GUT HEALTH OF POULTRY IN THE POST-ANTIBIOTIC ERA: ELUCIDATING THE MECHANISMS UNDERLYING SUCCESSFUL FEED SUPPLEMENTS

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ABSTRACT

The recognised risk of antibiotic-resistant bacteria is a global concern that has put increased pressure on non-EU countries to decrease their use of antibiotic growth promoters and EU countries to reduce their therapeutic antibiotic usage in poultry. Therefore, viable non-antibiotic feed supplements are needed to improve bird gut health, immunity and performance, whilst reducing the proposed risk to human health. Many supplements are being produced with this aim, but limited understanding of how they work hampers improvements to efficacy. A natural carbohydrate fraction (NCF) refined from the cell wall of *Saccharomyces cerevisiae* yeast has been recently developed and proposed to improve gut health, immunity and bird performance. The aim of this research project was to examine possible modes of action behind this feed supplement on gut health, immunity and performance in broiler chickens. A total of 3 bird trials were performed to achieve this aim.

Chapter 3 reports on a 6 week trial (Trial 1), designed to examine the performance and gut health of birds fed NCF with a commercial stocking density. The effects of NCF on gut development were examined only at 6 weeks in order to not alter stocking density. The results of this trial demonstrated a positive effect of NCF on performance and limited effects on gut health at 6 weeks. Performance improvements were also age-dependent with younger birds showing greatest response to supplementation. Following the conclusion drawn from Trial 1, Trial 2 (Chapter 4) was conducted using weekly sampling from each bird pen to examine the effects of NCF on development of the gut and intestinal morphology in broilers prior to 6 weeks. Trial 2 investigated the effects of NCF on microscopic changes in the gastrointestinal tract (GI tract) from hatch to slaughter and relate effects to performance. In contrast to Trial 1, data from Trial 2 did not show any pronounced effect of NCF on gut health or bird performance. This may be due to the lack of pathogenic challenge or physiological stress on the birds resulting

from the decrease in stocking density over the trial. In addition, activity of NCF in the manufactured diets could not be dependably verified to show the intended incremental increases between treatments, which may be due to an issue with the assay or supplement batch itself.

Trial 3 (Chapter 5) was performed to investigate the effects in young (0-3 week) broilers of two commercial forms of butyrate salts on gut health, performance, immunity and digestive enzyme activity, both singly and in combination with NCF. Butyrate salts are reported to have similar effects to NCF on gut health and performance but thought to engage different response mechanisms. Again, it was seen that NCF with or without the inclusion of butyrate did not show a pronounced effect on the gut health parameters measured or bird performance in this trial.

This project aimed to clarify mechanisms behind the observed effects of gut health supplements in broilers by investigating parameters spanning from the overall performance down to individual gene expression. Chapter 6 reports on upstream factors with the potential to subsequently influence gut health and performance. While NCF had no effect on the digestive enzyme activity or serum immunoglobulin measured, a substantial effect on the gene expression of pathway involving immune response and metabolic processes was observed. Furthermore the project provides evidence that some of the immune adaptations are borne out through alteration to mucin production by goblet cells. It was also indicated in this chapter that the mechanism underlying the effects of NCF depend on the inclusion level: 200g/t NCF altered immune and metabolism genes, 400g/t NCF altered mostly immune genes, whereas 800g/t altered mostly metabolism genes. From the alterations seen in the gene expression and mucin capacity when feeding NCF, it appears that NCF is increasing immune defences to prevent growth impedance, rather than any metabolic, growth promoting effect. From

this it may be concluded that performance improvements are more likely to be observed when some level of challenge is placed on the bird.

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CHAPTER 1: REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Intensive broiler production began in the United States and came to the United Kingdom in the 1950s (Sainsbury, 1999), resulting in the UK poultry production industry rapidly increasing. In 2010, the UK broiler production population had increased to over 105 million (DEFRA, 2010). However in-between 2011 and 2012, broiler numbers declined to 74 million (DEFRA, 2012), thought to be due to increasing feed costs. If this reduction in numbers of broilers is due to feed cost, it is economically important for the poultry industry that the broiler can maximally absorb and utilise feed provided to efficiently reach its target weight. The poultry industry has, over the past few decades, made notable developments in management, genetic engineering, nutrition and disease control, thus improving the efficiency of growth performance and total yield output. However, the intensive systems used in the poultry industry together with genetic selection for growth have led to a number of negative effects on broiler health. Negative effects caused by genetic selection for growth include; compromised immune system; pulmonary hypertension syndrome resulting in ascites (accumulation of oedematous fluid with in the abdominal cavity); lameness due to bone, tendon and ligament defects; tibial dyschondroplasia; and sudden death syndrome (flipover) (Julian 1998; Rauw *et al.*, 1998).

Alongside genetic selection for rapid growth, the use of antibiotics was recognised in 1946 as a tool to help maintain and support growth of the birds (Moore *et al.,* 1946; Bozkurt *et al*., 2009). This led to reliance on antibiotics to maintain efficient production methods (Nasir and Grashorn 2010). Antibiotics have been shown to reduce the burden of pathogens (Leitner *et al*., 2001) and increase feed conversion efficiency (Dibner and

Richards, 2005) in birds that often have compromised immune function due to intensive farming methods (Heckert *et al*., 2002) and aggressive genetic selection (Emmerson, 1997; Rauw *et al.,* 1998). On the 1st January 2006 a ban on using antibiotics as growth promoters was put in place throughout Europe (Regulation (EC) No 1831/2003), due to the increasing awareness of the appearance of antibiotic resistance in certain pathogenic bacteria (Diarra *et al.,* 2007). Therefore in-feed antibiotics cannot now be introduced to an animal's feed in order to improve their growth performance (Baurhoo *et al*., 2007b). Despite the decrease in the uses of antibiotic growth promoters (APGs) in recent years the use of therapeutic antibiotics is believed to have increased. The over-use of antibiotics is postulated to potentially lead to an increase in antibiotic-resistant infections among humans (Bates *et al*., 1994; Van den Bogaard and Stobberingh, 2000; Roe and Pillai, 2003). This poses a significant threat to human health, as antibiotic-resistant bacteria infections are harder to overcome (Baurhoo *et al.,* 2007). This is recognised as a global priority and has put increasing pressure on non-EU countries to decrease their use of antibiotic growth promoters, and EU countries to reduce their therapeutic antibiotic usage in poultry.

The EU-wide ban on AGP has resulted in increased incidence of endemic diseases in poultry (Chee, 2008) as well as performance problems such as decreased body weight (BW), increased feed conversion ratio (FCR), and higher disease challenges (Thomke and Elwinger, 1998; Engster *et al*., 2002; Huyghebaert *et al*., 2011). This has resulted in either, substantial economic losses and severe negative welfare implications for broilers, or increased use of therapeutic antibiotics. There is now a drive for a viable feed supplement, which will improve gut health, immunity and bird performance, whilst reducing the proposed risk to human health.

There are a number of naturally derived feed supplements that have been reported to have beneficial effects on bird health, such as immunomodulators, organic acids and prebiotics. These supplements are thought to have similar beneficial effects to each other and AGP on performance, however they have different modes of actions. Organic acids, such as sodium butyrate, are bacterial growth inhibitors. Prebiotics, such as mannan oligosaccharides (MOS) are indigestible feed supplements that encourage the growth or activity of non-pathogenic bacteria species in the digestive tract (Bozkurt *et al*., 2009). MOS have been suggested to have a similar, positive effect on gut heath to AGP, through a different action of binding to pathogens and modulating immune function (Ewing, 2008).

MOS have been shown to improve gut health by changing the population of bacteria in the gastrointestinal tract (GI tract) and decreasing levels of pathogenic bacteria, as well as increasing performance parameters (Apajalahti *et al*., 2006). MOS have also been indicated to modulate the immune response and inhibit pathogenic bacterial proliferation, thus leading to an improvement in the birds gut health and growth performance (Iji *et al*., 2001; Bozkurt *et al.*, 2009). The beneficial effects of MOS on the growth performance of broiler chickens have been investigated by both meta- and holo-analysis, suggesting that MOS has an overall positive effect. However this does not take into consideration private research where the data has been withheld from being published (Hooge, 2004; Rosen, 2007). Additionally, reports of the effects of MOS are variable, with some reports showing that MOS have no effect on bird performance or gut health (Iji, *et al.*, 2001). Due to the limited knowledge about their modes of action, there is no clear understanding why the results are variable. Possible contributing factors are differences in the composition of the MOS products investigated, alterations in rearing environment and the level of pathogenic challenge to

which the birds are subjected.

Recently a refined prebiotic, Actigen™ (Alltech Inc.) has been derived by isolating the active component of an existing MOS product. This means that it may have greater ability to bind pathogens and modulate the immune function than MOS. As Actigen™, a natural carbohydrate fraction (NCF), is derived by isolating the active part of an existing MOS supplement, it is possible the inclusion level may be lowered due to possible higher efficiency. The aim of this research is to quantify the efficacy of feed supplements, concentrating on NCF in promoting gut health.

1.2 GUT STRUCTURE AND FUNCTION IN POULTRY

Poultry are classified as monogastric animals (Ewing, 2008). The major function of the gastro-intestinal tract is the absorption of nutrients from the feed to support health and growth. The development of the GI tract is associated with the early gastric experience of an animal (Ewing, 2008). After the birds have hatched, in order to survive they must make the transition from using the energy obtained from the yolk (endogenous) to the utilisation of carbohydrate-rich feed (exogenous) via the GI tract (Sklan, 2001). The degree of access to food in the immediate post-hatch period affects the level of development of the GI tract.

1.2.1 Gross morphology of the poultry gut

The digestive tract in poultry comprises of the oesophagus, crop, proventriculus, gizzard, small intestine, caeca, and large intestine (figure 1.1). The development of the GI tract in the post-hatch period in terms of gross morphology is measured in growth; i.e. weight and length.

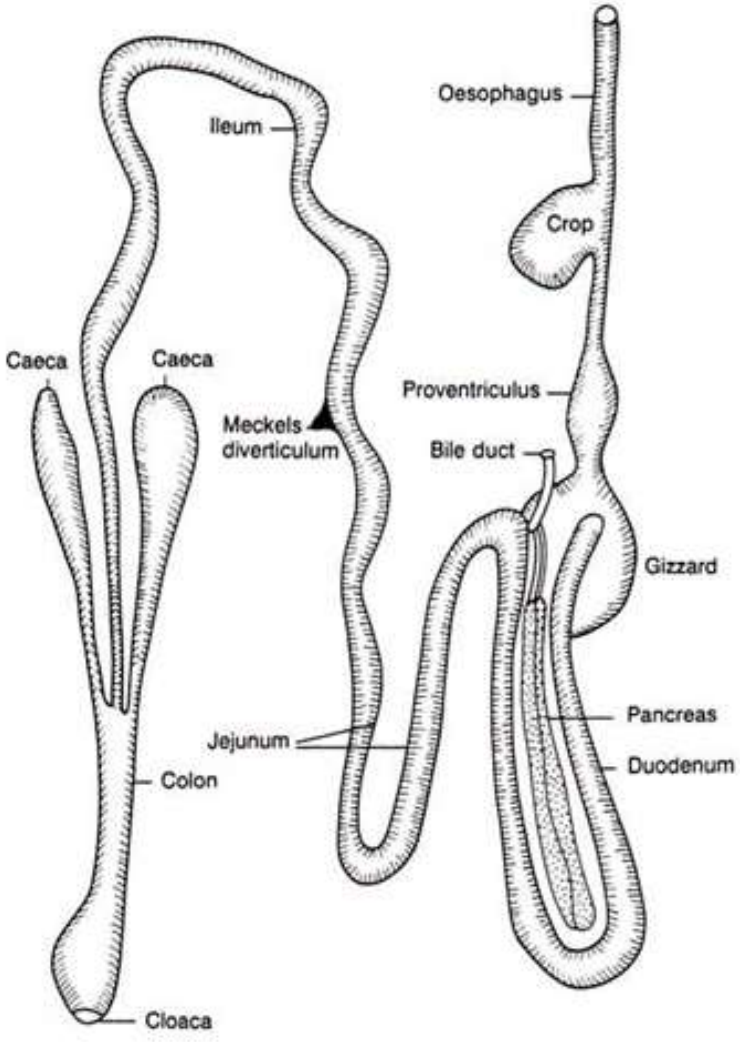

Figure 1.1: Anatomy of the gastro-intestinal tract in galliforms (Larbier and Leclercq, 1992)

1.2.1.1 Oesophagus and crop

Food enters the oesophagus, through the buccal cavity, which is the area within the beak of the chicken, containing a narrow triangular tongue. The numbers of taste buds a chicken has (312) is relatively few in number when compared to a mammals, however the number of taste buds to their bite size is higher than in mammals (Roura et al, 2008). Therefore, taste appears to play a role in consumption. Food is taken in and mixed with saliva, produced by salivary glands throughout the buccal cavity, a bolus is formed which moves down the oesophagus. In a mature chicken the oesophagus is about 20-

21cm long and enters the crop (Sturkie, 1976; Hill, 1976). The food bolus moves in to the crop, which is well developed in the chicken. The bolus may then be stored in the crop if the proventriculus is full/ The crop enables the bird to consume large quantities of food at one time. It also serves as a reservoir which regulates the transit time of digesta. There are three muscle types in the crop allowing for it to contract, mix and pass the food on to the proventriculus. In addition a small amount of softening and fermentation will occur in the crop.

1.2.1.2 Proventriculus and gizzard

The proventriculus and gizzard have complementary roles and make up the stomach of the chicken. The cells that line the proventriculus, the chief cells, secrete hydrochloric acid and pepsinogen, therefore it has a secretory role, whereas the gizzard has a mechanical role. The proventriculus starts the process of breaking down protein into amino acids (Grist, 2006). However due to the fast transit of the food from the proventriculus most of this occurs in the gizzard. The food in then held in the gizzard, which is covered by a hard yellow, ridged lining called the cutica gastrica. The cutica gastrica grinds food which is assisted by the extensive muscle development and grit that the bird has ingested. Under normal *ad libitum* feeding conditions the content of the proventriculus and gizzard are acid, due to the gastric secretions from the proventriculus. After the mixing and pepsin digestion the food/digesta is in the form of a paste, known as chyme, which passes on to the small intestine.

1.2.1.3 Small intestine

The small intestine is the principal site of digestion and absorption of soluble nutrients. Digestion in the small intestine involves enzymes from both intestinal and pancreatic origins. Intestinal juices are secreted by the brush border of the small intestine and

contain enzymes, although most of the digestion occurs here through the action of bile and pancreatic juices. The small intestine wall consists of an inner lining (the mucosa), a secondary layer (the submucosa), an inner circular layer of muscles and an outer layer of longitudinal muscles. The small intestine of the chicken is approximately 120-140cm long in adult birds and is divided into four regions, the duodenum, jejunum and ileum (Grist, 2006).

The duodenum follows the gizzard in the digestive tract, it a horseshoe-shaped loop surrounding the pancreas (Larbier and Leclercq, 1992). The pancreas secretes substantial concentrations of bicarbonate ions, peptidases, amylases, saccharidases and lipases. The bicarbonate ions neutralise the chyme so that the enzymes can digest protein, carbohydrates and lipids. The production of pancreatic juices is stimulated by secretin, which is produced by the intestinal wall when food reaches the duodenum (Duke, 1986b). Bile also enters the small intestine in the duodenum though two ducts from the liver. Bile aids the pancreatic lipase and the absorption of lipids by emulsifying the lipids (Sturkie, 1976). The duodenum is continuous with the jejunum, the jejunum starts where the bile and pancreatic ducts enters the small intestine. The jejunum is also continuous with the ileum which looks very similar, although the two segments are clearly distinguished by the presence of the Meckel's diverticulum (Chee, 2008), which is the remnants of the yolk sac. The jejunum and ileum constitute the major absorptive region of the digestive tract. The ileum runs to the ileo-caecal junction where the two caeca branch off.

1.2.1.4 Large intestine

The large intestine consists of the caeca and colon. The caeca are two 20cm long sacs at the junction between the small and large intestine. They are involved in the reabsorption

of water from the digesta (Grist, 2006), and contain micro-organisms which breakdown undigested nutrients. The microflora of the intestinal tract is very important to the health of the bird, through their effects on gut structure, nutrition, intestinal disease, and immunity (Lu *et al.*, 2003). Therefore it is very important for the beneficial population of micro-organisms in the caeca to establishment early, which will assist in the competitive exclusion of pathogenic bacteria.

1.2.2 Microscopic morphology of the gut

The gastrointestinal tract is made up of five distinct layers, mocosa, muscularis mucosae, submucosa, muscularis externa and serosa (figure 1.2). The mucosa is made up of the epithelium and the underlying lamina propria, which is a structural network containing nerve fibres, lymph and capillaries (Larbier and Leclercq, 1992; Grist, 2006; Koutsos and Arias, 2006). The muscularis mucosae is involved in the motion of the villi through a layer of circular and longitudinal muscle. The submucosa and the lamina propria, contain blood vessels, nerves and lymph tissue. The muscularis externa is a muscle layer containing circular muscle involved in motility and longitudinal muscle involved in maintaining integrity, and the serosa is the outer most layer containing a layer of squamous epithelium cells.

The epithelial luminal surface of the gastrointestinal tract in chickens has finger-like projections called villi, predominantly made up of enterocytes. The villi increase the surface area of the luminal surface and enterocytes have microvilli on the luminal surface, further increasing the surface area. The microvilli become less numerous and shorter toward the distal end of the small intestine. Goblet cells are also present in the epithelial layer, which secrete mucin. Mucin is the principle component of the mucus that lines the intestinal tract and acts as a lumenal lubrication. The villi are lined with

epithelial cells and are continuously being regenerated, which occurs near the bottom of the crypt. The crypts of Leiberkuhn and the cells produce epithelial cells that move up the villus and are eventually sloughed off the tip. Villi length are affected in response to a number of factors, such as diet and enteric micro-organisms. consequently, the turnover rate of the villus epithelial layer varies and is related to crypt mitotic activity.

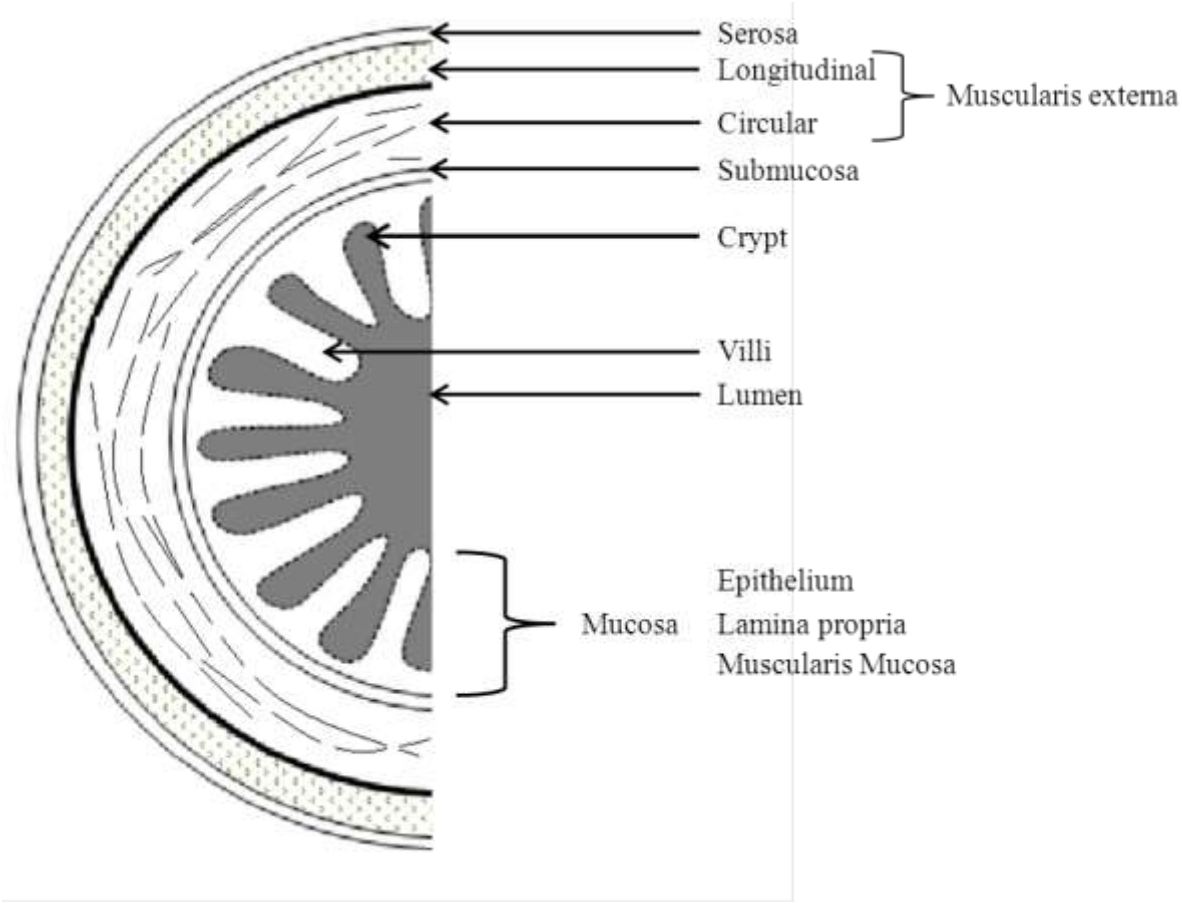

Figure 1.2: Diagram of the cross section of the gastrointestinal tract of a chicken.

1.3 DIGESTIVE ENZYMES

The main site of digestion in the bird is the small intestine, because most enzyme secretion occurs into the proventriculus or duodenum, Table 1.1 shows the location of digestive enzyme production and their substrates in the chicken. Figure 1.3 shows

location of digestive enzyme excretion in the digestive tract. Amylase, trypsin and chymotrypsin are secreted into the duodenum from the pancreas. The pancreatic ducts enter the latter portion of the ascending branch of the duodenum (Larbier and Leclercq, 1992). Amylase is secreted in the saliva as well as the duodenum, however most carbohydrate digestion occurs in the jejunum (Osman 1982). Alpha-amylase hydrolyses 1,4 α-linkages on both sides of the 1,6 branching points of starch, producing small branched oligosaccharides which are further broken down into glucose molecules (Leeson and Summers 2001).

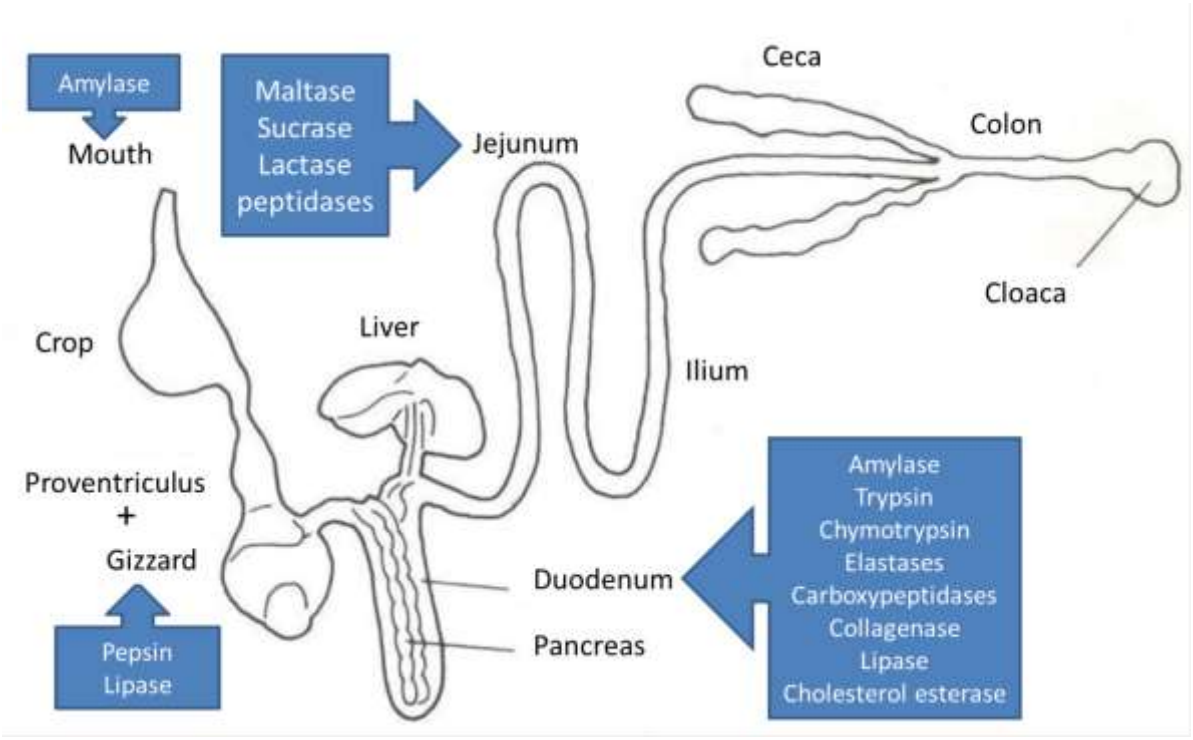

Figure 1.3: Digestive enzyme location in the chicken

Protein is broken down in the bird by a number of specific enzymes, which are produced by the pancreas in inactive forms, such as trypsinogen, which is activated to trypsin in the duodenum by enterokinase. Once trypsin is activated, it activates a cascade of other proteolytic enzymes meaning it is central to protein digestion.

Proteolysis is initiated by pepsin producing polypeptides that are broken down further by trypsin and chymotrypsin releasing terminal peptide bonds. In the intestinal content of chickens the trypsin activity increases from hatch to 15 days of age by a factor of 10 (Leeson and Summers 2001). Lipase produced in the pancreas breaks down fat that has been emulsified by bile salts into fatty acids, monoglycerides and glycerol.

Table 1.1: Digestive enzyme location and substrate in the chicken (Leeson and Summers 2001)

Location	Enzyme	Substrate
Mouth	Amylase	Starch
Gizzard and proventriculus	Pepsin	Protein
	Lipase	Triglyceride
Duodenum	Amylase	Starch
	Trypsin	Protein/peptides
	Chymotrypsin	Protein/peptides
	Elastases	Protein/peptides
	Carboxypeptidase	Peptides
	Collagenase	Collagen
	Lipase	Fat
	Cholesterol esterase	Cholesterol esters
Jejunum	Maltase	Maltose
	Sucrose	Sucrose
	Lactase	Lactose
	Peptidase	Peptides

1.4 INTESTINAL MICROFLORA AND GUT DEVELOPMENT

The GI tract of the newly hatched chick is sterile and chicks are relatively immuno-incompetent (Dibner *et al.,* 2008). This means that a chick when hatched is highly susceptible to having pathogen colonisation of their GI tract (Nurmi and Rantala, 1973).

It is vital that a chick develops a normal intestinal microbial population and resistance against enteric pathogens, as the gut microflora plays a significant role in maintaining optimal animal health (Lee *et al.,* 2010). It has been found that when an animal is germ free, it is highly susceptible to intestinal infection (O'Hara and Shanahan, 2006) and not able to establish an effective antibody response until their gut microflora has developed (Rhee *et al.,* 2004; Lee *et al.,* 2010). The development of the enteric microflora in a chick helps prevent a sudden growth of potentially lethal pathogens that are inevitably introduced at some point during the life of a bird. The mechanism of protection from the development of enteric microflora is thought to be through competition for binding site, competition for nutrients, production of antibacterial substances and immuno-stimulation (Perry, 2006).

Colonization by microbial populations proceeds immediately after hatch; normally the GI tract becomes populated quickly by bacteria from the hatchery. Therefore commercial birds are already populated by microorganisms before arriving at the farm (Dibner *et al.,* 2008). Development occurs through a series of colonisation steps that are similar across species (Dibner *et al.,* 2008). First of all, aerobic and facultative organisms colonise the GI tract; these organisms change the condition of the GI tract, making colonization for anaerobes easier. This is thought to be through lowering the oxidation reduction potential in the intestine (Dibner *et al.,* 2008). The small intestine microflora of a bird is developed within the first two weeks of its life. However the cecal flora, mainly composed of obligate anaerobes, require up to 30 days to become established (Amit-Romach *et al.,* 2004). The GI tract of poultry is composed of generally 10^7 to 10^{11} bacteria per gram of gut contents, but can be up to 10^{13} (Apajalahti *et al.,* 2004; Perry, 2006; Lee *et al.,* 2010). The healthy GI tract microbiota consists of approximately 400 known species. However as there is a large proportion

that cannot be cultured the total amount is unclear (Dibner *et al.,* 2008).

The commensal bacterial populations in a healthy bird can vary greatly depending on its age, environment, region of intestine, feed particle size and feed composition (Rehman *et al.,* 2007). Also, as not all bacteria can be cultured or detected, it is difficult to define an ideal population for gut health, as current knowledge of the intestinal bacteria is inadequate to support a definition (Rehman *et al*., 2007). However Rehman *et al.,* (2007) reviewed literature of indigenous bacteria in the gastrointestinal tract of broiler chickens and reported the following; the crop had mainly gram-positive facultative anaerobic bacteria particularly *Lactobacillus spp*.; Lactobacilli, enterococci, lactose-negative enterobacteria and coliform bacteria were the principal organisms in the gizzard; in the duodenum, jejunum and ileum, lactobacilli streptococci and *E. coli* were the primary organisms; and the caecal bacteria contained anaerobes comprised of mainly anaerobic cocci, *Eubacterium spp., Lactobacillus spp., Clostridium spp., gemmiger, Fusobacterium spp. and Bacteroides spp.*

Not only is the colonisation of the microflora population important in the development of the young birds GI tract but also the timing of the first feed after hatch. The timing of the first feed and the makeup of that feed can change the level of development of the small intestine (Perry, 2006). The GI tract is developmentally very active in the period just after hatching in birds (Uni *et al.,* 2000). Delayed access to feed may result in the late development of the small intestine mucosal layer (Uni *et al.,* 1998; Geyra *et al.,* 2001). Crypts form on the day of hatch, become defined in the first 48 to 96 h and continue growing quickly during the first 7 days after hatching (Uni *et al*., 2000). The intestinal villi increase significantly in diameter and length during the first 7 to 10 d after hatching (Sklan, 2001; Solis de los Santos *et al.,* 2007)

As the GI tract is the main area for nutrient absorption and infection in the bird, gut health is very important to the bird's overall wellbeing. When considering gut health, many aspects can be assessed from gross morphology of the intestine down to digestive enzyme production and subsequently integrity of cellular tight junctions. In addition to these aspects of gut health, the micro-organism population of the intestinal tract must also be taken into account, as they affect gut structure, nutrition, intestinal disease, and the immune status of the bird (Yegani and Korver 2008). Preferably, a bird would have ideal gut health by optimising the following factors; gut length, mucin production (mucosa thickness), commensal bacteria population, villus height, villus width, crypt depth, turnover rate of the epithelial layer, digestive enzyme production, gut wall thickness and ph. Factors that affect these parameters include; feed composition, particle size, feed conditioning, processing and presentation, and environmental bacteria population and conditions (Yegani and Korver 2008).

1.5 GASTROINTESTINAL TRACT AND IMMUNE SYSTEM OF THE BIRD

The GI tract lumen can be considered as being part of the external environment. One of the most important roles of the gut is providing a barrier between the internal and external environment, as the GI tract is the site of colonisation and invasion of many pathogenic bacteria. Through this and several other functions, the GI tract has an important role in the immune function of a bird (Patterson and Burkeholder, 2003; Beal *et al.,* 2006). Post hatching is a vital time for the development of the gut immune system of the bird. The immune system has to differentiate between food, beneficial bacteria and harmful pathogens (Perry, 2006), so there is a fine balance between tolerance and response to antigens. This is fundamental to gut health as a prolonged immune response can cause problems such as villus atrophy (Perry, 2006).

Newly hatched chickens have some degree of immune resistance via the innate immune mechanisms, which involve macrophages, dendritic cells, heterophils and natural killer cells, which recognise and control pathogens (Powell, 1987; Kogut, 2009). The innate immune system is non-specific or broadly specific and has no memory. Conversely, the adaptive immune system is specific and has memory, and is mediated by both B and T lymphocytes. Macrophages are part of the innate and adaptive immune system of the broiler and can destroy pathogens directly (Qureshi, 2003;). Macrophages also can secrete cytokines that act in the inflammatory process. Lymphoid cells, such as natural killer cells, T cells and B cells, can be found in key immune tissues in the GI tract called the gut-associated lymphoid tissue (GALT). GALT is crucial in developing the immune system of the GI tract and is the largest component of the birds entire immune system (Casteleyn et al 2010).

The Bursa of Fabricius, Peyer's Patches and cecal tonsils are all parts of the GALT (figure 1.4). The Bursa of Fabricius is a sac like structure located between the cloaca and sacrum. The Bursa of Fabricius is the key lymphoid organ of the bird, and responsible for the development and maintenance of the B-cell (Alitheen *et al* 2012; Wu *et al*., 2013). Its removal during embryonic life can prevent the development of B cells and the production of antibodies (Ratcliffe 2006). Peyer's Patches are made up of lymphoid tissues situated at numerous sites along the small intestine (Makala *et al.,* 2002). One Peyer's Patch is always situated in the ileum, 5 to 10 cm from the ileum-caecal junction (Casteleyn *et al.,* 2010). Peyer's Patches have been reported to be vital in local protection in the GI tract as regulators of the gut immune response in mammals against intestinal pathogens. little is known about the role of Peyer's Patches in gut immunity of the chicken. However they do contain T and B cells and are the main source of precursors of IgA-producing cells (Muir *et al.,* 2002; Davison *et al.,* 2008;

Holt *et al.,* 2011). Caecal tonsils are similar to Peyer's patches, containing T and B lymphocytes and are located in each caeca where they join the ilium. It has been shown that under a pathogenic infection caecal tonsils have increased B lymphocyte and macrophages numbers (Setta *et al.,* 2012) and therefore are assumed to be important in the bird's immune response to pathogens.

Newly hatched chickens also have a lot of maternal antibodies, which are transmitted from hen yolk (Lee *et al.,* 2010) Following hatching, the adaptive immunity of a chicken will take at least three weeks to mature and development (Beal *et al.,* 2006). Therefore, it is crucial that a bird is assisted through management and dietary supplements intervention in the early stages of life to give some protection against the microbial pathogens, when a bird is exposed to pathogens when it first eats, to minimise the negative effects of pathogenic bacteria on bird health, welfare and performance.

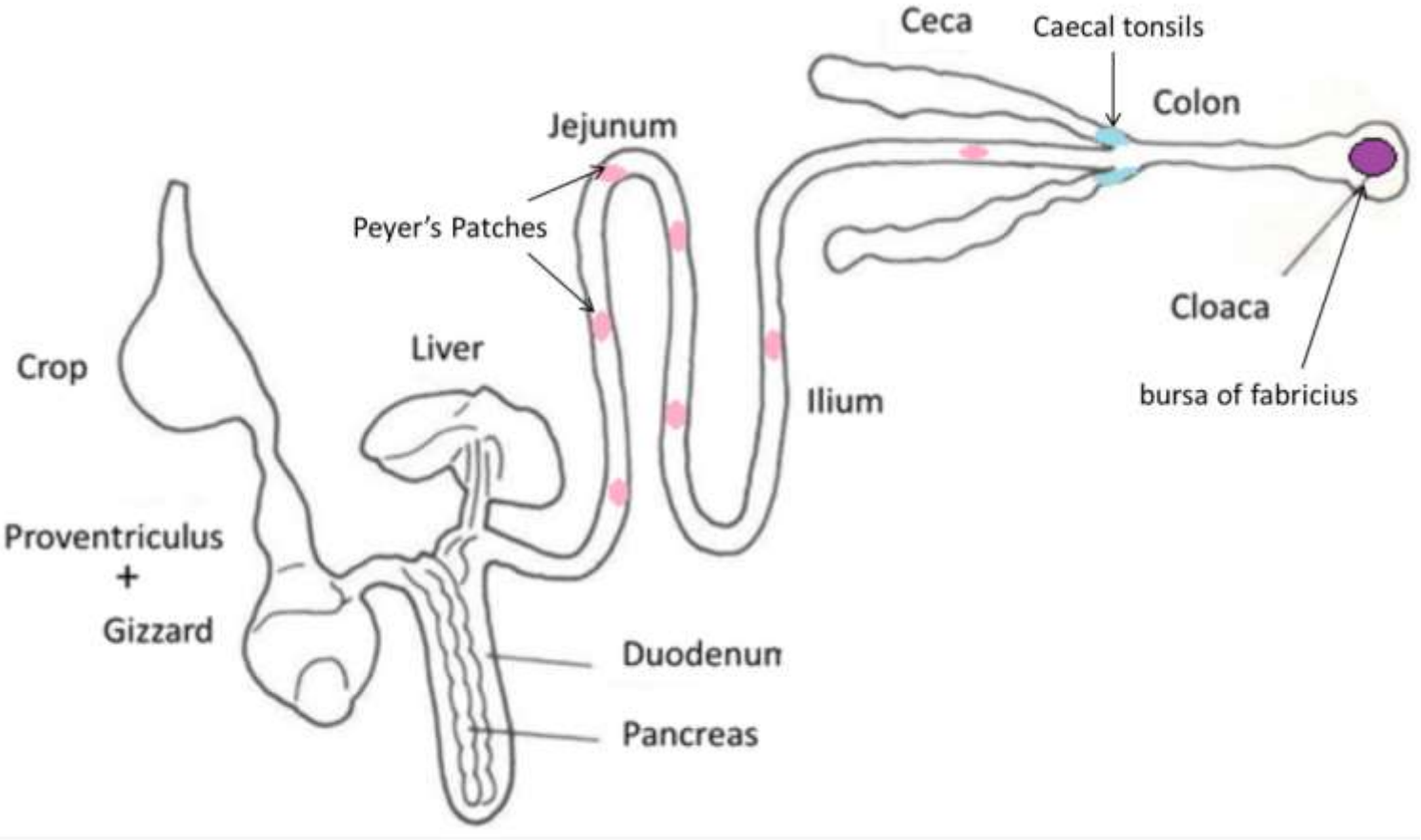

Figure 1.4: Diagram showing the locations of Peyer's Patches, Caecal tonsils and Bursa of Fabricius in a chicken.

1.6 DIFFERENCES BETWEEN COMMERCIAL AND RESEARCH BROILER PRODUCTION ENVIRONMENTS

In a new commercial poultry house it is possible to sensitively control the birds' environment. This is due to good heating, ventilation systems, water and wind tight roofing and walls. However, many commercial poultry houses were built decades ago, meaning that some houses have roofs that are not watertight, have cracked concrete, poor heating and ventilation systems. Consequently, birds are put under varying levels of environmental stress due to fluctuation in temperature, and cracked concrete can harbour pathogenic bacteria. Generally, poultry research facilities are kept to a high standard of repair, meaning that the likelihood of leaks, draughts and cracked concrete are low. Also, as research facilities are smaller and purpose built to maintain tightly controlled temperatures, fluctuations in temperature are small.

The maximum stocking density in the Nottingham Trent University poultry research facility is 24kg/m2, whereas in a commercial farm it is 34kg/m2 (DEFRA 2013). This means that birds that are in a commercial farm have a different amount of physiological stress from birds in a research facility, as there is more competition for space at the feed and drinker lines. Another factor that decreases the stress of birds in research facilities is the use of pen systems. Figure 1.5 shows the avian research facility of Scotland's Rural College and Nottingham Trent University's poultry research facility. Use of larger pens of 40 birds at the avian research facility of Scotland's Rural College provides an environment closer to a commercial condition than Nottingham Trent University. In commercial farms it is also common practice to over stock at the beginning of a crop, so that they 'thin' (remove a number of the birds) before the final harvesting of birds. This distresses the birds, as well as increasing the chance of pathogenic bacteria being brought in to the poultry house. Biosecurity on commercial farms can vary greatly, and

is generally dictated by the competence of the manager making sure correct protocols are followed for footwear and vehicles coming on to the farm.

The quality of floor litter can have a substantial effect on the birds, as they are constantly in contact with it. In a research facility it is very unlikely to have a capped litter (solid crust of excreta and litter stopping the mixing of the litter) due to the good environmental controls and the ability to top up litter easily. However in commercial situations the litter is more likely to cap due to stocking density, environmental conditions, management and condition of the building. Quality of feed can also vary substantially between batches, in regards to nutrient availability, presence of anti-nutrient factors and contaminants. This is due to the variability in crops nutrient composition and the price of crops, as formulation are made to balance cost with nutrition. Pellet quality can also vary and cause stress on birds' digestive systems.

Another area in which commercial situation varies from research is that the range of flock age of the parent flock that research will accept is normally tighter than the commercial range. This means that the evenness of commercial flocks may be worse that research flocks, and they are more likely to get 'problem' flocks. Therefore in commercial broiler production it is more likely that one or more of these conditions; presence of unfavourable organisms, extremes in ambient temperature, diseases, crowding and poor management will be present (Midilli *et al*., 2008). All of these factors above which interfere with the birds normal growth development and wellbeing are commonly referred to as challenges.

Figure 1.5: Pen sizes of poultry research facilities, Left Scotland's Rural College avian research facility, right Nottingham Trent University's poultry research facility.

