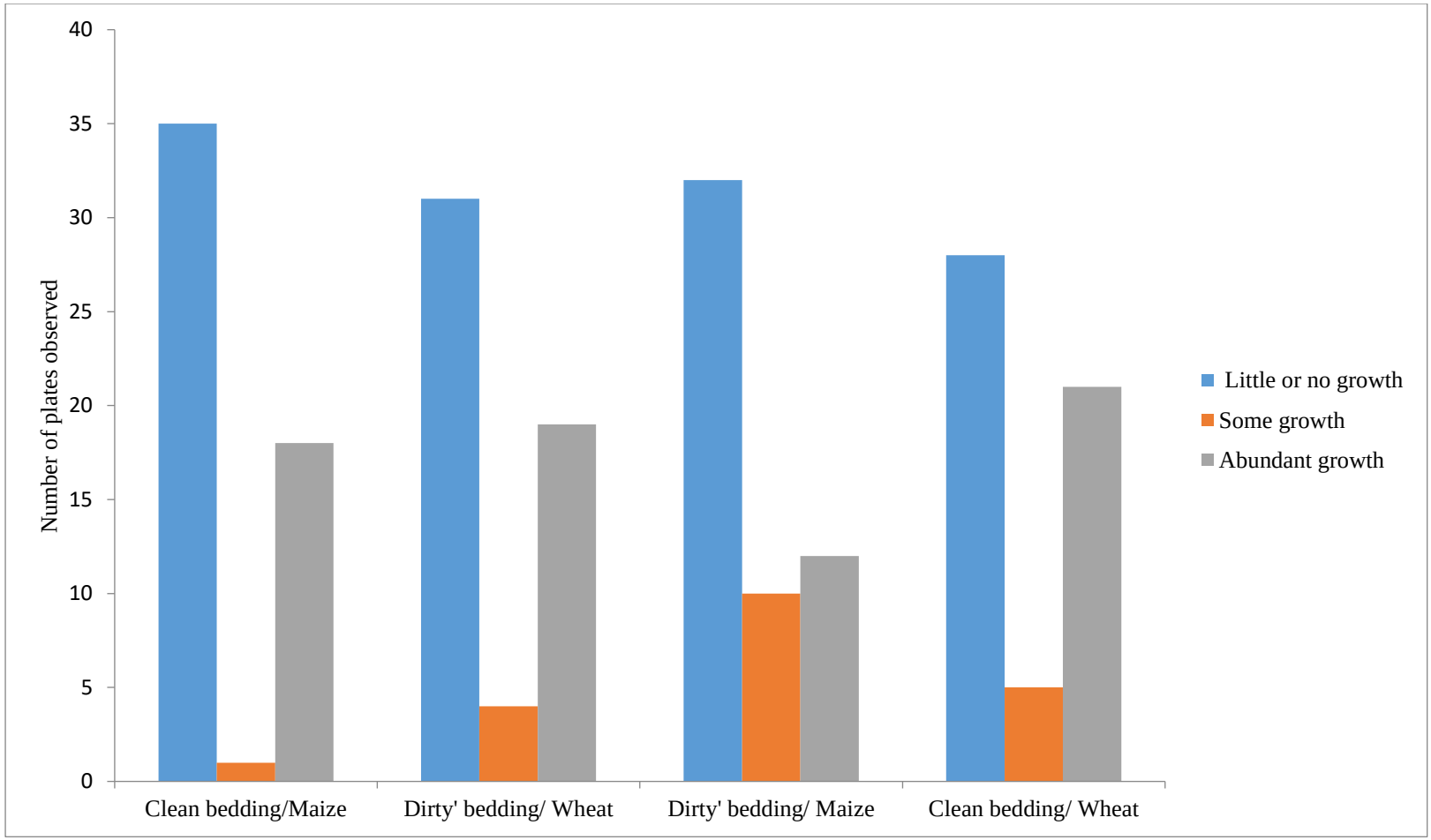


Figure 5.4: Effect of treatment on the relative growth of ampicillin (20 µg/ml) resistant *coliforms* isolated from samples taken from the gizzard, small intestine and caecum of growing broilers reared on either fresh bedding or bedding to which poultry excreta was added, and fed either a wheat or maize based diet