1.7 BROILER FEED COMPOSITION AND SPECIFICATION

It is important that a broiler diet contains the correct concentration and ratios of nutrients, particularly energy, protein, minerals and vitamins. In most poultry diets, starch from maize or wheat forms the major source of energy. The energy needs of the bird depends on the strain and type breed of broiler. For example, a Ross 308 strain broiler needs 12.65MJ of metabolisable energy between 0-10d, whereas a Cobb 500 strain needs 12.70MJ. Between 11-24d Ross 308 needs 13.20MJ/kg diet and Cobb500 needs 13.00MJ/kg diet; and between 25 and slaughter Ross 308 needs 13.40MJ/kg diet and Cobb 500 needs 13.30MJ/kg diet of metabolisable energy (Aviagen, 2007; Cobb, 2013). If a diet is low in energy, then the bird will increase its feed consumption to meet its energy need. However in the case of the maintenance energy needs not being met, the bird will lose weight. If a diet has a large excess of metabolisable energy relative to protein content required for normal growth, activity and maintenance, the bird will reduce its intake. This will result in the bird not taking in enough protein and vitamins to meet its needs and will show signs of protein and vitamin malnutrition.

Vegetable proteins are used as the main protein source in poultry diets. These include soybean meal, canola, field beans, peas and lupins. A Ross 308 broiler diet needs to contain 22-25% crude protein between 0-10d, 21-23% crude protein between 11-24d and 19-23% crude protein between 25d and slaughter (Aviagen 2007). The broiler requirement for crude protein actually describes the requirements for amino acids, the building blocks of protein. In order for a bird to reach its genetic potential it must be provided with the right balance of amino acids, avoiding excess or deficiencies. The amino acid requirement for a broiler chicken is made up by the maintenance requirement and the growth requirements of the chicken, Table 1.2 shows the amino acid specifications for Ross 308. The requirements for amino acids for both maintenance and growth change depend upon the age, genetic growth rate, carcass composition, and body weight of the chicken. In poultry diets the main limiting amino acids are lysine, methionine and threonine. Therefore, supplementation of these amino acids into poultry diets may allow for an increase in the efficiency of protein utilisation, and a decrease in nitrogen excretion. The requirements for amino acids are affected by the supply of others, so increasing the supply of one amino acid (AA) may improve performance only if other amino acids are not limiting. This can be overcome by expressing the amino acid requirements as ideal ratio to lysine because, although the AA requirements change due to the factors above, the ideal ratio of essential AAs to lysine will be only slightly affected within a specific age range. (Mack *et al*, 1999; Schutte and De Jong, 1999).

Table 1.2 Amino acid specifications for Ross308 as hatched (Aviagen 2007)

AMINO ACIDS	0-10 days	11-24 days	25- slaughter
Lysine %	1.43	1.24	1.09
Methionine and Cystine %	1.07	0.95	0.86
Methionine %	0.51	0.45	0.41
Threonine %	0.94	0.83	0.74
Valine %	1.09	0.96	0.86
iso-Leucine %	0.97	0.85	0.76
Arginine %	1.45	1.27	1.13
Tryptophan %	0.24	0.20	0.18

Minerals are inorganic nutrients and are classified as major or trace elements. The major minerals in broiler diets include calcium, phosphorus, potassium, sodium, chlorine, sulphur and magnesium. Calcium (Ca) and available phosphorus (P) are closely related in metabolism and bone formation. Most calcium in broilers is used for bone formation. The ratio of calcium to phosphorus is important, with a ratio of around 2:1, Ca:P typically being needed. It has been seen that a ratio of 3.3:1 Ca:P will result in rickets and leg problems. For optimum bone integrity in young chicks they may need a ratio greater than 2.2:1 (Leeson and Summers 2001). Both Ross 308 and Cobb 500 nutrition specifications have ratios of Ca:P between 2:1 and 2.1:1. A diet deficient in calcium will reduce growth, cause leg weakness, decrease feed intake, activity and produce increased urine volume. The signs of phosphorous deficiency and calcium toxicity is similar to calcium deficiency due to the ratio between calcium and phosphorus. Potassium, sodium and chlorine have primarily roles in electrochemical functions, to maintain acid-base balance and osmotic regulation. Trace minerals added to broiler diets include iron, iodine, copper, manganese, zinc and selenium. Vitamins are routinely

supplemented in most poultry feeds and can be classified into either water-soluble or fat-soluble. Water-soluble vitamins include the B-complex vitamins and vitamin C. Vitamins classified as fat-soluble include A, D, E and K. The fat-soluble vitamins can be stored in the liver and other parts of the body. Vitamins are naturally occurring in plant based feed ingredients, although content can vary due to crop location, soil type, fertiliser use, variety, stage of growth at harvest, and drying and storage conditions. in addition, the fat soluble vitamin content can be affected by mould. As levels of naturally occurring vitamins are variable birds diets are supplemented with synthetic vitamins. Vitamin D3 is important in broiler diets as its involved in calcium and phosphorus balance. Vitamin D3 stimulates the active transport of calcium and phosphorous across the GI tract of broilers, its uptake and release from bones and its recirculation via the kidneys. If a diet is deficient in vitamin D the signs are similar to deficiencies in calcium; reduced growth and leg weakness.

Fibre is also present in broiler diets, and represents a group of carbohydrates that are resistant to digestion and absorption in the birds small intestine, with complete or partial fermentation in the large intestine. This is due to the bird lacking the enzymes capable of digesting fibre. Fibre can either be soluble or insoluble and includes lignin, non-starch polysaccharides, resistant starch, inulin and non-digestible oligosaccharides. Soluble fibre has been seen to increase luminal viscosity, the solubility of non-starch polysaccharides (NSP) is a major anti-nutrient factor as increased viscosity means that it is less likely for the enzymes to have contact with the nutrients in the digesta.

1.8 ANTIBIOTIC USE IN POULTRY DIETS

Antibiotic growth promoters (AGP), avoparcin, virginiamycin, bacitracin zinc, tylosin phosphate, spiromycin, avilamycin and flavomycin, have been used since the mid-1940s

in agricultural animal production (Dibner and Richards, 2005; Yegani and Korver, 2008). Their use in animal feed grew as it was reported that AGP improved animal performance and health status (Miles *et al*., 2006; Yegani and Korver, 2008). The routine inclusion of AGP in feed negated the requirement to select genetic strains of broiler with disease resistance. This combined with the physiological stress induced by their immense potential for fast growth has led to compromised immune systems in modern strains of broilers used in intensive poultry production (Emmerson, 1997; Heckert *et al*., 2002). Antibiotics have their effect through reducing the birds pathogenic burden (Leitner *et al*., 2001). However pathogens can become resistant to antibiotics in specific situations. Therefore there has been an increased awareness of the link between the use of antibiotics in animal production to antibiotic resistant pathogens in humans, as a number of AGP used in animal production systems belong to classes of drugs used in human medicine (Emborg *et al*., 2002).

The use of antibiotics gave rise to the possible problem of antibiotic resistant pathogenic bacterial strains (Budino *et al*., 2005) and residual components entering the food chain (Chen *et al.,* 2005). It has been shown in many reports that there is an increase in the prevalence of resistance to antibiotics in pig and poultry *enterococci, Escherichia coli* and *Campylobacter spp.* when feeding antibiotics (Linton *et al*., 1985; Mathew *et al.,* 1998; Taylor *et al*., 2008). Ramos *et al.* (2012) found that in *E. coli* in faecal samples of 73 sheep, 71 pigs and 54 beef cattle, 76.6% were resistant to one or more antimicrobial agents. Antibiotic-resistant pathogens can be spread from animals to humans in several ways. Firstly through foodborne infection via humans having contact with meatcontaminated with the resistant bacteria, secondly, through direct contact with the infected animals, and thirdly through contact with bacteria in the animal manure (van den Bogaard and Stobberingh, 2000). Due to the possible undesirable negative effects of

AGP on human health, the agriculture industry has been under increasing pressure to reduce and eliminate their use of AGP. This led to the steady voluntary withdrawal of AGP from animal feed in many EU countries. On 1st January 2006, the use of antibiotics as growth promoters was prohibited in the EU (Regulation (EC) No 1831/2003).

It has been reported that AGP exerts growth promoting effects through several mechanisms. Antibiotics can influence the harmful effects of micro-organisms by stabilising the intestinal microbial flora and decreasing gram negative bacteria (Gunal *et al*., 2006), and decreasing competition for nutrients, such as simple sugars and amino acids ideally utilised by the host, but also available for utilisation by the bacteria population of the GI tract (Dibner, and Richards, 2005; Apajalahti, 2005; Brisbin *et al*., 2008). Antibiotics also have been observed to increase nutrient digestibility (Dibner and Richards, 2005; Miles *et al*., 2006); Knarreborg *et al.* (2004) found that ileal absorption of fatty acids were greatly improved by salinomycin and avilamycin in broilers; whereas Sayrafi *et al.,* (2011) found improvements in villus height when feeding bacitracin methylene disalicyate. In addition in-feed antibiotics can result in a reduction of microbial metabolites that depress growth (Feighner and Dashkevicz, 1987; Knarreborg *et al*., 2004; Dibner, and Richards, 2005), the quantity of opportunistic pathogens and levels of subclinical infection (George *et al.*, 1982; Brennan *et al*., 2003; Butaye *et al.,* 2003; Dibner and Richards, 2005).

Common types of antibiotics used in broiler production are effective against Gram-positive bacteria. However, these antibiotics also inhibit the gram-positive commensal bacteria, thought to be beneficial to the bird (Apajalahti and Kettunen, 2013). It has been shown that antibiotics can decrease the number of bacteria in the GI tract, however

with this change the percentage of beneficial bacteria in the population decreases and the percentage of harmful bacteria in the bacterial population of a chicken increases. Therefore, it is thought that antibiotics have their beneficial effect on bird GI tract and growth performance by decreasing the total number of bacteria, not by selectively altering and shifting the microbial populations to reduce pathogenic load (Apajalahti and Kettunen 2006). This has led to the view that the gut of the bird fed antibiotic is not in fact a healthy gut, although it is an efficient gut (Apajalahti and Kettunen, 2013).

The ban of AGP in poultry diets in the EU is thought to have led to reduced efficiency in feed conversion and a rise in the incidence of certain animal diseases, such as (subclinical) necrotic enteritis (Wierup, 2001; Dibner and Richards, 2005). These issues have been cited as causing substantial economic losses, and may have been due to changes in the gut microbial composition (Yegani and Korver, 2008), which may have increased the pathogenic burden on the bird and therefore decreased performance due to poor gut health. In Demark, the FCR of 6,179 flocks were calculated and it was reported that they found that the average FCR was significantly higher (0.016 kg/kg ($P<0.001$)) in the period after the voluntary removal of AGPs that before (Emborg *et al.,* 2002). Additionally, there are conflicting reports that suggest that the beneficial effects of AGP on weight gains are not adequate to offset the cost of the AGP (Graham *et al.,* 2007; Yegani and Korver, 2008). Despite the conflicting views around the economic impact of removing AGPs, there is consensus that bird gut health has deteriorated since their removal. Following the ban on the use of AGP in animal feeds, many believe that the use of therapeutic antibiotics have increased in livestock. The total poultry only veterinary antibiotics sold in the UK in 2006 was 17 tonnes, which increased to 50 tonnes in 2010 (Veterinary Medicines Directorate, 2012). However this has decreased since, which may be due to a drive to decrease the use of therapeutic antimicrobials in

poultry.

1.9 MANNAN OLIGOSACCHARIDES

Due to the increased pressure from consumers to reduce the use of sub-therapeutic antibiotics in poultry diets, there is an increased interest in natural feed supplements in the animal feed industry (Benites *et al*., 2008). Prebiotics are non-digestible food ingredients that selectively stimulate the growth and/or activity of bacteria in the digestive system (Bozkurt *et al*., 2009). This is in contrast to antibiotics which decrease the total level of bacteria in the GI tract. One class of prebiotics are mannan oligosaccharides, which have been isolated by enzymatic hydrolysis or autolysis of the cell wall of the *Saccharomyces cerevisiae* yeast (figure 1.6), a common yeast in feed supplements (Vondruskova *et al.,* 2010). MOS consist of a mannan and a glucan component. The structure of the mannan component resembles that of the surface glycoproteins containing mannose present on the mucosal surface of the intestine. The mannans act as high-affinity ligands for the mannose- specific type-1 fimbriae of pathogenic bacteria (Ofek, *et al.*, 1977; Spring *et al.*, 2000; Miguel *et al.,* 2004) thus rendering them inert. The glucans component is a polymer of glucose linked by β-glycosidic bonds and is discussed later in this chapter (1.10).

It has been reported that MOS may have a number of beneficial effects, similar to those of AGP, on growth, intestinal development and immunity (Fernandez *et al.,* 2000; Spring *et al.,* 2000; Iji *et al.,* 2001). It has been indicated that MOS may modify the microbial gut ecosystem by adhering to the receptors on the intestinal epithelium, thereby blocking the binding of specific pathogens exhibiting mannose-sensitive glycoproteins and the colonization of the pathogens (Spring *et al.,* 2000; Chee, 2008). MOS are also thought to contribute to the establishment of a stable gastrointestinal

microbial balance and have a favourable effect on the intestinal microflora (Lyons and Bourne, 1995; Gaggia *et al.,* 2010). It has been reported that MOS can stimulate the local immune system by acting as a non-pathogenic antigen to increase the activities of macrophages and T-lymphocytes (Lyons and Bourne, 1995; Kelly, 2004; Ewing, 2008; Vondruskova *et al.,* 2010) In addition, MOS have been reported to improve the functions and health of the GI tract by improvement in the intestine morphology (Iji *et al.,* 2001; Chee, 2008) and therefore improving nutrient digestibility and level of apparent metabolisable energy (Biggs *et al.,* 2007)

However the effects of MOS on the bird are variable; Iji *et al.* (2001), Midilli *et al.* (2008) and Brummer *et al.* (2010), showed no significant effects of MOS on bird weight and feed intake. Yang *et al.* (2007) also reported broiler feed conversion ratio (FCR) to be unaffected by MOS supplementation. It is thought that MOS is most effective under conditions of physiological stress (Baurhoo *et al.,* 2009), which are frequently present at some level in a commercial broiler production systems. However in a research environment the physiological challenge to the birds is typically lower (Midilli *et al.,* 2008).

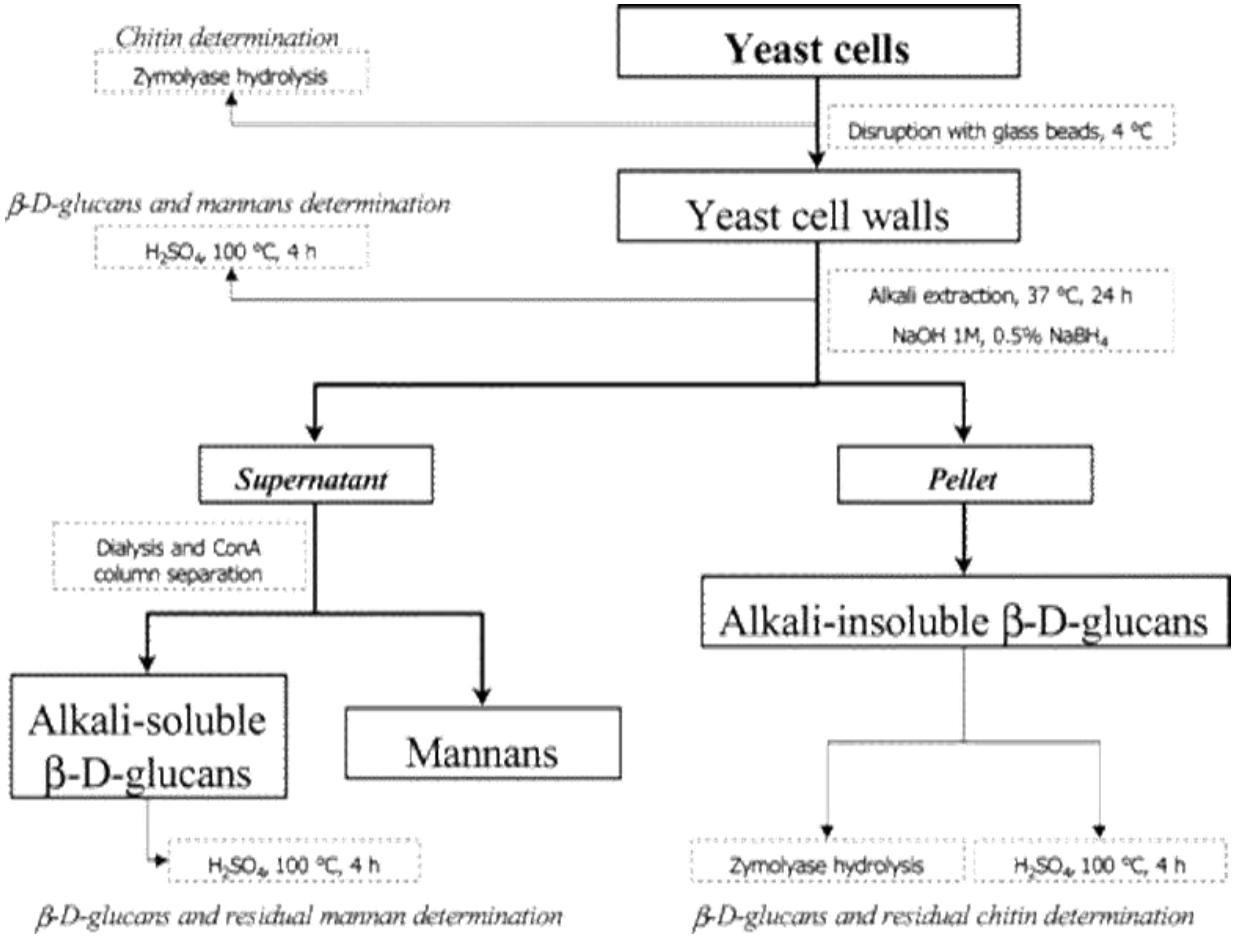

Figure 1.6 Schematic outline of the preparation procedure and analysis of yeast cell walls and extraction and analysis of alkali-soluble and alkali-insoluble fractions (Yiannikouris *et al.*, 2004)

1.10 BETA-GLUCANS

β-glucans are polymers of glucose linked by β-glycosidic bonds. There are many types of β-glucan with different structures (Bohn and BeMiller, 1995); β-1,3/1,6-glucan is from the cell wall of S. *cerevisiae* (figure 1.7). β-1,3/1,6-glucan is recognised by the immune systems of mammals, fish, and birds (Huff *et al.,* 2007). It is thought that the ß-glucans present in yeast cell wall stimulate macrophages through binding to macrophages via specific cell receptors, ß-1-3 or ß-1-6 links (Ferreira *et al.,* 2011). Macrophages are considered the first line of immune defence in birds (Qureshi, 1998; Ferreira *et al.,* 2011), as they form part of both the innate immune system and adaptive immune system (Qureshi, 2003; Ferreira *et al.,* 2011). It has been reported that β-glucan can have a number of stimulatory effects on the immune systems in pigs, poultry and some marine animals (Reynolds *et al.,* 1980; Bohn and BeMiller, 1995; Cleary *et al.,* 1999; Sohn *et al.,* 2000; Tzianabos, 2000; Suphantharika *et al*., 2003; Mao *et al.,* 2005; Huff *et al.,* 2006; Leblanc *et al.,* 2006; Morales-Lopez, 2009). Many authors suggest that *β*- glucans have their immunomodulating effects through increasing functional activity of macrophages and neutrophils (Reynolds *et al.,* 1980; Cleary *et al.,* 1999; Tzianabos, 2000; Guo *et al.,* 2003; Yun *et al.,* 2003; Cheng *et al*., 2004). Reynolds, (1980) reported a protective effect in a number of disease challenge studies. Lowry *et al.* (2005) indicated that *β*-glucan, present in the yeast walls, can decrease enteric *Salmonella enteritidis* in 7 day old chicken tissues and stimulate phagocytosis, bacterial killing, and oxidative burst in chickens after oral challenge. These improvements in defences against pathogens may result in the birds having better immunity and may contribute to the increased weight gain concurrently observed. More recently, Huff *et al.* (2006) demonstrated that *β*-glucans can maintain FCR of birds challenged with *Escherichia coli* respiratory disease, and numerically improved mortality.

It has been reported in a number of studies that supplementation with yeast β-1,3/1,6-glucan improved performance in broilers (Chae *et al.,* 2006; Zang *et al.,* 2008) and nursery pigs (Dritz *et al.,* 1995). This was also found by Huff *et al.* (2006) who observed an improvement in BW and FCR of broiler fed β glucans and challenged with *Escherichia coli*, compared to a negative control. However, Huff *et al.* (2006) also found that non-challenged (positive control) chickens fed β-glucans had a reduced body weight. This reduction in growth may be due to the energy expenditure associated with increased immune stimulation by the dietary yeast β-glucans and therefore increased energy used by the immune system, which is not needed in healthy birds and simply diverts energy away from growth. This may be the reason why Huff *et al.* (2006) found a negative effect on body weight effects when birds were not challenged.

The endemic presence of β-glucan in the cells wall of *S. cerevisiae,* means that most yeast cell wall products will contain some level of β-glucan (Morales-Lopez *et al.,* 2009). Mannan oligosaccharides are also derived from the cell wall of *S. cerevisiae,* suggesting that most yeast cell wall supplements are likely a mixture of mannan oligosaccharides, β-glucan and other cell wall components despite being commonly referred to as a MOS supplement. From this it is key to note that when a yeast cell wall product is fed to an animal, any observed effects could be a result of either or both MOS and β-glucan. The typical composition of yeast wall is 30 to 60% polysaccharides (15 to 30% of β-1, 3/1, 6-glucan and 15 to 30% of mannan sugar polymers), 15 to 30% proteins, 5 to 20% lipids, and no more than 5% of chitin (Aguilar-Uscanga and Francois, 2003; Ferreira *et al*., 2011). This unmeasured and undeclared portion of β-glucan within so called MOS supplements may explain some of the variability observed in bird response when such supplements are commercially applied.

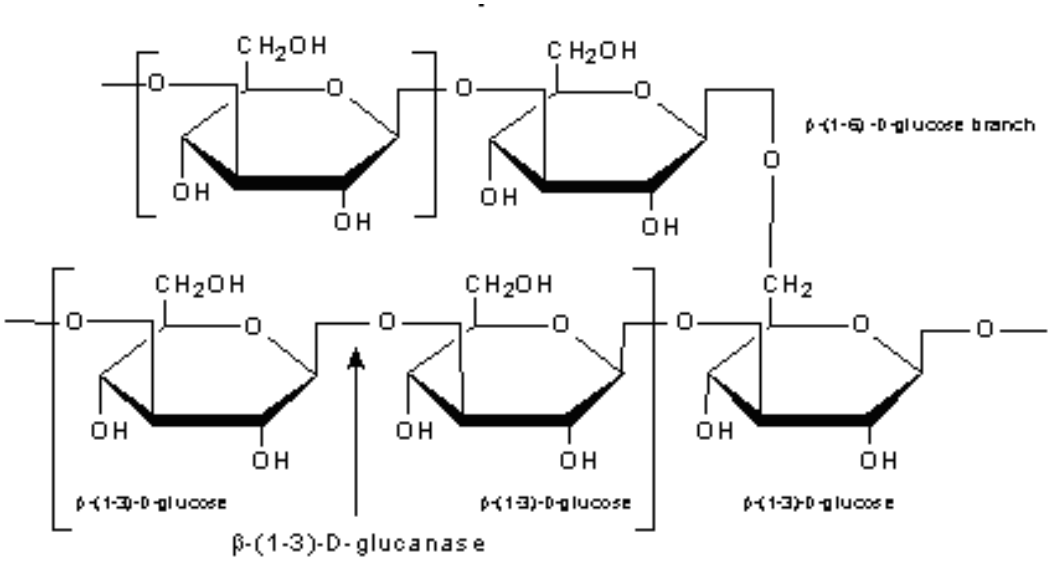

Figure 1.7 Yeast β- glucan (Sigma 2013)

1.11 SUPPLEMENTS BASED ON MANNAN RICH YEAST FRACTIONS

Actigen™ (Alltech) is a commercial form of mannan rich yeast fraction, which has been isolated from a MOS product. It is a soluble extract of the yeast cell wall of *S. cerevisiae* whereby the mannoproteins are separated from the other cell wall components. Actigen is a mannan rich fraction, which is produced to contain a greater concentration of mannan reactive units (alpha 1,3 mannan units) than previously produced yeast derived prebiotics such as BioMOS^{TM} (Alltech) (Mathis *et al.,* 2012). Alpha 1,3 mannans are involved in agglutination and recognition of phagocytic cells. It is therefore hypothesised that a NCF is more concentrated than traditional MOS supplements and therefore could have a similar action at a lower level of inclusion. At present there has been little work into the effect of NCF on poultry gut health and production, or their mode of action.

1.12 SUPPLEMENTS BASED ON BUTYRATE SALTS

Whilst prebiotic supplements modify the microflora in the birds GI tract, short chain fatty acids are thought to reduce pathogenic microorganisms without affecting the

beneficial intestinal microflora (Fernandez rubio et al., 2009). Sodium or calcium butyrates are the sodium or calcium salts of butyric acid, which is a short chain fatty acid (SCFA) (figure 1.8). Butyrate is a natural substance that can be found in the intestinal tract of birds, mainly produced by the bacterial fermentation of dietary fibre (Hamer *et al.,* 2008; Guilloteau *et al.,* 2010). In broilers, SCFA levels increase from untraceable levels to a stable concentration between 1 and 15 days, after 15 days the levels then stabilise (Guilloteau *et al.,* 2010). There are a number of reported beneficial effects of increasing levels of butyrate in poultry; butyrate can be used as an energy source (Jozefiak *et al.,* 2004), induce mucin synthesis, affect intestinal motility, regulates gene expression and inflammation (Hammer *et al.,* 2008; Louis and flint 2009), affects proliferation of intestinal cells (Kripke *et al.,* 1989; Friedel and Levine, 1992) and enhances disease resistance by inducing antimicrobial host defence peptide gene expression (Sunkara *et al.,* 2011).

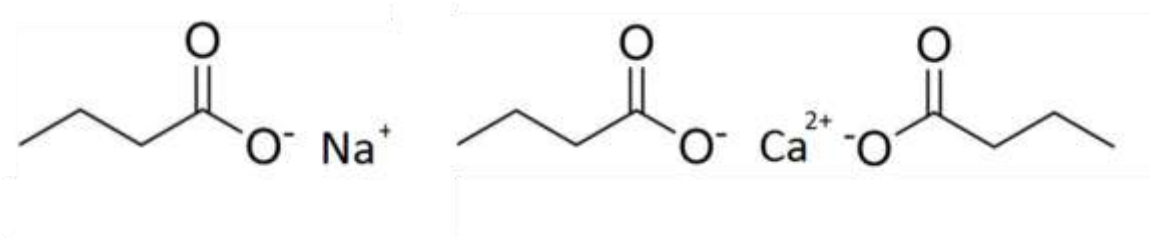

Figure 1.8 Sodium butyrate and calcuim butyrate chemical composition

Butyrates are fed as Na or Ca salt because they are easier to handle than the acid form in the feed manufacturing process (Guilloteau *et al.,* 2010). The Na or Ca butyrates can be covered in a fat coating, called microencapsulation, which protects the butyrate, allowing the molecules to be released further down the intestinal tract (Van Immerseel *et al.,* 2004; Fernandez Rubio *et al.,* 2009). Un-protected butyrate added to poultry feeds are metabolised in the upper GI tract, while microencapsulation reduces

dissociation of butyrate in the proventriculus, meaning more butyrate reached the distal part of the GI tract (Hu and Guo 2007; Zhang *et al.,* 2011). When butyrate is unprotected it is readily taken up by the GI tract epithelium, where it is metabolised for energy through the tricarboxylic acid (TCA) cycle (figure 1.9). This means that the host animal can get a significant source of energy from butyrate (Lawhon *et al.,* 2002; Guilloteau *et al.,* 2010), which may be one mechanism by which butyrate improves performance and gut integrity.

It is thought that sodium or calcium butyrate may have growth promoting effects due to their possible effects described, especially though providing energy for the epithelium and their antimicrobial activity. However, studies investigating butyrate growth promoting effects are inconsistent, the varied findings may be due to the type of environment and management conditions of the trial, particularly the microbial population to which the broilers or pigs were exposed. This suggest that broilers that are reared in a low pathogen environment do not have promoted growth due to butyrate supplementation, because the animals already have a high health status. A number of studies have found improvements in growth parameters in piglets when being challenged though weaning (Piva *et al.,* 2002; Manzanilla *et al.,* 2006; Le Gall *et al.,* 2009) and broilers when being challenged with coccidial oocytes, corticosterone or *E. coli* (Leeson *et al.,* 2005; Zhang *et al.,* 2011; Zhang *et al.,* 2011b) respectively, when feeding butyrate. However improvements in performance have also been seen in broilers when they have no recorded challenge (Smulikoska *et al.,* 2009). In contrast, other studies have found no effect of feeding butyrate on performance parameters in chickens (Leeson *et al.,* 2005; Zhang *et al.,* 2011b) and piglets (Biagi *et al.,* 2007; Tonel *et al.,* 2010) which were not challenged. It was also seen by Hu and Gao (2007) that feeding butyrate to unchallenged broilers negatively affected FCR.

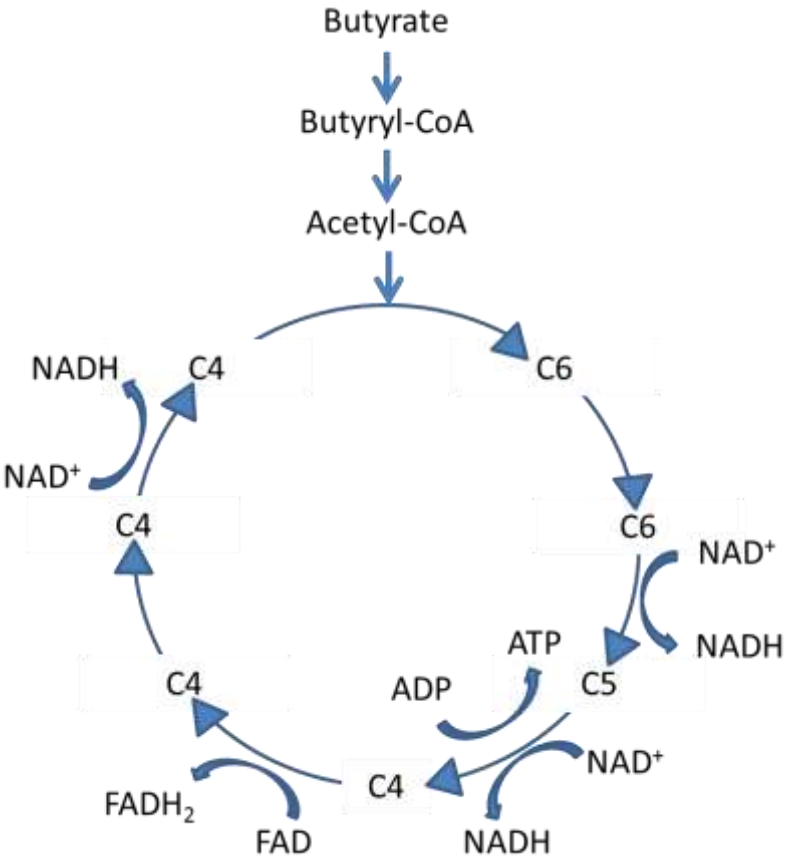

Figure 1.9 The tricarboxylic acid cycle (TCA cycle) is a series of enzyme-catalysed chemical reactions that form a key part of aerobic respiration in cells. (Donohoe *et al.*, 2011)

Sodium butyrate has been reported to prevent salmonella colonisation of the GI tract in broilers (Fernandez rubio *et al.*, 2009), layers (Van Immerseel *et al.*, 2005) and cultured chicken cecal epithelial cells (Van Immerseel *et al.*, 2004), by down regulating expression of bacterial genes involved in salmonella invasion of the GI tract (Gantois *et al.*, 2006). Sodium and calcium butyrate may also have antimicrobial activity due to their ability to acidify bacteria cell cytoplasm. Sodium and calcium butyrate can cross a bacterial cell wall when un-dissociated and dissociate in the more alkaline cell to H+ and anions inside, therefore bacteria that are unable to decrease intracellular pH

accumulate organic acid anions, causes a lowering in the pH of cytoplasm (Fernandez Rubio *et al.,* 2009).

Similarly to mannan oligosaccharides and other feed supplements, the feeding of butyrate seems to have variable effects on gut health parameters. Positive effects of butyrate on gut health may be through butyrate decreasing pathogenic load and therefore their harmful toxins, and/or increasing energy available to the epithelial cells. A lack of butyrate in the GI tract, whether from the diet or bacterial fermentation, can lead to nutritional deficiencies of the epithelium and result in atrophy (Hamer *et al.,* 2008). Manzanilla *et al.* (2006) found that pigs fed butyrate had a higher number of colonic goblet cells, whereas Le Gall *et al.* (2009) found no effect on intestinal goblet cell numbers. Manzanilla *et al.* (2006) also reported an increased jejunum crypt depth (CD) and Hu and Guo (2007) at 21 days found an improved VCR when feeding sodium butyrate. Leeson *et al.* (2005); Smulikowska *et al.* (2009) and Tonel *et al.* (2010) contradict these finding as they found no effect on gut morphology. As with supplements previously discussed, the variability reported in gut morphology when feeding butyrate may be due to differences in environmental and health statuses of the birds. Butyrate may only have an effect when the bird is not meeting its optimum performance due to some level of challenge.

The molecular composition of butyrate suggests that when fed to poultry, it may act as an acidifier, however the extremely low concentration of butyrate fed to poultry is not thought to be enough to change GI tract pH. This is supported by Manzanilla *et al.* (2006), Smulikowska *et al.* (2009), Zhang *et al.* (2011) and Czerwinski *et al.* (2012) who reported that GI tract pH was not affected when feeding sodium butyrate. However Biagi *et al.* (2007) did see an increase in pH of the cecum when feeding sodium butyrate.

There is little published data on the effects of feeding sodium or calcium butyrate on the digestive enzymes activity in broilers. Sileikiene *et al.* (2005) found that intra-ileal infusion of short chain fatty acids in pigs increased trypsin production. However this is contradicted by Le Gall *et al.* (2009) that found feeding sodium butyrate to pigs decreased the activity of chymotrypsin and trypsin in the GI tract.

In the poultry nutrition industry, there are numerous supplements that are marketed as having a regulatory effect on the immune status of the bird. The supplements are thought to provide a way to improve poultry health and production, sodium or calcium butyrate may mediate the immune system and result in performance improvements. Most studies looking into the effects of butyrate on intestinal inflammation are focused on humans, where it has been seen that SCFA can affect the recruitment of circulating leukocytes to the site of inflammation (Vinolo *et al.,* 2011). It has also been reported that SCFA can activate specific G protein coupled receptors that are involved in neutrophil migration, and suggested that SCFa may have an inhibitory effect on neutrophil migration (Vinolo *et al.,* 2011). Additionally, SCFA have been reported to affect human macrophage cytokine production and expression of Nitric oxide synthase (iNOS), Tumor necrosis factors (TNF)-α, and interleukin (IL)-6 induced by interferon (IFN)-γ, seeming to have an anti-inflammatory effect (Park *et al.,* 2007; Hamer *et al.,* 2008; Vinolo *et al.,* 2011). Similar to this, it has been noted in poultry that dietary sodium butyrate significantly inhibits the elevation of serum IL-6 and TNF- in chickens (Zhang *et al.,* 2011b)

1.13 THE EFFECT OF SUPPLEMENTS ON GUT TISSUE MORPHOLOGY

1.13.1 Length and weight

The addition of AGP has been reported to reduce intestinal weight and size. This effect is thought to be mediated through thinner intestinal villi, total gut wall and villus lamina propria as well as shortening of the gut (Thomke and Elwinger, 1998; Dibner and Richards, 2005). The reduction in the gut wall thickness has been used to explain the enhanced nutrient digestibility observed with AGP (Dibner and Richards, 2005). AGPs are also thought to decrease the intestinal muscularis weight, which is likely associated with a reduced need for gut motility to control microbial activity (Chee, 2008). A lower relative intestinal weight due to the reduced need for gut motility and a slower rate of epithelial cell renewal may have a favourable effect on basal energy expenditure and efficiency of nutrient utilisation (Chee, 2008). The energy spared from gut maintenance can subsequently be made readily available for growth (Iji *et al.*, 2001; Miles *et al.*, 2006; Chee, 2008). Increases in gut mass have been associated with inflammation following bacterial infection (Baurhoo *et al.*, 2007), and this response mechanism is supported by a study reporting that germ-free birds have thinner muscularis mucosae than conventional birds (Gordon and Bruckner-Kardoss, 1961).

The proposed theory that MOS influence the microbial population in the intestinal tract (Oyofo *et al.*, 1989; Sim *et al.*, 2004), suggests that MOS supplements may reduce intestinal weight by decreasing inflammation of the GI tract caused by pathogenic bacteria. A decrease in the weight of the intestine may be due to a decrease in the thickness on the muscularis. Ferket *et al.* (2002) reported that when turkeys were fed MOS, their muscularis thickness was significantly reduced. An increase in the intestinal weight has also been associated with inflammation following bacterial infection

(Baurhoo *et al.,* 2007). Therefore a reduction in the weight of the intestine may indicate a reduction in the volume of pathogenic bacteria in the GI tract. Chee (2008) concluded that MOS-supplemented birds have a thinner gut and therefore have a lower gut mass to maintain. This therefore would decrease the energy needed to maintain the gut and leave more energy for growth. However previous research is conflicting; Iji *et al.,* 2001 at 21 days, Yang *et al.,* 2007 at 28 days and Baurhoo *et al*. (2007) at 14, 28, 42 days all found that there was no effect of MOS on the weight of the intestinal tract. Chee (2008) found that MOS reduced the duodenal length at 28 days, in contrast Trevifio (1990) reported that birds fed oligosaccharide dried extract to 28 days had a longer relative length of jejunum, ileum and caeca than those fed on the control diet ($P< 0.05$). These observations demonstrate the variability in the effects reported when feeding a yeast cell wall product, which may be due to differences in the products or environments that the trials were performed.

1.13.2 Villus and crypt

The morphological structure of the GI tract offers key information to assessing gut health. Villus height (VH), width and CD are thought to reflect the health status of the GI tract of the bird. Longer, thinner villi are considered to indicate that the bird will have a better ability to absorb nutrients, due to the increased surface area (Gao *et al.,* 2008). Shorter villi and deeper CD have been associated with the presence of toxins (Yason *et al.,* 1987), decreasing the digestibility of nutrients (Zhang *et al.,* 2005). Increased CD is considered a negative indication of gut health because to compensate for villi atrophy the bird has to increase its tissue turn over and, as epithelial cells are produced in the crypts and migrate along the villi to the tip, it is thought that the higher turnover in the crypt cell cause it to become deeper (Gao *et al.,* 2008). Therefore shallower CD are considered a good indicator of gut health. Atrophy of villi can be due

to inflammatory response to pathogens and their toxins (Gao *et al.,* 2008). The higher turnover of epithelial cells during inflammatory response has an impact on the protein and energy requirement of the gut, leaving fewer nutrients for growth. VH and CD may be presented as a ratio of VH:CD as a single combined marker of overall intestinal health. However, an improved ratio can be achieved by solely the VH or CD changing; which may occur though different mechanisms.

Several studies have reported that MOS improves the overall intestinal health, when considered in terms of VH and CD. Iji, (2001) Santin, (2001), Sun, (2005); Zhang *et al*., (2005) and Baurhoo *et al.,* (2007) in broilers and Mourao *et al.,* (2006) in rabbits found that feeding yeast cell wall products increased VH over controls. The effect of MOS is not always shown throughout a study; Baurhoo *et al.* (2007) found that MOS improved VH at 28 days but not at 42 days and Baurhoo *et al.* (2009) again found that MOS increased duodenum VH only at 14 days. Conversely, Ferket *et al.* (2002), Yitbarek *et al.* (2012) and Sohail *et al*. (2012) in broilers and White *et al.* (2002) in pigs report no effect of feeding MOS on VH. There are also contradicting results over which region of the GI tract shows effects of MOS on VH. Santin *et al.* (2001) found that feeding yeast cell walls (likely to contain MOS type compounds) increased VH in the duodenum, jejunum, and ileum. However Baurhoo *et al.* (2009) found that feeding MOS increased VH on day 14 only in the duodenum and on day 24 VH was only increased in the jejunum.

Feeding of MOS has also been shown in some studies to decrease CD in broilers (Santin *et al*. 2001; Ferket *et al.* 2002). Santin *et al.* (2001) also found that villus:crypt ratio was increased in a dose dependent manner by dietary yeast cell wall treatment, but only in the jejunum. As with VH, the effects of MOS on CD are varied in terms of whether

there is any observed effect, which part of intestine is affected and at what bird age they have an effect. Iji *et al.* (2001), Zhang *et al.* (2005) Yitbarek *et al.* (2012) and Sohail *et al.* (2012) observed that MOS had no effect on CD in broilers; this was also found by Mourao *et al.* (2006) in rabbits. Changes in VCR may occur via a change in either or both VH and CD. Zang, (2005) found that birds fed yeast cell wall had increased VH and VCR but no change in CD; Ferket, (2002) found that MOS fed birds had decreased CD and increased VCR but no change in VH, whereas Santin, (2001) found that yeast cell wall fed birds had decreased CD and increased VH and VCR than that of the control. In contradiction, White (2002) found that the VH:CD ratios did not differ among dietary treatments when feeding MOS.

The term mannan oligosaccharides is used for a range of commercial products that are refined from yeast cell walls and are a variable mixture of mainly mannans and *β*-glucans. The terms yeast cell wall and MOS are used interchangeable in literature and the content of mannans and β-glucans depends on the extraction technique and yeast strain. The levels of mannans and β-glucans in a commercial product many be variable and might be contributing to the variability in the effects of MOS on VH and CD and therefore on the birds intestinal tract health.

The mechanism of action of MOS on intestinal microstructure is not fully understood and is complicated by the fact that many studies show that MOS had no effect on VH, CD or their ratio (Castillo *et al.*, 2008). However Bourhoo, (2009) suggested that the increase in villi length associated with MOS supplementation may be associated with increased lactobacilli and bifidobacteria colonization of broiler intestine. This is thought to be through the indigenous microbes stimulating vascularisation and villi development (Baurhoo *et al.*, 2007), but the exact mechanism underlying this effect is still not clear.

A more accepted theory is that the beneficial effect of MOS on intestinal morphology may be due to a reduction in the enterobacteria population (Castillo *et al.,* 2008), consequently reducing the presence of harmful toxins they produce. MOS is often attributed to reduce effects of pathogenic bacteria in the intestinal tract because it has been reported that MOS contains mannans that bind to pathogens with the mannose-specific type-1 fimbriae (Spring *et al.,* 2000). Therefore the pathogens cannot attach to the intestinal epithelial cells (Castillo *et al.,* 2008), and are evacuated from the intestine with other non-digested feedstuffs. This may reduce sloughing of villi in birds fed MOS as the pathogenic bacteria cannot colonise the GI tract and release toxins. These bacteria and their toxins can cause inflammation that in turn cause atrophy of the epithelial cells of the villi (Gao *et al.,* 2008), thus reducing the absorptive function of the gut through shorter VH and deeper CD (Yason *et al.,* 1987).

1.13.3 Goblet cells

Goblet cells are found in the epithelial layer along the villi of the avian gastrointestinal tract. Goblet cells are produced in the crypt cells and migrate up the villi to the tip, which takes 2 to 3 days (Uni *et al.,* 2003). Goblet cells produce and secrete mucin glycoproteins that make up part of the mucus layer, which protects the intestinal surface against damage by bacterial and environmental toxins, microorganisms and some coarse dietary components (Santos *et al.,* 2007). The mucus layer is also thought to be a medium for lubrication and transportation of nutrients between the lumen and the brush border (Uni, *el at.,* 2003; Smirnov *et al.,* 2004). While mucin can either be neutral or acidic, it has been suggested that acidic mucins in early life may be beneficial to the developing innate and intestinal immune system (Koutsos and Arias, 2006), through protecting against bacterial translocation in immature birds (Santos *et al.,* 2007).

There is no consensus on whether an increase in goblet cell numbers and area is considered an improvement in bird health. Increasing the number and area of goblet cells is thought to increase the volume of mucin stored in the GI tract and possibly its production (Brümmer *et al.*, 2010). Mucin is essential for a number of brush border processes, including facilitating the absorption of nutrients, enzymes carriage, lubrication and decreasing the binding and colonisation of pathogenic bacteria to the intestine (Blomberg *et al.*, 1993; Smirnov *et al.*, 2004). Therefore an increase in the level of mucin could have a beneficial effect on the first line of defence of the immune system (Baurhoo *et al.*, 2009) and the absorptive function of the gut. Conversely, overproduction of mucin may result in an negative effect, by increasing the mucus thickness on the GI tract wall to a level that might negatively affect the ability of nutrients to pass through to the gut epithelial to be absorbed (Smirnov *et al.*, 2004; Brummer *et al.*, 2010).

Baurhoo *et al.* (2007; 2009), Chee *et al.* (2010), Morales-lopez *et al.* (2010), Brummer *et al.* (2010) and Muthusamy *et al.* (2012) all found goblet cell numbers were increased by yeast cell wall product supplementation. However, an absence of response in goblet cell number to MOS was reported by Castillo *et al.* (2008) and Yitbarek *et al.* (2012), As with the other gut morphology measures, the effects of MOS on goblet cell number are variable between the different sections of the small intestinal tract and birds of different ages (Santos *et al.*, 2007; Baurhoo *et al.*, 2007; Castillo *et al.*, 2008; Baurhoo *et al.*, 2009). Published data on goblet cell area is limited; Uni and Smirnov, (2006) and Brummer *et al.* (2010) found that the area of goblet cells increased with supplementation of MOS.

An increase in goblet cell area is thought to show that the storage capacity of the goblet

cell for mucin has increased (Smirnov *et al*., 2005). The increase in mucin storage may suggest that the bird is more capable of forming a protective layer on the villi, thereby helping protect the intestine from damage caused by enteropathogens if there was a challenge from pathogenic bacteria (Smirnov *et al.,* 2006; Brümmer *et al.,* 2010). The mechanism by which MOS increases the goblet cell numbers or area is not clear, however one suggested mechanism of MOS on mucin production is through changing the gene expression of key genes through direct crosstalk between beneficial intestinal microbes and goblet cells, (Mack *et al.,* 1999; Freitas *et al.,* 2003; Smirnov *et al.,* 2005 Uni and Smirnov 2006; Chee, 2008).

1.14 PATHOGENIC COLONISATION OF THE GASTROINTESTINAL TRACT AND INHIBITION OF PATHOGENS

It is vital that a chick develops a normal intestinal microbial population and resistance against enteric pathogens, as the gut microflora plays a significant role in maintaining optimal immune defence (Lee *et al*., 2010). It has been found that when an animal is germ free it is highly susceptible to intestinal infection (O'Hara and Shanahan, 2006) and not able to establish an effective antibody response until its gut microflora has developed (Rhee *et al.,* 2004; Lee *et al.,* 2010). The development of the enteric microflora in a chick helps prevent a sudden growth of potentially lethal pathogens. The mechanism of protection from the development of enteric microflora is thought to be through competition for binding sites; competition for nutrients; production of antibacterial substances and immuno-stimulation (Perry, 2006).

One of the most important roles of the gut is providing a barrier between the internal and external environment, as the GI tract is the site of colonisation and invasion of many pathogenic bacteria (Patterson and Burkeholder, 2003; Beal *et al.,* 2006). The

mechanism by which a pathogen infects the GI tract and causes a disease can be through, the release of toxins and epithelial attachment, invasion, or local invasiveness and local toxicity. The way in which the host neutralises the organism depends on the type of mechanism of immunopathogenicity. When the mechanism is through toxins and epithelial attachment the host may only need to produce antibodies to neutralise them. However if the organism invaded the host cells, the host must destroy and degrade the organism by the cell-mediated immune response. When there is local toxicity, the host uses both antibodies and cell-mediated responses (Roitt *et al.,* 1998).

The first line of defence is the GI tract epithelial surface, which has a ciliary action, acidic pH and commensal bacteria as defences. The second line of defence is initially through the innate immune system, without B cells and T cells, and then through the adaptive immune system (Roitt *et al.,* 1998). Phagocytic cells that are not specific to a particular microorganism are part of the innate immune system, these include macrophages monocytes and neutrophils, which can internalise microorganisms and kill them (Qureshi, 1998; Ferreira *et al.,* 2011). Lymphocytes are part of the adaptive immune system and include B cells and T cells, B cell produce antibodies that are specific, whereas T cells can modulate B cell and antibiotic development, interact with phagocytic cells and recognise cells and destroy them. Immunoglobulins (Igs) are antibodies that are expressed and secreted by B cells, as part of the immune system. Igs have two functions; one binding to antigens; and a second having an effector function by activating complement pathways or binding to receptors. Binding to antigens causes agglutination, facilitates removal of toxins and blocks entrance of viruses into cells (McKay and Perdue 1993; Arnold *et al.,* 2006). Through the complement pathway, immunoglobulins can affect the inflammatory reaction of bringing leucocytes and immune molecules to a site of infection to eliminate it (Roitt *et al.,* 1998).

Pathogenic bacteria such as *Salmonella spp*., *Escherichia coli*, and *Clostridium perfringens* can bind to the GI tract, proliferate and cause an increased incidence of disease. Disease frequently only occurs at a sub-clinical level but this can lead to a decrease in the nutrients absorbed by the bird and therefore has economic consequences (Niewold, 2007; Jacob and Parsons 2009). Where the disease develops to a level that shows clinical signs in the bird, welfare is also severely affected. The development of a microflora population is very important to the bird as the presence of a balanced microflora helps to prevent pathogenic bacteria build up in the gut. *Bifidobacteria* and *Lactobacilli* are thought to be beneficial microorganisms for chickens, as they can create conditions unfavourable to the growth of pathogens, such as salmonella, enterobacteria and clostridia (Fernandez *et al.*, 2002). This is achieved by *Lactobacilli* and *Bifidobacteria* competing against pathogenic bacteria for nutrients and binding sites, and secreting compounds that are noxious to pathogenic bacteria. *Lactobacilli* secrete bacteriocins and *bifidobacteria* produce organic acids, such as butyrate, which can suppress the colonization of the intestines by pathogenic bacteria, thereby reducing the intestinal population of pathogens (Rolfe, 2000).

1.15 MOS AND THE INHIBITION OF PATHOGENIC COLONISATION

MOS is thought to work though competitively excluding pathogenic bacteria from the intestine. MOS has been shown to bind gram-negative pathogenic bacteria which use mannose-binding lectins to attach to the intestinal wall for colonization (Sims *et al*., 2004). The mannose-specific type-1 fimbriae that are found on some pathogenic bacteria such as *E. coli* can attach to MOS and then are excreted from the bird without being able to colonise the chicken gut (Oyofo *et al.,* 1989; Sim *et al.,* 2004). MOS are a non-digestible oligosaccharide and can therefore reach the lower intestinal area of the GI tract before fermentation and subsequent utilisation by only certain microbes such as

lactobacilli and *bifidobacteria* (Baurhoo *et al.,* 2007; Jung *et al.,* 2008; Jacob and Parsons, 2009). *Bifidobacteria* and *lactobacilli* are thought to be beneficial microorganisms for chickens, as they can create conditions unfavourable to the growth of pathogens, such as salmonella, enterobacteria and clostridia (Fernandez *et al.,* 2002). Therefore it is thought that feeding MOS may decrease pathogenic bacteria and increase beneficial microbes in the GI tract.

Spring *et al.* (2000) and Fernandez *et al*. (2002) found that birds challenged with Salmonella that were fed MOS had significantly reduced *Salmonella* colonisation. It has also been observed in broilers (Baurhoo, 2007) and turkeys (Zdunczyk *et al.,* 2005) that MOS reduced the population and therefore the growth of *E. coli*. Sim *et al.* (2004) also found a similar effect of MOS on *Clostridium perfringens* in turkeys. MOS has also been reported to have decreased the number of enterobacteria in pigs (Castillo *et al*., 2008). However, as with other measures, such as performance, in to the effects of MOS on poultry, studies are not consistent. Sims *et al.* (2004) reported that turkeys fed MOS did not differ from those fed a control when looking at the concentrations of *E. coli* and coliforms. White *et al.* (2002) reported that MOS had no effect on the numbers of coliforms, *E. coli* or *Clostridium perfringens* in pigs.

MOS effects on bacteria populations deemed beneficial are also variable. MOS has been reported to increase levels of *Bifidobacterium* and *Lactobacillus* in turkeys (Zdunczyk *et al.,* 2005) and broilers (Fernandez *et al.,* 2002; Baurhoo *et al.,* 2007). But this is contradicted by Jacob and Parsons, (2009) who found a significant decrease in caecal *Lactobacilli* populations in chicks fed yeast cell wall. Whereas Castillo *et al.* (2008) and Spring *et al.* (2000) found no difference when feeding MOS. When looking at these studies as a body of evidence no definitive conclusion can be made on how MOS affects

the bacterial colonisation of the GI tract.

1.16 YEAST CELL PRODUCTS AND IMMUNITY

Yeast cell products may contain several different components which affect immune response, including mannan oligosaccharides and β glucans. It has been shown that yeast cell products can modulate the immune system. However, it is presently not clear which component is causing the observed effect or the mechanisms behind it. Reynolds *et al.* (1980), Cleary *et al.* (1999) and Tzianabos, (2000) have all described the immunomodulating effects of *β*- glucan in mammals, reporting an increase in functional activity of macrophages and neutrophils.

It is also thought that improving the balance of microflora in the GI tract can positively affect the immune system, as some microflora can produce antibacterial substances and stimulate the immune defence system (Muir *et al.,* 2000). It has also been found that the right balance of microflora is vital for the development of acquired immune responses (Hooper and Gorden, 2001; Ewing, 2008) and that the bird may have a symbiotic relationship with some microorganisms through providing nutrients and defending against pathogenic bacteria (Gaskins *et al.,* 2008). The ingestion of prebiotics increases the numbers of *Lactobacillus* and *Bifidobacterium* in the GI tract. *Lactobacillus* has been reported to be an immunomodulator through affecting the levels of T cells and cytokines, which can increase resistance to disease (Muir *et al.,* 2002; Lee *et al.,* 2010). *Bifidobacterium* has also been reported to affect cytokine levels; Chichlowski *et al.,* (2007) found that feeding *Bifidobacterium* reduced intestinal mRNA of a pro-inflammatory cytokine (IL-1β and IL-6) and increased an anti-inflammatory cytokine (IL-10). Therefore feeding MOS may have an effect though this mechanism on the immune system.

Igs are found in the serum at varying concentrations depending on the immune status of the bird. IgG is the most abundant serum Ig, whereas the most abundant mucosal Ig, in terms of production, is IgA (McKay and Perdue 1993). IgM can also be found in considerable amounts in the serum (Arnold *et al.,* 2006). It has been seen that feeding MOS may affect circulating blood immunoglobulin level in poultry. However, as with other aspects of research investigating the effects of MOS on broilers, the effect of MOS on Ig levels in blood serum are variable. A number of studies have found no effect of feeding MOS on levels of IgG, IgM, and IgA in blood serum in broilers (Midilli *et al*., 2008; Janardhana *et al.,* 2009; Kim *et al.,* 2011), horses (Gurbuz *et al.,* 2010) and pigs (White *et al.,* 2002; Castillo *et al.,* 2008). These previous studies that showed no effect on Ig's level placed no disease or enivromental challenge on the animal, except Castillo *et al,* (2008) in which temperature stressed the pigs. In conditadiction, Savage *et al,* (1996) found feeding 0.11% MOS increased plasma IgG in turkeys and White *et al.* (2002) fingings that IgG levels in pigs fed yeast tended to be higher than controls. Conversely, Gao *et al* 2008 found no effect on serum IgG, increased IgM and decreased IgA levels when feeding yeast culture to broilers, whilst Sauerwein *et al,* (2007) found higher IgA serum concentrations in pigs fed yeast cell wall extract at 0.03% but not at 0.3%. Both Goa *et al,* (2008) and Sauerwein *et al,* (2007) found differences in immunoglobulins when feeding yeast cell wall or yeast culture, which will have different compositions and concentrations of mannans and β glucans to a more purified MOS as fed in the other studies.

1.17 EFFECT OF MANNAN OLIGOSACCHARIDES ON GENE EXPRESSION

Mannan oligosaccharides have been seen to have effects on broiler performance, gut morphology and the immune system, but the mechanisms by which these changes come

about are still unclear. The developing area of nutrigenomics may be used to increase understanding of how nutrients may affect gene expression and provide novel evidence on the molecular mechanisms behind the dietary modulation of immunity and metabolism. As many of the parameters measured in broiler studies are determined not only by genotype but environmental factors such as their nutrition, (Zhao *et al.,* 2004), this may allow nutritionists to modulate the diet of a host to positively affect immunity, physiology, and metabolism (Weaver *et al.,* 2011; Everard *et al.,* 2011; Yitbarek *et al.,* 2012). However, published literature on the effects of yeast cell wall-derived mannan-oligosaccharides on intestinal gene expression in broiler chickens is limited.

Xiao *et al.,* (2012) found that feeding MOS affected the gene expression of biological processes involving protein synthesis of broilers. However, is difficult to define the potential impacts of this change, due to the wide range of changes that can result from this process, as protein synthesis is critical for many functions. Xiao *et al.,* (2012) also reported effects of MOS on immune processes in broilers, such as Apolipoprotein (APO)-A1 (component of high-density lipoprotein) which may be involved in neutralising toxicity from gram-negative bacteria. In addition, it has been observed that MOS fed to broilers can up regulate the transcription of lysozyme, which is thought to be part of the first line of defence against bacteria (Xiao *et al.,* 2012). Another effect seen by Xiao *et al.,* (2012) in broilers when fed MOS is on oxidative phosphorylation, which is important in the energy released by nutrients to produce ATP.

It has been reported that *in ovo* feeding of MOS to chickens can increase levels of mucin (MUC)-2 mRNA, which is involved in production of mucin in the GI tract, as well as effecting the expression of brush-border enzymes amino peptidase and sucrase isomaltase (Cheled-Shoval *et al.,* 2011). This may mean that MOS improved the defence of the birds to bacterial population and improve digestive capacity but this link has yet to be demonstrated. Brennan *et al.,* (2012) also reported that feeding MOS to

chickens resulted in changes in gene expression that correspond to slower gut cell turnover and therefore increased energy preservation for growth. In addition, it has been demonstrated that MOS can affect the gene regulation of toll-like receptors (TLR2 And TLR4) (Cheled-Shoval *et al.,* 2011; Yitbarek *et al.,* 2012) and interleukin-12p35, and interferon-γ (Munyaka *et al.,* 2012; Yitbarek *et al.,* 2012) in broilers.

In contrast, Munyaka *et al.,* (2012) showed that expression of TLR2b and IL-6 was not affected by MOS in broilers, although Munyaka *et al.,* (2012) did find that MOS down regulated expression of TLR4 and IL-10. Down regulation of IL-12p35 and IFN-γ cytokines may be explained by the reduced expression of TLR4 and result in an anti-inflammatory effect. This could suggest that that birds fed MOS may have a reduced energy cost of their immune system, leaving more for growth performance (Huff *et al.,* 2006; Munyaka *et al.,* 2012).

1.18 DIGESTIVE ENZYMES AND MANNAN OLIGOSACCHARIDES

There is very limited published data on the effects of prebiotics on broiler digestive enzymes. Iji *et al.* (2001) reported that broilers fed MOS has increased specific activities of maltase, leucine aminopeptidase and alkaline phosphatase in the jejunum, but not the ileum. Matur *et al*. (2010) found that feeding *Saccharomyces cerevisiae* extract increased amylase and chymotrypsin activity in the pancreas of layers but had no effect on activities in the duodenum or jejunum. Studies in fish have also found increased activities in a number of different digestive enzymes when feeding prebiotics in Atlantic salmon (Refstie *et al.,* 2006), carp (Xu *et al.,* 2009) and Caspian roach fry (Soleimani *et al.,* 2012).

1.19 EFFECTS OF MANNAN OLIGOSACCHARIDES ON BIRD PERFORMANCE PARAMETERS

Body weight has been frequently reported to be improved by supplementation of MOS (Santin *et al.,* 2001; Benites *et al.,* 2008). Chee, (2008) also found that MOS significantly improved body weight gain, but only in the starter phase of growth. However, research in poultry supplementation with MOS is contradictory: Midilli *et al.* (2008) and Brummer *et al*. (2010), found body weight in poultry was not affected by the supplementation of MOS. These conflicting findings have also been shown to be the case in other species (Dimitroglou *et al.,* 2010). It has been reported previously that growth response in broilers to MOS is age-dependent with younger birds responding more than the older ones (Yang *et al.,* 2007; Chee, 2008). This may be due to the fact that the gut microflora of younger birds is more transient in nature and thus less mature than that of older birds. This suggests feeding of MOS may increase the rate of maturation of the gut microflora, leading to improved growth performance (Yang *et al.,* 2007).

A key measure of performance is the feed conversion ratio. MOS has been shown to improve FCR in numerous studies (Santin *et al.,* 2001; Zhang *et al.,* 2005; Chee, 2008). Any improvement observed in growth response after MOS supplementation may be linked with a series of improvements in intestinal mucosal integrity, gut microflora and local intestinal immunoresponse (Spring *et al.,* 2000; Iji *et al.,* 2001; Santin, 2001; Uni and Smirnov, 2006; Chee, 2008). It has been indicated in studies that there may be an optimum time for the supplementation of MOS to increase the efficiency of the birds. This may be due to the fact that the gut microflora of younger birds is more transient in nature and less established than in older birds and therefore more susceptible to colonisation by pathogenic bacteria. Therefore MOS may shorten the time required to

create a beneficial microflora population if it is offered early in life. MOS was reported to significantly increase body weight gain in birds between 1 and 21 days (Santin *et al*., 2001) and Chee, (2008) found that the MOS significantly improved body weight gain in the starter phase ($P<0.01$). However, similar studies have found variable early performance effects (Zhang *et al.,* 2005; Iji *et al.,* 2001; Sun *et al.,* 2005; Midilli *et al.,* 2008; Dimitroglou *et al*., 2010).

1.20 PROJECT AIMS

1.20.1 Overall Aims

The aim of this project is to investigate the effect of a novel feed supplement (Actigen^{TM}) on broilers by measuring a range of responses spanning whole bird performance to alteration in specific gene expression levels. By linking whole bird response to gross gut development, microscopic gut development, enzyme activity, immune function and gene expression, this project aims to clarify some of the mechanisms behind the observed effect of gut health supplements in broilers.

The overarching hypothesis of this project is that Actigen will improve bird growth performance and efficiency through improving gut health. The first posed mechanism supporting this hypothesis encompasses increasing the gut surface area and increasing digestive enzyme production. The second posed mechanism of this project is that feeding Actigen will improve the immune defences against pathogens whilst decreasing damage from adverse inflammation.

1.20.1.1 Specific objectives

To assess the effect of adding a novel feed supplement to broiler diets on performance.

To assess the effect of adding a novel feed supplement to broiler diets on the gross development of the digestive system.

To assess the effect of adding varying levels of a novel feed supplement to broiler diets on microscopic morphology of the gut.

To determine the effect of feeding a novel feed supplement on the expression and activity of broilers' digestive enzyme

To determine the effect of feeding a novel feed supplement on the expression and activity of broilers' immune cells

To compare the effect of feeding a novel feed supplement with butyrate salts both fed alone and in combination.

CHAPTER 2: GENERAL METHODS

2.1 INTRODUCTION

Three trials were completed, the first trial investigated the efficacy of the Actigen™ (NCF) at 200g/t, 400g/t and 800g/t inclusion rates on the performance of the broiler in a research facility with a commercial stocking density over 6 weeks with measurements of gut development at 6 weeks. The second trial evaluated the effect of NCF at 200g/t, 400g/t and 800g/t inclusion rates on the gut development and health of the broilers over the 6 weeks at 7, 14, 21, 28 and 42 days of age, whilst linking these to performance, and the third trial investigated the effect of NCF in combination with either of two organic acids, Ca butyrate or Na butyrate, on GI tract health parameters, digestive enzyme activity and immune parameters. Trial design and recorded parameters are given in table 2.1 with chapter numbers.

Table 2.1 Trial design and recorded parameters.

Trial details		Trial 1	Trial 2	Trial 3
Design	Trial duration	6 weeks	6 weeks	3 weeks
	Supplements	NCF	NCF	NCF, Ca and Na Butyrate
Recorded Parameters	Whole bird response - Performance	Chapter 3	Chapter 4	Chapter 5
	Gross gut development	Chapter 3	Chapter 4	Chapter 5
	Microscopic gut development	Chapter 3	Chapter 4	Chapter 5
	Digestive enzymes activity	Chapter 6	N/A	Chapter 6
	Immune function	Chapter 6	N/A	Chapter 6
	Gene expression	Chapter 6	N/A	N/A

2.2 DIET FORMULATION

Birds were fed starter diets from 1 to 14 days, grower from 15 to 28 days and finisher from 29 to 42 days. In Trials 1 and 2, a basal diet was formulated and made by Target Feeds (Coton, Whitchurch, Shropshire, UK), the basal diet for Trial 3 was formulated by Target but made in house at Nottingham Trent University. The dietary treatments were then added to the basal diet, which was free of antibiotics, according to a mixing protocol at Nottingham Trent University, for Trials 1 and 2 the dietary treatments were: 1) control diet (NCF free), 2) NCF at 200g per tonne, 3) NCF at 400g per tonne, and 4) NCF 800g per tonne. Dietary treatments for the Trial 3 were: 1) Control diet (NCF free), 2) NCF at 200g per tonne, 3) Calcium Butyrate (300g/t Globamax performant), 4) Sodium Butyrate (Starter:1000g/t, Grower:750g/t Adimix 30 Coated) 5) Calcium Butyrate (300g/t) plus NCF (200g/t), and 6) Sodium Butyrate (st:1000g/t, gr:750g/t) plus NCF (200g/t).

When adding the small amounts of supplements to the basal diet the following procedure was followed to ensure homogenous mixing. Firstly, three quarters of the basal diet was added to the ribbon mixer (Rigal Bennett, Goole, UK). Secondly, the supplement was then added to 2.5kg of basal diet, mixed in a bucket, then sprinkled over the top of the basal diet in the mixer. Thirdly, another 2.5kg of basal diet was then added to the bucket to rinse it and this with the last quarter of feed was added to the mixer and mixed for 5 minutes. Feed samples were taken for analysis of nutrient composition from the top and bottom of the mixer and then mixed together as a representative sample of the diet.

2.3 ANIMALS, HOUSING AND MANAGEMENT

All trials used Ross 308 male chicks aged 1 day supplied by P.D.Hook Hatcheries Ltd

(Cote, Bampton, Oxfordshire, UK). The birds were vaccinated at the hatchery with IB/H120 against infectious bronchitis. The birds were placed in pens (area = 0.43m^2), in a sanitary environment (biosecurity, disinfected facility on clean dry litter and good management). The room plan for each trial is given in figure 2.1. Birds were housed in an environmentally controlled room following a standard temperature regimen that gradually decreased from 31°C at bird height to 21°C by 21 days of the study. Heating was supplied as whole room heating using a mixture of fan heaters and heat lamps. Lighting was supplied by fluorescent strip lighting and on days 1 to 6 the birds had an increasing amount of darkness time during a 24 hour period. This started with 1 hour of darkness on day 1 and increasd consecutively until 6 hours of darkness on day 6. Then the lights were set to automatically give 6 hours of darkness from day 6 to end of trial, at the following times: 10pm-12am and 2am-6am. Ventilation was provided by a programmable fan and vent system. Ventilation was increased throughout the trial to meet the needs of the birds. Feed was provided on an *ad libitum* basis in a trough at the front of the pen and water was supplied by either bell drinkers or nipple drinkers.

All procedures undertaken in this thesis have been approved by the Nottingham Trent University College of Arts and Science Ethics committee, and did not require a licence under the Animal Scientific Procedures Act (ASPA, 1986).

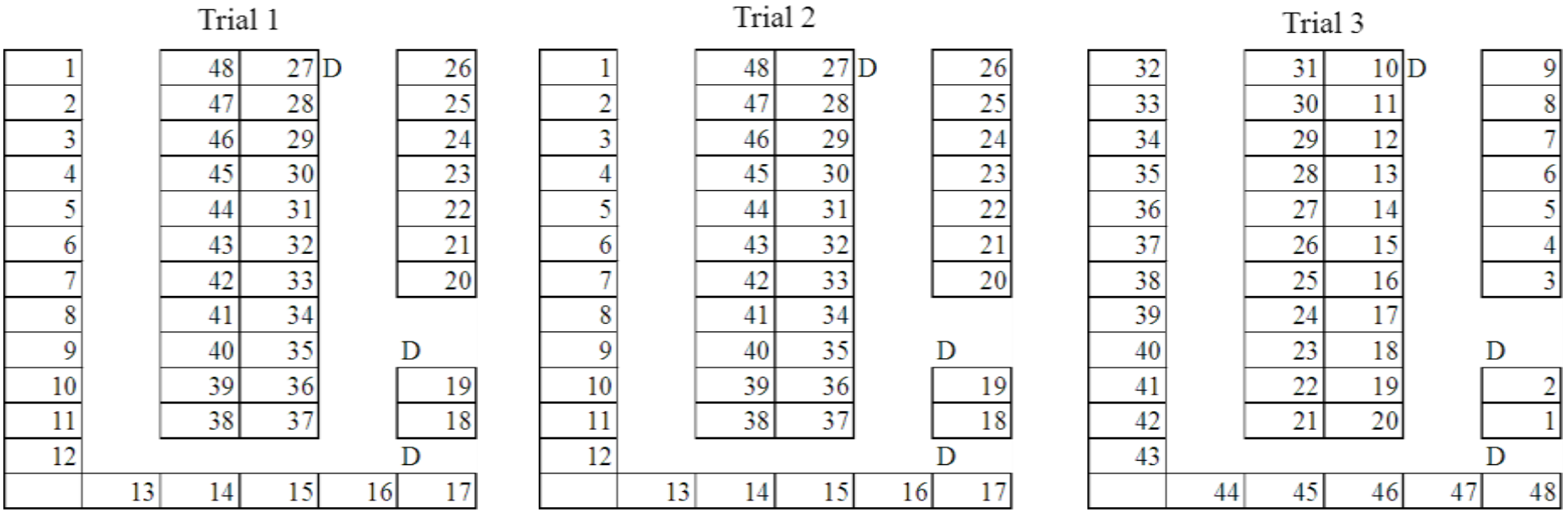

Figure 2.1 Room pen plan for each trial, D = door

2.4 TRIAL PERIOD

2.4.1 Feed Intake

Each pen of birds was fed from an individual pre weighed bag of diet. Feed intake was calculated weekly by weighing back uneaten feed. Troughs were positioned to minimize spillage and trays were placed under the troughs in the second trial to catch spilt feed in the first week.

2.4.2 Bird Weights

Birds were weighed by pen on day 1 and weekly, except on day 21 and 42 of Trial 1, when they were weighed individually. The birds were weighed using a top pan balance to one decimal place in grams (Mettler Toledo, Leicester, UK).

2.4.3 Foot Pad Scores

Foot pad scores were measured weekly using the method stated by Thomas, *et al.,* (2004) to determine foot pad dermatitis. Briefly, this involved giving the birds a score of 1-3, where a score of 1 is when there are no burns, a score of 2 is mild burns and a score of 3 is severe burns, as shown in figure 2.2.

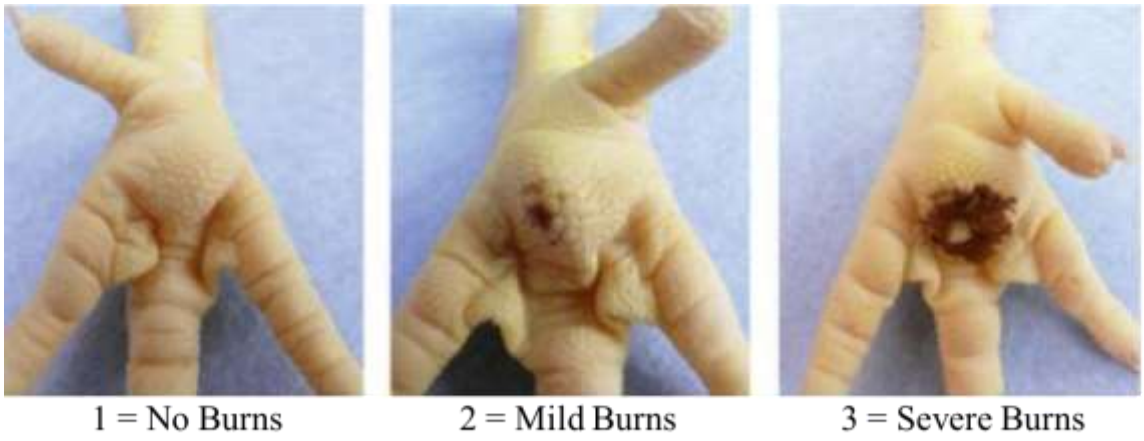

Figure 2.2 Foot pad score (Poultry World, 2013)

2.4.4 Litter Quality

Litter quality was quantified by collecting litter samples at 1, 13, 27 and 41 d to determine dry matter content via the method published by Thomas *et al.*, (2004). Samples were taken from 5 areas in the pen and mixed to get a more accurate measurement, as shown in figure 2.3.

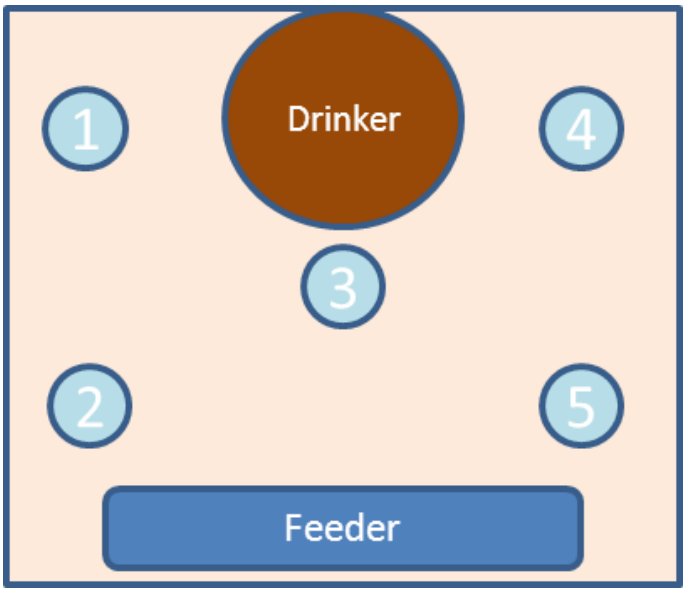

Figure 2.3 Diagram of areas of litter collection.

2.4.5 Histology

Histological samples were taken to measure the gut morphology of the bird, including villus height, crypt depth and goblet cells. One bird per pen was culled via cervical dislocation. The gastrointestinal tract was excised and the last 2.5cm of the descending section of the duodenum loop was removed before the loop around the pancreas and a 2.5cm section of the jejunum just before the Meckel's diverticulum were removed (figure 2.4). The sections of tissue were immediately flushed with cold phosphate buffered saline (PBS) with a 10ml syringe (pH 7.4). After flushing, the sections were fixed in Bouins fixative solution for 6 hours, and then transferred

to 70% ethanol for storage until further processing.

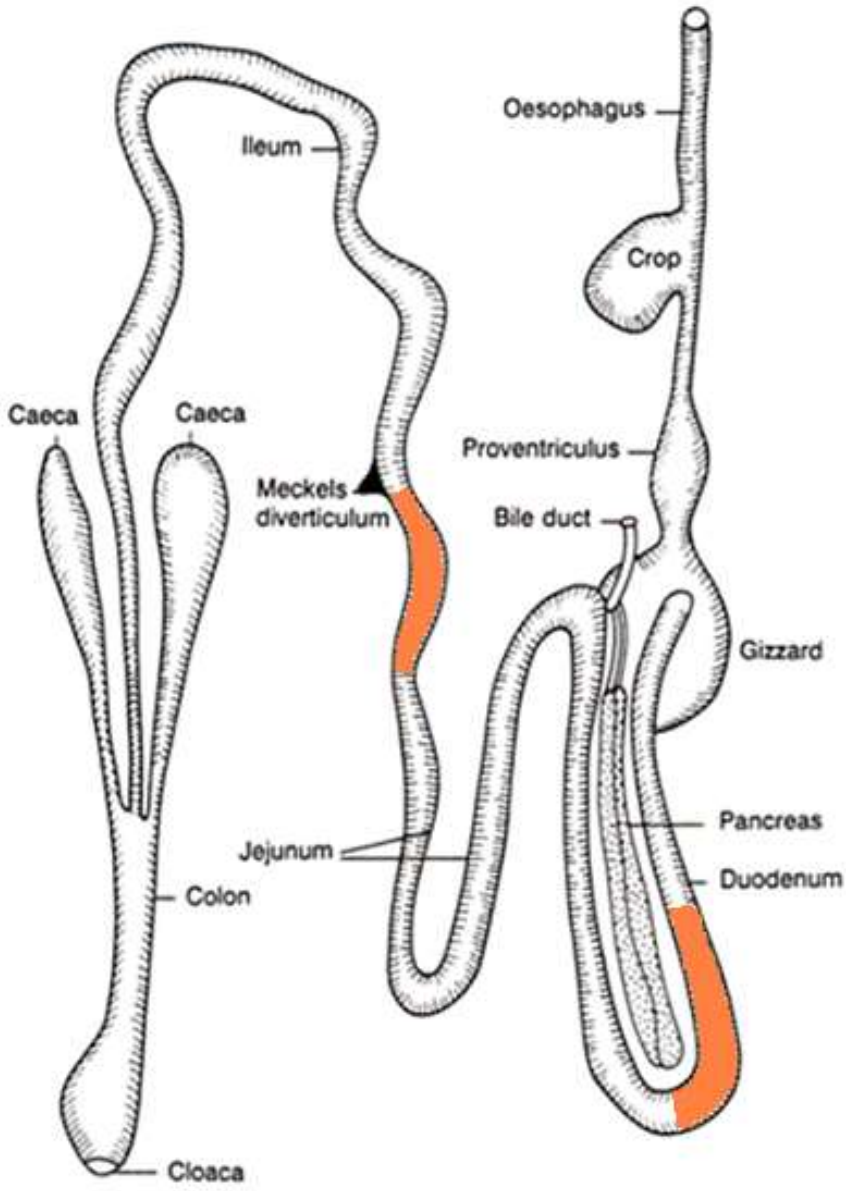

Figure 2.4. Diagram showing the sections of duodenum and jejunum removed for histology (Larbier and Leclercq, 1992)

2.4.6 Blood Serum

Blood was taken from the heart immediately after death with a 10ml syringe and transferred in a plain 15ml centrifuge tube. The blood was allowed to stand for 2-6 hours before it was centrifuged at 2000g for 10 minutes and the supernatant (serum)

was subsequently transferred in to a 10ml blood tube and stored at -20^{o}C.

2.4.7 Tissue for RNA extraction

A 0.2cm section of the duodenum was removed from the descending section of the duodenum loop before the loop around the pancreas and a 0.2cm section of the jejunum just before the Meckel's diverticulum were taken from the same bird and placed in a micro centrifuge tube containing RNA stabilization reagent, RNAlater (Qiagen, Venlo, Netherlands). This was then stored at 4^{o}C overnight and then stored in a -20^{o}C freezer.

2.4.8 Intestinal Weights

The whole intestine was removed from a second bird from each pen, the intestine was exercised from the start of the duodenum (including the pancreas) to the ileal-caecal junction. The combined and separate empty sections of the intestine, including duodenum, jejunum, ileum and pancreas, were weighed and their length measured

2.4.9 Digestive Enzyme Samples

The jejunum, duodenum and pancreas were removed from the bird, the digesta was squeezed into a single pot and frozen at -20^{o}C. The mucosa was scraped using a glass slide into a pot and frozen at -20^{o}C. The intestinal tissue left was placed into separate pots and frozen at -70^{o}C.

2.4.10 Viscosity

Digesta samples were removed from the first bird from the ileum for determination

of digesta supernatant viscosity. Samples were collected in pots, mixed and transferred to Micro centrifuge tubes for centrifugation at 10,000g for 10 minutes in a microcentrifuge (SLS, Goole, UK) The viscosity of the supernatant collected was then measured using a Brookfield DV-II+ Pro spinning disc viscometer with a cone and plate attachment (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA). Viscosity measurements were taken immediately after centrifugation of digesta at chicken body temperature (41^{o}C).

2.4.11 Intestinal tract pH

Immediately post euthanasia the gizzard, duodenum, jejunum, ileum and caeca were removed intact and a digital pH meter (Mettler-Toledo, UK) with a spear tip piercing pH electrode (Sensorex, California, USA) was directly inserted into the digesta in the lumen of the GI tract, whilst ensuring the pH electrode did not touch the walls, and the pH was recorded in duplicate. The pH meter was calibrated with pH standards as stated in the operation manual of the pH meter.

2.5 ANALYTICAL PROCEDURES

2.5.1 Determination of Dry Matter

The dry matter of the diets were determined as follows: 4-5g of finely ground sample were weighed accurately into pre-weighed crucibles and dried for 72 hours in an oven at 105^{o}C. The samples were then removed from the oven, cooled in a desiccator and reweighed.

2.5.2 Determination of Nitrogen/Crude Protein

Diets were analysed for nitrogen content using the kjeldhal method (AOAC official method 2001.11). Briefly, 1g of sample was digested in a tube with concentrated sulphuric acid, copper and selenium catalyst tablets at 450^{o}C for 45 minutes in a Kjeltec digestion unit (Foss Tecator, Cheshire, UK). The tubes were then cooled before distillation in a 2100 Kjectec distillation unit (Foss Tecator, Cheshire, UK), with sodium hydroxide. The resulting ammonia was expelled into a conical flask containing boric acid solution with an indicator, changing the liquids colour (orange to blue). Post distillation, the boric acid solution was titrated with a weak hydrochloric acid solution until the colour changed back to orange. Crude protein was calculated from nitrogen content by multiplication by a conversion factor of 6.25.

2.5.3 Determination of Ether Extract

Determination of percentage ether extractable oil content of the feed samples was performed by Soxhlet extraction, according to the method outlined by the Association of Official Analytical Chemists (2003.05). Briefly, 4.5g of the sample was refluxed with petroleum ether at 40-60^{o}C through soxhlet thimbles for 18 hours, the resulting petroleum ether and extracted fats were then heated to evaporate the petroleum ether leaving the fat. The percentage of ether extractable oil content of the feed samples was calculated as the total fat extracted divided by total original sample weight.