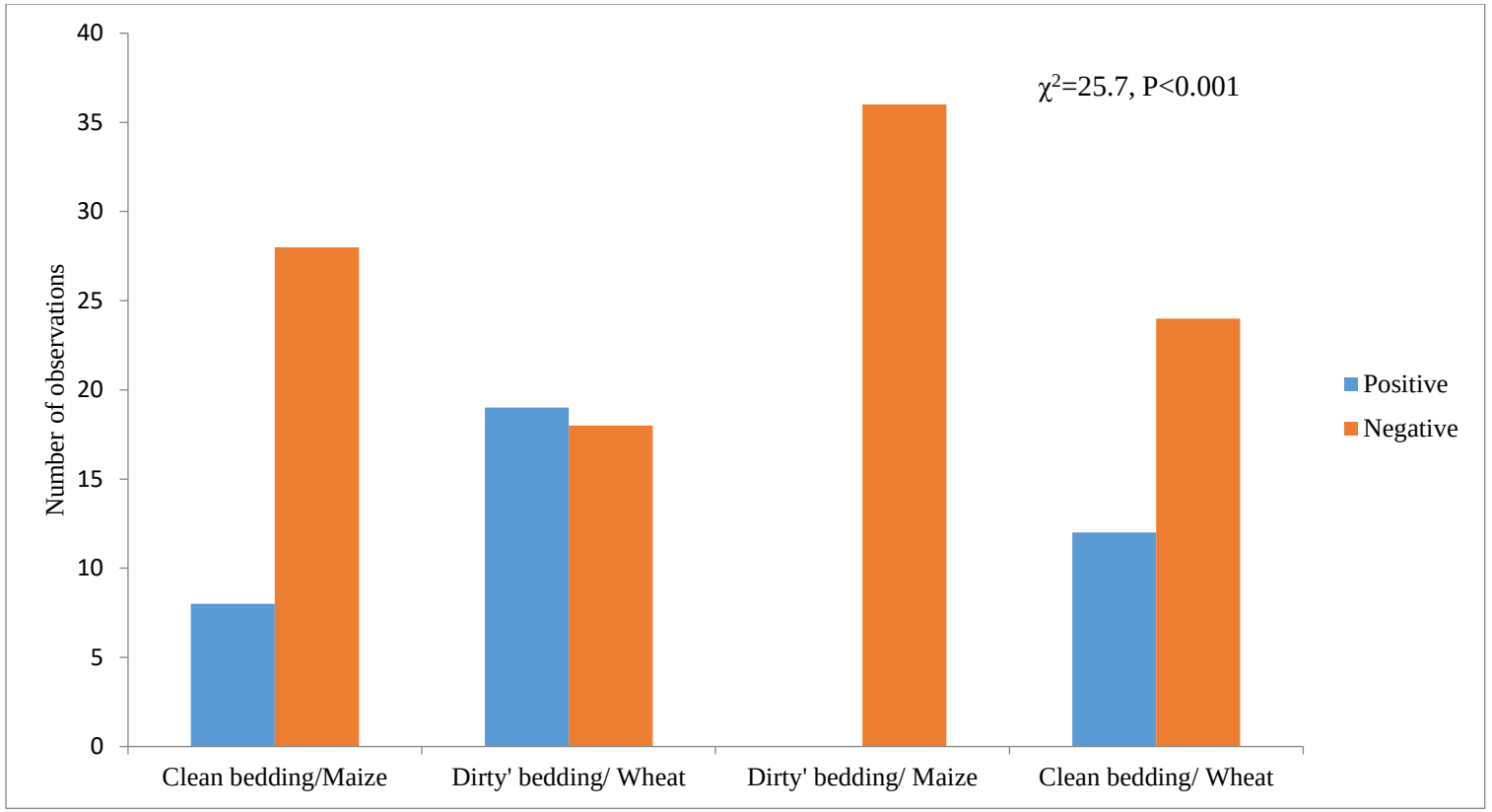


Figure 5.5: Effect of treatment on the number of negative and positive observations of ampicillin resistant (20µg/ml) *E coli* isolated from samples of digesta taken from broilers reared on either fresh bedding or bedding to which poultry excreta was added, and fed either a wheat or maize based diet

5.7.11 Strains of ampicillin resistant *E. coli*

Differences in which C sources could be utilized by isolated colonies of *E coli* were taken as evidence of differences in the strain of *E coli* cultured. On this basis, six different strains were isolated, but there was no evidence that the frequency of observation of these strains changed in *E coli* samples isolated from the litter as the birds got older (χ^2=3.96, P=0.50, Table 5.15). Most strains could utilize dulcitol, with or without the ability to also utilize sucrose, sorbose or both. 2/17 strains could utilize sucrose alone, and no strains could utilize sorbose to the exclusion of other C sources. 3/27 samples could not utilize any of the C sources. The profile of Amp^R *E coli* strains in the litter was not affected by the birds' diet (χ^2=4.96, P =0.75) or treatment (χ^2=18.5, P>0.1). However, bedding quality did affect it (χ^2=12.03, P<0.05) with samples isolated from litter in pens that had poultry excreta added to them having no strains able to utilize sucrose and dulcitol while those from pens with fresh bedding had 4/11 samples able to utilize these two C sources.

Table 5.15 : The effect of bird age, diet and bedding quality on the frequency of observations of C source utilization in samples of ampicillin resistant E. coli isolated from the litter on which the birds were kept

C sources	Bird age 23 d				Bird age 36 d			
Added to M9 medium	Clean bedding/ Maize	Dirty' bedding/ Maize	Clean bedding/ Wheat	Dirty' bedding/ Wheat	Clean bedding/ Maize	Dirty' bedding/ Maize	Clean bedding/ Wheat	Dirty' bedding/ Wheat
None						1		2
Dulcitol			1	1	1		1	
Dulcitol and sorbose		1					1	
Sucrose				1			1	
Sucrose and Dulcitol	2		1				1	
Sucrose, sorbose and dulcitol	1				1			

Unlike litter samples, samples of *E coli* isolated from digesta samples did change in their C utilization profile as birds aged (χ^2=16.6, P<0.01). *E. coli* isolated from younger birds (23 d) were more likely to utilize sorbose and dulcitol, or only sucrose, whereas samples from older birds were more likely to utilize dulcitol alone or sucrose and dulcitol (Table 5.16)

Table 5.16 : The effect of bird age ((χ^2=16.6, P<0.01) on the number of times an isolate of ampicillin resistant *E. coli* taken from the digesta of the bird was observed to utilise different combinations of carbon sources

C source utilized	Bird age (d)	
	22	36
None	3	5
Dulcitol	1	7
Dulcitol and sorbose	4	0
Sucrose	11	3
Sucrose and Dulcitol	0	3
Sucrose, sorbose and dulcitol	1	1

The site (gizzard, small intestine or caecum) did not affect the profile of C utilization (χ2= 9.39, P=0.50, Table 5.16), but the cereal source the birds were fed did (χ^2=11.6, P<0.05) with samples taken from birds fed maize being more likely to utilize only dulcitol (Table 5.17)

Table 5.17 : The effect of site of digesta collection ($\chi 2$= 9.39, P=0.50) on the number of times an isolate of ampicillin resistant *E. coli* taken from the digesta of the bird was observed to utilise different combinations of carbon sources

C source utilized	Site of digesta collection		
	Gizzard	Small intestine	Caecum
None	2	3	3
Dulcitol	2	4	2
Dulcitol and sorbose	2	0	2
Sucrose	2	6	6
Sucrose and Dulcitol	1	1	1
Sucrose, sorbose and dulcitol	0	0	2

Table 5.18: The effect of the birds' diet's cereal source ($\chi 2$= 11.6, P<0.05) on the number of times an isolate of ampicillin resistant *E. coli* taken from the digesta of the bird was observed to utilise different combinations of carbon sources

C source utilized	Cereal source used in the birds' diet	
	Maize	Wheat
None	1	7
Dulcitol	5	3
Dulcitol and sorbose	0	4
Sucrose	2	12
Sucrose and Dulcitol	0	3
Sucrose, sorbose and dulcitol	0	2

As with the litter samples, the bedding on which the birds were kept was also associated with a change in the profile of Amp^R *E. coli* (χ^2=14.6, P<0.05), but the association that was observed was the opposite of that observed with the litter samples. Birds kept on bedding to which poultry excreta had been added produced digesta samples that were able to utilize dulcitol and sorbose, and this was more frequently observed in these birds than those that had been reared on 'clean' bedding (Table 5.19)

Table 5.19 : The effect of the birds' bedding quality (χ2= 14.6, P<0.05) on the number of times an isolate of ampicillin resistant *E. coli* taken from the digesta of the bird was observed to utilise different combinations of carbon sources

C source utilized	Cereal source used in the birds' diet	
	'Clean'	'Dirty'
None	3	5
Dulcitol	5	3
Dulcitol and sorbose	0	4
Sucrose	9	5
Sucrose and Dulcitol	3	0
Sucrose, sorbitol and dulcitol	0	2

Unlike the litter samples, there was a significant effect of treatment on the profile of C source utilization (χ^2=25.7, P<0.01), with birds fed maize and reared on fresh bedding being more likely to produce samples of Amp^R *E. coli* that were only able to utilize dulcitol (Table 5.20). No samples of Amp^R *E coli* were isolated from birds fed maize and reared on the bedding which had had poultry excreta added.