2.5.4 Determination of Ash

Dry samples of feed were weighed (W2) in to a pre-weighed ceramic crucible (W1)

before placing in a muffle furnace at 650^{o}C for 13 hours. Crucibles were allowed to cool in a desiccator and reweighed (W3), allowing percentage of ash to be calculated using the following equation ((W3-W1) / (W2-W1)) x 100 = % ash

2.5.5 Determination of Energy

Feed was analysed for gross energy content using a bomb calorimeter, as per the manufacures instructions (Instrument 1261, Parr Instruments, Illinois, USA). Pellets were made using a pellet press (Parr Instruments, Illinois, USA) and dried overnight at 105^{o}C. Pellets were weighed and placed in the bomb combustion chamber which is surrounded by water, a 10cm piece of fuse wire was placed as so to be touching the sample. After completely combusting the pellet in pure oxygen, the colorimeter measures the temperature change of the water and calculates the energy given off in KJ/kg.

2.6 HISTOLOGY

2.6.1 Tissue Fixation and Wax Embedding

A 2.5cm section from both the jejunum and duodenum was fixed in Bouins fixative solution for 6 hours, and then transferred to 70% ethanol for storage until further processing. The tissue was the removed from the 70% ethanol and a section of 5mm or less was cut and placed into a histology cassette. The intestinal samples were then dehydrated by immersion in an ascending series of aqueous alcohol solutions from 70% to 100% IMS. Following dehydration the tissue was cleared with histoclear, a substance which is miscible with both alcohol and paraffin wax. The sample was them placed in molten wax. The timings for this process are shown in table 2.2. After the samples had been infiltrated with wax, they were moved into warm metal moulds,

which was then filled with histology grade paraffin wax. The cassette lid was placed on top and the sample cooled immediately to 15°C to prevent the wax fracturing. The samples were left to harden completely and then removed from the moulds and stored at room temperature in cassette trays.

Table 2.2. Schedule of paraffin wax embedding of gut tissue

Chemical/Solution	Time
70% IMS	Overnight
70% IMS	1 Hour
85% IMS	1 Hour
95% IMS	1 Hour
100% IMS	1 Hour
100% IMS	1 Hour
Histoclear	1 Hour
Histoclear	1 Hour
60°C Paraffin Wax	1 Hour
60°C Paraffin Wax	1 Hour

2.6.2 Sectioning and Mounting

After embedded the samples were cooled in an ice bath and sectioned to a width of 8µm in the first trial and 6 µm for the second and third trials using a rotary microtome (1512 microtome, Leica, Wetzlar, Germany). To ensure that individual sections presented different villi, only every twentieth section was kept for mounting. At least 15 sections were collected for each sample. The cut sections were placed on slides, and then floated using 30% IMS on a hot plate set to 40°C to allowed the

samples to flatten. Once flattened on the slides the samples were left on the hot plate until they had dried and then stored in slide boxes.

2.6.3 Staining

Once mounted onto slides the samples were run down a series of alcohols to rehydrate them prior to staining. The staining method used is laid out in table 2.3. Once the staining procedure was complete, the slides were then protected with a cover slips. Cover slips had DPX synthetic resin applied to them and were carefully placed on the slides with gentle, even pressure being applied until no visible air bubbles remained. Finally, they were placed on a hot plate set to 40°C in order to dry and set and left overnight.

Table 2.3 Tissue staining process

Chemical/Solution	Time
Xylene	5 minutes
Xylene	5 minutes
100% IMS	2 minutes
100% IMS	2 minutes
70% IMS	2 minutes
Distilled Water	3 dips
Alcian Blue pH 2.5	30 minutes
Running Tap Water	5 minutes
Distilled Water	3 dips
1% Periodic Acid	5 minutes
Distilled Water	3 dips
Schiff's Reagent	20 minutes
Running Tap Water	5 minutes
Distilled Water	3 dips
Haematoxylin	3 minutes
Running Tap Water	5 minutes
Borate	2 dips
Running Tap Water	5 minutes
70% IMS	2 minutes
100% IMS	2 minutes
100% IMS	2 minutes
Xylene	5 minutes
Xylene	5 minutes

2.6.4 Microscopic analysis

Slides were analysed using an Olympus BX51 microscope fitted with an Olympus DP71 camera (Olympus, Pennsylvania, USA). Olympus Cell F software was used to identify and measure villus height, villus width and crypt depth. For each of the 15 sections cut from each sample two villi were measured for height and width and two crypts were measured for depth. Where possible the measurements were taken from opposite quarters and, if that was not possible, from different halves. Villi width was measured from either edge of the epithelial cells half way down the length of the villi, and height was measured from the highest epithelial cells across the top if the villi, down the centre, to the point where the crypt began. Crypt depth was measured from the centre of the opening down to the centre of the innermost epithelial cells.

2.7 GENE EXPRESSION

2.7.1 RNA Purification

The 0.2cm sections of tissue were taken out -20 ^{o}C freezer and placed in an ice box. The tissue was then removed from the RNA stabilization reagent (RNAlater, Qiagen, Venlo, Netherlands), and placed in a pestle and mortar with liquid nitrogen and homogenised. The RNA was then purified using a purification kit (RNeasy Plus Mini Kit, Qiagen, Venlo, Netherlands) via the method in table 2.4.

2.7.2 RNA Quality and Quantity

RNA quality and quantity was accessed using an Agilent 2100 bioanalyzer (Agilent, California, USA) at Alltech headquarters, (Nicholasville, Kentucky, USA) using the method in table 2.5. RNA Integrity Number (RIN) of 7 or above was required, which is a quality measure of the RNA.

Table 2.4 The RNA purification method using an RNeasy Plus Mini Kit (Qiagen)

Step	Procedure
1	Weigh 20-30mg of the homogenized tissue into 2ml micro centrifuge tubes.
2	Suspend the homogenised tissue in 600µl of RLT buffer.
3	Apply onto Qiagen shredder column.
4	Centrifugation at 10,000g for 2 minutes at RT.
5	Keep the flow through and discard the column
6	Transfer liquid to gDNA column
7	Centrifuge for 30s at 8000xg or 10,000rpm
8	Keep the flow through and discard the column
9	Add 600µl of 70% ethanol to flow through and mix by pipetting
10	Transfer 600µl liquid to RNeasy column.
11	Centrifuge at 10,000g for 15s at RT. Discard flow through
12	Repeat step 10. and 11.
13	Add 700µl of RW1 buffer to the same column and tube
14	Centrifuge at 10,000g for 1 minute at RT and flow through discarded.
15	Add 500µl of the RPE buffer to the same column and tube
16	Centrifuge at 10,000g for 1 minute at RT and flow through discarded.
17	Add 500µl of the RPE buffer to the same column and tube
18	Centrifuged at 10,000g for 1 minute at RT and discard flow through
19	Centrifuge at 10,000g for 3 minute at RT in a new tube
20	Put the column into a fresh 1.5ml tube (Provided with the kit)
21	Add 35µl of RNase free water (Qiagen) on to membrane
22	Let it stand for 3 min at RT
23	Centrifuge at 10,000g for 3 minute.
24	The flow through contain the RNA and were stored in -80ºC.

Table 2.5 RNA quality and quantity method using an Agilent 2100 bioanalyzer.

	Steps
	Preparing the Gel
1	Pipette 550µl of RNA 6000 Pico gel matrix into a spin filter.
2	Centrifuge at 1500g ± 20 % for 10 minutes at room temperature.
3	Aliquot 65µl filtered gel into 0.5 ml RNase-free microfuge tubes.
	Preparing the Gel-Dye Mix
1	Allow the RNA 6000 Pico dye concentrate to equilibrate to rt for 30 min.
2	Vortex RNA 6000 Pico dye concentrate for 10s, spin down and add 1µl of dye into a 65µl aliquot of filtered gel.
3	Vortex solution well. Spin tube at 13000g for 10 min at rt.
	Loading the Gel-Dye Mix
1	Put a new RNA 6000 Pico chip on the chip priming station.
2	Pipette 9.0 µl of gel-dye mix in the well marked
3	Make sure that the plunger is positioned at 1 ml and then close the chip priming station.
4	Press plunger until it is held by the clip.
5	Wait for exactly 30 seconds then release clip.
6	Wait for 5s. Slowly pull back plunger to 1ml position.
7	Open the chip priming station and pipette 9.0 µl of gel-dye mix in the wells marked.
8	Discard the remaining gel-dye mix.
	Loading the RNA 6000 Pico Conditioning Solution and Marker
1	Pipette 9.0µl of the RNA 6000 Pico Conditioning Solution in well marked CS.
2	Pipette 5µl of RNA 6000 Pico marker in all 11 samples and the marked well.
	Loading the Diluted Ladder and Samples
1	Pipette 1 µl of the heat denatured and aliquoted ladder in the well marked .
2	Pipette 1 µl of sample in each of the 11 sample wells. Pipette 1 µl of RNA 6000 Pico Marker in each unused sample well.
3	Put the chip in the adapter of the IKA vortexer & vortex for 1min at 2400 rpm.
4	Run the chip in the Agilent 2100 bioanalyzer within 5 min.

2.7.3 Microarray

After the 7 samples per treatment were assessed for RNA quality and quantity, the samples were assessed for gene expression. This was performed at Alltech headquarters, (Nicholasville, Kentucky, USA) using the standard protocol from the GeneChip® Expression Analysis Technical Manual (Affymetrix Inc., Santa Clara, CA). Double-stranded cDNA was first synthesised, purified, and used as a template for amplification and label by using the GeneChip Expression 3′-Amplification IVT Labeling Kit (Affymetrix Inc., Santa Clara, CA). Then, cDNA was used to synthesize cRNA, which was hydrolysed to produce fragmented cRNA in the 35- to 200-nucleotide size range for proper hybridisation. The fragmented cRNA was labelled and further hybridised to the Affymetrix GeneChip Poultry Genome Array. Gene microarrays were run to assessed gene expression using a GeneChip Fluidics Station, and were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, California) at Alltech headquarters (Nicholasville, Kentucky), using the GeneChip Chicken Genome Array. Samples were prepared using a GeneChip 3'IVT Express Kit. Fold change and t-tests were then run using GeneSpring GX, significance was set at $P < 0.05$.

2.8 DIGESTIVE ENZYMES

2.8.1 Enzyme Extraction

Intestinal samples were removed from the -80^{o}C freezer and kept in liquid nitrogen until processed. The intestinal sample was placed in a pre-cooled pestle and mortar with liquid nitrogen and ground until homogenized. The homogenized tissue was then mixed with cold phosphate buffer saline (pH 7.4) at 9 ml per 1g of tissue. This

was then centrifuged for 15 minutes at 10,000g. The supernatant was subsequently aliquoted and stored at -70^{o}C until analysed for enzyme activity.

2.8.2 Amylase

The extracted enzyme aliquots were taken out of the freezer and placed on ice. Whilst 2.0% (w/v) Soluble Starch Solution was equilibrated to 25^{o}C. 50µl of the extracted enzymes was added to 200µl of water, then 50µl of this diluted enzyme extract was added to an micro centrifuge tube. Then 50µl of the starch solution was added to the micro centrifuge tube, mixed and incubated for 3 minutes at 25^{o}C. Immediately after incubation 100µl of 3,5-Dinitrosalicylic Acid Solution was added to the micro centrifuge tube, at this point the enzyme extract was added to the blank rather than at the beginning. The micro centrifuge tube was then incubated at 100^{o}C for 15 minutes and then cooled to room temperature. Finally, 1ml of deionized water was added to the micro centrifuge tube, mixed and absorbance measured at a wave length of 530nm using a UV spectrophotometer (Cecil CE3410, Cecil intruments, Cambridge, UK). A standard curve was made using the concentration given in table 2.6 in replacement of the enzyme. Concentration of amylase was calculated using the following equation;

$$\text{Amylase activity (units/ml)} = M/1.08 \times df$$

(M = mg of Maltose released, df = dilution factors)

Table 2.6 Amylase standard curve.

Standard	Maltose (µl)	Distilled Water (µl)
1	1	40
2	2	48
3	3	47
4	4	46
5	5	45
6	10	40
7	20	30
8	30	20
9	40	10
10	50	0
11	0	50

2.8.3 Trypsin

While Nα-Benzoyl-DL-Arginine-p-Nitroanilide (BAPNA) was equilibrated to 37^{o}C, 100µl of enzyme extract was pipetted in to a micro centrifuge tube with 100µl distilled water. Then 700µl of BAPNA was added into the micro centrifuge tube and incubated at 37^{o}C for 10 minutes. Immediately after 10 minutes 100µl of 30% acetic acid was added to the micro centrifuge tube to terminate the reaction. For the blank, the enzyme extract was added to the micro centrifuge tube at this point rather than previously. Absorbance was measured at a wave length of 410nm using a UV spectrophotometer (Cecil CE3410, Cecil intruments, Cambridge, UK). 0.01 optical density unit represents 1 trypsin unit in 10ml. A standard curve was made by using the following solutions in replacement of the enzyme extract (table 2.7).

Table 2.7 Trypsin standard curve

mg trypsin/ml	Trypsin solution (ml)	0.001M HCL (ml)
200	1.000	0.000
170	0.850	0.150
140	0.700	0.300
110	0.550	0.450
80	0.400	0.600
60	0.300	0.700
50	0.250	0.750
40	0.200	0.800
30	0.150	0.850
25	0.125	0.875
20	0.100	0.900
15	0.075	0.925
10	0.050	0.950
5	0.025	0.975
0	0.000	1.000

2.8.4 Chymotrypsin

While N-glutaryl-L-phenylalanine p-nitroanilide solution (GPNA) was equilibrated to 25^{o}C, 100µl of enzyme extract pipetted into a micro centrifuge tube with 100µl water. Then 1000µl of GPNA was added into the micro centrifuge tube and incubated at 25^{o}C for 10 minutes. Immediately after 10 minutes, 200µl of 30% acetic acid was added to the micro centrifuge tube to terminate the reaction. For the blank, the enzyme extract was added to the micro centrifuge tube at this point rather than

previously. Absorbance was measured at a wave length of 410nm using a UV spectrophotometer (Cecil CE3410, Cecil intruments, Cambridge, UK). 1.0 optical density unit represents 1.02 x 10^{-5}M chymotrypsin. A standard curve was made by using the solutions in table 2.8 in replacement of the enzyme extract.

Table 2.8 Chymotrypsin standard curve

mg Chymotrypsin/ml	Chymotrypsin solution (ml)	0.001M HCL (ml)
1000	1.000	0.000
750	0.750	0.250
500	0.500	0.500
400	0.400	0.600
300	0.300	0.700
200	0.200	0.800
100	0.100	0.900
75	0.075	0.925
50	0.050	0.950
25	0.025	0.975
0	0.000	1.000

2.9 SERUM IMMUNOGLOBULINS

Levels of IgM, IgA and IgG were measured in serum of the bird using an enzyme linked immunosorbent assay (ELISA) micro titre plate for the detection of Chicken IgM, IgA or IgG in serum from Bethyl Laboratories Inc. (Montgomery, TX, USA).

2.9.1 IgM, IgA and IgG

Chicken IgM, IgA or IgG present in the sample was measured by their binding to anti-chicken IgM, IgA or IgG antibody that had been pre-adsorbed onto the surface of microtiter wells. After sample binding the plate was washed to remove unbound molecules, and a detection antibody was added to the wells to bind to the IgM, IgA or IgG. A strepavidin-conjugated horseradish peroxidase (SA-HRP) was then added to catalyze a colorimetric reaction with the chromogenic substrate TMB (3,3',5,5'-tetramethylbenzidine). The colorimetric reaction produces a blue product, which turns yellow when the reaction is terminated by addition of dilute sulfuric acid. Absorbance was measured at a wave length of 450nm using a UV spectrophotometer (Cecil CE3410, Cecil intruments, Cambridge, UK). Samples were run in duplicate, where there was a variation of more than 10% between replicates repeats were run. The absorbance was proportional to the amount of IgM, IgA or IgG present in the sample. Before all samples were run optimisation was performed to obtain the optimal dilution to use, this resulted in the following dilution being used; IgA – 1/1000, IgG – 1/100000 and IgM – 1/10000. The IgM, IgA or IgG concentrations in the test samples were quantified using a standard curve generated in parallel with the samples, which was ran on every plate in triplicate. Typical standard curves are given in appendix 1,2 and 3, the full methods used are also given in appendix 4.

2.10 STATISTICAL ANALYSIS

Through out all studies, one pen was taken to as a single unit for analysis. All data analysed via ANOVA was using the SPSS software version 12 or later for Windows. All data was analysed for normality by a Kolmogorov-Smirnov test. If the data was normally distributed a one-way analysis of variance (ANOVA) was performed, if the

data was not normally distributed a Kruskal-Wallis test was performed. Treatment means were separated using Tukey's post hoc test, and statistical significance was declared at $P < 0.05$. Regression analysis was performance using JMP Pro 11 to examine the effects of rate of inclusion of NCF on all paramenters measured and to establish linear and non linear contrasts via linear, quadratic and cubic partitioning of the treatment of sum of squares. Further linear regression analyses were also performed via Pearson correlations to examine relationships between live body weight and intestinal lengths and weights using SPSS software.

CHAPTER 3: TRIAL 1- INVESTIGATION OF THE EFFICACY OF ACTIGEN™ (ALLTECH) AT VARYING INCLUSION RATES ON THE PERFORMANCE OF BROILERS AND THEIR GUT DEVELOPMENT AT SIX WEEKS OF AGE.

3.1 INTRODUCTION

This project seeks to investigate the effects of a feed supplement, Actigen (NCF), from a whole bird response, down to alterations in gene expression. Prebiotics are an important feed supplement in the poultry industry, with mannan oligosaccharides (MOS) being one of the main prebiotics used in broiler diets. MOS products have been associated with a number of improvements in whole bird response through effects on performance and health (Santin *et al.,* 2001, Yang *et al.,* 2007). However there is little evidence as to whether Actigen (NCF), which is derived from MOS, will have a similar effect on broiler performance and health. This study was designed in conjunction with the industrial sponsor to investigate the effects of NCF on the whole bird performance and examine any associated histological change. This was achieved through a 6 week performance trial, with stocking density close to commercial stocking density (DEFRA, 2013) to mimic the environmental challenges experienced by the bird in a commercial situation, with weekly bird weights and feed intakes recorded. The manufacturer's (Alltech Inc.) recommended inclusion level of NFC ranges from 200-800g/t of NFC and currently lacks specific giuldance on dosage related to bird age. In order to identify the optimum inclution level, NFC was feed at 200g/t, 400g/t and 800g/t. This trial allowed for the evaluation of the ability of the poultry research facility at Nottingham Trent University to produce data relevant to the UK commercial situation. The effects of NCF on gut health was only

examined at 6 weeks, as to not affect the number of birds per pen, weekly weights or feed intakes of the birds, through altered stocking density.

3.2 AIM

The aim of this trial was to investigate the efficacy of NCF at 200g/t, 400g/t and 800g/t inclusion rates on the performance of birds in the poultry unit at Nottingham Trent University, and gut development of broilers at 6 weeks.

The hypothesis of this trial was that bird age and dietary inclusion level of NCF would differentially improve bird performance. Specifically, the highest rate of NCF inclusion would show greatest improvement in the starter phase, through accelated maturation of the gut.

3.3 MATERIALS AND METHODS

3.3.1 Bird Husbandry

A total of 240 one day old male Ross 308 birds from a flock of 50 weeks of age were obtained from P.D.Hook Hatcheries Ltd (Cote, Bampton, Oxfordshire, UK). They were placed in 48 pens, each containing five birds and fed one of four dietary treatments. The diets were allocated using a stratified random allocation.. There were four dietary treatments with 12 replicates for each treatment, made up of 12 small floor pens (area = 0.43m^2). Birds were kept with a stocking density aiming for a commercial stocking density of 30kg per m^2 at the end of the trial. The litter provided was fine dry sand on a concrete floor at a depth of 7cm and was topped up if necessary to maintain adequate environmental welfare.

3.3.2 Experimental Diets

The rations were based on the nutritional requirements for the strain of bird and contained approximately 50% wheat but no pentosan degrading enzymes, in order to provide a digestive challenge. The basal diet was free of antibiotics and manufactured using the formulation in table 3.1. The trial lasted 42 days with a three phase feeding programme; starter diets were fed from 1 to 14 days; grower diets were fed from 15 to 28 days and finisher diets were fed from 29 to 42 days. The four dietary treatments were 1) control diet (NCF free), 2) NCF at 200g per tonne, 3) NCF at 400g per tonne, and 4) NCF 800g per tonne.

3.3.3 Determined Parameters

Birds weight and feed intake per pen were recorded weekly. The weekly FCR was calculated using this information for each phase. Foot pad scores, litter dry matter and digesta viscosity were taken on day 42 as described in chapter 2. Two birds per pen with weights closest to the mean weight for the pen were selected and slaughtered on day 42. The whole intestine was removed from one of the birds to weigh and measure the length of the part of the intestinal tract, as described in chapter 2. From the second bird, tissue samples for histological examination were taken and fixed in Bouins. Samples for RNA and digestive enzymes analysis were taken and stored as per chapter 2. The feed was analysed for NCF level at The Bioscience Centre in Lexington, USA by an assay determining the amount of NCF in grams per tonne (Appendix 5).

Table 3.1. Composition of basal diet and calculated analysis of the basal diet

Item	Starter	Grower	Finisher
Ingredients (%)			
Barley	10.61	8.46	7.23
Wheat	50.00	55.00	60.00
Soybean meal, 48% CP	26.00	23.00	19.00
Full fat soybean meal	5.00	5.00	5.00
L lysine HCL	0.31	0.26	0.25
DL methionine	0.38	0.35	0.33
L threonine	0.14	0.13	0.14
Soya oil	4.00	4.50	4.75
Limestone	1.25	1.25	1.25
Monocalcium phosphate	1.50	1.25	1.25
Salt	0.25	0.25	0.25
Sodium bicarbonate	0.15	0.15	0.15
Premix*	0.25	0.25	0.25
Calculated analysis			
ME MJ/kg	12.78	13.04	13.2
CP%	21.75	20.59	19.1
Lys %	1.37	1.25	1.13
Met + Cys %	1.01	0.95	0.89
Ca	0.96	0.90	0.90
Available P	0.48	0.42	0.65

*Premix content (volume/kg diet): Mn 100mg, Zn 80mg, Fe 20mg, Cu 10mg, I 1mg, Mb 0.48mg, Se 0.2mg, Retinol 13.5mg,Cholecalciferol, 3mg, Tocopherol 25mg, Menadione 5.0mg, Thiamine 3mg, Riboflavin 10.0mg, Pantothenic acid 15mg, Pyroxidine 3.0mg, Niacin 60mg, Cobalamin 30µg, Folic acid 1.5mg, Biotin 125mg

3.3.4 Data Analysis

All data were analysed using the SPSS software for Windows; a one-way ANOVA was used. One pen was used as a unit for analysis. Treatment means were separated using the Tukey post hoc test, and statistical significance was declared at $P < 0.05$. Regressions analysis was performed to identify any dose response effects.

3.4 RESULTS

3.4.1 Body weight and Live weight gain

The mean live body weights per bird for the four diets over the 6 weeks are shown in table 3.2. Table 3.2 shows that the mean body weight per bird on days 14, 21, 28, 35 and 42 were significantly different between treatments. When comparing body weight to breed target, the control birds were 400g behind by 42 day, whereas the birds fed NCF were on target or approaching target. This indicates that the birds were under physiological pressure through environmental challenge, which was over come by NCF supplementation. On day 14, 21, 28 and 35, diets including 200g/t and 800g/t of NCF significantly increased live weight over the control (P<0.001). On day 42, it was observed that NCF at 200g/t increased live weight over the control (P=0.005). In summary, from week two onwards the inclusion of NCF at 200g/t in the diet resulted in an increase in the body weight of the birds compared to the control. No effect of diet on body weights were observed at 7 days.

Table 3.2. Effect of NCF in broiler diets on the mean weekly body weight (g/bird)

Age	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
1 d	43.33	43.90	43.58	43.05	0.34	0.851	0.694
7 d	102.44	114.20	106.68	107.05	2.02	0.227	0.893
14 d	216.99 ^{C}	302.66 ^{A}	249.85 ^{BC}	290.68 ^{AB}	8.15	<0.000	0.030^{C}
21 d	524.63 ^{C}	741.19 ^{A}	599.76 ^{BC}	680.33 ^{AB}	20.42	<0.000	0.091
28 d	1026.17 ^{A}	1380.77 ^{A}	1198.38 ^{AB}	1287.23 ^{A}	31.17	<0.000	0.031^{Q}
35 d	1804.11 ^{C}	2179.73 ^{A}	1943.19 ^{BC}	2112.13 ^{AB}	36.98	0.001	0.023^{Q}
42 d	2593.70 ^{B}	2927.65 ^{A}	2654.53 ^{B}	2841.69 ^{AB}	38.69	0.005	0.073

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

The mean weekly live weight gains per bird for the four diets over the 6 weeks are in table 3.3. Table 3.3 illustrates that in the second, third and fourth weeks significant differences between treatments in mean live weight gain per bird are recorded. In week two it was shown that diets including 200g/t and 800g/t of NCF significantly increased live weight gain over the control ($P<0.001$). In week three it was demonstrated that diets including 200g/t of NCF significantly increased live weight gain over the control ($P=0.007$). In week four it was recorded that diets including NCF at all three level of inclusion significantly increased live weight gain over the control ($P<0.001$). Regression analysis of NCF rate of inclusion (ROI) against both body weight and weekly weight gain showed some complexcity in bird response. On day 14 there was a cubic response to increasing NCF levels for both parameters, but at later ages all obsevered significant responses were quadratic.

Table 3.3. Effect of NCF in broiler diets on the mean live weight gain (g/bird).

Age	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
7 d	59.11	70.30	63.10	64.00	1.97	0.150	0.665
14 d	114.55 ^{B}	188.45 ^{A}	141.26 ^{B}	183.63 ^{A}	6.61	<0.001	0.007^{C}
21 d	293.64 ^{B}	438.81 ^{A}	336.15 ^{AB}	281.66 ^{AB}	16.15	0.007	0.186
28 d	497.93 ^{B}	639.58 ^{A}	598.61 ^{A}	604.80 ^{A}	12.45	<0.001	0.009^{Q}
35 d	739.33	782.17	761.16	794.36	10.04	0.232	0.077^{Q}
42 d	751.03	747.55	711.13	681.35	13.49	0.226	0.482

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

3.4.2 Feed Intake

The mean weekly feed intake per bird for the four diets over the six weeks is shown in table 3.4, demonstrating that there was significant differences between the treatments in weeks two, three, four and five. However when further *post hoc* tests were performed significance was lost in week two and four. In weeks three and 5 birds fed 200g/t had higher feed intakes that the control birds. Regression analysis of NCF ROI against feed intake showed no dose response.

Table 3.4. Effect of NCF in broiler diets on the mean weekly feed intake (g/bird).

Attribute	Age	Dietary treatment				SEM	P value	Regression
		Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Feed	7 d	131.99	142.41	150.00	136.47	4.66	0.565	0.581
intake (g)	14 d	212.39	260.44	225.05	260.98	7.61	0.041	0.180
	21 d	569.43 ^{B}	688.72 ^{A}	616.65 ^{AB}	617.17 ^{AB}	14.23	0.024	0.781
	28 d	899.28	1030.96	976.69	1024.13	18.93	0.041	0.306
	35 d	1228.65 ^{B}	1372.97 ^{A}	1270.86 ^{AB}	1359.00 ^{AB}	20.08	0.025	0.307
	42 d	1395.48	1439.34	1369.34	1404.59	13.05	0.302	0.754

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$.

3.4.3 Feed Conversion Ratio

The mean weekly feed conversion ratio (FCR) per bird for the four diets over the 6 weeks is shown in table 3.6. Table 3.6 illustrates that the mean FCR per bird between diets for the second week was significantly different. In the second week it was shown that diets including 200g/t and 800g/t of NCF significantly decreased the FCR over the control (P=0.001). Simularly to body weight response, regression analysis of NCF ROI against FCR showed that on day 14 there was a cubic dose response to increasing NCF. Table 3.7 reports that the mean FCR between diets over the three feeding phases, during the first phase (week one and two) and the second phase (week three and four) there was significant differences between the treatments. In the first phase of feeding it was recored that diets including 200g/t and 800g/t of NCF significantly decreased the FCR over the control (P=0.003). In the second phase of feeding it was shown that all diets containing NCF significantly decreased the FCR over the control (P=0.011). When investigating the whole six week trial period the FCR was not significantly altered by dietary treatment (table 3.7).

Table 3.6. Effect of NCF in broiler diets on the weekly feed conversion ratio.

Age	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
7 d	2.19	2.10	2.46	2.07	0.08	0.231	0.800
14 d	1.92 ^{A}	1.40 ^{B}	1.68 ^{AB}	1.42 ^{B}	0.05	0.001	0.019^{C}
21 d	1.90	1.72	1.76	1.60	0.05	0.153	0.054
28 d	1.83	1.62	1.60	1.66	0.03	0.058	0.091
35 d	1.69	1.70	1.68	1.71	0.02	0.903	0.901
42 d	1.86	1.94	1.94	2.00	0.02	0.233	0.068

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

Table 3.7. Effect of NCF in broiler diets on the feed conversion ratio

Attribute	Age	Dietary treatment				SEM	P value
		Control	NCF 200g/t	NCF 400g/t	NCF 800g/t		
FCR	Wk1+2	2.03 ^{A}	1.59 ^{B}	1.91 ^{AB}	1.61 ^{B}	0.05	0.003
	Wk3+4	1.85 ^{A}	1.61 ^{B}	1.63 ^{B}	1.63 ^{B}	0.03	0.011
	Wk5+6	1.76	1.81	1.84	1.82	0.02	0.524
	Wk1-6	1.80	1.73	1.76	1.74	0.01	0.308

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

3.4.4 Litter Dry Matter

The dry matter of the litter was measured as a measure of litter quality, the higher the

dry matter content the better the litter quality. The mean dry matters of the litter per treatment over the six weeks are shown in table 3.8. Table 3.8 reports that the mean dry matter of the litter per treatment were not significantly different. However fresh sand was added to pens when litter deteriorated, to keep to welfare standards set by Nottingham Trent University.

Table 3.8. Effect of NCF in broiler diets on the mean dry matter of the litter (%)

Attribute	Age	Dietary treatment				SEM	P value	Regression
		Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Litter Dry	d 1	99.80	99.76	99.88	99.80	0.02	0.523	0.629
Matter (%)	d 13	98.62	98.51	98.53	98.02	0.10	0.151	0.066
	d 27	75.42	68.92	68.99	65.07	1.89	0.280	0.088
	d 41	58.62	62.88	67.12	57.88	1.46	0.085	0.837

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

3.4.5 Viscosity

The viscosity of the digesta was measured as a measurement of sticky dropping, which can cause poor litter quality. The mean viscosity of digesta per treatment is reported in table 3.9 and shows that there was no significant effect of NCF on the viscosity of the digesta.

Table 3.9. Effect of NCF in broiler diets on the mean viscosity of digesta

Attribute	Age	Dietary treatment				SEM	P value	Regression
		Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Viscosity	d 42	3.20	3.02	2.84	2.93	0.10	0.617	0.135

3.4.6 Foot Pad Score

Foot pad dermatitis in chickens is a condition that causes lesions on the surface of the footpad. The lesions can be scored to determine the level of foot pad dermatitis, which can be caused by poor litter quality. All foot pad scores throughout the treatment groups for the six weeks were all one. A score of one represents no tissue damage, therefore there was no foot pad dermatitis in this trial. However it should be noted that extra bedding was added as required to minimise risk of birds welfare becoming compromised.

3.4.7 Intestinal Weight and Length

The mean weight of the intestine per treatment is reported in table 3.10. Table 3.10 demonstrates that there was no dose response of NCF on intestinal weights and no significant effect of NCF on the mean weight of the intestine or pancreas. However when examining the weight of the intestines relative to bird body weight, it was recorded that the relative duodenum weight and total intestinal weight was significantly greater in control fed birds than birds fed 200g/t of NCF (P=0.032, P=0.010 respectively). Relative ileum weight was also significantly greater in control fed birds than birds fed 200g/t and 400 g/t of NCF (P=0.005).

Table 3.10. Effect of NCF in broiler diets on the weight of the intestine (g)

Attribute	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Duodenum wt	12.83	11.97	11.90	12.57	0.24	0.435	0.936
Duodenum wt /body v	0.50 A	0.41 B	0.46 AB	0.44 AB	0.01	0.032	0.242
Jejunum wt	27.90	29.71	27.86	27.03	0.41	0.111	0.238
Jejunum wt /body wt	1.14	1.01	1.06	0.98	0.02	0.096	0.051
Ilium wt	24.61	23.17	21.91	23.32	0.45	0.181	0.385
Ilium wt /body wt	0.98 A	0.81 B	0.80 B	0.86 AB	0.02	0.005	0.071
Total Intestinal wt	65.52	62.94	61.41	63.05	0.78	0.344	0.445
Total Intestinal wt/bod	2.58 A	2.14 B	2.35 AB	2.28 AB	0.05	0.010	0.065
Panc. wt	4.77	4.88	4.80	4.97	0.11	0.929	0.566
Panc. Wt /body wt	0.19	0.16	0.18	0.17	0.00	0.151	0.5304

Differing superscript letters within one week denote means are significantly different at $P < 0.05$. Intestinal weight to body weight units are g/100g

The mean lengths of the intestine per treatment are reported in table 3.11, demonstrating that the relative duodenum length was significantly greater in control fed birds than the birds fed 800g/t of NCF (P=0.038). However, further examination through regression analysis indicated a complex response; relative duodenum length showed a significant cubic response to NCF ROI. There was also a significant dietary effect of 200g/t of NCF increasing the length of the jejunum over the control but no overall relationship to ROI of NCF.

Table 3.11. Effect of NCF in broiler diets on the length of the intestine (cm)

Attribute	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Duodenum length	32.32	32.40	31.83	31.33	0.49	0.865	0.224
Duodenum/ body wt	1.32 ^{A}	1.14 ^{AB}	1.22 ^{AB}	1.11 ^{B}	0.03	0.038	0.050^{C}
Jejunum length	70.48 ^{B}	78.70 ^{A}	74.97 ^{AB}	72.76 ^{AB}	0.94	0.011	0.831
Jejunum/ body wt	2.86	2.69	2.87	2.57	0.05	0.140	0.176
Ilium length	75.55	78.80	78.08	74.23	0.92	0.264	0.562
Ilium/ body wt	3.02	2.77	3.00	2.69	0.06	0.090	0.129
Total length	177.89	189.90	184.88	178.31	1.84	0.056	0.446
Total/ body wt	7.21	6.66	7.09	6.31	0.13	0.043	0.095

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

3.4.8 Gut Morphology

Intestinal villus height and crypt depth were measured at 42 days to investigate the effects of NCF on gut health. Table 3.12 illustrates that there was no effect of NCF on the villus height, villus width, crypt depth or villus to crypt ratio in the duodenum or jejunum at 42 days.

Table 3.12. Effect of NCF on the gut morphology of the duodenum and jejunum (µm)

Attribute	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Duodenum							
Villus Height	2818.8	2790.8	2891.2	2703.9	52.6	0.682	0.718
Villus Width	274.3	264.8	262.0	281.9	5.5	0.575	0.728
Crypt Depth	209.4	209.1	210.3	212.9	5.6	0.995	0.429
Villus/Crypt Ratio	15.1	13.6	14.2	12.7	0.47	0.361	0.309
Jejunum							
Villus Height	1283.8	1284.5	1379.2	1314.8	25.5	0.528	0.345
Villus Width	246.7	235.2	222.7	248.6	5.5	0.351	0.940
Crypt Depth	162.5	151.1	151.6	153.3	2.4	0.304	0.882
Villus/Crypt Ratio	8.1	8.6	8.9	8.4	0.17	0.466	0.373

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$)

3.4.9 Goblet Cell Numbers

Investigation of goblet cell number and area in the duodenum (table 3.13), revealed that the diets including NCF did not significantly affect goblet cell area, goblet cell number per 165µm of villi or goblet cell measurements as a ratio. However in the jejunum 800g/t NCF significantly ($P<0.05$) increased goblet cell area over the control, but no other parameters were altered in the jejunum. Both goblet cell area villi was seen to have a cubic dose response, whereas goblet cell area per 165 µm has a linear response to ROI of NCF.

Table 3.13. Effect of NCF on the goblet cell of the duodenum and jejunum (μm^2)

Atribute	Dietary treatment				SEM	P value	Regression Pvalue
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			
Duodenum							
Goblet Cell Area (μm^2)	59.2	69.4	58.3	62.6	2.5	0.263	0.927
N° of Goblet Cell per 165µm	11.8	11.4	11.7	11.9	0.2	0.860	0.338
Goblet Cell Area per 165µm	683.1	747.0	664.4	719.3	23.6	0.314	0.582
Jejunum							
Goblet Cell Area (μm^2)	67.6 ^{B}	68.3 ^{B}	73.8 ^{AB}	82.8 ^{A}	2.2	0.041	0.047^{C}
N° of Goblet Cell per 165µm	12.4	12.2	12.0	11.8	0.2	0.741	0.444
Goblet Cell Area per 165µm	847.0	868.0	882.1	988.4	21.9	0.089	0.025^{L}

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

3.4.10 Mortality

Mortality for this study is show in table 3.14; there were no significant differences between treatments.

Table 3.14. Effect of NCF on mortality

Atribute	Dietary treatment				SEM	P value
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t		
Mortality %	6.67	6.67	3.33	5.00	0.01	0.308

3.5 DISCUSSION

This trial showed that performance of broilers was improved by the supplementation of NCF when a commercial stocking density was implemented in the poultry facility at Nottingham Trent University. Overall, supplementation with NCF significantly increased the weekly body weights compared to control from two weeks onwards.

This trial is in agreement with findings of Benites *et al*. (2008) who showed that diets supplemented with MOS significantly increased body weight at 21, 28, 35 and 42 day compared to a control. However Benites *et al.* (2008) did not find this increase in body weight resulted in an improvement in weekly FCR, which is in contrast with the present study. Other studies were in contrast with the present study as they found no significant difference in terms of bird weight when adding MOS (Baurohoo, *et al.,* 2007; Brummer, *et al*. 2010; Kim, *et al.,* 2011). These differences between both published work and current study may relate to differences in NCF ROI between studies. Where published work uses a single ROI, the current study examined 3 levels of NCF and identified biologically unusual cubic response at 14 days, before returning to a quadratic response to increasing NCF at 28 and 35 days. The depression in NCF response seen at 400g/t NCF is difficult to explain but may suggest differing mechanism underpin the positive responses to NCF at 200g/t and 800g/t. This hypothesis is further explored in the general discussion. The increase in the body weight over the control in this study may be due to an improvement in the microflora populations in the intestinal tract; the binding of pathogenic bacteria to MOS results in their evacuation from the intestine with other non-digested feedstuffs (Spring, *et al.,* 2000). This may have improved gut health of the birds fed MOS and contributed to increased performance compared to the control, due to an increased capacity to absorb nutrients (Sun, 2005).

Improvements in the body weight of birds do not always result in an improvement in FCR, this trial however showed improvements in both weekly body weights and FCR in the starter and grower phase. At 14 days this improvement in FCR was in a cubic response, which suggests at 14 days all three inclusion levels are improving

FCR, however 200g/t and 800g/t are having a greater effect. MOS improvements in body weight may not lead to an FCR effect in some studies, due to a lack of challenge on the birds (Benites, *et al.,* 2008; Bazkurt, *et al.,* 2009). The effects of MOS on the birds performance appears to be stronger if the birds are under some level of challenge, Nolet *et al.,* (2007) in broiler and Castillo *et al.* (2008) and Che *et al.* (2012) in pigs found that when challenged, animal performance improves when fed MOS. In the present trial the birds were kept at a higher stocking density than normally maintained in the research facility at Nottingham Trent University's poultry facility, which may have been a factor in the increased level of challenge on the birds shown through the reduced body weight of the control birds. This may be the reason why performance parameters were improved in this trial but not in other trial with no disease or stress challenge on the birds (Morales-Lopez, *et al.*, 2009).

Generally, it was demonstrated that NCF improved FCR in starter and grower feeding phases. This suggests that the inclusion of NCF in the diet in the starter and grower phase increases the efficiency of the birds. This is in agreement with research that found that feeding yeast cell walls in the diet of broiler chicks lowered the feed/gain ratio in 0-3wks ($P<0.05$) (Zhang, *et al.,* 2005). However this conflicts with other research that showed; no effect throughout a study; no effect until later in a study; or an increase FCR when feeding MOS (Iji, *et al*., 2001; Sun, *et al*., 2005; Midilli, *et al*., 2008 respectively). Improved FCR in the starter and grower phase may be due to an ideal supplementation time for MOS to support the developing gut. MOS has been shown to stimulate the immune system and inhibit pathogenic bacterial proliferation, therefore normally leading to an improvement in the birds health and growth performance (Iji, *et al*., 2001; Bozkurt, *et al.,* 2009). Improving

gut health should in theory increase performance, as the absorption status should be better. It has been reported that MOS in many trials has increased performance (Santin, *et al*., 2001; Zhang, *et al*., 2005; Chee, 2008). NCF has been developed from MOS so may be expected to induce similar modes of action. The performance data from this trial supports the hypothesis that NCF is improving the efficiency of the bird in the starter period. Hooge (2004) concluded in a meta-analysis improvments may be through; binding of pathogenic bacteria; improved intestinal function via increases in villus height, uniformity and integrity; and immune modulation. Despite extremely rigorous histologyical examination, the current study shows no effect of NCF on either VH or CD at 6 weeks post hatch. However, another mechasnism posed by Hooge (2004) is supported by the current study: the significnate increase goblet cell area indicates an improment in ability of the bird to mount an immune reponse. This means that the significant improvement observed in growth response after NCF supplementation may be linked with a series of improvements in intestinal mucosal integrity, gut microflora and local intestinal immunoresponse, as has been shown with MOS (Spring, *et al.,* 2000; Iji, *et al.,* 2001; Santin, *et al.,* 2001; Uni and Smirnov, 2006; Chee, 2008). The possible reason for the FCR being higher in the first week than the expected FCR of Ross 308 male broilers, may be due to a feed spillage problem. Birds were fed a mash diet, which resulted in the birds in week one being able to dust bathing in the fed causing the spillage.

NCF had significantly increased weekly weight gain on days 14, 21 and 28. However live weight gain was not significantly different in weeks 5 and 6, which may be due to a number of factors. One explanation is that the birds fed NCF had a heavier body weight than the control birds in the later stages of the trial and had

passed the point of inflection on the growth curve and therefore rate of weight gain is slower (Aviagen, 2007). It is also thought that there may be an optimum time for MOS to support the developing gut and this may be why the study showed greater benefit at the beginning of the trial. It has been reported previously that growth response in broilers to MOS is age-dependent with younger birds responding more than the older ones (Yang, *et al*., 2007; Chee, 2008). This may be due to the fact that the gut microflora of younger birds is more transient in nature and less mature than those older birds. Therefore it may be assumed that feeding of MOS may increase the rate of maturation of the gut microflora, leading to better growth performance (Yang, *et al.,* 2007). These findings in weight gain are in agreement with Santin *et al*., 2001 and Chee, 2008, however research in poultry supplementation with MOS is contradictory. Midilli, *et al*. (2008) found body weight gain in poultry was not affected by the supplementation of MOS. These conflicting findings have also been shown to be the case in other species. In fish; Grisdale-Helland *et al.*, (2008) and Dimitroglou, *et al*., (2010), found no effect of MOS supplementation, whereas Torrecillas *et al.*, (2007) found improvements in weight.

Whilst numerically NCF increased the cumulative feed intake for birds on all three treatment in every week, only NCF at the lowest inclusion level was shown to significantly increase feed intake over the control from 21 days. This may mean that NCF at the lowest inclusion level is a feed intake enhancer. An increase in feed intake was also seen by Morales-Lopez, *et al.* (2010) in broilers when feeding YCW, while it been reported that feeding MOS does not affect feed intake in broilers (Zhang, *et al*. 2005; Midilli, *et al*. 2008; Chee, *et al.* 2010) and in pigs (Castillo, *et al.* 2008). Whereas Baurhoo, *et al.* (2007) and Muthusamy, *et al.* (2012) reported a

decrease in feed intake when feeding MOS. This shows that effects of MOS on feed intake as well as growth of broilers is variable, and may also be variable when feeding NCF at different levels.

As MOS is generally believed to improve gut health it could be proposed that feeding MOS may improve litter quality, due to the bird being able to efficiently digest and absorb nutrients and to pass less urine. When analysing the dry matters of the litter as a measure of litter quality, it was seen that there was no effect of NCF on the quality of the litter. The dry matter results may have been affected by the small pen size, as the birds were not able to move around the room like in a commercial farm. In addition the disproportionately large bell drinker relative to pen size and their tendency to spill, resulted in the need to top up the litter with fresh sand. The viscosity of digesta showed that there was no significant effect of NCF on the viscosity of the digesta. The dry matter of the litter and viscosity results suggests that NCF has no effect on excreta. All foot pad scores throughout the 6 weeks were all scored as 1. This means that there were not foot pad problems for any of the birds in this trial. This is most probably due to good husbandry practices in this system and not diet related, as the study was conducted in a small pen system. This means that the whole pen area is relatively close to the water supply and can deteriorate quickly due to water spillage. Therefore in this study to keep a high standard of welfare, fresh sand was added if there was any water spillage, thereby confounding investigation of this parameter.

When looking at the gross gut development at 6 weeks, NCF at 200g/t caused an elongation of the jejunum. This was also found in a study by Trevifio (1990), who

found chicks receiving oligosaccharide dried extract had a larger relative length of jejunum, ileum and caeca than those fed on the control diet. However, Trevifio (1990) also found that generally, an increase in oligosaccharide content in the diet corresponded to an increase in the length of the different intestinal sections, but the present study did not find this. A reason for the increase in jejunum length may be due to an increase in the production of epithelial cells in the intestine but not their turn over. NCF may be having a combined effect by improving the gut health of birds through decreasing the sloughing of the villi caused by toxins from pathogens and by increasing the amount of energy available to epithelial cells by increasing butyrate levels due to NCF fermentation by microflora. In addition, 200g/t NCF decreased duodenum, ilium and the total intestinal weight relative to bird body weight, which may be due to a decrease in the thickness on the muscularis. The cubic response of duodenum length to body weight ratio to NCF ROI mirrors the reponse of several performance paramenters, suggesting duodenum development might have some involvement in the possible mechanistic differences occurring at 200g/t and 800g/t NCF inclusion. Ferket, *et al*. (2002) reported that when turkeys were fed MOS their muscularis thickness was significantly reduced. An increase in the duodenum weight has been associated with inflammation following bacterial infection (Baurhoo, *et al.*, 2007), therefore the reduced weight of the duodenum and the rest of the intestine may mean that NCF is reducing the pathogenic bacteria in the birds GI tract. Chee, (2008) concluded that MOS-supplemented birds have a thinner gut and therefore have a lower gut mass to maintain. This therefore would decrease the energy needed to maintain the gut and leave more energy for growth. Bozkurt, *et al.* (2009) also found that MOS decreases intestinal weight, however other research is conflicting and reports no effect of MOS on the weight of the intestinal tract in

broilers (Iji, *et al.*, 2001; Baurhoo, *et al.* 2007). Consideration of possible relationships between body weights and intestinal lengths and weights showed that there were little (0.10-0.29) or no correlations (<0.1). This means that the weights of the birds did not appear to affect the gross gut morphology.

When feeding NCF to broilers, it could be postulated that birds will have longer VH and shorter CD via a reduction in sloughing. Spring, *et al.* (2000) reported that pathogens with the mannose-specific type-1 fimbriae, such as some strains of *Escherichia coli*, *Salmonella typhimurium* and *Salmonella enteritidis*, are attracted to mannans, which are reported to be present in NCF (Che, *et al.*, 2012), and will readily bind with them instead of attaching to intestinal epithelial cells (Castillo, *et al.*, 2008). Consequently these pathogenic bacteria cannot colonise the GI tract and release toxins. These bacterium and their toxins can cause inflammation that in turn causes atrophy of the epithelial cells of the villi (Gao, *et al.*, 2008), thus reducing the absorptive function of the gut through shorter VH and deeper CD (Yason, *et al.*, 1987). If NCF binds the pathogenic bacteria and reduce the level of these bacteria in the GI tract, there will be less villi damage in the gut, therefore improving the gut health of the bird. To compensate for this atrophy the bird has to increase its tissue turn over, and as epithelial cells are produced in the crypts and migrate along the villi to the tip, it is thought that the higher turnover in the crypt cell cause it to become deeper (Gao, *et al.*, 2008). Therefore shallower CD are considered a good indicator of gut health. MOS has also been reported to increase levels of *Bifidobacterium* and *Lactobacillus* in broilers (Fernandez, *et al.*, 2002; Baurhoo, *et al.*, 2007), this increases the competition against pathogenic bacteria for nutrients and binding sites, and secreting compounds that are noxious to pathogenic bacteria. Lactobacilli

secrete bacteriocins and bifidobacteria produce organic acids, such as butyrate, which can suppress the colonization of the intestines by pathogenic bacteria, thereby reducing the intestinal population of pathogens (Rolfe, 2000). In addition butyrate can be used by the epithelial cells for energy (Jozefiak, *et al.*, 2004), which may contribute to an improvement in gut health parameters.

Morphometric analysis at 6 weeks revealed that NCF had no significant effect on the gut morphology of the birds at this time point. A possible reason why no effect was evident in the gut morphology and performance of birds fed NCF at 6 weeks, may be due to the birds not being challenged with pathogenic microbes at this time. This trial supports the VH findings of White, *et al.* (2002) in pigs and Yitbarek, *et al.* (2012) and Sohail, *et al.* (2012) in broilers when feeding MOS. However, Iji, *et al.* (2001) and Zhang, *et al.* (2005) found that birds fed yeast cell wall fractions had longer VH than control birds at 21 days. Similarly, Baurhoo, *et al.* (2007) measured VH and found that MOS improved VH at 28 days but not at 42 days. A similar, early age response may have occurred in this present study, but as histological measures were not taken at 21 or 28 days this cannot be verified.

When looking at CD, Zhang, *et al.* (2005), Yitbarek, *et al.* (2012) and Sohail, *et al.* (2012) found feeding MOS had no effect on CD in birds, which was also shown in this study. Santin, *et al.* (2001) also saw no effect of MOS at 42 days, however decreased CD was seen when feeding MOS at 7 days. In addition, this present trial found no effect on VCR, which can be used as a marker of overall intestinal health as it takes into account both CD and VH. This trial included a total of 240 measures for each treatment, which is a considerable amount and therefore it should be noted

that taking more measures to try and reduce associated error would have major time and cost implications. The inherent variability both in this study and other studies suggests that measuring VH and CD may not be an optimal approach to quantifying gut health.

This study showed that the greatest benefit of NCF on FCR occurred at the beginning of the trial, indicating there may be an optimum time for the supplementation of NCF to increase the efficiency of the birds. This may be due to the fact that the gut microflora of younger birds is more transient in nature and less established than in older birds and therefore more susceptible to colonisation by pathogenic bacteria. Therefore prebiotic intervention may shorten the time required to create a beneficial microflora population if it is offered early in life. In this study it was generally shown that NCF improved FCR over the starter and grower feeding phases. This suggests that the inclusion of NCF in the diet at both the starter and grower phase, increases the efficiency of the birds. This concurs with Zhang, *et al.* (2005), although, other studies have shown no early response (Iji, *et al*., 2001; Sun, *et al*., 2005; Midilli, *et al*., 2008). Therefore it could be hypothesised that the improvements observed in FCR early on in the study when feeding NCF may have been due to an increase in absorptive area, due to an increase in lactobacilli and bifidobacterial populations and a reduction in pathogenic bacteria. This has been seen in other studies where improvements in gut morphology is associated with increased lactobacilli and bifidobacterial populations (Baurhoo, *et al.,* 2009). However, improvements in gut health were not observed in this present study as histological measurements were only recorded at 6 weeks, when the microflora is already established with a population of beneficial bacteria. This means that birds on

all treatment groups were not under any challenge and it was seen that NCF had no effect on the performance in the last phase of the trial, which is consistent with the histological measurements at 6 weeks.

The variability seen within and between trials may be due to a number of reasons. Firstly the variability in the yeast cell wall products being fed in the trial might affect the levels of the compounds that induce the proposed effects of the yeast cell wall product. The composition of a yeast cell product depends on many factors including source and processing technique. Second, the variability between experimental conditions and the health status of the birds, may mean that the birds are under differing levels of environmental challenge and therefore will respond differently to a prebiotic.

There is no consensus on whether an increase in goblet cell numbers and area is considered an improvement in bird health. Increasing the number and area of goblet cells is thought to increase the volume of mucin stored in the GI tract and possibly its production (Brümmer, *et al.,* 2010). Mucin is essential for a number of brush border processes, including facilitating absorption of nutrients, containing enzymes, lubrication and decreasing the binding and colonisation of pathogenic bacteria to the intestine (Blomberg, *et al.,* 1993; Smirnov, *et al.,* 2004). Therefore an increase in the level of mucin could have a beneficial effect on the first line of defence of the immune system (Baurhoo, *et al.,* 2009) and the absorptive function of the gut. In contrast, overproduction of mucin may result in a negative effect, by increasing the mucus thickness on the GI tract wall to a level that might impair the ability of nutrients to pass through to the gut epithelial to be absorbed (Smirnov, *et al.,* 2004;

Brummer, *et al.*, 2010), as well as increasing the energy partitioned away from tissue growth to mucin production.

In this trial, the number and area of goblet cells in the duodenum were not affected by the supplementation of NCF to 42 days. This would suggest that NCF has no effect on the mucin profile of broilers in the duodenum in this study. This absence of response was also reported by Castillo, *et al.* (2008) and Yitbarek, *et al.* (2012), however Baurhoo, *et al.* (2007; 2009); Chee, *et al.* (2010); Morales-lopez, *et al.* (2010) and Muthusamy, *et al.* (2012) all found goblet cell numbers were increased by yeast cell wall product supplementation. In the jejunum it was seen that NCF increases the area of goblet cells and there was a trend for increasing goblet cell area per 165 μm of villi, suggesting that NCF at 800g/t affects the mucin profile of broilers in the jejunum. The responses of goblet cell area to increasing NCF were both cubic, showing that 200g/t and 800g/t were having a greater effect. Published data on goblet cell area is very limited: Brummer, *et al.* (2010) also found that the area of goblet cells increased with supplementation of MOS. Insight into the mechanisms behind this goblet cell response would be highly beneficial to understanding the effect of MOS supplementation on gut health.

An increase in goblet cell area is thought to show that the storage capacity of the goblet cell for mucin has increased (Smirnov, *et al.*, 2005). The increase in mucin storage may suggest that the bird is more capable of forming a protective layer on the villi, thereby helping protect the intestine from damage caused by enteropathogens if there was a challenge from pathogenic bacteria (Smirnov, *et al.*, 2006; Brümmer, *et al.*, 2010). One suggested mechanism of MOS on mucin

production is through changing the gene expression of key genes through direct crosstalk between beneficial intestinal microbes and goblet cells, (Mack, *et al.,* 1999; Freitas, *et al.*, 2003; Smirnov, *et al*., 2005; Uni and Smirnov 2006; Chee, 2008). The effects of NCF on the goblet cell area observed in the current study agree with this suggestion, that goblet cell area increased with increased supplementation level, but it is also possible that NCF is having a direct effect on mucin production and subsequently increasing bifidobacteria due to the increase in mucin production, as bifidobacteria can produce enzymes allowing them to utilise and proliferate on mucin glycoproteins (Katayama, *et al*., 2005; Jung, *et al.*, 2008; Ruas-Madiedo, *et al*., 2008).

3.6 CONCLUSION

In conclusion, this trial indicates that NCF had a positive effects on performance and limited effects on gut health at 6 weeks. Regarding performance, 200g/t and 800g/t NCF in particular improved FCR, but the growth response to NCF is age-dependent with the bird responding more at a earlier age than the later in it life. This has also been shown by Tucker, *et al.* (2003) using MOS, at a higher inclusion than NCF. This could be explained by the fact that the gut microflora of younger birds are less mature than older birds. Therefore it may be assumed that feeding of MOS may increase the rate of maturation of the gut microflora, leading to better growth performance (Yang, *et al.,* 2007). This trial showed that NCF improved body weight, body weight gain, FCR and performance of the birds to some degree earlier on in the trial, however this cannot be related to gut morphology of the bird as it was not measured earlier on in the trial. It may be postulated that NCF improved gut health of bird in the starter and growth phase of feeding as FCR was improved. However

without gut morphology being measured at this time point, this cannot be proved. The published research appears to be variable, which may be due to differences in the type of MOS product, experimental conditions, diet formulation and health status of the birds. The performance data from this trial supports the hypothesis that NCF is improving the efficiency of the bird in the starter period, but the dose response was not linear. The cubic response observed for many parameters measured suggest differing mechanisms underpin the improve performance as 200g/t and 800g/t NCF. Therefore, future studies are warranted to explore further the interactions between NCF and the development of the gut and modulation of intestinal morphology over time.

CHAPTER 4: TRIAL 2 - INVESTIGATION OF THE EFFICACY OF THE A NATURAL CARBOHYDRATE FRACTION (NCF) ACTIGEN AT VARYING DIETARY INCLUSION RATES ON THE GUT DEVELOPMENT OF BROILERS OVER THE TRIAL.

4.1 INTRODUCTION

After observing the performance improvements in Trial 1 in the starter and grower phases, but generally no effect of NCF on microscopic gut morphology at 6 weeks, it was deemed appropriate to investigate the effects of NCF on parameters prior to 6 weeks to detect possible modes of action of NCF in the starter and grower periods. Therefore Trial 2 was carried out to investigate the effects of NCF on microscopic changes in the GI tract over the life of the bird, as well as elucidate any links to bird performance. Trial 2 investigated the effects of NCF at 200, 400 and 800g/t on bird gut morphology at intervals throughout the trial period at 7, 14, 21, 28 and 42 days, to investigate the development of the gut over time. This study also examined the effects of NCF on gross gut development, linking it to microscopic gut development and performance. This trial continues to use the inclusion levels of 200g/t, 400g/t and 800g/t used previously, but sequentially examines bird development on a weekly basis to investigate the mechanisms behind the cubic responses seen in the previous trial.

4.2 AIM

The aim of Trial 2 was to investigate the efficacy of NCF at 200g/t, 400g/t and 800g/t inclusion rates on the microscopic gut development of broilers over the trial period, whilst linking this to performance and gross gut development.

The hypothesis of this trial was that improvements in performance seen with supplementation of NCF are linked to altered gut devolopment in early life, with differing developmetal responses to differeing ROI of NCF.

4.3 MATERIALS AND METHODS

4.3.1 Bird Husbandry

A total of three hundred and sixty 1 day old male Ross 308 birds from a flock of 43 weeks of age were obtained from P.D.Hook Hatcheries Ltd (Cote, Bampton, Oxfordshire, UK). They were placed in 36 pens, each containing 10 birds and fed on 1 of 4 dietary treatments. The diets were stratified allocated to remove any effect of the room environment on the study. There were 4 dietary treatments with 8 replicates per treatment, made up of 8 small floor pens (area = 0.43m^2) with one extra pen per treatment holding spare birds for replacement of birds culled due to health reasons during the trial. The litter provided was wood shavings on a concrete floor at a depth of 6cm topped up as necessary to maintain adequate environmental welfare.

4.3.2 Experimental Diets

Trial 2 lasted 42 days with a three phase feeding programme: starter diets were fed from 1 to 14 days, grower diets from 15 to 28 days and finisher diets from 29 to 42 days. The 4 dietary treatments were: 1) control diet (NCF free), 2) NCF at 200g per tonne, 3) NCF at 400g per tonne and 4) NCF at 800g per tonne. The rations were based on the nutritional requirements for the strain of bird (as defined by Aviagen) and contained rye and no pentosan degrading enzymes. The formulations were checked by an independent nutritionist. The basal diet was free of antibiotics and manufactured using the formulation in table 4.1, by Target Feeds, a commercial feed

mill.

Table 4.1. Composition of basal diet and calculated analysis of the basal diet

Item	Starter	Grower	Finisher
Ingredients (%)			
Barley	5.50	0.90	0.00
Rye	10.00	15.00	20.00
Wheat	45.00	47.00	47.00
Soybean meal, 48% CP	26.00	23.00	19.00
Full fat soybean meal	5.00	5.00	5.00
L lysine HCL	0.40	0.30	0.30
DL methionine	0.40	0.35	0.30
L threonine	0.15	0.15	0.15
Soya oil	4.00	4.50	4.75
Limestone	1.25	1.25	1.25
Monocalcium phosphate	1.50	1.25	1.25
Salt	0.25	0.25	0.25
Sodium bicarbonate	0.15	0.15	0.15
Premix*	0.40	0.40	0.40
Calculated analysis			
ME MJ/kg	12.75	12.99	13.11
CP%	21.75	20.49	18.91
Lys %	1.45	1.29	1.17
Met + Cys %	1.04	0.96	0.87
Ca	0.96	0.91	0.91
Available P	0.48	0.42	0.42

*Premix content (volume/kg diet): Mn 100mg, Zn 80mg, Fe 20mg, Cu 10mg, I 1mg, Mb 0.48mg, Se 0.2mg, Retinol 13.5mg,Cholecalciferol, 3mg, Tocopherol 25mg, Menadione 5.0mg, Thiamine 3mg, Riboflavin 10.0mg, Pantothenic acid 15mg, Pyroxidine 3.0mg, Niacin 60mg, Cobalamin 30µg, Folic acid 1.5mg, Biotin 125mg

4.3.3 Determined Parameters

Bird weight and feed intakes per pen were recorded weekly. The FCR was calculated using this information on a weekly basis and for each phase. On days 7, 14, 21, 28 and 42, two birds per pen were selected and slaughtered. The whole intestine from one of the birds was removed and weighed. The length of the separate parts of the intestinal tract as described in Chapter 2, were measured. A blood sample was taken from the second bird and its serum removed and frozen at -20^{o}C for later analysis. The second bird was then dissected to remove the small intestine tissue sections from the duodenum and jejunum for detailed intestinal and general intestinal structure examination. The tissue samples from the duodenum and jejunum were fixed in Bouins solution prior to histological procedures including staining with Periodic Acid Schiff's and Alcian Blue (pH 2.5). In addition tissue samples were taken and snap frozen in dry ice and industrial methylated spirits and stored at -80^{o}C for later analysis of digestive and immune markers. Standard feed analyses were carried out on the three basal diets (Appendix 6). The feed was also analysed for NCF levels at the Alltech's Bioscience Centre in Dunboyne, Ireland by an assay determining the amount of NCF in grams per tonne.

4.3.4 Data Analysis

All data were analysed using the SPSS software for Windows. A 1-way ANOVA analysis was applied. One pen was used as a unit for analysis. Treatment means were separated using the Tukeys post hoc test, and statistical significance was declared at $P < 0.05$. Regression analysis was also performed to identify any dose response to increasing levels of NCF.

4.4 RESULTS

4.4.1 Body Weight and Live Weight Gain

Whilst body weight and live weight gain was recorded, the pen size (80x80cm) and weekly reduction in stocking density limited the commercial relevance of this data. The mean weekly live body weights per bird for the 4 diets over the 6 weeks are shown in Table 4.2, with the mean weekly body weight per bird on days 1 and 21 showing a significant difference between treatments. On day 1, it was shown that the birds on diet 200g/t of NCF had a significantly heavier live weight over the control (P=0.025). On day 21 it was shown that NCF at 800g/t increased live weight over the control and 400g/t (P=0.023). The regession anaylsis showed no dose reponse to NCF level.

The mean weekly live weight gains per bird for the 4 diets over the 6 weeks are given in Table 4.3, illustrating that the mean weekly live weight gain per bird on days 21 and 35 was significantly different between the treatments. On day 21, 800g/t of NCF increased weight gain over the other two inclusion levels of NCF but not the control (P=0.008). Contrastingly, on day 35, 800g/t of NCF significantly decreased the live weight gain compared to the control (P=0.011). The regression analysis showed that on day 35 that increasing levels of NCF resulted in a negative linear response in weight gain. Weight gain do not make up the difference between weekly live body weights due to birds being culled weekly, this means that the end live weight of a pen for a week (e.g. 6 bird) is not the same at the start live weight for the next week (e.g. 4 birds (2 birds culled)).

Table 4.2. Effect of NCF in broiler diets on the mean weekly body weight (g/bird)

Age	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
1 d	42.7 ^{B}	44.2 ^{A}	43.5 ^{AB}	43.1 ^{AB}	0.18	0.025	0.810
7 d	142.0	150.9	143.0	152.7	1.99	0.135	0.717
14 d	409.4	429.9	416.3	442.0	4.59	0.055	0.911
21 d	871.0 ^{B}	880.9 ^{AB}	863.6 ^{B}	929.9 ^{A}	8.44	0.023	0.934
28 d	1455.2	1516.5	1491.1	1546.1	15.50	0.212	0.731
35 d	2203.0	2314.8	2253.5	2179.5	23.60	0.177	0.236
42 d	3040.1	3103.5	3046.3	3002.0	28.32	0.671	0.473

Differing superscript letters within one week denote means are significantly different at $P < 0.05$

Table 4.3. Effect of NCF in broiler diets on the mean weekly live weight gain (g/bird)

Age	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
7 d	99.3	106.7	99.5	109.7	1.97	0.159	0.709
14 d	267.0	279.7	272.9	290.3	3.37	0.089	0.925
21 d	464.1 ^{AB}	443.9 ^{B}	451.9 ^{B}	484.2 ^{A}	4.58	0.008	0.965
28 d	579.6	622.3	601.3	603.7	12.73	0.723	0.745
35 d	854.1 ^{A}	792.8 ^{AB}	756.6 ^{AB}	715.3 ^{B}	15.77	0.011	0.042^{L}
42 d	846.4	788.7	792.8	822.5	20.33	0.753	0.741

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

4.4.2 Feed Intake

The mean weekly feed intakes per bird for the 4 diets over the 6 weeks are shown in Table 4.4 indicating that 800g/t NCF increased feed intake over the control birds on

day 7. No other significant differences between the treatments were demonstrated and no dose response to NCF observed. The cumulative mean feed intakes per bird for the 4 diets over the 6 weeks are shown in Table 4.5 where a significant difference between the treatments in weeks 1 and 3 was evident as 800g/t NCF increased feed intake over the control.

Table 4.4. Effect of NCF in broiler diets on the mean weekly feed intake (g/bird)

Attribute	Age	Dietary treatment				SEM	P value	Regression
		Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Feed	7 d	115.1 ^{B}	126.3 ^{AB}	120.1 ^{AB}	132.2 ^{A}	2.22	0.032	0.884
intake (g)	14 d	376.2	392.7	390.8	401.0	4.19	0.216	0.984
	21 d	687.4	715.4	717.0	726.8	6.77	0.200	0.392
	28 d	1044.7	1078.8	1093.6	1072.9	12.78	0.609	0.394
	35 d	1440.3	1471.8	1461.7	1437.1	18.6	0.905	0.894
	42 d	1649.8	1651.7	1673.3	1649.4	22.60	0.981	0.891

Differing superscript letters within one week denote means are significantly different at $P < 0.05$

Table 4.5. Effect of NCF in broiler diets on the cumulative mean feed intake (g/bird)

Attribute	Age	Dietary treatment				SEM	P value
		Control	NCF 200g/t	NCF 400g/t	NCF 800g/t		
Feed	7 d	115.1 ^{B}	126.3 ^{AB}	120.1 ^{AB}	132.2 ^{A}	2.22	0.032
intake (g)	14 d	491.3	519.0	510.8	533.3	5.77	0.072
	21 d	1178.8 ^{B}	1234.4 ^{AB}	1227.8 ^{AB}	1260.0 ^{A}	10.47	0.041
	28 d	2223.5	2313.2	2321.4	2346.0	19.04	0.109
	35 d	3663.7	3785.0	3783.1	3816.3	32.16	0.362
	42 d	5313.5	5436.7	5456.3	5477.1	43.77	0.568

(Differing superscript letters within one week denote means are significantly different at $P < 0.05$.)

4.4.3 Feed Conversion Ratio

The mean weekly FCRs per bird for the 4 diets over the 6 weeks are shown in Table 4.6 with the mean weekly FCR per bird between diets not showing a significant difference, except on day 21 where 200g/t increased FCR over the control (P = 0.010). Table 4.6 also shows there was no dose effect of NCF inclusion level on FCR. Table 4.7 illustrates that there was no significant difference between dietary treatments on the mean FCR per phase of feeding. Furthermore throughout the whole 6 week trial period the FCR was not significantly altered by dietary treatment.

Table 4.6. Effect of NCF in broiler diets on the weekly feed conversion ratio.

Attribute	Age	Dietary treatment				SEM	P value	Regression
		Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
FCR	7 d	1.17	1.18	1.21	1.23	0.02	0.540	0.149
	14 d	1.42	1.40	1.43	1.38	0.02	0.808	0.810
	21 d	1.48 ^{B}	1.61 ^{A}	1.59 ^{AB}	1.50 ^{AB}	0.02	0.010	0.328
	28 d	1.84	1.74	1.83	1.78	0.03	0.717	0.908
	35 d	1.84	1.86	1.93	2.01	0.03	0.070	0.239
	42 d	1.94	2.17	2.11	2.13	0.05	0.396	0.548

Differing superscript letters within one week denote means are significantly different at $P < 0.05$

Table 4.7. Effect of NCF in broiler diets on the phase feed conversion ratio

Attribute	Age	Dietary treatment				SEM	P value
		Control	NCF 200g/t	NCF 400g/t	NCF 800g/t		
FCR	Wk1+2	1.35	1.34	1.37	1.35	0.01	0.848
	Wk3+4	1.67	1.69	1.72	1.66	0.02	0.597
	Wk5+6	1.92	1.98	2.02	2.08	0.03	0.236
	Wk1-6	1.77	1.79	1.83	1.84	0.01	0.144

4.4.4 Intestinal Weight and Length

The mean weekly intestinal weight, lengths and ratios to body weight for the 4 diets are shown in tables 4.8, 4.9, 4.10, 4.11 and 4.12, the pancreas weights and ratios to body weight are in table 4.13. Quadratic dose responses to NCF were seen on day 14 in duodenum, jejunum and ilium length to body weight ratio and on day 21 in duodenum weight to body weight ratio. Generally on day 14 and 21 NCF increased length on the intestine. There was also a quadratic dose response on day 14 for pancrease weight and jejunum weight. A significant difference was observed in the intestinal measurements on day 42, when analysing the length of the jejunum as a ratio of cm per 100g body weight. It was found that 800g/t NCF increased the jejunum over 200g/t NCF (P=0.034). Two other significant differences were observed one at day 7 and one at day 28, however significance was lost at post hoc level.

Table 4.8 Effect of dietary treatment in broiler diets on the length and weight of the intestine at 7 days

	Duodenum				Jejunum				Ileum				Total	
Diet	Weight	Length	DWBWR	DLBWR	Weight	Length	JWBWR	JLBWR	Weight	Length	IWBWR	ILBWR	Weight	Length
NCF 0g/t	1.64	15.98	11.09	1.13	2.76	36.65	25.50	1.92	1.91	35.23	24.56	1.43	6.48	87.85
NCF 200g/t	1.89	16.43	10.48	1.21	3.35	37.89	25.21	2.07	2.49	37.34	24.29	1.60	7.73	90.64
NCF 400g/t	1.46	15.74	10.89	1.06	2.77	37.53	27.64	1.99	2.23	33.95	24.87	1.60	6.05	88.63
NCF 800g/t	1.44	15.54	10.75	0.98	2.56	35.67	25.12	1.77	1.86	34.39	24.17	1.22	5.94	88.05
SEM	0.07	0.30	0.28	0.04	0.13	0.56	0.43	0.07	0.11	0.62	0.47	0.07	0.26	1.03
p values	0.052	0.773	0.647	0.110	0.142	0.551	0.394	0.612	0.111	0.218	0.660	0.162	0.044	0.769
Regression	0.247	0.324	0.577	0.883	0.218	0.740	0.671	0.383	0.314	0.088	0.743	0.947	0.753	0.430

Weight (g), Length (cm), DWBWR = grams of duodenum tissue per 100g bird body weight, DLBWR = centimetre of duodenum tissue per 100g bird body weight, JWBWR = grams of jejunum tissue per 100g bird body weight, JWBWR = centimetre of jejunum tissue per 100g bird body weight, IWBWR = grams of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight, IWBWR = centimetre of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight.

Table 4.9 Effect of dietary treatment in broiler diets on the length and weight of the intestine at 14 days

	Duodenum				Jejunum				Ileum				Total	
Diet	Weight	Length	DWBWR	DLBWR	Weight	Length	JWBWR	JLBWR	Weight	Length	IWBWR	ILBWR	Weight	Length
NCF 0g/t	4.09	20.81	5.10	1.00	7.25	51.33	12.65	1.78	6.09	50.33	12.40	1.50	17.43	122.46
NCF 200g/t	4.15	21.18	4.93	0.96	7.49	51.25	11.94	1.74	6.22	49.59	11.55	1.44	17.86	122.01
NCF 400g/t	4.30	20.61	4.96	1.03	8.36	50.11	12.06	2.02	6.44	49.36	11.88	1.56	19.39	120.09
NCF 800g/t	4.68	21.80	4.90	1.06	8.70	51.46	11.64	1.91	6.68	47.94	10.84	1.51	20.05	121.14
SEM	0.14	0.36	0.09	0.03	0.26	0.79	0.24	0.06	0.20	0.86	0.26	0.05	0.54	1.80
p value	0.464	0.743	0.878	0.775	0.156	0.933	0.537	0.404	0.763	0.825	0.216	0.876	0.286	0.971
Regression	0.112	0.594	0.833	0.021^{Q}	0.025^{Q}	0.870	0.800	0.010^{Q}	0.294	0.387	0.354	0.005^{Q}	0.726	0.057

Weight (g), Length (cm), DWBWR = grams of duodenum tissue per 100g bird body weight, DLBWR = centimetre of duodenum tissue per 100g bird body weight, JWBWR = grams of jejunum tissue per 100g bird body weight, JWBWR = centimetre of jejunum tissue per 100g bird body weight, IWBWR = grams of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight, IWBWR = centimetre of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

Table 4.10 Effect of dietary treatment in broiler diets on the length and weight of the intestine at 21 days

	Duodenum				Jejunum				Ileum				Total	
Diet	Weight	Length	DWBWR	DLBWR	Weight	Length	JWBWR	JLBWR	Weight	Length	IWBWR	ILBWR	Weight	Length
NCF 0g/t	7.67	24.39	2.81	0.90	13.99	66.07	7.32	1.58	10.66	60.06	7.13	1.23	32.32	150.24
NCF 200g/t	7.85	24.84	2.82	0.89	13.77	62.16	7.06	1.55	10.99	57.93	6.44	1.25	32.59	143.67
NCF 400g/t	8.14	25.37	2.89	0.94	13.31	63.16	7.34	1.55	10.39	59.01	6.85	1.23	31.98	147.05
NCF 800g/t	9.09	26.10	2.81	0.98	14.40	66.68	7.12	1.55	11.37	59.43	6.56	1.22	34.86	149.59
SEM	0.24	0.38	0.04	0.03	0.31	0.92	0.12	0.03	0.30	0.85	0.12	0.03	0.71	2.17
P value	0.188	0.451	0.905	0.675	0.660	0.238	0.790	0.977	0.734	0.863	0.160	0.994	0.523	0.718
Regression	0.180	0.047^{L}	0.042^{Q}	0.231	0.852	0.317	0.826	0.346	0.634	0.267	0.693	0.324	0.790	0.341

Weight (g), Length (cm), DWBWR = grams of duodenum tissue per 100g bird body weight, DLBWR = centimetre of duodenum tissue per 100g bird body weight, JWBWR = grams of jejunum tissue per 100g bird body weight, JWBWR = centimetre of jejunum tissue per 100g bird body weight, IWBWR = grams of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight, IWBWR = centimetre of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight. In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

Table 4.11 Effect of dietary treatment in broiler diets on the length and weight of the intestine at 28 days

	Duodenum				Jejunum				Ileum				Total	
Diet	Weight	Length	DWBWR	DLBWR	Weight	Length	JWBWR	JLBWR	Weight	Length	IWBWR	ILBWR	Weight	Length
NCF 0g/t	9.88	28.44	1.97	0.68	19.54	66.78	4.61	1.26	15.34	67.18	4.65	1.07	45.77	165.64
NCF 200g/t	10.48	28.21	1.86	0.69	18.70	65.13	4.30	1.23	15.40	63.11	4.27	1.01	44.48	154.60
NCF 400g/t	9.30	26.88	1.81	0.63	18.12	68.48	4.60	1.22	15.10	69.84	4.71	1.01	42.51	164.53
NCF 800g/t	11.17	28.87	1.87	0.72	19.53	67.06	4.31	1.32	15.98	66.76	4.11	1.04	46.68	163.50
SEM	0.35	0.36	0.03	0.02	0.56	0.96	0.07	0.03	0.76	1.12	0.09	0.03	1.27	1.66
p value	0.265	0.237	0.398	0.622	0.786	0.695	0.209	0.687	0.905	0.237	0.041	0.935	0.700	0.071
Regression	0.811	0.886	0.621	0.915	0.874	0.534	0.990	0.734	0.718	0.990	0.999	0.537	0.686	0.985

Weight (g), Length (cm), DWBWR = grams of duodenum tissue per 100g bird body weight, DLBWR = centimetre of duodenum tissue per 100g bird body weight, JWBWR = grams of jejunum tissue per 100g bird body weight, JWBWR = centimetre of jejunum tissue per 100g bird body weight, IWBWR = grams of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight, IWBWR = centimetre of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight.

Table 4.12 Effect of dietary treatment in broiler diets on the length and weight of the intestine at 42 days

	Duodenum				Jejunum				Ileum				Total	
Diet	Weight	Length	DWBWR	DLBWR	Weight	Length	JWBWR	JLBWR	Weight	Length	IWBWR	ILBWR	Weight	Length
NCF 0g/t	13.04	32.53	1.07	0.43	27.85	80.01	2.63^{AB}	0.92	23.72	82.53	2.72	0.78	65.35	195.06
NCF 200g/t	13.86	33.73	1.09	0.45	27.67	75.95	2.42^{B}	0.89	22.56	77.46	2.50	0.73	64.10	183.91
NCF 400g/t	11.10	31.89	1.05	0.37	25.98	75.03	2.47^{AB}	0.85	23.02	80.85	2.65	0.76	60.96	187.76
NCF 800g/t	13.22	31.79	1.07	0.45	26.91	82.64	2.77^{A}	0.91	23.09	84.77	2.82	0.77	63.20	198.71
SEM	0.39	0.45	0.02	0.01	0.90	1.21	0.05	0.03	0.68	1.04	0.45	0.02	1.79	2.42
p value	0.423	0.416	0.910	0.103	0.890	0.075	0.035	0.899	0.953	0.094	0.070	0.851	0.862	0.122
Regression	0.181	0.318	0.146	0.797	0.391	0.337	0.258	0.706	0.978	0.669	0.793	0.932	0.408	0.447

Weight (g), Length (cm), DWBWR = grams of duodenum tissue per 100g bird body weight, DLBWR = centimetre of duodenum tissue per 100g bird body weight, JWBWR = grams of jejunum tissue per 100g bird body weight, JWBWR = centimetre of jejunum tissue per 100g bird body weight, IWBWR = grams of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight, IWBWR = centimetre of ileum tissue per 100g bird body weight, g/cmPBWR = grams/centimetre of pancreas tissue per 100g bird body weight.

Table 4.13 Effect of dietary treatment in broiler diets on the weight of the pancreas over time

	Day 7		Day 14		Day 21		Day 28		Day 42	
Diet	Weight	PBWR	Weight	PBWR	Weight	PBWR	Weight	PBWR	Weight	PBWR
NCF 0g/t	0.49	3.39	1.40	3.43	2.90	3.32	3.82	2.62	5.39	1.78
NCF 200g/t	0.65	4.17	1.38	3.21	2.97	3.38	3.57	2.35	5.72	1.84
NCF 400g/t	0.56	3.94	1.58	3.81	2.63	3.04	3.92	2.64	5.05	1.66
NCF 800g/t	0.53	3.53	1.75	3.99	3.17	3.40	3.74	2.53	5.11	1.75
SEM	0.03	0.20	0.06	0.12	0.09	0.09	0.11	0.07	0.15	0.05
p values	0.266	0.358	0.052	0.097	0.265	0.515	0.708	0.522	0.364	0.664
Regression	0.905	0.753	0.021^{Q}	0.726	0.741	0.790	0.768	0.686	0.505	0.408

Weight (g), PBWR = grams of pancreas tissue per 100g bird body weight In regression analysis significant relationships are identified as follows, Linear (L), Quadratic (Q) and Cubic (C))

4.4.5 Gut Morphology

The mean weekly jejunum VH, VW, CD and VCR for weeks 1, 3 and 6 for the 4 diets are shown in Tables 4.14, 4.15 and 4.16 respectively. Significant differences were only observed in week 3. Control birds had longer VH than birds fed 400g/t NCF and wider VW than birds fed 800g/t NCF. No dose responses to NCF were seen when regression analysis were undertaken.

Table 4.14. Effect of NCF on the gut morphology of the jejunum in week 1 (µm)

Attribute	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Jejunum							
Villus Height	1027.6	961.0	885.8	1090.7	50.5	0.537	0.876
Villus Width	185.4	171.6	180.3	188.8	4.9	0.650	0.627
Crypt Depth	127.1	126.1	123.9	140.2	4.0	0.508	0.645
Villus/Crypt Ratio	8.1	7.8	7.2	8.1	0.3	0.635	0.713

Table 4.15. Effect of NCF on the gut morphology of the jejunum in week 3 (µm)

Attribute	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Jejunum							
Villus Height	2307.1 ^{A}	2241.5 ^{AB}	2025.0 ^{B}	2115.3 ^{AB}	36.5	0.017	0.402
Villus Width	238.5 ^{A}	216.6 ^{AB}	205.9 ^{AB}	200.9 ^{B}	5.1	0.047	0.052
Crypt Depth	166.4	175.0	170.3	173.7	2.8	0.745	0.845
Villus/Crypt Ratio	12.6	13.3	12.1	13.0	0.3	0.667	0.821

Differing superscript letters within one week denote means are significantly different at $P < 0.05$

Table 4.16. Effect of NCF on the gut morphology of the jejunum in week 6 (µm)

Attribute	Dietary treatment				SEM	P value	Regression
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t			P value
Jejunum							
Villus Height	2665.2	2641.3	2804.1	2591.3	40.0	0.284	0.514
Villus Width	234.1	241.4	245.1	255.1	4.5	0.417	0.496
Crypt Depth	201.1	197.9	207.3	199.2	7.4	0.974	0.914
Villus/Crypt Ratio	14.1	14.1	14.5	13.4	0.6	0.932	0.793

4.4.6 NCF Levels

Diets were analysed for NCF levels at the Alltech Bioscience Centre in Dunboyne, Ireland by an assay determining the amount of NCF in grams per tonne, using the control as a comparative base line (method was not supplied due to Patent application in process). The first assay results produced unexpected NCF levels in the diet, therefore a further two assays were carried one at Dunboyne, Ireland and one at The Alltech Bioscience Centre in Lexington, USA. These further assays also showed unexpected results that were not consistent as illustrated in Table 4.17. This could be due to a number of factors: firstly the assay may not be reliable, secondly there is a factor in the feed interacting with the NCF or assay, thirdly there was a problem with the NCF and finally the mix of NCF in the feed was not homogenous.