Table 5.20: The effect of treatment ($\chi 2$= 25.7, P<0.01) on the number of times an isolate of ampicillin resistant *E. coli* taken from the digesta of the bird was observed to utilise different combinations of carbon sources

Bedding quality	Treatment		
	'Clean' bedding, maize based diet	'Dirty' bedding, wheat based diet	'Clean' bedding, wheat based diet
None	1	5	2
Dulcitol	5	3	0
Dulcitol and sorbose	0	4	0
Sucrose	2	5	7
Sucrose and Dulcitol	0	0	3
Sucrose, sorbose and dulcitol	0	2	0

5.7.12 Effect of diet or bedding on the present or absence on different bacterial species in litter

There were no differences between feed sources (wheat or maize) or bedding (clean or dirty) on the presence or absence of different bacterial species in the litter (χ^2= 69.215; P = 0.965; χ^2= 83.219; P = 0.754) respectively. There were also no differences between treatments (clean maize, dirty maize, clean wheat, and dirty wheat; χ^2= 208.633; P = 0.907) Table 5.21.

Table 5.21: Effect of diet or bedding on the present or absence on different bacterial species.

Family	Clean maize	Dirty maize	Clean wheat	Dirty wheat
Aerococcaceae	3	3	3	3
Alcaligenaceae	3	2	5	2
Bacteroidaceae	1	1	2	1
Bacillaceae	0	3	2	0
Bifidobacteriaceae	0	0	0	2
Brevibacteriaceae	0	1	2	0
Brucellaceae	0	0	1	0
Burkholderiaceae	0	1	0	0
Clostridiaceae	6	7	3	7
Comamonadaceae	1	3	0	1
Corynebacteriaceae	8	4	10	2
Dermabacteraceae	1	2	3	1
Dietziaceae	0	0	1	0
Enterobacteriaceae	75	99	76	78
Enterococcaceae	14	11	8	9
Erysipelotrichaceae	0	0	0	1
Erwiniaceae	1	0	0	0
Flavobacteriaceae	2	1	0	2
Hafniaceae	0	0	1	0
Lachnospiraceae	4	8	3	7
Lactobacillaceae	19	18	21	17
Leuconostocaceae	0	1	0	1
Moraxellaceae	14	9	13	12
Morganellaceae	2	1	1	1
Nocardiopsaceae	0	0	1	0
Odoribacteraceae	2	0	1	3
Oscillospiraceae	2	3	1	3
Propionibacteriaceae	0	2	0	0
Pseudomonadaceae	6	5	5	7
Planococcaceae	0	1	0	0
Ruminococcaceae	0	1	0	2
Staphylococcaceae	15	23	22	10
Streptococcaceae	8	8	5	6
Sphingobacteriaceae	3	1	2	1
Unclassified Bacteria	4	5	3	4
Xanthomonadaceae	4	3	2	2
Yersiniaceae	4	2	2	3
All	202	229	199	188

*Maize: A maize based diet

*Wheat: A wheat based diet

**Clean: Birds were bedded on new wood shavings

**Dirty: Birds were bedded on new wood shavings, each pen mixed with 500 g of excreta

5.8 Discussion

This experiment investigated the effects of cereal source and litter quality on bird performance, foot health, litter quality, ampicillin resistant *E.coli* and the composition of the litter microbiome. All diets were formulated to meet the birds' nutrient requirements, so on that basis it is not surprising that cereal source had no effect on feed intake. These results agree with (Crouch et al., 1997) who also observed similar levels of feed intake in broilers fed either wheat based or maize based diets. However, a study by (Mathlouthi et al., 2002) reported that the water soluble non- starch polysaccharides (NSP) in wheat have anti-nutritive properties and caused growth depression in broilers. In Kiarie et al. (2014) study, the concentration of soluble NSP in wheat diets were observed to be only half those of maize (18.3 and 39.4 g/kg) respectively, but the concentrations of soluble xylose and arabinose in wheat were 9.1 and 5.8 g/kg respectively compared with concentrations in maize of 1.7 and 0.8 g/kg respectively. Although the wheat diets had slightly lower concentrations of insoluble NSP (97.5 compared with 107 g/kg) compared with maize diets, wheat diets had a greater total concentration of NSP compared with maize (137 and 125 g/kg respectively. NSP were not determined in our experiment, but (using literature estimates of NSP contents) it is estimated that there was little difference in the NSP contents of the diets, with the wheat diets having slightly lower concentrations of insoluble NSP (97.5 compared with 107 g/kg) but slightly greater total concentration of NSP compared with maize (137 and 125 g/kg respectively). The concentration of protein in the diet may also impact growth as the fermentation of protein in the caecum can produce toxic substances. This occurs with protein fermentation more than with the fermentation of carbohydrates (Apajalahti and Vienola, 2016). The impact of these antinutritive factors in wheat agrees with the findings of (Kiarie et al., 2014; Munyaka et al., 2015) who observed that birds fed wheat diets significantly grew faster and had better body weight gain than those fed

maize diets. The results of bird performance in this experiment were not expected to be significantly different between wheat and maize because of the small difference in water soluble and viscous NSP content between the two diets. The differences in bird performance (growth rate and FCR) were because of differences in protein intake.

Feeding maize was associated with a significant reduction in the litter ammonium-N content, which would improve the air quality within the housing facility (Ferguson et al., 1998b) and because of the lower protein content of the maize diet (and lower protein intake associated with maize diets) since litter ammonia concentration increases with increasing protein content (Ferguson et al., 1998a; Qaisrani et al., 2015). The concentration of ammonia in the air of a poultry facility increases with increasing litter pH (Carr et al., 1990) since when litter pH is below 7 the release of ammonia from litter is negligible; release starts when the pH is near 7.0 and reaches high levels at 8.0 and above (Reece et al., 1979). In this experiment, the litter pH was lower than 7.0 which would reduce the release of ammonia from the litter but would not protect the birds' feet from exposure to ammonia. Reece et al., (1979) investigated the effect of exposing chickens to 0, 50, 100 and 200 ppm of ammonia in the atmosphere during 0-28 days age. At market age, birds exposed to ammonia weighed significantly less and ammonia exposure adversely affected FCR, growth rate and mortality. Generally exposure to increased ammonia concentrations have an adverse effect on chicken performance (Beker et al., 2004). The results in this experiment were in contrast to these findings, as bird performance was slightly improved when litter ammonium-N concentrations were higher. This may be because the ammonium-N was trapped in the litter as ammonium, rather than being released into the atmosphere as ammonia.