Table 4.17. Levels of measured NCF in feed

Attribute	Supposed levels		
	NCF 200g/t	NCF 400g/t	NCF 800g/t
Assay 1			
Starter	152	434	467
Grower	307	247	431
Finisher	95	183	349
Assay 2			
Starter	182	322	734
Grower	213	240	389
Finisher	65	112	380
Assay 3			
Starter	218	431	570
Grower	488	597	1033
Finisher	87	283	745

4.4.7 Mortality

There was no difference between diets in mortality for this study as shown in Table 4.18.

Table 4.18 Effect of NCF on mortality

Attribute	Dietary treatment					P value
	Control	NCF 200g/t	NCF 400g/t	NCF 800g/t	Total	
Mortality (%)	0.7	0.4	1.1	0.0	2.2	0.780

4.5 DISCUSSION

Due to the design of Trial 2 (weekly sampling days) it is difficult to draw a conclusion from the bird weight data, limiting its commercial application. This is due to the requirement to cull birds each week for the sample. Selection of the birds occurred randomly to limit the potential to skew the data by choosing either the smallest, largest or middle sized birds. Therefore little importance can be placed on the body weight or gain, although some significances were found. At 1 day old the birds were weighed and placed into pens with other birds within 10g of each other, however as the bird uniformity was very good the mean pen weights ranged from 41.8g to 46.2g, which is commercially regarded as a small range in newly hatched chickens. This meant that the small difference of 1.5g between control birds and 200g/t NCF birds on day 1 showed up as significantly different. Supplementation of 800g/t NCF significantly increased the weekly body weights on days 21 over control and 400g/t NCF. This is supported by the findings of Benites *et al*. (2008) who showed that diets supplemented with MOS significantly increased body weight at 21 days. However Benites *et al*. (2008) also found significant differences at 28, 35 and 42 days, which conflicts with the present study where significant differences after day 21 were not observed. The significant difference illustrated between body weights of 800g/t and 400g/t NCF fed birds, with 800g/t heavier may be due to different mechanisms occurring at varying levels of NCF. This indicates that there may be two mechanisms behind the observed response to supplementation; one occurring at the lowest/middle inclusion and one occurring at the highest inclusion. A possible mechanism that may be having the apparent antagonist effects at the middle inclusion level, is an increase in the readiness of the immune system, causing a higher energy partition to immune rather than growth.

When looking at body weight gain at 21 days, 800g/t NCF increased weight gain over 200g/t and 400g/t NCF. This may be due to mechanisms at lower inclusion levels causing an increase in energy requirements of the birds, however as explained earlier the performance data may not be reliable due to weekly sampling of birds. At 35 days weight gain had a negative quadratic reponse to increasing NCF, 800g/t had lower weight gains than birds on the control. This may be due to 800g/t birds being significantly heavier at 21 days and numerically at 28 days than control birds, meaning that they were further along their developmental growth curve and therefore the rate of bird growth had started to slow in the week up to 35 days (figure 4.1).

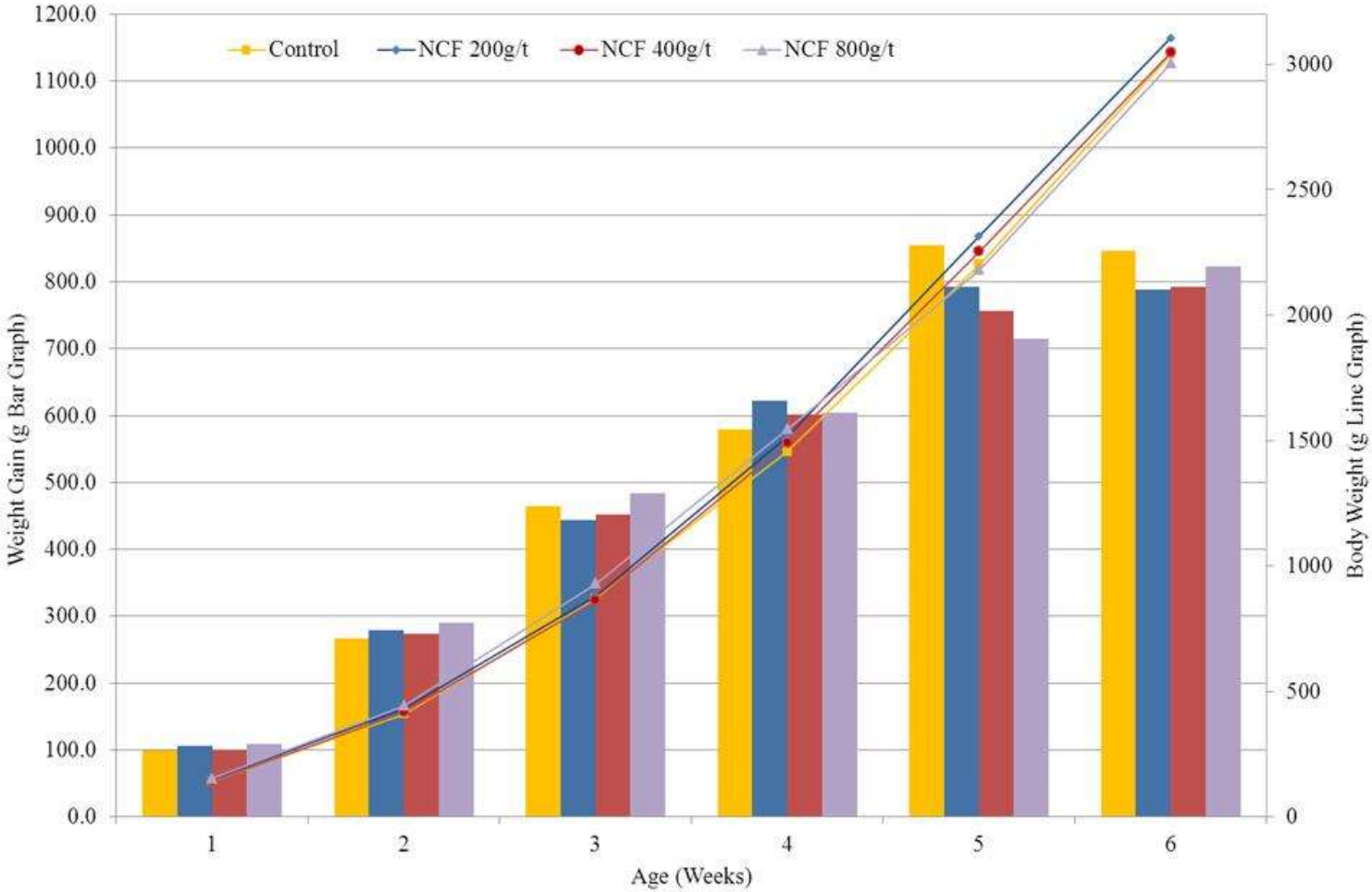

Figure 4.1 NCF effect on live body weight and weight gain.

NCF generally had no effect on FCR throughout Trial 2, which may be due to the environment of the birds not being challenging, due to reduced stocking density. Dietary raw materials may also contribute a physiological challenge to fast growing strains of broiler, so a dietary challenge was implemented to compensate for the reduced stocking density. Rye exerts a dietary challenge on broilers through high levels of pentosans which result in decreased digestive efficiency and therefore increase risk of pathogenic chanllenge to the gut (Antoniou, *et al.* 1981). However the birds grew ahead of breed target and were not affected by rye. Another factor that may have contributed to the performance improvements seen between trial 1 and 2 was the type of litter used. In trial 1 sand was used as standard for this poultry unit (to avoid ingestion of litter), but by the second trial standard practice had altered to use of wood shavings to improve bird welfare.

One significant difference in FCR was observed in this trial: at 21 days that 200g/t increased FCR over control. As 24 FCR significance values were generated for this study, it is possible that significant finding is simply a Type 1 Error. However if this result is a true finding, it may be related to an immune response to NCF inclusion. One possible mode of action of MOS is to modulate the immune system to aid in response to a pathogenic challenge to prevent a negative response in bird performance. Therefore, although MOS is commonly cited as leading to an improvement in the health and growth performance of the bird (Iji *et al*. 2001; Bozkurt *et al.* 2009), it would be more correct to state MOS prevents a performance deteriation under pathogenic challenge. This outlook on MOS is further supported by Briggs *et al.* (2007); Baurhoo, *et al.* (2009) and Brummer *et al*. (2010), found that where there was no pathogenic challenge, there was no significant effect of feeding

MOS on bird performance. This may be due to MOS stimulating the immune system when there is no need, and negatively affecting performance, due to redirection of energy from growth to immune response (Huff, et al., 2006). In these situations the energy cost associated with immune response results in overall gain to the bird, by preventing pathogenic damage to the digestive capacity. This argument is further verified by studies artificially invoking the reverse situation: Nollet, *et al*. (2007) in broilers and Che, *et al.* (2012) in pigs found improvements in performance when feeding MOS over a control when animals were dosed with a pathogenic bateria. This shows that the effect of MOS and NCF supplementation on bird performance is variable.

Supplements that are thought to work through changing the bacterial load of an animal or through up-regulating the immune system, have been observed to have variable effects on performance. This may be due to the environment and management of the birds ranging from high pathogenic challenge through to a moderate to low challenge. Therefore if the pathogenic challenge of the bird alters, the effectiveness of feeding a gut health supplement will change. An animal with a healthy GI tract, with no pathogenic infection that is fed on a supplement that will modify the microflora population or immune defence, will gain little benefit from a supplement. This will occur whether it is through an antimicrobial mechanism or a pre/probiotic mechanism as no impediment exists to prevent the bird from meeting its growth potential. However, the supplement may in fact reduce growth due to the partitioning of energy away from growth to the immune system. Therefore the NCF effects, as with other supplements on the performance of the bird, appear to be stronger if the birds have some level of challenge or stress.

NCF at the highest inclusions level was shown to significantly increase cumulative feed intake at day 7 and 21. This may mean that NCF is promoting an increase in feed intake, which was also numerically higher in Trial 1. An increase in feed intake was also illustrated by Morales-Lopez, *et al.* (2010) in broilers when feeding yeast cell wall. However, previous findings observed that feeding MOS does not affect feed intake (Zhang, *et al*., 2005; Midilli, *et al*., 2008).

These results show that whilst NCF appears not to affect intestine, pancreas or pancreas to body weight ratio, it may cause elongation of the jejunum. This was also found in a study by Trevifio (1990), who found chicks receiving oligosaccharide dried extract had a larger relative length of jejunum, ileum and caeca than those fed on the control diet ($P< 0.05$). However they also found that in general, an increase in oligosaccharide content in the diet corresponded to an increase in the length of the different intestinal sections. Trial 2 did not find this as the increase in length of the jejunum when feeding 800g/t NCF was not significantly longer than the control, only in the birds fed 200g/t NCF. Increasing the amount of MOS in the diet may have an effect on the amount of SCFA in the intestine available for the epithelial cells to use as an energy source. This is due to the fermentation of MOS in the caecal, which may result in higher turnover of cells and longer, heavier GI tract (Juskiewicz, *et al.,* 2003). However several studies found no effect of MOS on the weight or length of the intestinal tract (Iji, *et al.,* 2001; Juskiewicz, *et al.,* 2003; Baurhoo, *et al.,* 2007; Bozkurk, *et al.,* 2009). The lack of effect of NCF on intestinal length and weight may be due to the fact that there was no stocking density challenge on the birds in Trial 2, as in Trial 1. However Castillo, *et al.* (2008) in pigs and Iji, *et al.* (2001) in broilers found no effect of MOS on the intestine gross gut development even with a

challenge.

When investigating whether there was a correlation between the birds body weight and intestinal lengths and weights, it was seen that there were larger correlations between body weight and intestine length and weight when birds were younger. It was also observed that the weight of the intestine had larger correlations than length of the GI tract.

Table 4.19 Regressions of the correlation between live body weight and intestinal lengths and weights.

Day	Weight					Length			
	Duodenum	Jejunum	Ileum	Total	Panc	Duodenum	Jejunum	Ileum	Total
7	0.617**	0.556**	0.605**	0.562**	0.481**	0.525**	0.580**	NS	0.726**
14	0.493**	0.593**	0.374*	0.560**	0.535**	NS	NS	NS	NS
21	NS	0.543**	NS	0.441*	0.485**	NS	NS	NS	NS
28	NS	0.437*	NS	NS	NS	NS	NS	NS	NS
42	NS	0.350*	0.356*	0.376*	NS	NS	NS	NS	NS

**means are significantly different at $P < 0.01$, * means are significantly different at $P < 0.05$

It has been reported previously that growth response in broilers to MOS is age-dependent with a greater response in younger birds than older ones (Yang, *et al.*, 2007; Chee, 2008). This is due to earlier maturation of the gut microflora improving the integrity of the GI tract of birds. Therefore it could be postulated that birds fed NCF will have longer VH and shorter CD via a reduction in sloughing, because pathogenic bacteria cannot colonise the GI tract and release toxins. However, if there

are no or little unwanted bacteria in the GI tract, then adding NCF to the bird's diet will have no effect on the gut morphology. In Trial 2 NCF had little effect on VH or CD, which is supported by the findings of White, *et al.* (2002) in pigs and Yitbarek, *et al.* (2012) and Sohail, *et al*. (2012) in broilers when feeding MOS. However, others have reported that VH is longer with MOS supplementation (Iji, *et al.*, 2001; Zhang, *et al.*, 2005; Baurhoo, *et al.*, 2007). When investigating CD, Zhang, *et al.* (2005), Yitbarek, *et al.* (2012) and Sohail, *et al.* (2012) found feeding MOS had no effect on CD in birds, which was also shown in Trial 2.

There was little effect of NCF inclusion on the birds' performance, gross gut or microscopic development. This may be due to a problem with the NCF in the diet as the analysed levels of NCF in the diets produced unexpected results. This inconsistency in NCF may be due to a number of factors: the assay may not be reliable; there is a factor in the feed interacting with the NCF or assay; there was a problem with the NCF; or the mix of NCF in the feed was not homogenous. As shown in figure 4.2, the standard deviation of the average Actigen assay results are very high, therefore the reliability of data generated from Trial 2 may be questionable. Rye was added to the diets in this trial, which may be a factor in the diets causing an interaction with the assay causing the unpredicted results. However as this assay method is not in the public domain it is difficiult ascertain whether rye was considered in the methods validation.

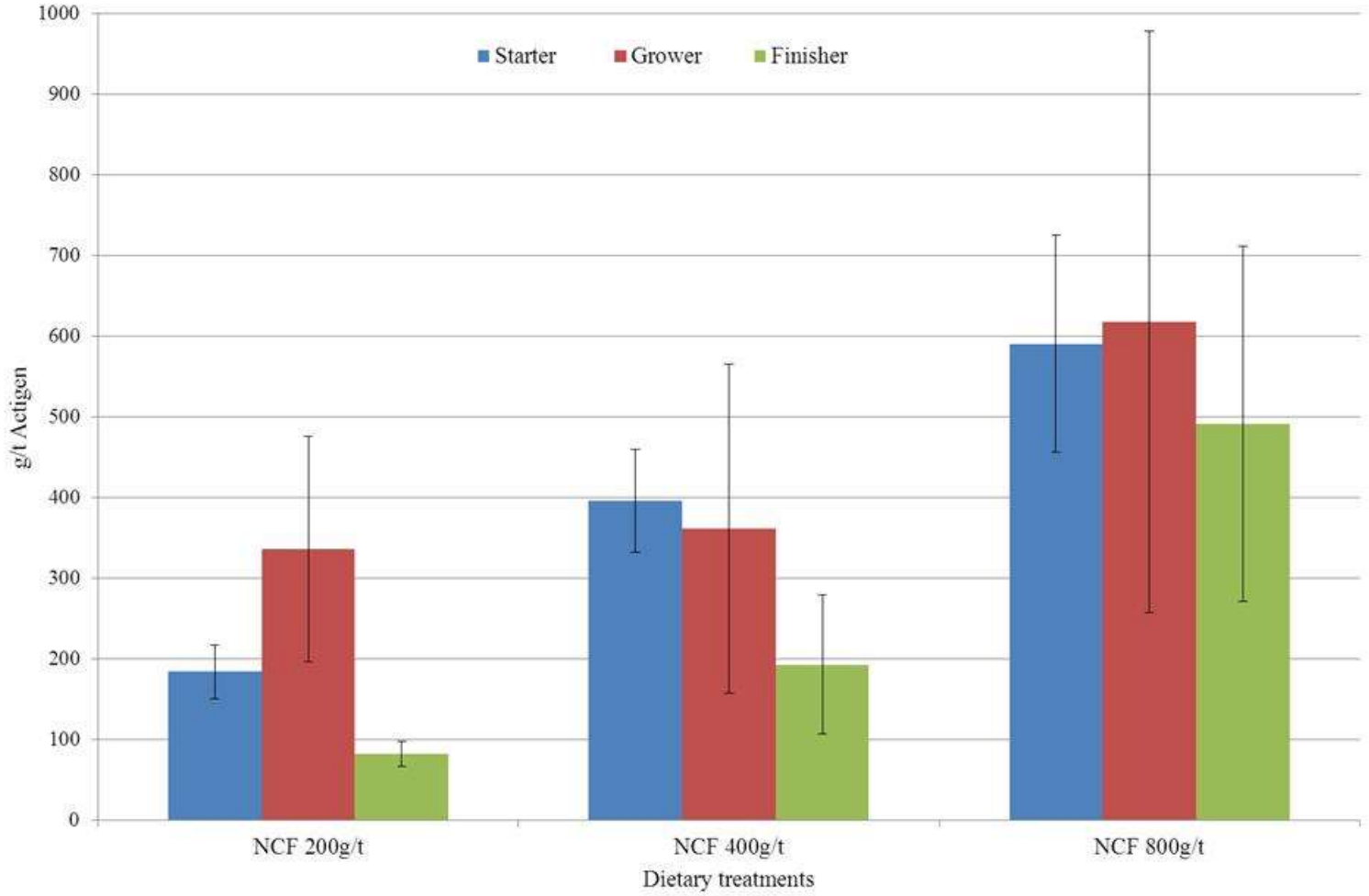

Figure 4.2 Average levels of measured NCF in feed with standard deviations.

4.6 CONCLUSION

These data indicate that, while some significant differences between treatments were observed, NCF did not show a pronounced effect on bird performance in Trial 2. This may be due to the lack of pathogenic challenge or stress on the birds in Nottingham Trent University poultry facility. There have been many studies showing that MOS improves performance and there are several theories into the mode of action of MOS in relation to improved gut health and performance. However, the published research appears to be variable with some trials demonstrating that MOS has no effect or a negative effect on performance. This may be due to differences in the type of MOS product, experimental conditions, diet formulation and health status of the birds. Therefore Trial 2 illustrates that the observed variability in the effects of MOS in other studies, is also evident when feeding NCF, however, whether this is due to product variability or environmental conditions, is still unclear.

CHAPTER 5: TRIAL 3 - INVESTIGATION OF THE EFFICACY OF A NATURAL CARBOHYDRATE FRACTION (NCF), CALCIUM BUTYRATE, SODIUM BUTYRATE AND THEIR COMBINATIONS.

5.1 INTRODUCTION

This chapter studies the effects of 2 commercial forms of butyrate salts on broiler gut health, performance, immune status and digestive enzyme activity, both singly and in combination with NCF. Butyrate salts have been reported to have similar effects to NCF on gut health and performance. However their modes of action are thought to be through different mechanisms. Trial 3 may help to illustrate the mechanisms involved when feeding these supplements and demonstrate if their combined treatment has a greater effect on the birds, either positive or detrimental. The inclusion levels of the butyrate salts in the diets were set by manufacturers' recommendations, however NCF was added at 200g/t, as in previous trials (chapter 3 and 4) this level showed the greatest effect. This trial was designed to investigate the effects in young broilers of NCF compared to another type of feed supplement also thought to improve gut health (butyrate salts). This trial used the same diet as trial 1 as to eliminate any possible effects of rye NCF on activity highlighted in trial 2.

5.2 AIM

The aim of Trial 3 was to investigate the effect of butyrate addition on efficacy of the NCF. Therefore Trial 3 compared the effects of feeding NCF, calcium butyrate (Ca But) or sodium butyrate (Na But) alone or using a butyrate salt in addition to NCF on bird gut morphology through the trial period at 7, 14, and 21 days post hatch and attempted to link microscopic gut development and performance.

The hypotheses of this trial was that the butyrates and NCF may have similar effects on the bird performance when fed singally, but show incompletely additive response when fed in combination, through sharing modes of action.

5.3 MATERIALS AND METHODS

5.3.1 Bird Husbandry

A total of 384, one day old male Ross 308 birds from a flock of 35 weeks of age were obtained from P.D.Hook Hatcheries Ltd (Cote, Bampton, Oxfordshire, UK). They were placed in 48 pens, each containing 8 birds and fed on 1 of 6 dietary treatments. The diets were allocated using a stratified random allocation. There were 6 dietary treatments with 8 replicates for each treatment, made up of 8 small floor pens (area = 0.43m^2). The litter provided was wood shavings on a concrete floor at a depth of 6cm, which was topped up if necessary to maintain adequate environmental welfare

5.3.2 Experimental Diets

Trial 3 lasted 21 days with a 2 phase feeding programme: starter diets were fed from 1 to 14 days and grower diets from 15 to 21 days. The 6 dietary treatments are shown in table 5.1 and were: 1) control diet, 2) NCF (Actigen), 3) Ca But (Globamax performant), 4) Na But (Adimix 30 Coated) 5) Ca But plus NCF and 6) Na But plus NCF. The rations were based on the nutritional requirements for the strain of bird (as defined by Aviagen) and contained no pentosan degrading enzymes in order to provide some digestive challenge. The formulations were checked by an independent nutritionist to ensure accuracy. The basal diet was formulated by Target feeds (the basal diet details are given in Table 5.2) and made at Nottingham Trent University.

Table 5.1 Inclusion levels of the dietary treatments

Dietary Treatment	Supplement inclusion level	
	Starter period	Grower period
Control	None	None
NCF	200g/t NCF	200g/t NCF
Ca But	300g/t Ca But	300g/t Ca But
Na But	1000g/t Na But	750g/t Na But
NCF/Ca But	200g/t NCF+300g/t Ca But	200g/t NCF+300g/t Ca But
NCF/Na But	200g/t NCF+1000g/t Na But	200g/t NCF+750g/t Na But

5.3.3 Determined Parameters

Bird weight and feed intakes per pen were recorded weekly. The FCR was calculated using this information on a weekly basis and for each phase. On days 7, 14 and 21, two birds per pen were selected and slaughtered. The whole intestine from one of the birds was removed. The length and weight of the separate parts of the intestinal tract were measured as described in Chapter 2. A blood sample was taken from the heart of the second bird using a 10ml syringe, its serum was collected and frozen at -20^{o}C for later analysis. The second bird was then dissected to remove the small intestine tissue sections from the duodenum and jejunum for detailed intestinal and general intestinal structure examination. Tissue samples from the duodenum and jejunum were fixed in Bouins solution prior to histological procedures, including staining with Periodic Acid Schiff's and Alcian Blue (pH 2.5), as described in chapter 2. In addition, tissue samples were taken and snap frozen in dry ice and industrial methylated spirits and stored at -80^{o}C for later analysis of digestive and immune markers. An extra bird per pen was culled on day 21 for digesta pH measurements of the gizzard, duodenum, jejunum, ileum and caeca.

Table 5.2. Composition of basal diet and calculated analysis of the basal diet

Item	Starter	Grower
Ingredients (%)		
Barley	10.61	8.46
Wheat	50.00	55.00
Soybean meal, 48% CP	26.00	23.00
Full fat soybean meal	5.00	5.00
L lysine HCL	0.31	0.26
DL methionine	0.38	0.35
L threonine	0.14	0.13
Soya oil	4.00	4.50
Limestone	1.25	1.25
Monocalcium phosphate	1.50	1.25
Salt	0.25	0.25
Sodium bicarbonate	0.15	0.15
Premix*	0.25	0.25
Calculated analysis		
ME MJ/kg	12.78	13.04
CP%	21.75	20.59
Lys %	1.37	1.25
Met + Cys %	1.01	0.95
Ca	0.96	0.90
Available P	0.48	0.42

*Premix content (volume/kg diet): Mn 100mg, Zn 80mg, Fe 20mg, Cu 10mg, I 1mg, Mb 0.48mg, Se 0.2mg, Retinol 13.5mg,Cholecalciferol, 3mg, Tocopherol 25mg, Menadione 5.0mg, Thiamine 3mg, Riboflavin 10.0mg, Pantothenic acid 15mg, Pyroxidine 3.0mg, Niacin 60mg, Cobalamin 30µg, Folic acid 1.5mg, Biotin 125mg

5.3.4 Data Analysis

All data were analysed using a incomplete 3 way ANOVA using JMP software for Windows. One pen was used as a unit for analysis. Treatment means were separated using the Tukeys post hoc test, and statistical significance was declared at $P < 0.05$.

5.4 RESULTS

5.4.1 Body Weight and Live Weight Gain

Whilst body weight and live weight gain were recorded, the pen size (80x80cm) and weekly reduction in stocking density limit the relevance of this data. The mean weekly live body weights per bird for the 6 diets over the 3 weeks are shown in Table 5.3. The most notable effect in performance was observed at 21 days when feeding Na But, where an abormally low body weight was achieved. Additionally, an interaction response between NCF and Na But was observed at 21 days, which indicates that feeding NCF and Na But together improved body weight over NCF and Na But fed individually. Little difference was seen between dietary treatments other than the weekly body weigh on days 21. On day 21, it was shown that the birds fed Na But were significantly lighter than birds fed any diet containing NCF (P=0.001).

Table 5.3. Effect of dietary treatment in broiler diets on the mean weekly body weight (g/bird)

Attribute	Age	Dietary treatment						SEM	P value	Interaction	
		Control	NCF 200g/t	Ca But	Na But	NCF/Ca	NCF/Na			NCF*Ca B	NCF*Na B
Body	1	35.8	36.1	36.5	36.9	36.4	37.4	0.25	0.412	0.928	0.755
weight (g)	7	127.3	123.3	122.2	129.6	131.7	129.3	1.71	0.570	0.120	0.663
	14	373.9	388.8	370.0	408.0	378.8	386.4	4.39	0.178	0.808	0.423
	21	761.1^{AB}	782.8^{A}	766.9^{AB}	660.3^{B}	774.4^{A}	832.6^{A}	12.24	0.001	0.787	0.004

Differing superscripts within a week denote means are significantly different P< 0.05.

The mean weekly live weight gained per bird for the 6 diets over the 6 weeks are given in Table 4. There was interactions seen when NCF and Na But were fed in combination between 15-21 days and 0-21 days, which indicates that feeding NCF and Na But together improved body weight gain over NCF and Na But fed individually. As with live body weight, there were minimal significant differences between treatments, other than the mean live weight gain per bird on day 21 and over the whole trial period. On day 21, Na But had a lower weight gain over all the other diets (P=0.001), this was also seen over the whole trial period.

Table 5.4. Effect of dietary treatment in broiler diets on the mean weekly live weight gain (g/bird)

Attribute	Age	Dietary treatment						SEM	P value	Interaction	
		Control	NCF 200g/t	Ca But	Na But	NCF/Ca B	NCF/Na B			NCF*Ca B	NCF*Na B
Weight	1-7	90.5	86.1	85.0	91.9	94.3	91.8	1.68	0.575	0.103	0.756
gain (g)	8-14	245.1	263.7	248.9	274.2	247.1	255.0	3.88	0.242	0.298	0.474
	15-21	381.9 A	428.9 A	423.2 A	277.0 B	414.7 A	443.8 A	10.41	0.000	0.937	0.002
	1-21	724.3 AB	745.7 A	729.7 A	622.6 B	737.0 A	794.8 A	12.24	0.001	0.792	0.006

Differing superscript letters within one week denote means are significantly different at $P < 0.05$

5.4.2 Feed Intake

The mean weekly feed intakes per bird for the 6 diets over the 6 weeks are shown in Table 5.5. In a similar pattern to that seen for other performance parameters, there was an interaction seen when NCF and Na But were fed in combination between 15-21 days, which indicates that feeding NCF and Na But together increased feed intake over NCF and Na But fed individually. Feed intake was generally not affected by dietary treatments other than on day 21 where birds fed Na But had a significantly

lower feed intake than birds fed NCF or NCF/Na But.

Table 5.5. Effect of dietary treatment in broiler diets on the mean weekly feed intake

Attribute	Age	Dietary treatment						SEM	P value	Interaction	
		Control	NCF 200g/t	Ca But	Na But	NCF/Ca B	NCF/Na B			NCF*Ca B	NCF*Na B
Feed	1-7	104.0	98.7	102.9	106.5	118.0	107.7	2.17	0.179	0.054	0.531
intake (g)	8-14	322.8	329.3	317.6	337.7	330.9	337.8	3.50	0.504	0.895	0.402
	15-21	609.9^{AB}	635.4^{A}	615.6^{AB}	535.9^{B}	609.7^{AB}	655.5^{A}	9.74	0.007	0.878	0.034
	1-21	825.2	830.0	860.2	810.2	894.7	889.1	12.76	0.275	0.631	0.235

Differing superscript letters within one week denote means are significantly different at $P < 0.05$

5.4.3 Feed Conversion Ratio

The mean weekly FCRs per bird for the 6 diets over the 6 weeks are shown in table 5.6. The mean weekly FCR per bird between diets showed that there were no effects of dietary treatment in the first two weeks, however there were significant differences between dietary treatments on day 21 and over the whole 3 week trial period. There was interactions seen when NCF and Na But were fed in combination, which indicates that feeding NCF and Na But together improved FCR over NCF and Na But fed individually at 15-21 days and over Na But between 0-21 days. In the third week it was shown that birds fed Na But had significantly increased FCR over the other treatment groups (P=0.001), and over the whole trial period birds fed Na But had significantly increased FCR over NCF, Ca But and Na But treatment groups (P=0.001).

Table 5.6 Effect of dietary treatment in broiler diets on the weekly FCR.

Attribute	Age	Dietary treatment						SEM	P value	Interaction	
		Control	NCF 200g/t	Ca But	Na But	NCF/Ca B	NCF/Na B			NCF*Ca B	NCF*Na B
FCR	1-7	1.15	1.16	1.22	1.16	1.25	1.17	0.01	0.053	0.161	0.669
	8-14	1.34	1.31	1.33	1.34	1.34	1.32	0.01	0.901	0.263	0.453
	15-21	1.56 B	1.49 B	1.49 B	1.95 A	1.46 B	1.39 B	0.03	<0.001	0.420	0.001
	1-21	1.21 AB	1.11 B	1.18 B	1.30 A	1.22 AB	1.13 B	0.01	0.001	0.070	0.006

Differing superscript letters within one week denote means are significantly different at $P < 0.05$

5.4.4 Intestinal Weight and Length

The mean weekly intestinal weight and lengths and pancreas weights for the 6 diets on day 7, 14 and 21 are shown in tables 5.7, 5.8 and 5.9 respectively. There were interactions observed at 7 days between NCF and Ca But, as the trend toward significant improvement in both duodenal weight and DWBWR observed with Ca But supplementation is lost when fed in combination with NCF. On day 7 birds fed solely Ca But supplement had significantly heavier jejuna than birds fed NCF (P=0.039). Also on day 7 birds fed the control diet and the Na But/NCF diet had lighter mean PBWR than birds fed diets just containing Ca But or Na But (P = 0.001). On day 14 there were no significant differences between diets on intestinal weights or lengths. There were interactions observed at 21 days between NCF and Ca But, where numerical decreases compared to control in ileum and total intestinal length observed when feeding NCF but not Ca But alone, were also observed when feeding Ca But and NCF together. A second interation was also observed at 21 days between NCF and Na But; Na But increased the pancrease weight relative to body weight, but this effect was not seen when fed in combination with NCF. On day 21 birds fed Na But had significantly longer DLBWR than birds fed NCF, Na But and

Ca But/Act. Ca But fed birds also had significantly shorter DLBWR than control birds (P=0.006). Also on day 21 JLBWR was significantly different between treatments; birds fed Na But had a longer jejunum than birds fed NCF, Ca But/Act and Na But/Act. Birds fed Na But similarly had a significantly longer ILBWR than other treatment groups. Birds at 21 days fed Na But and the control diet had heavier PBWR than birds fed Ca But/Act or Na But/Act. However effects of Na But at 21 days on gross gut morphology may be unreliable due to the unusual growth performance which suggested that the Na But diet composition may be incorrectly manufactured. However feed samples could not be analysed for composition due to samples getting wet during storage, causing mould growth.

Table 5.7 Effect of dietary treatment in broiler diets on the length and weight of the intestine and pancreas development at 7 days

	Duodenum				Jejunum				Ileum				Total		Pancreas	
Diet	Weight	Length	DWBWR	DLBWR	Weight	Length	JWBWR	JLBWR	Weight	Length	IWBWR	ILBWR	Weight	Length	Weight	PBWR
Control	1.40	14.7	1.21	11.91	2.93^{AB}	32.9	2.46	26.26	2.06	33.0	1.65	27.20	6.35	81.5	0.50	3.9^{B}
200g/t NCF	1.67	14.5	1.32	12.33	2.90^{B}	32.3	2.36	27.23	1.80	30.5	1.44	26.83	6.37	78.6	0.54	4.4^{AB}
Ca But	1.97	15.3	1.49	12.15	3.42^{AB}	32.3	2.68	25.82	2.16	33.2	1.67	25.74	7.93	81.7	0.62	5.0^{A}
So But	1.79	15.8	1.35	11.88	3.18^{AB}	33.8	2.61	26.46	2.11	32.0	1.62	25.15	7.31	81.5	0.61	5.0^{A}
NCF/Ca But	1.65	15.2	1.23	11.31	4.00^{A}	33.3	2.82	25.59	2.26	34.0	1.67	25.58	8.03	83.3	0.54	3.8^{B}
NCF/Na But	1.54	13.7	1.46	12.46	3.10^{AB}	33.7	2.76	29.63	1.71	31.5	1.52	28.39	6.59	78.1	0.53	4.7^{AB}
SEM	0.06	0.24	0.03	0.28	0.11	0.46	0.06	0.54	0.07	0.49	0.04	0.59	0.23	0.90	0.02	0.11
P value	0.075	0.209	0.090	0.890	0.039	0.905	0.234	0.285	0.226	0.355	0.628	0.627	0.087	0.530	0.195	0.001
NCF*Ca B	0.597	0.212	0.031	0.417	0.191	0.340	0.566	0.252	0.180	0.209	0.552	0.510	0.276	0.193	0.826	0.112
NCF*Na B	0.685	0.365	0.929	0.906	0.644	0.735	0.934	0.750	0.783	0.522	0.369	0.359	0.629	0.563	0.470	0.854

Weight (g), Length (cm), DWBWR = grams of duodenum tissue per 100g bird body weight, DLBWR = centimetre of duodenum tissue per 100g bird body weight, JWBWR = grams of jejunum tissue per 100g bird body weight, JLBWR = centimetre of jejunum tissue per 100g bird body weight, IWBWR = grams of ileum tissue per 100g bird body weight, ILBWR = centimetre of ileum tissue per 100g bird body weight, PBWR = grams of pancreas tissue per 100g bird body weight. Differing superscript letters denote means are significantly different at $P < 0.05$

Table 5.8 Effect of dietary treatment in broiler diets on the length and weight of the intestine and pancreas development at 14 days

	Duodenum				Jejunum				Ileum				Total		Pancreas	
Diet	Weight	Length	DWBWR	DLBWR	Weight	Length	JWBWR	JLBWR	Weight	Length	IWBWR	ILBWR	Weight	Length	Weight	PBWR
Control	4.89	20.1	1.31	5.41	8.96	45.5	2.41	12.23	5.96	44.3^{C}	1.61	11.74	19.81	108.6	1.41	3.8
200g/t NCF	4.65	19.6	1.21	5.13	9.20	47.7	2.38	12.81	5.87	47.6^{ABC}	1.53	12.70	19.72	113.1	1.49	3.9
Ca But	4.55	20.9	1.19	5.72	8.06	49.0	2.35	12.82	5.77	47.8^{AB}	1.61	13.00	18.37	118.3	1.73	4.6
So But	4.18	20.0	1.19	5.18	9.30	47.1	2.42	11.62	6.04	49.0^{AB}	1.56	12.14	19.90	116.1	1.60	4.1
NCF/Ca But	4.68	19.5	1.24	5.17	8.67	45.0	2.45	11.87	6.37	45.7^{BC}	1.69	12.10	20.36	110.2	1.54	4.1
NCF/Na But	4.79	20.6	1.23	5.34	9.40	47.5	2.43	12.31	6.83	49.8^{A}	1.77	12.77	21.02	119.2	1.76	4.5
SEM	0.12	0.29	0.03	0.11	0.31	0.62	0.07	0.20	0.17	0.51	0.05	0.17	0.55	1.22	0.05	0.12
P value	0.675	0.719	0.914	0.636	0.841	0.463	0.999	0.439	0.468	0.015	0.683	0.233	0.849	0.060	0.121	0.155
NCF*Ca B	0.734	0.743	0.560	0.874	0.816	0.212	0.634	0.477	0.999	0.992	0.840	0.888	0.830	0.502	0.685	0.685
NCF*Na B	0.608	0.213	0.394	0.170	0.558	0.787	0.758	0.710	0.802	0.908	0.532	0.609	0.885	0.844	0.799	0.885

Weight (g), Length (cm), DWBWR = grams of duodenum tissue per 100g bird body weight, DLBWR = centimetre of duodenum tissue per 100g bird body weight, JWBWR = grams of jejunum tissue per 100g bird body weight, JLBWR = centimetre of jejunum tissue per 100g bird body weight, IWBWR = grams of ileum tissue per 100g bird body weight, ILBWR = centimetre of ileum tissue per 100g bird body weight, PBWR = grams of pancreas tissue per 100g bird body weight. Differing superscript letters denote means are significantly different at $P < 0.05$

Table 5.9 Effect of dietary treatment in broiler diets on the length and weight of the intestine and pancreas development at 21 days

	Duodenum				Jejunum				Ileum				Total		Pancreas	
Diet	Weight	Length	DWBWR	DLBWR	Weight	Length	JWBWR	JLBWR	Weight	Length	IWBWR	ILBWR	Weight	Length	Weight	PBWR
Control	7.64	25.0	1.02	3.33 AB	15.30	56.8	2.05	7.57 AB	12.09	60.1	1.61	7.63 B	35.03	141.9	2.77	3.6 AB
200g/t NCF	6.91	25.4	0.89	3.18 BC	14.72	54.2	1.89	6.99 B	11.33	55.8	1.45	7.17 B	32.95	134.7	2.42	3.1 BC
Ca But	7.53	25.1	1.05	3.31 ABC	16.88	59.0	2.25	7.80 AB	12.52	60.3	1.75	7.51 B	36.29	144.4	2.29	3.0 BC
Na But	7.49	25.3	1.16	3.69 A	15.10	57.0	2.32	8.71 A	10.55	58.0	1.62	8.87 A	33.13	140.2	2.58	3.9 A
NCF/Ca But	6.79	23.4	0.88	2.89 C	14.45	55.1	1.87	6.98 B	10.52	56.1	1.48	7.25 B	31.93	133.4	2.23	2.9 C
NCF/Na But	7.77	24.9	0.93	3.05 BC	15.26	57.0	1.82	6.83 B	11.71	59.2	1.41	7.08 B	34.73	141.0	2.39	2.8 C
SEM	0.19	0.29	0.03	0.06	0.30	0.77	0.06	0.18	0.24	0.82	0.05	0.16	0.58	1.56	0.05	0.10
P value	0.580	0.443	0.138	0.006	0.266	0.594	0.067	0.019	0.082	0.467	0.285	0.005	0.311	0.280	0.057	0.002
NCF*Ca B	0.996	0.745	0.269	0.142	0.884	0.994	0.274	0.161	0.573	0.046	0.506	0.088	0.898	0.049	0.173	0.983
NCF*Na B	0.086	0.504	0.492	0.594	0.634	0.131	0.942	0.948	0.077	0.642	0.343	0.689	0.167	0.860	0.412	0.021

Weight (g), Length (cm), DWBWR = grams of duodenum tissue per 100g bird body weight, DLBWR = centimetre of duodenum tissue per 100g bird body weight, JWBWR = grams of jejunum tissue per 100g bird body weight, JLBWR = centimetre of jejunum tissue per 100g bird body weight, IWBWR = grams of ileum tissue per 100g bird body weight, ILBWR = centimetre of ileum tissue per 100g bird body weight, PBWR = grams of pancreas tissue per 100g bird body weight. Differing superscript letters denote means are significantly different at $P < 0.05$

5.4.5 Gut Morphology

The mean jejunum VH, VW, CD and VCR for weeks 1 for the 6 diets are shown in table 5.10. There were no interactions seen when NCF and Na But or Ca But were fed in combination. No significant differences were observed between dietary treatments, however there was a trend for NCF/Ca But to have higher VCR (P=0.068). The mean jejunum VH, VW, CD and VCR for weeks 2 and 3 for the 6 diets are shown in table 5.11 and 5.12, which shows no significant differences between dietary treatments.