Regardless of treatment, most birds had FPD score of 0 which indicates a healthy foot and there were no birds with a score of 6 and 7. Ruiz et al. (2008) reported no

significant differences in FPD scores between new and used litter. However, while some differences in foot pad score were observed, this was not reflected in the birds' gait score, as most birds had a gait score of 0 which is the score for normal mobility and there was only one bird with a score of 2 in week 4 of the study.

The antimicrobial resistance of the *E coli* from the litter changed dramatically after the arrival of the birds. There was an absence of antimicrobial resistant *E. coli* that could be cultured from the bedding before the birds were placed on it, but all subsequent samples produced highly resistant strains of *E. coli*. This change could have resulted from colonization of the chicks by resident environmental *E.coli* or from some kind of a selection pressure which induced the overgrowth of *E.coli* strains which originated from a minority of chicks before they arrived in the poultry hall. In this experiment, resistant isolates of *E. coli* were observed in both the litter and the gut of chickens.

In the absence of ampicillin, there was more growth of bacteria, but few Gram negative bacteria were observed in the gizzard, and bacterial abundance increased lower down the digestive tract being obviously most abundant in the caecum. Small bacterial populations in the gizzard would be expected because of its low pH. It was encouraging that bacterial growth was much reduced in the presence of ampicillin but the prevalence of ampicillin resistant *E coli*, especially in the caecum and persisting in the litter, was concerning. This was especially so since this resistance was observed from the first time point (when the birds were 14 d old), and the selective advantage of ampicillin resistance was unclear since the birds were never exposed to any antibiotic. This might suggest that the plasmid (or other mobile genetic element) coding for ampicillin resistance also codes for other traits (such as sucrose and/or dulcitol utilisation) that confers a selective advantage on the host bacterium. This is an area

worthy of future research, which would require the characterisation of the resistant genome.

In this experiment, the number of isolated colonies of ampicillin resistant *E. coli* was much greater when birds were fed wheat rather than maize. This may be a consequence of the (assumed, based on literature values of cereal NSP contents) high NSP content of wheat altering the composition of the microbial community in the small intestine. The inefficiencies of digestion and absorption associated with NSP reduce feed efficiency and the unabsorbed nutrients flow to the caecum stimulating the growth of caecal microbiota (Branton et al., 1987; Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992). However, while that may be the case, there was no evidence of any difference in the composition of the litter microbiome when birds were fed wheat rather than maize. The wheat diet being beneficial to bacterial growth is not a new concept; the same findings were made when a study was undertaken into the effect of diet on *Campylobacter* levels in broilers (Heres et al., 2004; Hilmarsson et al., 2006; Hermans et al., 2010). Their theory was that the wheat formed a more viscous digesta in the gut, meaning slower travel through the gut, allowing bacterial fermentation to take place. In one experiment by Branton et al. (1987), it was reported that feeding broilers wheat (rather than maize) led to a significant increase in mortality from necrotic enteritis associated with *Clostridium perfringens.*

The treatment of litter varies from country to country, but it seems that the microbiota in the broiler gut and the microbiota in the litter affect each other. Fresh litter is associated with more environmental bacteria, whereas re-used litter had an increased number of bacteria that came from intestinal origin (Cressman et al., 2010). In this experiment, ‘dirty’ bedding was associated with an increase in the number of different strains of Amp^R *E. coli* in the litter, although there was no significant difference in the

total microbiome profile. However, the fact that there were more types of *E. coli* found on the 'dirty' bedding in this experiment may support the finding of (Newell and Fearnley, 2003) that re-using litter can risk increasing exposure to pathogenic strains of bacteria.

There was evidence of a change with time in the strain of *E. coli* present. Sucrose utilising strains were clearly more prevalent on day 15, but, dulcitol utilising strains had taken over by day 22. Utilisation of sorbose and dulcitol that was present on day 15 was not present by day 22. This shows that recolonization of the gut occurs during growth of the bird and that the balance of bacteria, with regard to *E. coli,* alters as the chicken matures. This could be due to the carbon sources present in the diet favouring some strains, as the birds changed from the starter to the grower feed. These diets had different compositions, and so altered the substrates present. Both diets increased the concentrations of wheat or maize respectively, consequently altering the proportion of energy from these sources in the diet. When the composition of the diet altered some *E. coli* strains were able to adapt, this is supported by previous studies that showed some strains adapt better than others (Westermayer et al., 2016; Aidelberg et al., 2014). This supports the findings of (Awad et al., 2015) in relation to *C. jejuni* and (Rehman et al., 2007) that the microbiota in the chicken GIT varies with age. Cressman. *et al.,* (2010) also found that the complexity of the caecal bacterial composition increased with age, in line with similar studies by (Lu et al., 2003a) and (Wielen et al., 2002).

An important finding in this experiment was the effect of diet (and its interaction with environment) on the prevalence of ampicillin resistant *E. coli* in the digesta of chickens. Chickens fed a maize based diet had a low prevalence of Amp^R *E coli*, particularly if they were reared on bedding to which poultry excreta had been added. Other ampicillin resistant bacteria were present, but the random selection of colonies did not isolate any

E. coli. In this study, Amp^R *E coli* was isolated throughout the digestive tract, although, as would be expected, the largest populations were in the caecum since bacterial growth is low in the gizzard, because of its low pH, which inhibits bacterial activity (Rehman et al., 2007) However, the isolation of antibiotic resistant *E. coli* from the gizzard implies that the birds are eating it (presumably from the bedding and via horizontal transfer from infected birds) rather than from mechanisms such as cloacal drinking. Ampicillin resistance may also be evolving and establishing within each bird as a result of conjugation, but the question remains as to what selective advantage there is for *E coli* in acquiring Amp^R in circumstances where no ampicillin is administered. Changes in the ability to utilise different C sources may provide some of the answer, particularly the unusual ability to utilise sucrose. Interestingly, the sugar content of the wheat based grower/finisher diet was 33% higher than the maize grower/finisher diet, and the number of positively identified isolates of Amp^R *E coli* was much greater if birds were fed wheat rather than maize. The ability to metabolize sucrose as a carbon source is a highly variable feature among *E. coli* strains (Trevino et al., 2007). *Escherichia coli* W (ATCC 9637) grows especially quickly on sucrose and is the only safe laboratory or industrial strain that can utilize sucrose (Archer et al., 2011). Suriana et al., (2013) Reported that sucrose utilization can be used to improve *E. coli* ^{W}.