Table 5.10. Effect of NCF on the gut morphology of the jejunum in week 1 (µm)

Attribute	Dietary treatment						SEM	P value	Interaction	
	Control	NCF 200g/t	Ca But	Na But	NCF/Ca But	NCF/Na But			NCF*Ca B	NCF*Na B
Jejunum										
Villus Height	725.3	814.4	795.0	774.4	885.4	788.0	23.3	0.533	0.614	0.205
Villus Width	183.2	172.9	176.8	181.6	174.8	182.1	2.9	0.881	0.467	0.354
Crypt Depth	125.1	129.3	136.8	135.4	130.2	126.1	1.9	0.398	0.203	0.338
VCR	5.9	5.6	5.9	5.9	6.9	6.1	0.12	0.063	0.902	0.366

Table 5.11. Effect of NCF on the gut morphology of the jejunum in week 2 (µm)

Attribute	Dietary treatment						SEM	P value	Interaction	
	Control	NCF 200g/t	Ca But	Na But	NCF/Ca But	NCF/Na But			NCF*Ca B	NCF*Na B
Jejunum										
Villus Height	1187.2	1305.0	1158.2	1263.2	1221.3	1201.9	32.8	0.830	0.694	0.200
Villus Width	204.0	218.2	190.6	209.8	192.1	212.2	4.0	0.245	0.405	0.403
Crypt Depth	155.6	172.4	168.4	167.6	161.5	171.0	3.4	0.743	0.140	0.421
VCR	7.8	7.7	7.0	7.6	7.8	7.2	0.12	0.357	0.240	0.395

Table 5.12. Effect of NCF on the gut morphology of the jejunum in week 3 (µm)

Attribute	Dietary treatment						SEM	P value	Interaction	
	Control	NCF 200g/t	Ca But	Na But	NCF/Ca But	NCF/Na But			NCF*Ca B	NCF*Na B
Jejunum										
Villus Height	1346.3	1302.6	1453.0	1396.4	1375.6	1348.6	25.4	0.660	0.793	0.597
Villus Width	261.6	245.4	243.5	227.4	236.9	234.8	4.4	0.301	0.686	0.321
Crypt Depth	162.3	150.4	159.2	171.7	153.2	155.7	2.9	0.331	0.114	0.829
VCR	8.5	8.9	9.1	8.5	8.8	9.0	0.18	0.888	0.204	0.880

5.4.6 Goblet cells

Investigation of goblet cell number and area in the jejunum (table 5.13), revealed that there were no interactions seen when NCF and Na But or Ca But were fed in combination, and that diets including supplements did not significantly affect goblet cell area, goblet cell number per 165µm of villi or goblet cell measurements as a ratio. However in the jejunum calcium butyrate tended ($P<0.1$) to increased goblet cell area per 165µm of villus over the NCF/Na But treatment group.

Table 5.13. Effect of NCF on the goblet cells of the jejunum in week 1 (µm^2)

Atribute	Dietary treatment						SEM	P value	Interaction	
	Control	NCF	Ca But	Na But	NCF/Ca B	NCF/ Na B			NCF*Ca B	NCF*Na B
Jejunum										
GC Area (µm^2)	75.5	73.4	81.4	80.9	76.7	62.5	2.1	0.108	0.332	0.596
N^o of GC per 165µm	12.7	14.1	15.0	12.5	13.9	13.5	0.3	0.154	0.058	0.724
GC Area per 165µm	949.0	1031.7	1193.0	980.6	1038.6	834.8	34.8	0.072	0.568	0.607

GC= Goblet cell

5.4.7 Intestinal pH

The mean intestinal pH per bird for the 6 diets at 21 days is shown in Table 5.14,

which shows that there were no interations or significant effect of dietary treatment on the pH of the separate parts of the intestine.

Table 5.14. Effect of dietary treatment on the intestinal pH.

Tissue	Dietary treatment						SEM	P value	Interaction	
	Control	NCF	Ca But	Na But	NCF/Ca But	NCF/Na But			NCF*Ca B	NCF*Na B
Gizzard	2.70	2.70	2.52	2.56	2.66	2.66	0.105	0.996	0.829	0.871
Duodenum	6.06	6.11	5.97	6.06	6.00	6.08	0.033	0.417	0.289	0.261
Jejunum	6.03	5.93	5.99	5.96	6.03	5.93	0.021	0.611	0.615	0.835
Ileum	6.80	6.87	6.98	6.94	6.59	6.91	0.077	0.733	0.359	0.604
Caeca	5.87	5.58	5.74	5.77	5.70	5.50	0.052	0.372	0.144	0.342

5.4.8 Mortality

There was no difference between diet treatments in mortality for this study, shown in Table 5.15.

Table 5.15. Effect of dietary treatment on mortality

Attribute	Dietary treatment						
	Control	NCF 200g/t	Ca But	Na But	NCF/Ca But	NCF/Na But	Total
Mortality (%)	1.8	1.3	0.5	1.3	0.0	0.8	5.7

5.5 DISCUSSION

Performance parameters, in general were not affected by dietary treatment. The only significant performance effects seen between diets were on day 21, where Na But substantially decreased body weight, weight gain, feed intake and increased FCR over some or all of the other diets. At this time point it was also recorded that there was an interaction when feeding NCF and Na But, which showed an increased body weight, weight gain, feed intake and improved FCR over NCF and Na But fed separately. This could be due to a negative effect of Na But on the bird, but this degree of negative response seems unlikely in a licenced feed product, and is more

likely due to a diet manufacturing fault resulting in the grower diet of birds fed Na But being deficient in some way. The lack of treatment differentiation in terms of performance effects in the starter phase of Trial 3 may be due to the lack of challenge on the birds. This is in agreement with other studies in chickens (Leeson, *et al*., 2005; Zhang, *et al*., 2011b; Czerwinski *et al* 2012) and Piglets (Biagi, *et al*., 2007; Tonel, *et al*., 2010), that demonstrated no effect on performance from either sodium butyrate, calcium butyrate or butyric acid when there were no challenges placed on the animal. When a challenge was present in other studies improvements in growth parameters were reported in piglets (Piva, *et al*., 2002; Manzanilla, *et al*., 2006; Le Gall, *et al*., 2009) and broilers (Leeson, *et al*., 2005; Zhang, *et al*., 2011; Zhang, *et al*., 2011b) by feeding sodium butyrate. It is thought that sodium or calcium butyrate may have a growth promoting effects through providing energy for the epithelium and altering antimicrobial activity. Conversely, performance improvements have been observed in one study where broilers were fed sodium butyrate without any recorded challenge (Smulikoska, *et al*., 2009). In further contradiction, Hu and Gao (2007) reported that feeding butyrate to unchallenged broilers negatively affected FCR, which may explain the effects seen when feeding Na But at 21 days. These studies show that the growth promoting effects of butyrate salts are inconsistent, probably due to variability in the type of environment and management conditions of the trials.

It is possible that the growth inhibiting effect of high pathogen burden associated with a poor environment is mitigated by butyrate usage. This suggests that broilers reared in a low pathogen environment do not have ‘promoted’ growth due to butyrate supplementation, because the animals already have a high health status and

are therefore performing at optimum growth rate. When butyrate is not protected by a fat coating it is readily taken up by the GI tract epithelium, where it is metabolised for energy though the TCA cycle. This means that the host animal can gain a substantial supply of energy from butyrate (Lawhon, *et al.*, 2002; Guilloteau, *et al*., 2010), which may lead to increased proliferation of intestinal cells (Kripke, *et al*., 1989; Friedel and Levine, 1992). Therefore the negative performance effects observed when feeding Na But up to 21 days may in part be due to an increase in the maintenance needs of the GI tract from increased proliferation of intestinal cells. However, this is unlikely to be the main cause of the negative performance effects, as the negative effect seen was abnormally large and may be due to problems with the feed.

Investigation of gross gut morphology showed showed little effect overall, however there were interactions observed at 7 days between NCF and Ca But, as the trend toward significant improvement in both duodenal weight and DWBWR observed with Ca But supplementation is lost when fed in combination with NCF. The improvement observed when feeding Ca But alone may be due increased proliferation of duodenal epithelium cells in response to additional energy supplied via butyrate ions (Jozefiak *et al.*, 2004). The contrasting decrease in weight observed when feeding Ca But in combitation with NCF may be due to diversion of this energy from epithelial proliferation to upregulation of immune system (Spring *et al.*, 2000).

Additionally, on day 7 birds fed the control diet and the Na But/NCF diet had lighter mean PBWR than birds fed diets just containing Ca But or Na But. This may mean

that Ca But and Na But have a greater ability to produce pancreatic enzymes than control birds or Na But/NCF. On day 14 there were no significant differences between treatments on gross gut morphology. However on day 21 birds fed Na But had significantly longer DLBWR, jejunum and ILBWR than some or all other treatment groups. This increase in intestinal length could be due to the birds fed Na But diverting too much energy in to development of the intestinal tract rather that carcass growth, as growth performance was negatively affected in the same birds. However this may also be due to a problem with the diet, meaning that the birds have compensated by increased the length of their intestine to try increase their ability to absorb nutrients. PBWR at 21 days was heavier in birds fed Na But and the control than birds fed Ca But/NCF or Na But/NCF, this may be due to the birds fed Ca But/Act or Na But/Act having heavier body weight. Also at 21 days Ca But fed birds had significantly shorter DLBWR than control birds. When examining this body of evidence as a whole, no consistent pattern of effect emerged. The few significant differences recorded may be due to type 1 errors, which would be expected when examining so many parameters.

As there were no clear treatment patterns in the gross gut development, data from all diets was pooled to examining the relationship between body weight and organ development over the first three weeks post hatch (table 5.15). When investigating whether there was a relationship between the bird body weight and intestinal lengths and weights, it was seen that there were stronger correlations between body weight and intestine length and weight when birds were younger. It was also seen that the weight of the intestine had stronger correlations to body weight than length of the intestine, as seen in Trial 2. Similarly, pancreas weight to body weight ratio was also

significantly correlated at day 7 and 14 but not at day 21.

Table 5.15 Regressions of the correlation between live body weight and intestinal length and weight.

Day	Weight					Length			
	Duodenum	Jejunum	Ileum	Total	Panc	Duodenum	Jejunum	Ileum	Total
7	0.591**	0.719**	0.606**	0.664**	0.634**	0.350*	0.529**	0.280	0.351*
14	0.137	0.236	0.130	0.209	0.301*	-0.141	-0.180	0.001	0.057
21	-0.130	-0.080	0.008	-0.076	-0.030	-0.090	-0.140	-0.024	-0.101

**means are significantly different at $P < 0.01$, * means are significantly different at $P < 0.05$

Microscopic gut morphology at day 7 revealed no significant differences between treatments, however a trend ($P = 0.058$) was seen when expressing villus height and crypt depth as a ratio, VCR. NCF/Ca But birds tended to have higher VCR than birds fed NCF, suggesting that NCF/Ca But have better gut health, as higher VCR indicates longer villi and shallower crypts. This indicates improved gut health, as longer villi are considered to indicate that the bird will have a better ability to absorb nutrients, due to the increased surface area (Gao, *et al*., 2008). Conversely, deeper CDs are considered a negative indication of gut health because, to compensate for villi atrophy, the bird has to increase rate of tissue turn over. As epithelial cells are produced in the crypts and migrate along the villi to the tip, it is thought that the higher turnover in the crypt cell causes it to become deeper (Gao, *et al*., 2008). A higher VCR of birds fed NCF/Ca But may be a result of Ca But providing extra energy to the epithelium cells of the jejunum, thus allowing them to proliferate and increase their VCR, in combination with NCF decreasing the pathogenic load of the birds and therefore reducing presence of toxic products. Smulikowska *et al*. (2009)

also found no significant effect of feeding sodium butyrate on the microscopic gut morphology of 8 day old broilers. However they did not measure VCR, but did see a numerical increase in VH and decreases in CD, which may have resulted in a higher VCR as seen numerically in this trial.

Mean jejunum VH, VW, CD and VCR at week 2 and 3 for the 6 diets showed no significant differences. Leeson *et al*. (2005) at 21 day and Czerwinski *et al.* (2012) at 30 day in broilers and Tonel *et al*. (2010) at 56 day in piglets supports this finding, as these authors also found no effect of butyrate on gut morphology. However, Manzanilla *et al*. (2006) reported an increased jejunum crypt depth (CD) in piglets and Hu and Guo (2007) at 21 days found an improved VCR when feeding sodium butyrate to broilers. In common with mannan oligosaccharides and other feed supplements, feeding butyrate seems to have variable effects on gut health parameters. Positive effects of butyrate on gut health may be through butyrate decreasing pathogenic load and therefore harmful toxins, and/or increasing energy available to the epithelial cells (Sunkara et al., 2011). A lack of butyrate present in the GI tract, whether from the diet or bacterial fermentation, can lead to nutritional deficiencies of the epithelium and result in atrophy (Hamer, *et al*., 2008). As with additives, such as MOS, the variability reported in gut morphology when feeding butyrate may be due to differences in environmental and health statuses of the birds. Butyrate may only have an effect when the bird is not meeting its optimum performance due to some level of challenge. In the present trial there was no environmental challenge applied to the birds, and hence no impairment to gut development, which may be the reason why no effects were observed at 21 days of dietary treatment on the microscopic gut morphology.

When investigating the effects of the supplements on goblet cells it was seen that there were no significant differences between diets, however there was a trend demonstrated when investigating the goblet cell area per 165µm of villus. It was seen numerically that both Ca But and NCF/Ca But treatment groups increased goblet cell area per 165µm of villus over Na But and NCF/Na But. This suggests that calcium butyrate is having a larger effect on the bird than sodium butyrate. This may be due to calcium butyrate containing a different level of butyric acid, calcium butyrate is bound to two butyric acids whereas sodium is bound to one. This suggested increase in goblet cell area per 165µm of villus may mean that there is an increased storage and production of mucin, which may be considered an improvement in bird health. Mucin is essential for a number of brush border processes, including facilitating absorption of nutrients, containing enzymes, lubrication and decreasing the binding and colonisation of pathogenic bacteria to the intestine (Blomberg, et al., 1993; Smirnov, et al., 2004). Therefore an increase in the level of mucin could have a beneficial effect on the first line of defense of the immune system (Baurhoo, et al., 2009) and the absorptive function of the gut. In contrast, overproduction of mucin may result in a negative effect, by increasing the mucus thickness on the GI tract wall to a level that might impair the ability of nutrients to pass through to the gut epithelial to be absorbed (Smirnov, et al., 2004; Brummer, et al., 2010), as well as increasing the energy partitioned away from tissue growth to mucin production.

In this trial, there were no significant differences between diets when investigating the number and area of goblet cells in the jejunum at 7 days, only a trend. This would suggest that the supplements had only a small or no effect on the mucin

profile of broilers in the jejunum in this study, which links to the lack of performance effect seen. This absence of response was also reported by Castillo, et al. (2008) and Yitbarek, et al. (2012), however Baurhoo, et al. (2007; 2009); Chee, et al. (2010); Morales-lopez, et al. (2010) and Muthusamy, et al. (2012) all found goblet cell numbers were increased by yeast cell wall product supplementation. To the authors knowledge there is no published studies on the effects of butyrate salts on goblet cells in broilers, however it has been seen in one paper that sodium butyrate had no effect on goblet cell number in pigs (Manzanilla et al., 2006). In contrast to this Nahrling et al (2010) reported that butyrate in piglets increased MUC2 expression, which is key in mucin production. The lack of data in this area makes it difficult to drawn conclusion, however it suggests that butyrate may be affecting mucin production.

Sodium and calcium butyrate have antimicrobial activity due to their ability to acidify bacteria cell cytoplasm (Sunkara et al., 2011). Butyrate salts can cross a bacterial cell wall when bound but dissociate once in the more alkaline content of the cell to proton which disrupts the internal pH, and the anion, causes a lowering in the pH of cytoplasm (Fernandez Rubio, *et al*., 2009). The molecular composition of butyrate salts suggest that when fed to poultry, it may act as an acidifier. Indeed, Biagi *et al*. (2007) reported an increase in pH of the caecum when feeding sodium butyrate, and Czerwinski, *et al.* (2012) saw an increase in pH in the ileal digesta but not in the caecal digesta. However, Trial 3 showed no significant effect of diet on the pH of the intestine on day 21. This is likely be due to the dietary inclusion levels of butyrate salt not reaching a level that would not be considered an acidifier; acidifiers can be added into water or feed and have been added at inclusion levels of 3-9g/kg of

feed (Brzóska, et al., 2013). This finding is supported by Manzanilla, *et al*. (2006); Smulikowska, *et al*. (2009) and Zhang, *et al*. (2011) who all similarly reported that GI tract pH was not affected when feeding sodium butyrate.

The effect of NCF on performance parameters in Trial 3 are similar to observed results in Trial 2. The data indicate that 200g/t NCF did not show a marked effect on bird performance in Trial 2 or 3. This may be due to the weekly sampling making it difficult to assess the performance data, limiting its commercial application. However, the effect of feeding 200g/t NCF on other measurement parameters in Trial 2 and 3 also show little difference to the control, which may be due to a lack of pathogenic challenge or stress on the birds in Nottingham Trent University's poultry facility.

5.6 CONCLUSION

These data indicate that, while some significant differences between treatments were observed, NCF, Ca But and Na But did not show a pronounced effect on bird performance, gross GI tract morphology, microscopic GI tract morphology or digesta pH in Trial 3. This may be due to the lack of pathogenic challenge or stress on the birds in Nottingham Trent University's poultry facility. There have been many studies showing that MOS, Ca But and Na But improves performance and there are several theories into the mode of action in relation to improved gut health. However, the published research appears to be variable with some trials demonstrating they have no effect or a negative effect on performance. This may be due to differences in the type of product, experimental conditions, diet formulation and health status of the birds.

CHAPTER 6: INVESTIGATION INTO THE EFFECTS OF NCF AND BUTYRATE SUPPLEMENTS ON POTENTIAL EARLY DETERMINANTS OF GUT HEALTH AND PERFORMANCE.

6.1 INTRODUCTION

This thesis seeks to provide insight into the mechanisms behind the effects of the feed supplements, NCF, Ca and Na butyrate, by investigating parameters from a whole bird response down to alterations in gene expression. Where previous chapters have examined direct performance measures and development of the gut itself, this chapter reports on upstream factors with the potential to subsequently influence gut health and performance. Measures reported in the present chapter include; gene regulation of immune and metabolic processes in birds fed NCF; digestive enzymes activity in broilers fed NCF, sodium butyrate and calcium butyrate supplements; and immunoglobulin levels in broilers fed NCF, sodium butyrate and calcium butyrate supplements. Therefore this chapter explores the genetic and molecular changes resulting from NCF supplementation that may elucidate the observed whole bird performance effects. This chapter reports on the expression of genes associated with immune and metabolic processes from Trial 1, and any changes in concentration of immunoglobulins (IgG, IgM and IgA) and activity of amylase, chymotrypsin and trypsin digestive enzymes from Trials 1 and 3.

6.2 AIM

The aim of the work reported in this chapter was to identify the key immune and metabolic gene expressions, which are affected by NCF supplementation in broilers and to examine the effects of NCF, sodium butyrate and calcium butyrate on

digestive enzyme activity and immunoglobulin levels in broilers.

This chapter investigates two distinct mechanisms that may support previously observed performance responses. The first hypothesis is that NCF alters immune-related gene expression and increases immunoglobulin levels in the blood to support bird defence against pathogenic bacteria. The second hypothesis is that NCF increases levels of digestive enzymes in the GIT due to improved gut morphology and development.

6.3 MATERIALS AND METHODS

6.3.1 Experimental design

Bird husbandry for Trial 1 is as stated in Chapter 3 and for Trial 3 as stated in Chapter 5. Trial 1 lasted 42 days with a 3 phase feeding programme, providing starter diets from 1 to 14 days, grower diets from 15 to 28 days and finisher diets from 29 to 42 days. Trial 3 lasted 21 days with a 2 phase feeding programme, providing starter diets from 1 to 14 days and grower diets from 15 to 21 days. Trial 1 had four dietary treatments containing increasing levels of NCF, whereas Trial 3 had six dietary treatments of various combinations of NCF and butyrate salt supplements. The diets in both trials were allocated using a stratified random allocation. The litter provided was fine dry sand in Trial 1 and shavings in Trial 3, both on a concrete floor to a depth of 6cm. Bedding substrate was added to maintain adequate environmental welfare standards.

6.3.2 Experimental Diets

The diets were based on the nutritional requirements for the strain of bird and

contained approximately 50% wheat but no pentosan degrading enzymes; in order to provide a small digestive challenge. The basal diets for both trials were free of antibiotics and manufactured using the formulation in table 6.1. The four dietary treatments in Trial 1 were 1) control (NCF free), 2) NCF at 200g per tonne, 3) NCF at 400g per tonne, and 4) NCF 800g per tonne. The 6 dietary treatments in Trial 3 were 1) Control diet (NCF free), 2) NCF at 200g per tonne, 3) Ca But (300g/t), 4) Na But (Starter:1000g/t, Grower:750g/t) 5) Ca But plus NCF, and 6) Na But plus NCF.

Table 6.1. Composition and calculated analysis of the basal diet for Trial 1 and 3.

Item	Starter	Grower	Finisher
Ingredients (%)			
Barley	10.61	8.46	7.23
Wheat	50.00	55.00	60.00
Soybean meal, 48% CP	26.00	23.00	19.00
Full fat soybean meal	5.00	5.00	5.00
L lysine HCL	0.31	0.26	0.25
DL methionine	0.38	0.35	0.33
L threonine	0.14	0.13	0.14
Soya oil	4.00	4.50	4.75
Limestone	1.25	1.25	1.25
Monocalcium phosphate	1.50	1.25	1.25
Salt	0.25	0.25	0.25
Sodium bicarbonate	0.15	0.15	0.15
Premix*	0.25	0.25	0.25
Calculated analysis			
ME MJ/kg	12.78	13.04	13.20
CP%	21.75	20.59	19.10
Lys %	1.37	1.25	1.13
Met + Cys %	1.01	0.95	0.89
Ca	0.96	0.90	0.90
Available P	0.48	0.42	0.65

*Premix content (volume/kg diet): Mn 100mg, Zn 80mg, Fe 20mg, Cu 10mg, I 1mg, Mb 0.48mg, Se 0.2mg, Retinol 13.5mg,Cholecalciferol, 3mg, Tocopherol 25mg, Menadione 5.0mg, Thiamine 3mg, Riboflavin 10.0mg, Pantothenic acid 15mg, Pyroxidine 3.0mg, Niacin 60mg, Cobalamin 30µg, Folic acid 1.5mg, Biotin 125mg

6.3.3 Determined Parameters

For immunoglobulin measurement, one bird per pen was selected and slaughtered on day 42 in Trial 1. In Trial 3, one bird per pen was selected and slaughtered on days 7, 14 and 21. Immediately after death blood was removed from the bird and allowed to clot for 2-4 hours. The blood was then centrifuged and the serum poured off and stored at -20^{o}C. Levels of IgM, IgA and IgG were measured in serum of the bird using an enzyme linked immunosorbent assay (ELISA) micro titre plate for the detection of chicken IgM, IgA or IgG in serum from Bethyl Laboratories Inc. (Montgomery, TX, USA) as described in Chapter 2.

In order to measure digestive enzyme activity, one bird per pen was selected and slaughtered on day 42 in Trial 1 and on days 7, 14 and 21 in Trial 3. Tissue samples were taken from two intestinal regions, the duodenum and the jejunum. Details of sample removal and storage are given in chapter two. The tissue samples were later analysed to determine the activity levels of trypsin, chymotrypsin and amylase enzymes.

Tissue samples from the jejunum were taken from one bird per pen in Trial 1 on day 42 and stored for microarray analysis as per the method stated in Chapter 2. The RNA was purified and its quality and quantity measured prior to conducting gene microarrays to assess gene expression using a GeneChip Fluidics Station, and were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, California, USA) at Alltech headquarters (Nicholasville, Kentucky, USA), using the GeneChip Chicken Genome Array. The gene expression data produced was analysed using Genespring GX software (Affymetrix, Santa Clara, California, USA), IPA

(Ingenuity Systems Inc, California, USA) and LifeGenDB.

6.3.4 Data Analysis

GeneSpring GX a molecular analysis software package was used to identify significant differences between the control birds gene expression and the treatment birds, significance was set at $P<0.05$. GeneSpring produced a fold change value from the control bird for each gene and a measure of significance (P value) based on an unpaired t-test. Immunoglobulin and digestive enzyme data was analysed via 1-way ANOVA using the SPSS software for Windows. was used. Statistical significance was declared at $P<0.05$ and treatment means were separated using the Tukey post hoc test.

6.4 RESULTS

6.4.1 Gene Expression

Change in gene expression was measured in birds fed varying levels of NCF against a control to illustrate any immune or metabolic effects of the supplement on the birds.

6.4.1.1 Heat Map

In Trial 1 transcription changes in intestinal samples of broilers fed NCF were analysed. Figure 6.1 shows a cluster analysis in the form of a microarray heat map of the four treatment groups. The heat map uses colours to represent the intensity of expression of a gene and shows any similarities between samples using a dendrogram to hierarchically cluster genes (a tree diagram). Figure 6.1 shows that each treatment group had a similar effect on the gene expression of the birds within the separate treatments as they have been grouped together. Figure 6.2 is also a

microarray heat map which shows the similarities between the average gene expressions of the dietary treatment group. It shows that the control and 800g/t treatment group had higher correlation between their changes in gene expression than between control, 200g/t NCF and 400g/t NCF dietary treatment gene expressions.

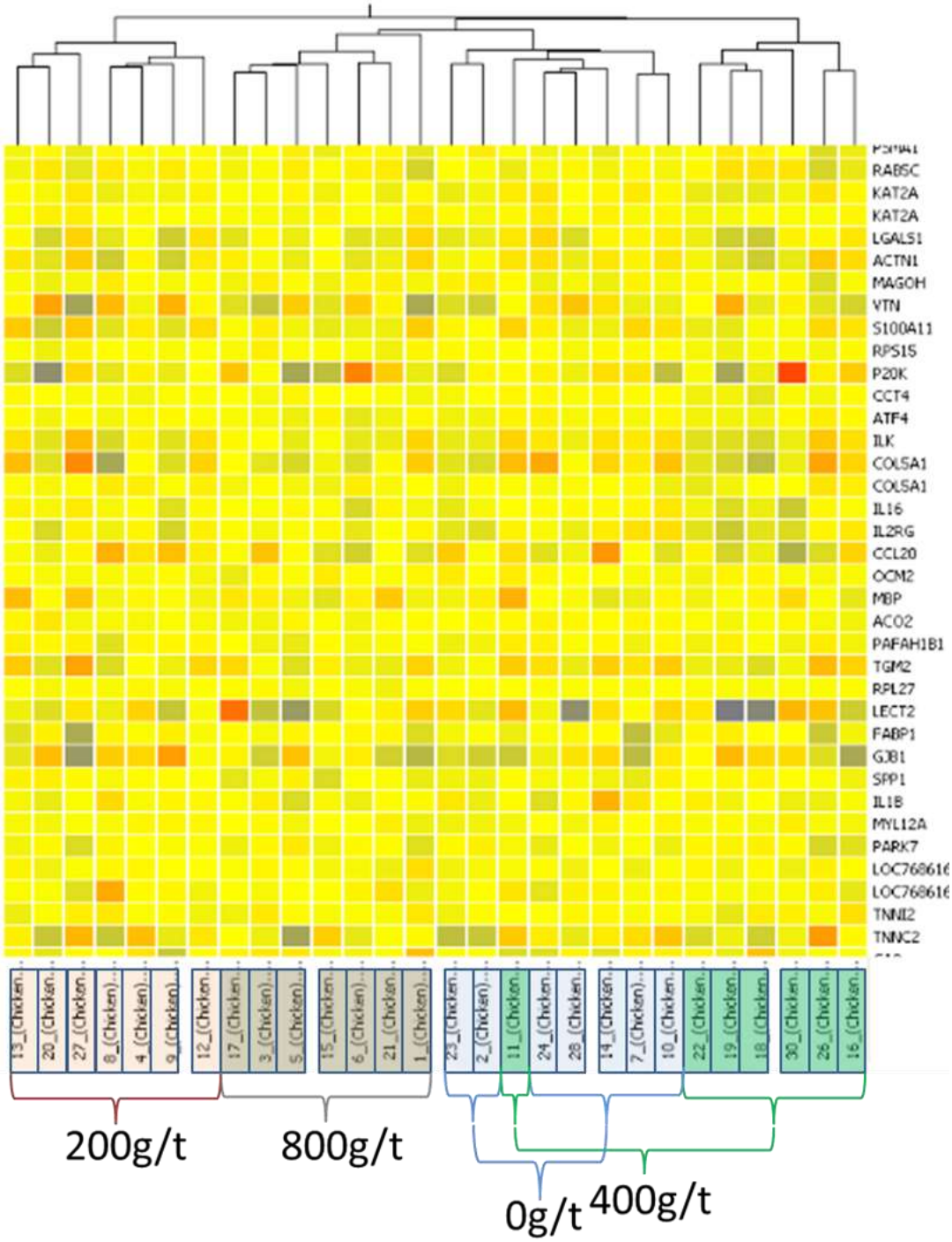

Figure 6.1. Sectional image of approximately 30 of the 28,000 genes used in cluster analysis of the gene expression of the 10,000 of birds fed one of the four treatments, representing the similarities between samples using a dendrogram.

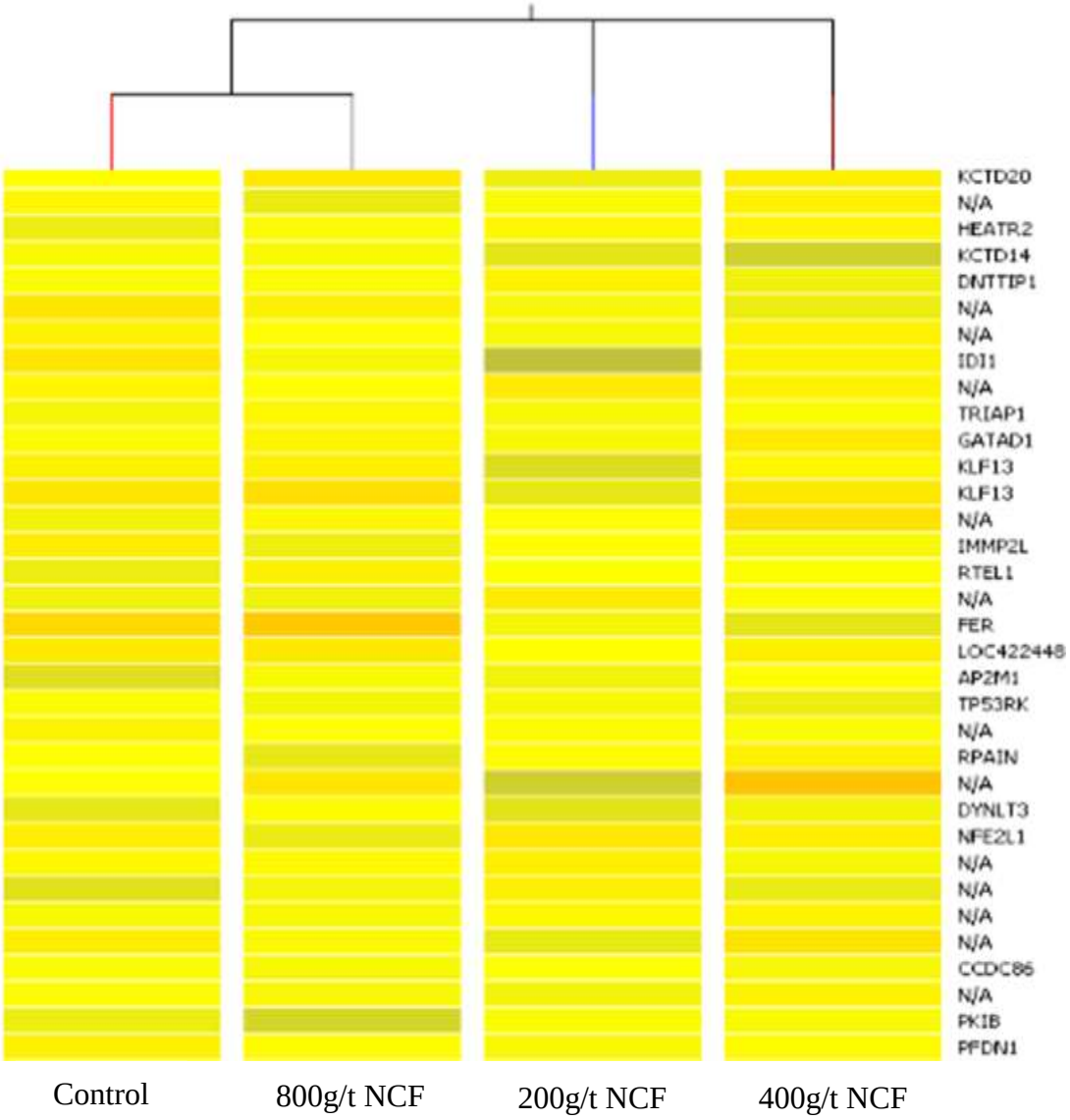

Figure 6.2. Sectional image of approximately 30 of the 28,000 genes used in cluster analysis of the gene expression of the 10,000 of birds fed one of the four treatments, representing the similarities between samples using a dendrogram.

6.3.1.2 Venn diagram and Volcano Plot

Figure 6.3 is a Venn diagram showing gene expression changes and over laps between dietary treatments. It demonstrates that expression of 41 genes were altered with all supplementation levels of NCF compared to the control birds.

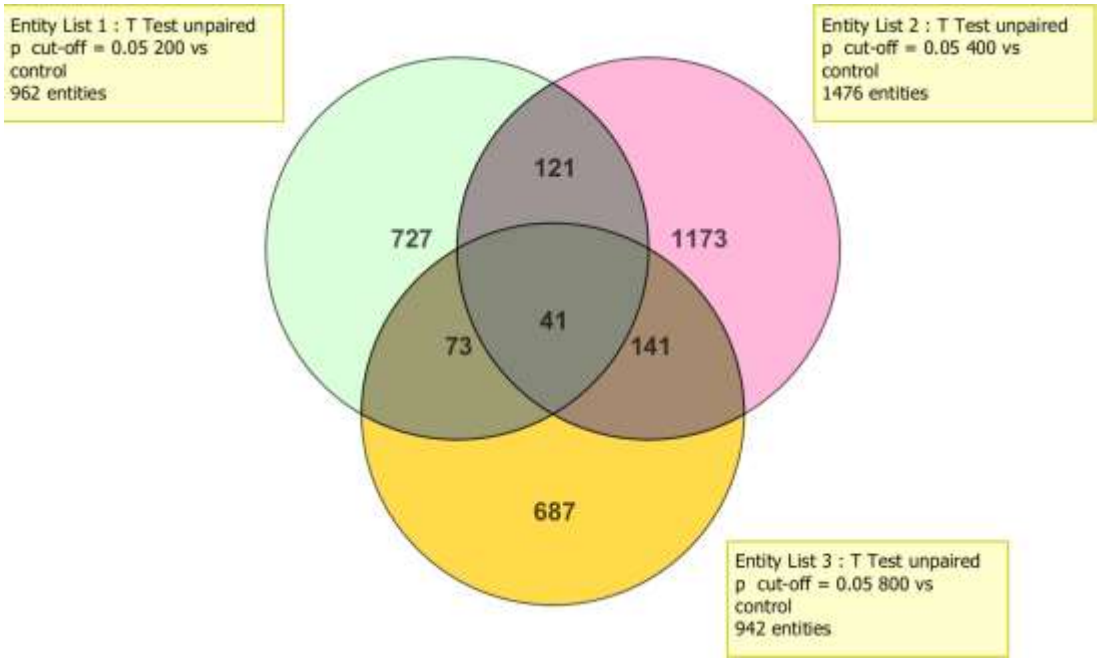

Figure 6.3 Venn diagram comparing changes in gene expression when broilers are fed varying levels of NCF over a control.

Of the 962 genes changed when feeding 200g/t NCF from the control, 395 were found to be down regulated and 567 up regulated. Of the 1476 genes changed when feeding 400g/t NCF, 432 were found to be down regulated and 1044 up regulated, and of the 942 genes changed when feeding 800g/t NCF, 375 were found to be down regulated and 567 up regulated. When further analysis was performed on the data, it was seen that just 40, 33 and 28 genes showed a difference of greater than 1.5 fold change from the control. when birds were fed 200g/t, 400g/t and 800g/t respectively. The volcano plots shown in figures 6.4, 6.5 and 6.6 highlight clearly that, whilst many genes are significantly altered in their expression (left image), few are altered more than 1.5 fold (right image).

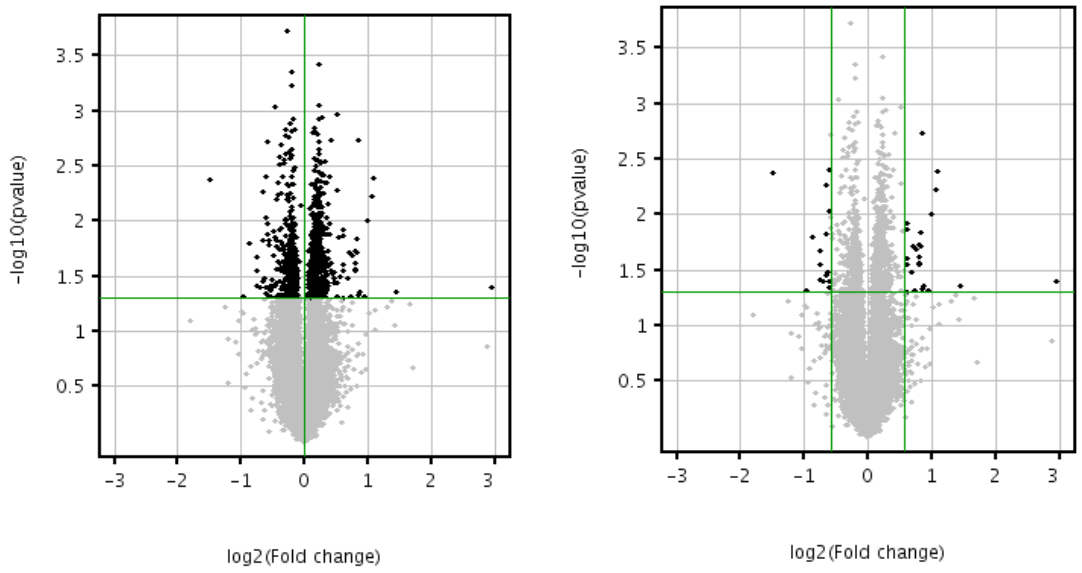

Figure 6.4. Volcano plot representing the distribution of genes expressed in intestine for 200g/t vs. 0g/t NCF. Right image illustrates genes significantly altered (P<0.05) and left image illustrating genes significantly altered more than 1.5-fold.

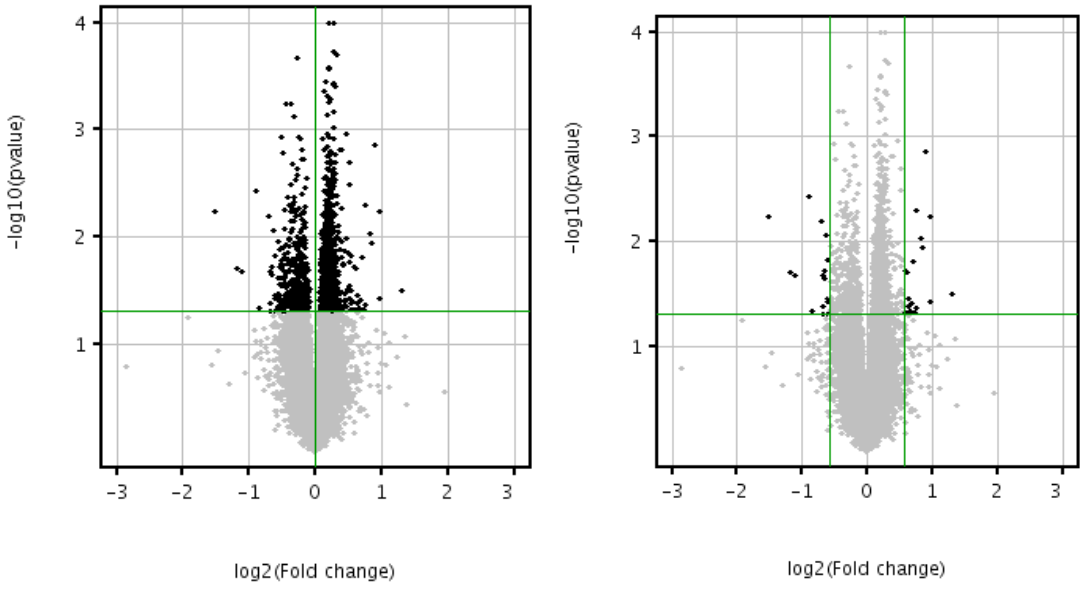

Figure 6.5. Volcano plot representing the distribution of genes expressed in intestine for 400g/t vs. 0g/t NCF. Right image illustrates genes significantly altered (P<0.05) and left image illustrating genes significantly altered more than 1.5-fold.