The caecum has the densest population of bacteria. The caecum therefore is likely to have been the source of the majority of excreted ampicillin resistant *E. coli*. This study has shown that *E. coli* is capable of colonising the entire GIT, however it was observed that prevalence of the number of colonies of Gram negative bacteria isolated was less in the upper GIT compared to the caecum. This has been seen previously in studies by (Barnes et al., 1972), when *E. coli* was seen to be most prevalent in the caeca. Similarly, levels of other enteritis producing bacteria are higher in the caeca (Lu *et al*., 2003; Rehman *et al*., 2007). This is most likely because their proliferation is encouraged by

microbial fermentation (Choct *et al*., 1996). There was no difference between the strains of *E. coli* at different locations, this means that there was no specific benefit of location to any strain of *E. coli*. This suggests that the diet the chickens were fed provided sufficient of the chosen carbon sources for the *E. coli* throughout, with higher levels further down the GIT being due to level of nutrition available or conditions in the tract.

The composition of the poultry litter microbiome has not been extensively studied with the exception of potential flock or human pathogens (Lu et al., 2003b). Some studies on microbial communities in chicken litter have focused on the detection of specific pathogens, such as *Campylobacter*, *Salmonella*, *Escherichia coli* and *Clostridium perfringens* (Martin et al., 1998; Bennett et al., 2005; Omeira et al., 2006). Microbial diversity has been shown to vary with poultry litter (Aktan and Sagdic, 2004), but little is known regarding the direct impact of litter material on the poultry intestinal microbiota and, as mentioned before, poultry consume as much as 4% of their diet as litter (Malone et al., 1983). It is clear that diet affects gut microbiota composition (Apajalahti et al., 2001; Torok et al., 2008) and it seems likely that it would also affect the composition of the litter microbiome. However, in this experiment, no significant effect of the birds' diet or bedding quality on the composition of the litter microbiome was observed. On the basis of the number of reads (although it is recognised that this would be confounded by the composition of the database on which identification is based), the most abundant family was *Enterobacteriaceae*. This would include genera and species such as *Escherichia coli, Enterobacteria, Salmonella, Shigella, Klebsiella pneumoniae, Escherichia fergusonii,* and *Enterobacter.* It is not at all surprising that this is the dominant family in poultry litter, particularly in a sample of litter taken at the end of the experiment. It would have been interesting to have taken a sample of litter (both 'clean' and 'dirty') at the beginning of the experiment as well, to determine the

impact of the excreta contribution to litter on the composition of its microbiome. The addition of poultry excreta (to make the 'dirty' bedding) was associated with a reduction in the prevalence of Amp^R *E coli*, but any changes the addition of this excreta had made to the litter microbiome to achieve this were not evident by the time the litter sample was taken for microbiome determination. If this experiment were to be repeated, it would be interesting to use an alternative, semi-quantitative technique (other than number of reads) of microbiome analysis, such as 16S RNA sequencing. This would enable a more complete picture of the profile of the microbiome to be constructed to determine whether particular families or genera were associated with the encouragement or suppression of antimicrobial resistance.

5.9 Conclusion

The feeding of wheat or maize to boilers with either fresh litter or litter to which poultry excreta had been added did not affect feed intake. There was no evidence of foot problems in this experiment. However, feeding broilers maize rather than wheat reduced litter ammonia content (which would be expected to improve air quality and foot health) as ammonia is an irritant to the skin and so decreasing litter ammonia content would improve foot health. Feeding broilers maize also reduced the prevalence of Amp^R *E. coli* (if not the total count of ampicillin resistant coliform bacteria). There was no evidence that feeding wheat rather than maize or using fresh litter compared with 're-used' litter affected the composition of the litter microbiome. This study has shown that, even in situations where no antimicrobials are administered to the birds, there is a high level of ampicillin resistance by *E. coli* isolated from both the broiler gut and the litter on which the broilers are reared. This persists throughout the birds' life, and appears to originate from the bird. Since ampicillin is a critically important antibiotic, this is of huge concern.

Chapter 6 General discussion

This thesis investigated different management and nutritional strategies that might improve gut health, thereby reducing the moisture content of poultry excreta. The rationale behind this was wetter excreta would result in wetter litter, and thereby increase the risk of the bird developing foot pad dermatitis. In the first two experiments, turkeys were used and the intervention that was investigated was the inclusion of whole grain wheat (WGW) in the diet. In the first experiment, turkeys were offered WGW in a free choice system, whereas in the second experiment, the WGW was mixed with the pelleted diet. In Experiment 1, the proportion of WGW in the actual diet was variable when the birds had free choice indicating considerable variation in the voluntary consumption of WGW. When comparing the data in the two experiments, it was observed that when the WGW was mixed birds ate less feed but grew more quickly $P < 0.001$ so that their FCR was lower (better) than those birds offered WGW in a free choice method. This may be because mixing WGW in the diet reduced the consumption of WGW as birds could not choose to consume more WGW. A more balanced diet was therefore consumed, leading to improved growth rate and FCR as mentioned in Chapter 3; Section 3.12. Alternatively, it is quite likely that the improved ventilation that was achieved (by keeping the turkeys in an open fronted shed with natural ventilation) was responsible for the improved performance in Experiment 2.

In the third experiment, two different bedding materials (Envirobed and wood shavings) were evaluated with broilers. Although bird weight was greater with birds reared on wood shavings there were no other differences observed in bird performance. The consumption of bedding material was greater in birds reared on Envirobed, but this did not affect the availability of nutrients in the diet. The increased consumption of bedding was also associated with the birds' digesta being drier, which would suggest that birds

reared on Envirobed would be at less risk of developing foot pad dermatitis. In the final experiment, two different cereal sources (wheat and maize) and the use of either fresh bedding (wood shavings) or wood shavings to which poultry excreta was added were compared. Differences in bird performance, but not on foot health were observed, there were some interesting data on litter quality and the prevalence of antimicrobial resistance. The litter ammonia content was lower when birds were fed maize, and this was also associated with a lower prevalence of ampicillin resistant *E. coli* in both the litter and the birds' digesta The viability of E.coli was decreased when the concentration of ammonia increased (Park and Gonazalez, 2003). Himathongkham et al. (2000) observed that ammonia could inactivate E.coli in litter and reported that the most important factors to kill pathogenic bacteria is the accumulation of free ammonia. Ammonia is capable of inhibiting and killing microorganisms in litter (Turnbull and Snoevenbos, 1973).

In all these experiments, the aim was to investigate factors that might give rise to wetter droppings in poultry and therefore increase the risk of them developing foot pad dermatitis (FPD). The actual incidence of FPD that was observed was very low or non-existent in all studies. However, it is known that the incidence of FPD is decreased when litter moisture content is reduced, and so this discussion will focus on what interventions affected the quality of the digesta and litter, (and thus gut health), and so might have altered the risk of FPD, even if no differences in FPD occurrence were observed. Litter dry matter was determined in the first and final experiment, it really should have been measured in each experiment, but as the litter was dry and the focus (at the time) was on the link between gut health and FPD, and as there was no evidence of any gut abnormalities in experiments 2 and 3, litter quality was not considered as closely as it should have been. An observation that was made in all experiments was that birds consume litter. The amount actually consumed was not quantified in these

experiments, but it has been reported by (Malone et al., 1983) that around 4% of intake may consist of litter. The objective of 2 experiments was to indirectly estimate bedding intake but a more systematic determination of litter intake would be worthwhile to determine the real intake of bedding material, and the amount of variation between birds in litter consumption. Some litter was observed in the samples of crop contents that were taken from turkeys in the experiments reported in Chapter 3, as well as in the samples taken from broilers in the experiment reported in Chapter 4. In the modern poultry industry, birds are provided with diets that supply all their nutrient requirements, so this raises the question as to why birds are consuming so much litter. Are they trying to satisfy an unmet nutritional requirement, and if so, what would that nutrient be? If it is not a nutrient requirement, is it instead evidence of an innate behavioural need to forage that drives them to peck at and consume the litter on which they are kept? Birds may have a requirement for dietary fibre that is not met by the proprietary pelleted feed, and an insufficient supply of fibre may result in abnormal behaviour such as consumption of litter and feather pecking (Hetland et al., 2005). Laying hens' response to the inclusion of dietary fibre is a decrease in feather pecking and improved behaviour (Hetland et al., 2005). This may be because of the satisfaction of a previously unmet nutritional requirement or because of the satisfaction derived from foraging activities (Mateos et al., 2012). Reflecting on the fibre supplied in the diets used in this thesis, it is noted that the calculated crude fibre concentration of the diets differed between experiments; in Chapter 4 (the comparison of Envirobed and wood shavings as bedding) crude fibre contents were 33.3 g/kg in the grower diet and 32.0 g/kg in the finisher diet, but in Chapter 5 (comparison of wheat and maize), crude fibre contents were 38.4 and 63.1 in the starter diet and 36.6 g/kg and 63.7 g/kg in the grower/finisher diets for maize and wheat respectively. The birds with high crude fibre content diets in Chapter 5 appeared to consume less litter than those fed less crude fibre

(birds fed maize in Chapter 5, and the birds in the experiment reported in Chapter 4) suggesting that the consumption of litter may be an attempt to meet a requirement for fibre, but robust assessments of bedding (and fibre) intake were not made.

Broiler requirements for crude fibre (CF) are usually considered to be low and it is usually recommended that crude fibre contents of broiler chick diets should be less than 30-40 g/kg depending on the age of birds (Swennen et al., 2010). However, as with any monogastric animal, poultry require a minimal amount of fibre for the actual functioning of digestive organs and the response to fibre inclusion depends on the source and concentration of dietary fibre and, indeed, on the health of the bird. It has been observed that bird growth was improved if the fibre content was 20-30 g/kg compared with a higher concentration (Mateos et al., 2012). Increasing the fibre content above 30 g/kg might have a detrimental effect on nutrient digestibility and performance (Mateos et al., 2012; Sadeghi et al., 2015; Jiménez-Moreno et al., 2013). Increasing the inclusion rate of fibre rich feeds such as sugar beet pulp or oat hulls from 25 to 75 g/kg diet resulted in lower daily weight gain in broilers from day 1 to 6 of age (Jiménez-Moreno et al., 2013). Increasing the crude fibre content of the diet from 30 to 90 g/kg reduced growth performance and impaired nutrient retention in turkeys (Sklan et al., 2003). Fibre is therefore generally considered an antinutritive factor for birds, and yet the birds appear to seek it out. It may be that it is the physical structure of the fibre that is more important than its chemical composition, and certainly changes in the physical structure of fibre have different effects on the broiler gut and bird performance (González-Alvarado et al., 2008; Rao et al., 2000; González-Alvarado et al., 2007). Certainly in ruminant nutrition, physical characteristics of dairy rations such as 'scratch factor' are essential to obtain proper ruminal fermentation and for animal production, and it is possible that the physical characteristics of poultry diets should be considered as well to ensure optimal caecal health. Envirobed and wood shavings are both derived

from wood, and so would have similar chemical characteristics (although some chemical components would have been removed during paper making) but with very different physical characteristics. Wood shavings have longer and wider particles, whereas Envirobed is more powdery. The 'structural fibre' content of wood shavings would therefore be expected to be higher than that of Envirobed, and so (if it is the physical structure of the fibre that is important) wood shavings may be expected to be more attractive. However, it was Envirobed that appeared to be consumed in larger amounts. The increased intake of Envirobed was associated with potentially improved gut health, in that digesta moisture content was reduced, and so the consumption of litter may well improve gut health but it is unclear whether it is the physical or chemical characteristics of the litter which appeals to the bird.

Allowing birds to eat to appetite when energy intake needs to be restricted (as in the case with broiler breeders) is one of the most important factors associated with the physical form of fibre (Mirzaei-Aghsaghali and Maheri-Sis, 2011). Recent studies have defined in detail the beneficial effects of fibre for poultry when fed at moderate amounts to improve digestive organ development (González-Alvarado et al., 2007). Fibre stimulates HCl, bile acids and enzyme secretion (Svihus, 2011; Hetland et al., 2003; Jiménez-Moreno et al., 2009) which in turn leads to improved gastrointestinal tract health (Correa-Matos et al., 2003; Perez et al., 2011; González-Alvarado et al., 2010), improved gizzard function (Mateos et al., 2012) nutrient digestibility (Amerah et al., 2009; Rogel et al., 1987; Jiménez-Moreno et al., 2013) and growth performance (González-Alvarado et al., 2007; Mateos et al., 2012; González-Alvarado et al., 2010; Jiménez-Moreno et al., 2013). The profile of the microbiota in the distal part of the gastrointestinal tract -is also likely to be affected, but will obviously depend on the amount and type of dietary fibre that is fed (Shakouri et al., 2006; Amerah et al., 2009).

The growth of pathogenic microorganisms and the incidence of digestive disturbances evidenced by wet litter can be reduced by the consumption of dietary fibre; the three key physicochemical properties of fibre sources that affect microflora diversity and colony counts in the gastrointestinal tract are solubility, viscosity and fermentation capability, and depend on diet composition and the nature of the fibre (Mateos et al., 2012). The beneficial influence of dietary fibre on nutrient digestibility might be associated with increased HCl and digestive enzyme secretion and by greater gizzard development leading to improved gastrointestinal tract motility (Duke, 1992; Svihus et al., 2004).

The viscosity of dietary fibre (DF) produced by polysaccharides such as arabinoxylans, pectin and glucans has an impact on the gastrointestinal tract as they are not hydrolysed by the gastrointestinal enzymes of poultry, but are fermented by microflora to produce short chain fatty acids (SCFA) in the caecum which may inhibit the growth of pathogenic bacteria (Józefiak et al., 2004). Diets containing wheat or barley have a high concentration of arabinoxylans and are associated with high digesta viscosity leading to increased transit times in the intestine, which may be responsible for the direct correlation between clostridial counts and intestinal viscosity (Annett et al., 2002), and perhaps for the direct correlation observed in Chapter 3 between whole wheat intake and *Clostridium perfringens* counts.

'Dietary' fibre coming from a bedding of wood shavings (or Envirobed) supply xylooligosaccharides (XOS) rather than arabinoxylans. XOS are considered prebiotics and are xylose based oligomers obtained from xylan rich hemicelluloses (Samalal et al., 2015). In monogastric animals XOS promote a positive influence on the composition and activity of gastrointestinal microbiota (Aachary and Prapulla, 2011) . For example, broilers fed straw derived XOS showed an increase of 9.44 % in body weight gain

compared with controls (Zhenping et al., 2013). An increased number of *Bifidobacteria* in the caeca of chickens were observed after they had been fed XOS for two weeks, but there was no effect on the number of *Enterobacteria* or *Lactobacilli* (Courtin et al., 2008; Samalal et al., 2015). These beneficial effects of XOS may explain the improved gut health (in terms of digesta moisture content) observed when birds consumed more Envirobed compared with wood shavings.

When birds consume litter, in addition to the consumption of fibre, they will also consume bacteria, but it is not clear what the dose and which particular orders of bacteria are consumed. In addition to consumption through the beak, the cloaca of turkeys and chickens undertake a typical sucking movement whenever some watery substance is dripped onto the cloacal lips. This action has been referred to as cloacal drinking (Van der Sluis et al., 2009) and is another means by which bacteria from the litter will be consumed by the bird. The intestinal tract of the broiler chicken consists of different sections inhabited by particular specialist microbiota adapted to the physiochemical conditions, available nutrients and host physiology of the specific habitat (Apajalahti and Vienola, 2016). Broilers have a complex intestinal microbiota like all homoeothermic animals, with their composition and metabolism varying at different sites within the intestinal tract associated with the very different physiochemical microenvironments, and perhaps by the route of entry of the bacteria (Pan and Yu, 2014).

Chicken litter is a reservoir of many families of bacteria, and in Chapter 5; Section 5.7.12 of this study it was observed that the dominant families were *Enterobacteriaceae, Lactobacillaceae* and *Staphylococcaceaeae*. It is necessary and beneficial to understand the bacterial composition of litter when trying to improve the environmental conditions of birds (Terzich et al., 2000). There is an immediate

multiplication of bacteria when chicken droppings are added to litter. In poultry litter the number of viable bacteria were found to be 10^{10}-10^{11}/g fresh weight and this was little affected by factors such as age of litter, moisture, temperature and pH (Schefferle, 1965). Undoubtedly, the quality of litter in the poultry house is rarely given enough attention. However, birds are in continuous contact with litter from day old until market age. Therefore, awareness of litter quality is important regarding bird welfare and performance as the quality of feed, water, chicks and chicken meat are major concerns of researchers and producers. Warm temperatures, high pH and high humidity provide a favourable environment for pathogen proliferation in litter. In contaminated litter bacterial disease can spread easily, and in reused litter, fungi may produce mycotoxins which cause increased mortality in flocks (Ritz et al., 2009).

Some of the bacteria consumed by birds from the litter may be resistant to particular antibiotics and the transfer of antimicrobial resistance between birds is considerable, as evidenced by the high prevalence of ampicillin resistant *E. coli* in Chapter 5. In the experiment reported in Chapter 5, it seemed most likely that the source of the ampicillin resistant *E. coli* was the birds, but the consumption of litter by penmates would ensure that even if the prevalence of antimicrobial resistance was initially very low, it would quickly spread. (Jiménez-Belenguer et al., 2016) concluded that the high percentage of resistant *E. coli* in day old chicks not exposed previously to any antibiotic was the result of vertical transmission from the parent flock. Vertical transmission of resistant *E. coli* strains from parent to broilers has also been reported by (Bortolaia et al., 2010). A high prevalence of ampicillin resistance was observed in the experiment reported in Chapter 5 in 15 d old birds even though they had not been exposed to any antibiotics. The source of this resistance is not known but might well have been present in the chicks when they were brought from the hatchery. Subsequent experiments have observed a high prevalence of antimicrobial resistance in both the caecum and yolk sac of day old

birds (Lee et al., 2017), and even a single bacterium with ampicillin resistance is able to transfer its genes to other bacteria, as observed by (Gillings and Stokes, 2012; Pleydell et al., 2007). A similar experiment reported by Jiménez-Belenguer et al. (2016), reported antibiotic resistant bacteria in birds even when environmental exposure was limited by maintaining hygienic conditions, and they suggested that AMR bacteria arrived in the birds, feed or litter. As the birds grew, ampicillin resistant *E. coli* increased in excreta samples (Pleydell et al., 2007). Controlling this spread of AMR bacteria is clearly important, and identifying management strategies to assist in this control is necessary. The potential for a maize based diet, and possibly the reuse of litter or an appropriate probiotic to reduce the prevalence of AMR bacteria (as was observed in Chapter 5 with ampicillin resistant *E coli*) is an area that merits future work.

One means of manipulating the gut microbiome to improve gut health and potentially reduce the prevalence of AMR is by altering the supply of nutrients to the gut. Bacterial requirements for nutrients depend on their species and strain. Those bacteria that can use substrates of all available sugars and minerals will proliferate easier than those bacteria that need more complex nutrients such as amino acids and vitamins. *E. coli* are able to proliferate in the small intestine as they require only sugars and minerals (Morishita et al., 1981; Zaldivar and Ingram, 1999; Zhou et al., 2006), but as was observed in Chapter 5, there is variation between *E coli* in terms of the sugars they can metabolise. Most *E coli* are unable to utilise sucrose, but most of the (ampicillin resistant) *E coli* that were isolated in this experiment could utilise sucrose, as well as other atypical C sources. If this is the selection advantage that the AMR *E coli* have, then identifying means of altering the chicken's diet so that the supply of these sugars is reduced may be one means of reducing the prevalence of the AMR bacteria. In addition, the presence of non starch polysaccharides as mentioned in this discussion

reduces pathogenic bacteria in the gastrointestinal gut of chickens because of the decreased passage rate of digesta through the digestive tract, enabling commensal bacteria to ferment more of the digesta (Pan and Yu, 2014). A conceptual framework for the manipulation of the intestinal microbiome by the management of the bird, diet and litter is illustrated in Figure 6.1

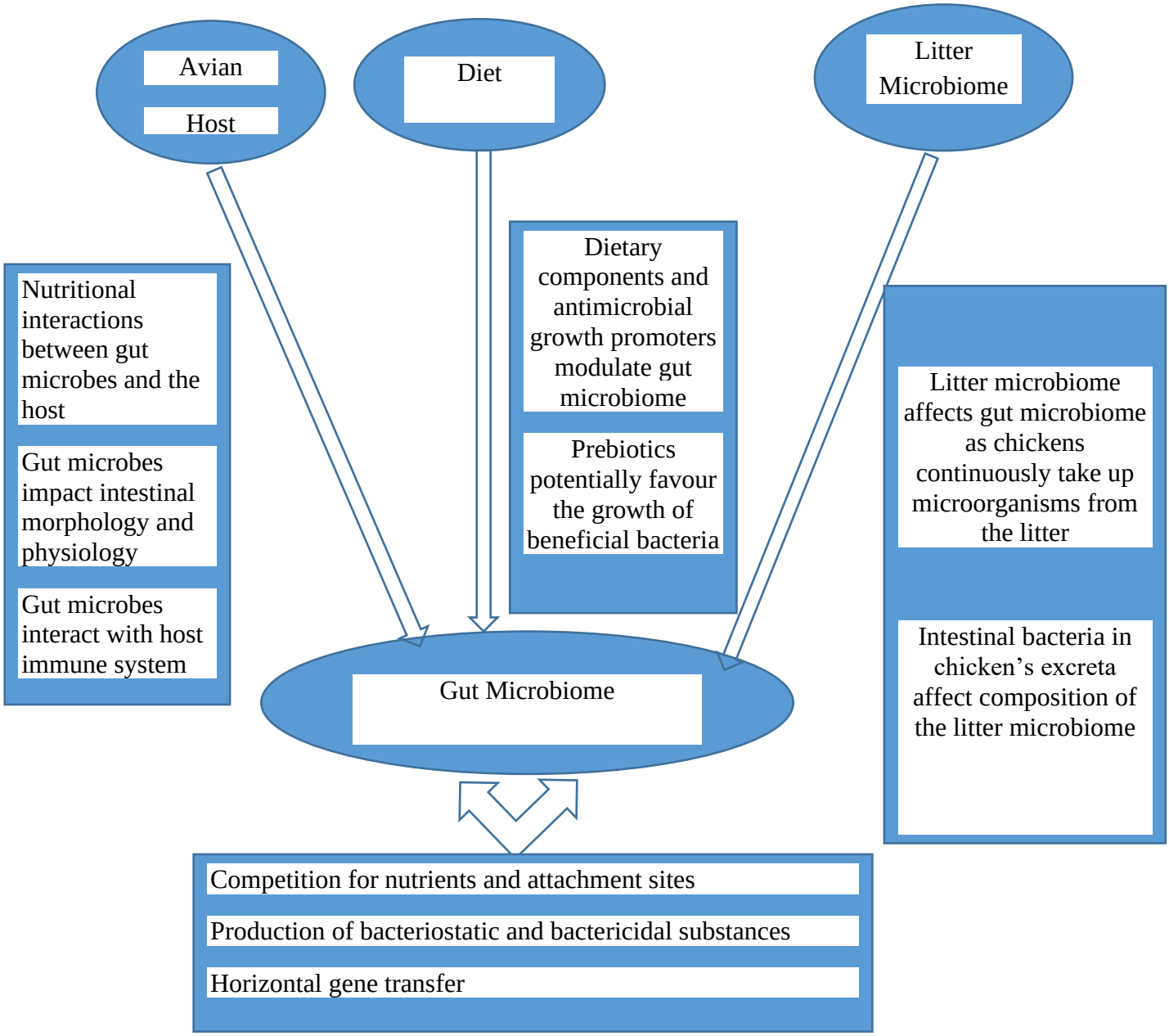


Figure 6.1: The interactions in the conceptual model between the gut microbiome, diet, avian host and litter microbiome by (Pan and Yu, 2014).

It could be hypothesised that birds kept in close contact with their litter are more likely to consume more litter. Therefore, it may be more beneficial to reduce the contact

between bird and litter. This could be achieved by cleaning out houses after each batch and always using fresh litter or clean bedding. Within batches, the provision of perches so that the birds are not lying in the litter may be another means of reducing contact. If there are benefits to the birds eating clean litter, then clean bedding could be offered in a separate feeder, although if the benefits of litter consumption are behavioural and a means of satisfying the birds' need to forage, this would not meet that need.

The maintenance of gut health to improve litter quality (and therefore foot health) can be achieved by the management of both diet and the environment. This study has identified a number of potential nutritional and management interventions that merit further study. 'It was not possible to prove or disprove the original hypotheses because overall the severity.....'. The recommended research requirements are:

- A need to further investigate and compare the response of turkeys when fed diets with lower protein and/or fibre contents in terms of nutrient digestibility, gut health, foot health and presence or absence of pathogenic bacteria in gut and litter.

- A need to investigate the effect of cleaning out litter and using fresh litter every day compared with reused litter on nutrient digestibility and prevalence of AMR bacteria in both gut and litter.

- A need to investigate the effect of offering clean bedding materials in one feeder separately from the feed to determine the voluntary intake of bedding materials and their effect on digestibility, gut health, and composition of the microbiome in both gut and litter.

- A need to confirm the beneficial effect of maize (relative to wheat) on the prevalence of ampicillin resistant E. coli and identify the mechanism by which this effect is achieved.

- A need to characterise the interaction between the litter microbiome and the birds' gut health.

- A need to cetermine the effect of genotype and gender on gut and foot health.

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