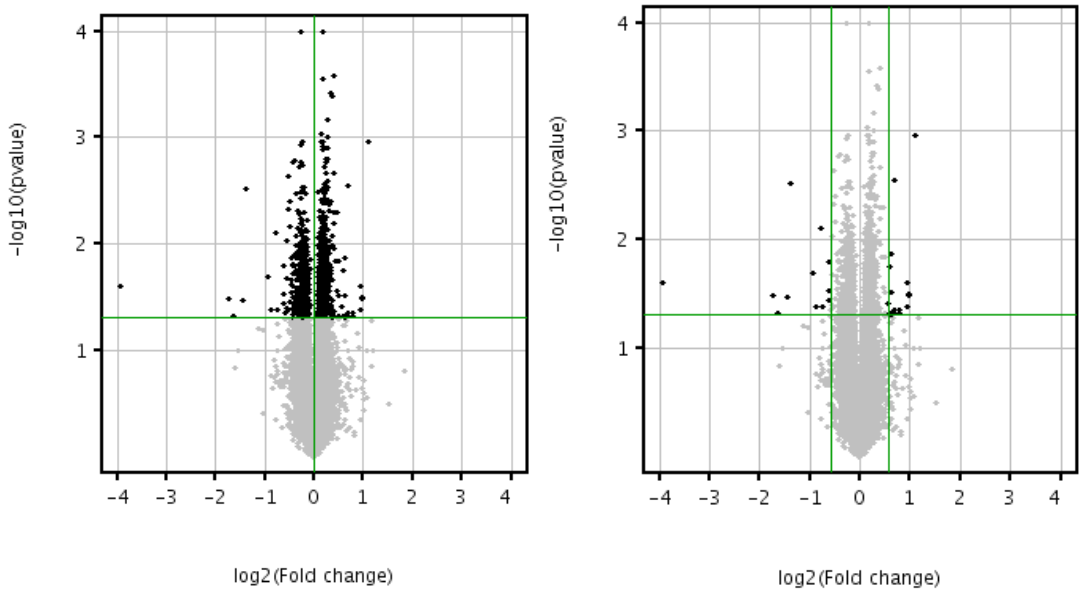

Figure 6.6. Volcano plot representing the distribution of genes expressed in intestine for 800g/t vs. 0g/t NCF. Right image illustrates genes significantly altered (P<0.05) and left image illustrating genes significantly altered more than 1.5-fold.

6.4.1.3 GO Analysis

Metabolic activities were highly represented in the 400g/t treatment group, with primary metabolic processes (GO:0044238; 760 probes) and nitrogen compound metabolic processes (GO:0006807; 377 probes). In the 200g/t treatment group responses were seen in glutamine family amino acid metabolic processes (GO:0009064; 15 probes) and steroid biosynthetic processes (GO:0006694; 11 probes). In the 800g/t treatment group responses were only seen in helicase activity (GO:0004386; 24 probes) (figure 6.7). Immune response terms were also identified, 400g/t had an effect on immune system development (GO:0002520; 65 probes; FDR 0.38) and 800g/t on cytokine stimulus (GO:0034097; 26 probes; FDR 0.40) and defence response to bacterium (GO:0042742; 23 probes; FDR 0.40). However as these genes had a false discovery rate (FDR) over 0.1, they may indicate a noteworthy effect but these data cannot be relied upon as representing a true effect.

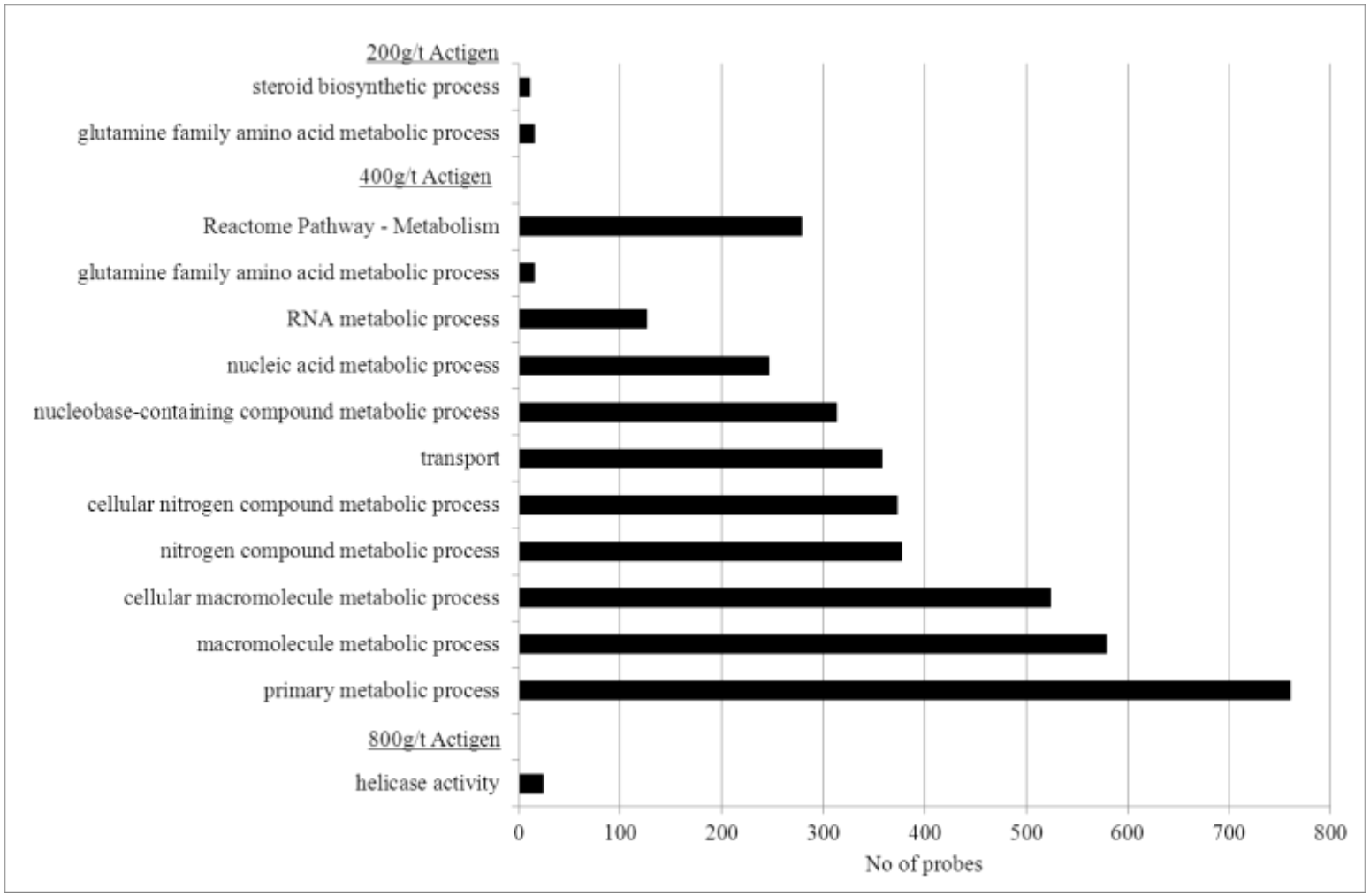

Figure 6.7 Effects of dietary treatments on biological processes and molecular functions.

6.4.1.4 IPA Canonical Pathways

When looking at more specific canonical pathways using IPA software analysis, it was shown that immune and metabolic pathways were affected by NCF, which is shown in table 6.3. These pathways contain numerous genes that were up or down regulated by NFC that can have interaction with other processes and pathways.

Table 6.3 Specific canonical pathways affected by NFC supplementation.

Diet	Canonical Pathway
200g/t NCF	Biosynthesis of steroids
	Role of Wnt/GSK-3b Signalling in the Pathogenesis of Influenza
	Role of IL-17F in Allergic Inflammatory Airway Diseases
	Maturity Onset Diabetes of Young (MODY) Signalling
	Urea Cycle and Metabolism of Amino Groups
400g/t NCF	G-Protein Coupled Receptor Signalling
	T Cell Receptor Signalling
	Atherosclerosis Signalling
	Reg. of IL-2 Expression in Activated & Anergic T Lymphocytes
	Phospholipase C Signalling
800g/t NCF	Glyoxylate and Dicarboxylate Metabolism
	Linoleic Acid Metabolism
	G-Protein Coupled Receptor Signalling
	IL-17A Signalling in Fibroblasts
	Citrate Cycle

6.4.2 Immunoglobulins

Immunoglobulins were measured periodically in birds fed NCF, Ca But and Na But as a gauge to investigate their effects on the immune system over time. It was seen that there were no interactions between feeding NCF and Na But and no significant differences between treatment on broiler immunoglobulin levels in serum at days 7, 14, 21 or 42 (tables 6.4 and 6.5). However there was interaction observed at 21 days between NCF and Ca But, Ca But feed on its own did not alter IgG levels, where as a trend was seen when NCF was fed on it only and in combination with Na But to decreased IgG levels.

Table 6.4 Effect of dietary supplements on immunoglobulin levels in serum (mg/ml).

Attribute	Age	Dietary treatment						SEM	P value	Interaction	
		Control	NCF 200g/t	Ca But	Na But	NCF/Ca But	NCF/Na But			NCF*Ca B	NCF*Na B
IgA	7	0.085	0.064	0.082	0.081	0.097	0.074	0.006	0.708	0.306	0.765
IgM		0.070	0.069	0.067	0.061	0.074	0.070	0.005	0.988	0.658	0.588
IgG		1.906	1.553	1.555	1.387	1.515	1.718	0.077	0.466	0.698	0.170
IgA	14	0.161	0.130	0.172	0.154	0.211	0.151	0.009	0.137	0.404	0.915
IgM		0.118	0.104	0.172	0.152	0.166	0.122	0.009	0.185	0.556	0.916
IgG		1.058	1.098	1.040	1.045	1.126	1.101	0.085	1.000	0.571	0.866
IgA	21	0.225	0.194	0.237	0.189	0.232	0.224	0.011	0.718	0.975	0.550
IgM		0.080	0.080	0.107	0.119	0.103	0.131	0.010	0.595	0.733	0.884
IgG		2.976	2.656	2.961	4.456	2.418	2.662	0.209	0.058	0.047	0.410

Table 6.5 Effect of NCF on the immunoglobulin levels in serum of broilers (mg/ml).

Attribute	Age	Dietary treatment				SEM	P value
		Control	NCF 200g/t	NCF 400g/t	NCF 800g/t		
IgA	42	0.239	0.217	0.240	0.212	0.012	0.802
IgM		0.214	0.261	0.262	0.230	0.017	0.735
IgG		2.822	2.748	1.928	3.211	0.191	0.108

Figure 6.8, 6.9 and 6.10 shows the immunoglobulin levels in serum of broilers over time in Trial 3. Levels of IgA increased with age, whereas IgM increased from 7 to 14 days, then decreased at 21 days before IgM substantially increased in Trial 1 to its highest level at 42 days. In contrast, IgG decreased from 7 to 14 days, before greatly increased to its highest level at 21 days and was approximately maintained at 42 days in Trial 1.

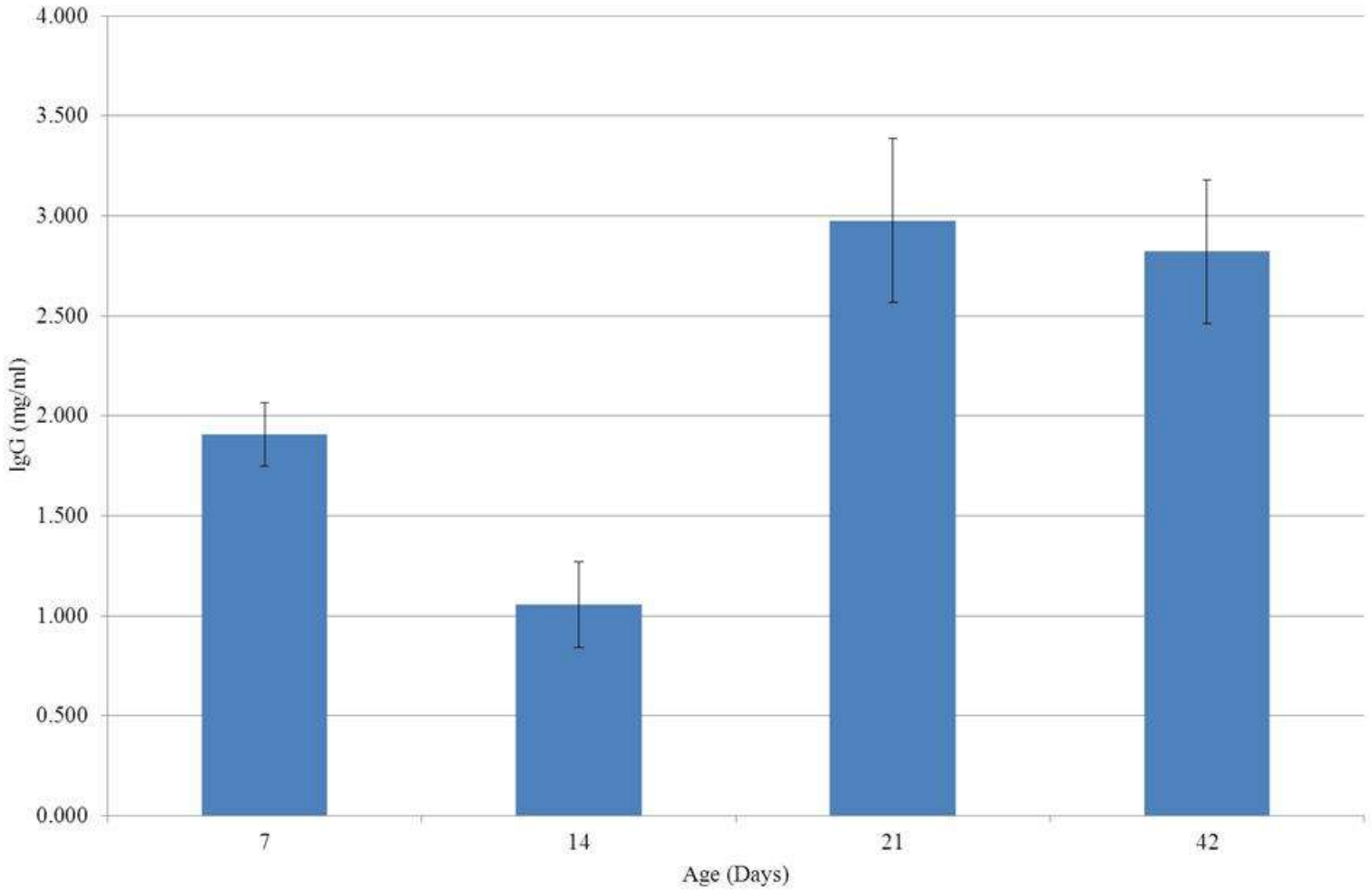

Figure 6.8 Immunoglobulin G levels in serum of broilers of increasing ages.

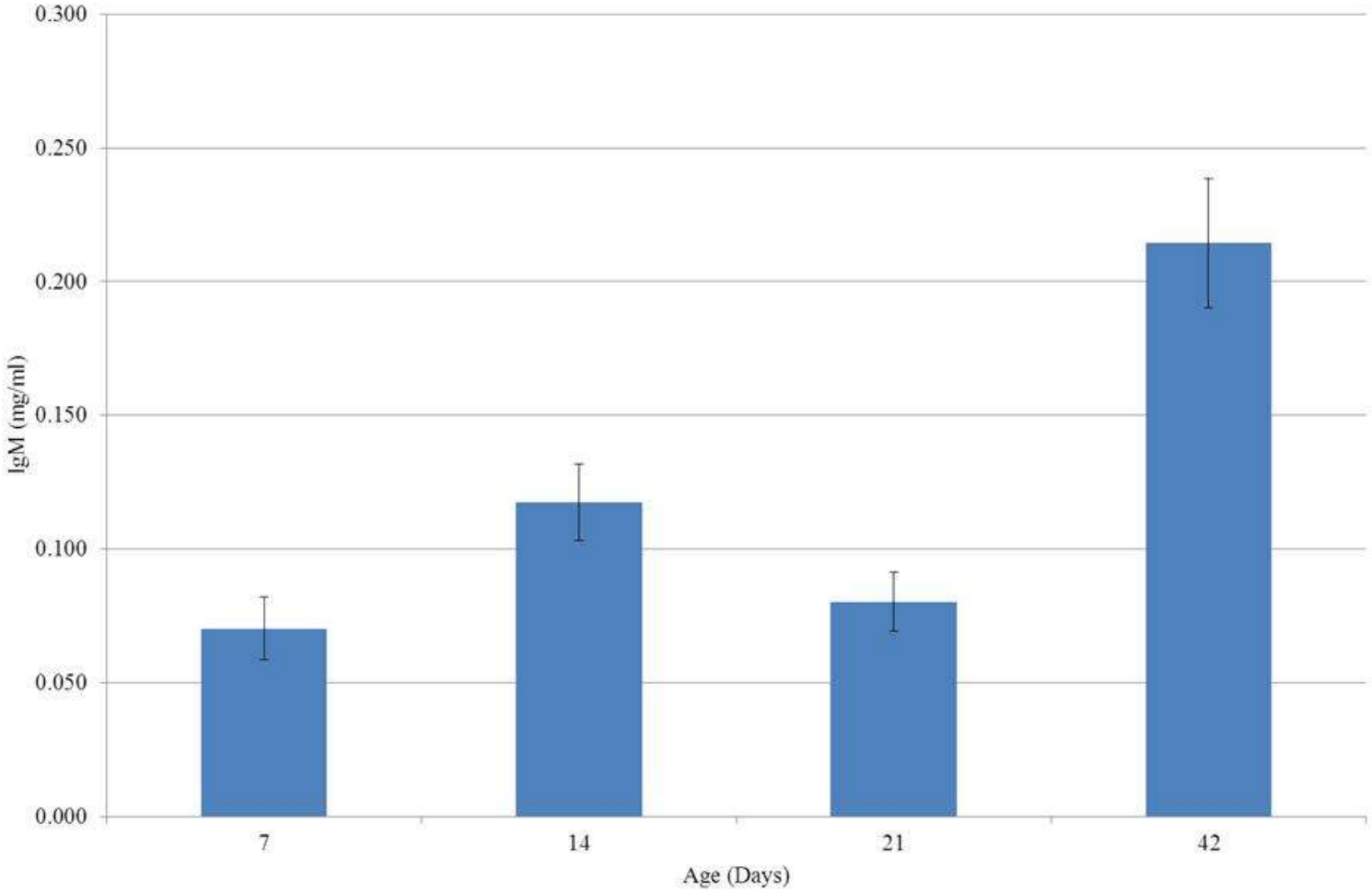

Figure 6.9 Immunoglobulin M levels in serum of broilers of increasing ages.

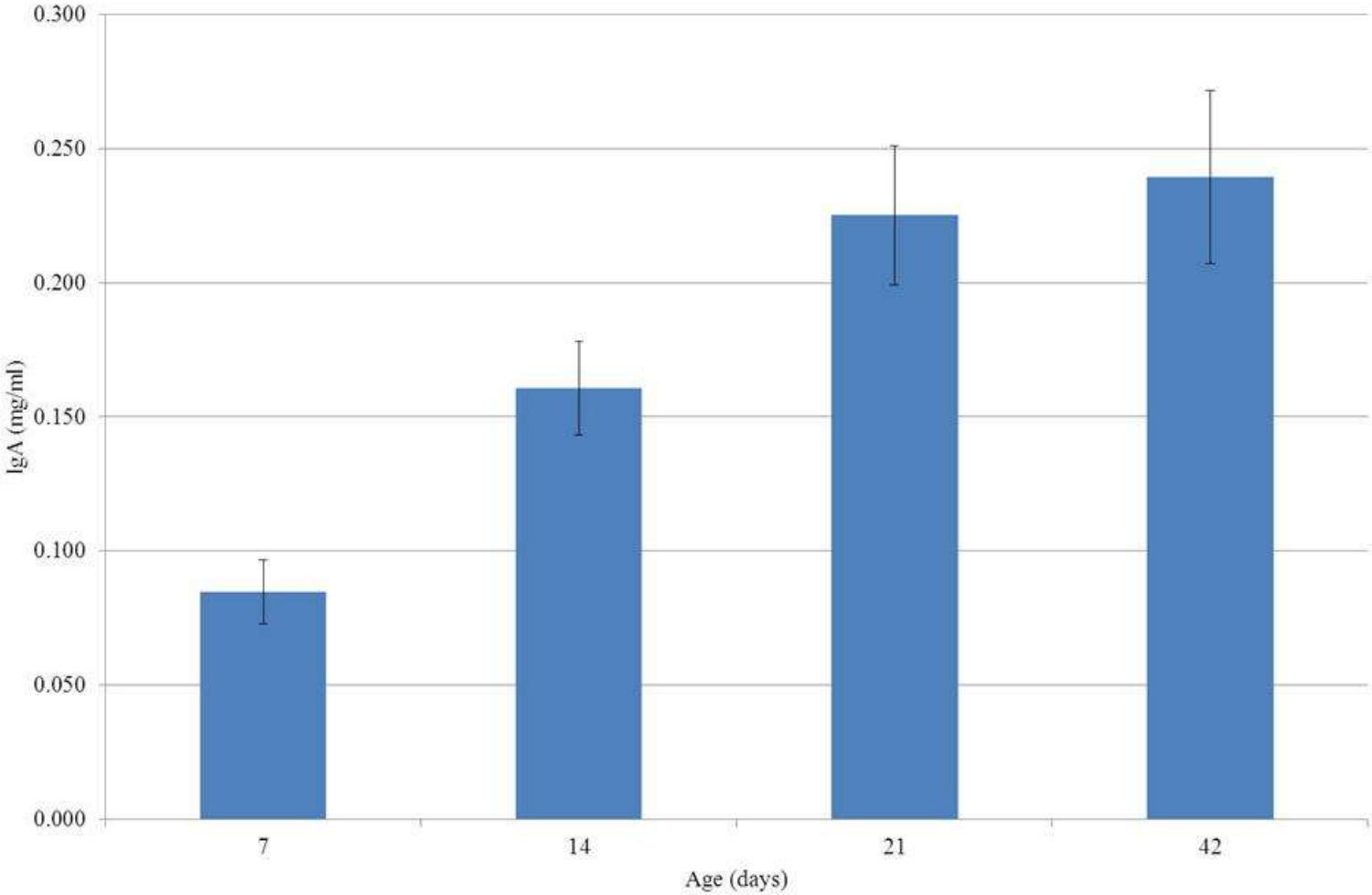

Figure 6.10 Immunoglobulin A levels in serum of broilers of increasing ages.

6.4.3 Digestive Enzymes

Feeding NCF, Ca But and Na But had no significant effect on activity of the broiler digestive enzymes; amylase, chymotrypsin and trypsin in the duodenum and jejunum at days 7, 14, 21 or 42 (tables 6.6, 6.7, 6.8, 6.9). However, it could be seen that amylase, chymotrypsin and trypsin levels were higher in the jejunum than the duodenum up to day 21.

Table 6.6 Effect of fed supplements at 7d on digestive enzymes activity of broilers (units/g protein)

	Duodenum			Jejunum		
	Amylase	Chymotrypsin	Trypsin	Amylase	Chymotrypsin	Trypsin
Control	11.6	48.2	1.4	22.6	139.6	2.8
NCF 200g/t	8.8	53.7	1.7	21.9	142.3	2.7
Na But	7.9	51.6	1.3	19.7	125.6	2.7
Ca But	10.4	69.9	2.3	18.7	144.1	2.7
Na But/NCF	8.7	48.1	1.3	17.1	155.6	2.8
Ca But/NCF	7.0	68.3	2.4	19.3	134.8	2.8
SEM	0.7	3.8	0.2	1.1	7.0	0.2
P value	0.451	0.378	0.556	0.763	0.900	1.000

Table 6.7 Effect of fed supplements at 14d on digestive enzymes activity of broilers (units/g protein)

	Duodenum			Jejunum		
	Amylase	Chymotrypsin	Trypsin	Amylase	Chymotrypsin	Trypsin
Control	11.3	83.5	0.9	17.7	128.8	5.4
NCF 200g/t	8.5	79.2	0.8	15.6	126.8	6.8
Na But	10.4	63.7	1.1	14.5	110.6	5.5
Ca But	10.3	61.5	0.6	20.1	129.4	6.4
Na But/NCF	9.8	82.6	1.1	17.9	125.8	6.2
Ca But/NCF	11.7	89.1	1.1	18.5	137.1	7.0
SEM	0.6	4.2	0.1	1.1	7.4	0.4
P value	0.698	0.315	0.701	0.734	0.955	0.823

Table 6.8 Effect of fed supplements at 21d on digestive enzymes activity of broilers (units/g protein)

	Duodenum			Jejunum		
	Amylase	Chymotrypsin	Trypsin	Amylase	Chymotrypsin	Trypsin
Control	5.2	51.7	0.4	6.8	57.9	5.1
NCF 200g/t	4.5	59.3	0.4	6.9	54.7	5.9
Na But	4.1	53.3	0.4	7.1	53.1	4.9
Ca But	4.1	52.1	0.4	8.1	72.1	5.1
Na But/NCF	4.3	65.5	0.5	7.1	45.1	4.3
Ca But/NCF	4.8	68.5	0.5	7.5	48.5	5.5
SEM	0.2	3.3	0.03	0.3	3.5	0.4
P value	0.383	0.544	0.924	0.897	0.311	0.915

Table 6.9 Effect of fed supplements at 42d on digestive enzymes activity of broilers (units/g protein)

	Duodenum			Jejunum		
	Amylase	Chymotrypsin	Trypsin	Amylase	Chymotrypsin	Trypsin
Control	13.1	82.0	1.9	11.3	56.9	2.9
NCF 200g/t	11.1	77.3	2.0	12.1	67.5	3.4
NCF 400g/t	11.3	66.6	2.1	12.4	63.5	3.1
NCF 800g/t	9.2	64.7	2.2	12.8	61.4	2.9
SEM	1.4	3.4	0.2	1.0	3.8	0.2
P value	0.967	0.215	0.880	0.964	0.805	0.668

6.5 DISCUSSION

The effects of varying levels of NCF in the diet of broilers on the regulation of gene expression of Trial 1 are discussed in this section, as well as the effects of NCF and butyrate salt supplementation in Trial 1 and 3 on immunoglobulin serum levels and digestive enzyme activities.

6.5.1 Gene expression

The cluster analysis (heat map – figure 6.1) showed similarities in the gene expression of individual birds on the same diet and clear differentiation between groups of birds on the same diet. This suggests that the diets and hence differing inclusion levels of NCF are having a consistent effect on the regulation of gene expression. The cluster analysis of the average gene expression of the dietary treatment group (figure 6.2) indicates that the control and 800g/t treatment group had higher correlation between their regulation of gene expression than between the

other dietary treatment gene expressions. This suggests that 200g/t and 400g/t dietary inclusions of NCF are having a greater effect on gene expression compared to the control birds than 800g/t NCF. However, a cluster analysis only provides a general view of gene expression changes. The Venn diagram shows that a total of 962,1476 and 942 genes were significantly up or down regulated compared to the control with inclusion of 200g/t, 400g/t and 800g/t NCF, with a number of genes changing in two or all three NCF dietary treatment groups. This suggests that feeding NCF at differing inclusion levels had a similar effect on the regulation of a number of genes. However, it also showed an altered regulation of different genes depending on the NCF inclusion level. When these are plotted on a volcano plot it can be seen that the levels of change in expression relative to the control varies; only 40, 33 and 28 genes showed an expression difference of greater than 1.5 fold change from the control. This indicates that the changes in individual gene expression from the control in the NCF treatment groups are small, suggesting that NCF supplementation may only be having small effects on gene expressions. It has been reported by Ibuki et al, (2010) that when feeding a prebiotic that there were 171 genes up or down regulated by more than 1.5 fold change, which is substantially more than were found in the present trial.

The gene ontology analysis describes gene changes in terms of their associated biological processes, cellular components and molecular functions. In this study, it was seen that metabolic activities (relating to carbohydrate, lipid, protein and cellular amino acid metabolic processes) were highly represented. In the 400g/t treatment group, primary metabolic processes and nitrogen compound metabolic processes were flagged by Lifegen as greatly affected, suggesting that the main effects on

performance of birds fed of 400g/t NCF may be through modifying the metabolic processes of the broiler.

In the 200g/t treatment group, changes were shown to affect the chemical reactions and pathways involving amino acids of the glutamine family, comprising of arginine, glutamate, glutamine and proline. Steroid biosynthetic processes were also affected by 200g/t NCF supplementation, which suggest impact via bile acid, vitamin D and glucocorticoid biosynthetic processes. Glucocorticoid has been reported to have effects on inflammation and immunity (Proszkowiec-Weglarz and Porter 2010), thus providing a possible mechanism for 200g/t NCF to affect inflammation and immunity of the birds fed this level of NCF supplement. However it not possible from the available data and published literature to determine whether this change will invoke an overall positive or negative effect on immune status of the bird.

In contrast to the lower NCF supplementation levels, the 800g/t treatment group only showed responses in helicase activity. This catalyses the reaction: NTP + H2O = NDP + phosphate, to drive the unwinding of a DNA or RNA helix, suggesting that 800g/t NCF may be affecting cell proliferation (AmiGO: helicase activity Details. 2013). Immune responses were also identified, although the false discovery rates (FDR) were over 0.1 which means that they have been identified but are not unreliable. It was seen that 400g/t may have had a small effect on immune system development (FDR 0.38) and 800g/t on cytokine stimulus (FDR 0.40) and defence response to bacterium (FDR 0.40).

When looking at more specific canonical pathways using IPA it was shown that

immune and metabolic pathways were affected by NCF. These pathways contain numerous genes that were up or down regulated by NFC that can have interaction with other processes and pathways. Key genes that are up or down regulated in these pathways are shown in table 6.10 and discussed later.

Table 6.10 Key genes effected by NCF supplementation

Gene	Up/down regulated	Function/effect
200g/t NCF		
IDDI	↓	Increased activation of γδ T cells
NCoA-1	↑	Effect IL4 mediated biological response
Act1	↑	Decreases numbers of B cells
C-RAF	↑	Effect inflammation, increased tight junction proteins & improved barrier function
HNF-1α	↑	Increase glucose uptake by the liver
INSR	↑	Improved uptake of glucose
		Effect epithelial cell proliferation
CPS-I	↓	Decrease in inflammation
400g/t NCF		
Syk	↓	Decrease B cell development
Egr-1	↓	Suppressed immune system
NFATc3	↑	Cytokines transcription
NF-kB1	↓	Increased immune factors
C-RAF	↑	Effect inflammation, increased of tight junction proteins and improved barrier function of the intestine
SLP-76	↓	Decreased development of thymocytes
TIFA	↑	Innate immunity
PDE7A	↓	Suppress the immune system
Gs-coupled receptor	↓	Movement of natural killer cells and cytotoxic abilities
800g/t NCF		
ACO2	↓	TCA cycle
HOGA	↑	Metabolism of 4-hydroxyproline, glyoxylate, and pyruvate - TCA cycle
MTHFD1	↓	Folate metabolism
PLA2G10	↓	Decreases pro-inflammatory intermediates
FADS1	↑	Increased levels of the pro-inflammatory arachidonic acid
Gs-coupled receptor	↑↓	Movement of natural killer cells and their cytotoxic abilities
Gi coupled receptor	↑↓	production of nitric oxide
PDE-5A/11A	↑	increase immune and inflammation response

6.5.1.1 Biosynthesis of steroids

When investigating in further detail, one of the pathways that was affected when NCF was supplemented at 200g/t was the steroid biosynthesis pathway. This pathway has interaction with the function, expansion of gamma-delta T lymphocytes (γδ T cells). γδ T cells are thought to be an important element in the innate immune system and may be the first line of defence (Price *et al*., 2006; Chen *et al*., 2005). In cattle it had been shown that production of interferon (IFN)-C by γδ T cells post-infection may have a role in linking the innate and adaptive immune responses (Price *et al*., 2006). The interaction of this pathway with γδ T cells in this study may be through the down regulation of Isopentenyl-diphosphate Delta-isomerase (IDDI) (-1.446, P = 0.0397), which is involved in the reaction of isopentenyl diphosphate ↔ dimethylallyl diphosphate. Isopentenyl diphosphate (IPP) has been reported to activate γδ T cells in humans (Chen *et al*., 2005). Activation of γδ T cells leads to the production of cytokines and chemokines involved in the initiation of inflammatory and immune responses, as well as in the generation of T cells with cytotoxic activity (Cipriani *et al.*, 2000; Chen *et al*., 2005). Therefore, the down regulation of Isopentenyl-diphosphate Delta-isomerase may mean that there is an increased level of IPP and increased activation of γδ T cells. This may lead to the increase in readiness of the immune function of birds fed 200g/t NCF, resulting in a decrease in the time for the bird to defend itself against a pathogenic challenge.

6.5.1.2 Role of Wnt/GSK-3b Signalling in the Pathogenesis of Influenza

NCF at 200g/t was also shown to have an effect on a pathway involving Wnt/glycogen synthase kinase 3 (GSK3)-β signalling (Figure 6.10). Figure 6.10 also indicates links between genes in this pathway to other signalling pathways and

functions. The Wnt signalling pathway has been reported to trigger a β-catenin/TCF-induced IFN production (Ingenuity Systems, 2013), which allows for communication between cells to trigger the protective defences of the immune system to fight the pathogen. The pathway also has interaction with the protein kinase A signalling and integrin-linked kinase (ILK) signalling pathways. One of the genes significantly up regulated in this pathway is Nuclear Receptor Coactivator 1 (NCoA-1), part of p160 steroid receptor coactivator (1.102 P=0.0339). NCoA-1 acts as a co-activator for signal transducer and activator of transcription 6 (STAT6) (Nuclear receptor coactivator 1, 2013). STAT6 is activated in response to interleukin–4 (IL-4) and interleukin–13 (IL-13), and has a crucial role in exerting IL4 mediated biological response. IL-4 regulates immune and anti-inflammatory responses, through promoting the differentiation of T helper precursors, inhibiting T helper 1 development and stimulates B-cells to change Immunoglobulins (Ig) to IgE isotype (Litterst and Pfitzner., 2001). Therefore, the up regulation of NCoA-1 by 200g/t NCF may have had significant effects on the immune system of the birds.

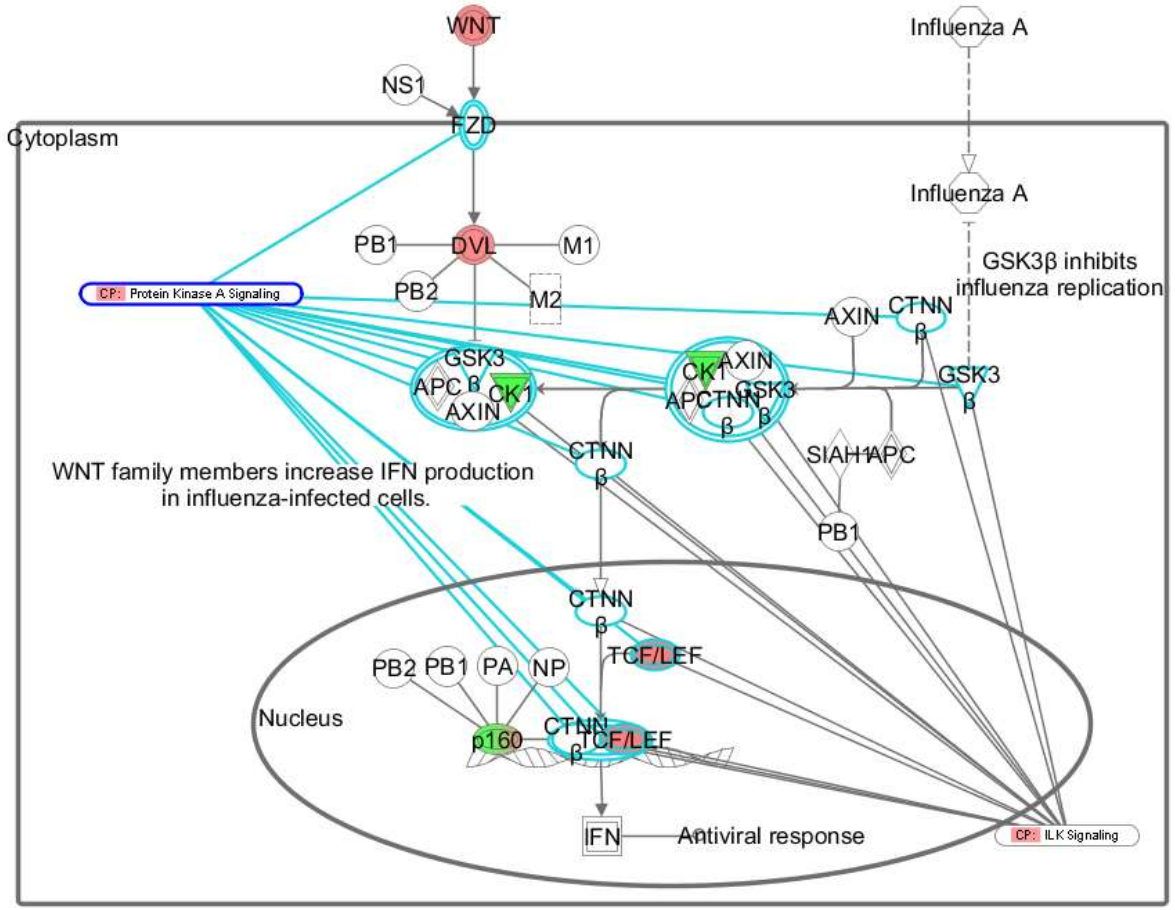

Figure 6.10. Wnt/glycogen synthase kinase 3-β signalling pathway. CP = canonical pathway, each line between a canonical pathway and a gene indicates a link between the pathway and a gene with at least one published paper indicating a link.

6.5.1.3 Role of IL-17F in Allergic Inflammatory Airway Diseases

Another canonical pathway that was significantly affected by 200g/t NCF involves IL-17F, which has interactions with the inflammatory response and attraction of leukocytes. IL-17F has also been shown to be important in the pathogenesis of allergic airway inflammation, and has been associated with inflammatory bowel disease (Chen *et al.,* 2009). Interleukin-17 has been reported to stimulate the production of other cytokines and chemokines such as IL-1β, IL-6, IL- 8 (Shaw *et al*., 2011). However, the mechanisms and function of IL-17F in the intestine is unclear. In mice, gut microflora has been reported to induce T helper cells 17 (Th17), which produce IL-17, playing an important role in the maturation of the gut immune system (Crhanova *et al*., 2011). Interestingly, IL-17 has been shown to be important for defence against bacterial and fungal pathogens and maybe regulating the immune response as IL-17 receptors have been found on macrophages and T lymphocytes (Crhanova *et al*., 2011).

The IL-17F pathway also has interaction with the inflammatory response function through the gene Act1, which was up regulated (1.769 P= 0.0243). Act1 is a key component in IL-17A signalling and has been linked to CD40 mediated B cell survival. It has been shown that Act1-deficient B cells mice have increased CD40, which indicates that Act1 negatively regulates B cell survival (Qian *et al*., 2004). This suggest that the up regulation of Act1 in birds fed 200g/t NCF decreases numbers of B cells, and may have decreased the energy need for the immune system. C-RAF was also up regulated (1.196 P=0.0028), which is a functional protein that activates mitogen-activated protein kinase (MEK1/2). MEK1/2 is involved in activating extracellular-signal-regulated kinases (ERK1/2). Interleukin-1 is also

involved in the activation of ERK1/2 to regulate certain pro-inflammatory genes (Perera *et al*., 2010). This suggests that the up regulation of C-FAF may have an effect on inflammation. In addition, ERK 1/2 is thought to be (Takahashi, *et al.*, 2012) involved in the proliferation and differentiation of cells. It has been seen in the lungs that a reduction in ERK-1/2 signalling correlates with an inhibition of lung branching morphogenesis *in vitro*. This infers that ERK-1/2 activation may mediate lung growth and development (Kling *et al*., 2002). This may also be the mechanism of ERK 1/2 in the intestine on the development of the intestinal wall. It has been proposed that ERK1/2 may be involved in the regulation of tight junctions and that a decreased ERK1/2 activity may lead to an increased level of tight junction proteins and improved barrier function of the intestine (Zimmerman *et al*., 2011).

6.5.1.4 Maturity Onset Diabetes of Young (MODY) Signalling

200g/t NCF also had a significant effect on the maturity onset diabetes of young (MODY) signalling pathway, and a number of these genes have interaction with metabolism and transport of carbohydrates, homeostasis of D-glycogen and the transport of fatty acids. One of these genes is Hepatic Nuclear Factor 1 (HNF-1α), which was up regulated (1.210 P=0.0147) and is a key component of a pathway that is essential for normal glucose-induced insulin secretion (Shih *et al*., 2002). It has been reported that mice deficient in HNF-1α have a reduced uptake of glucose by the kidney (Pontoglio *et al*., 2000). This suggests that feeding NCF may increase glucose uptake by the liver. Another key gene that was up regulated in this pathway was INSR (1.052 P=0.0312), which encodes for an insulin receptor, that plays an important role in the regulation of glucose homeostasis and metabolism (Kim *et al*., 2001). The binding of insulin to INSR results in the uptake of glucose; therefore, the

up regulation of INSR may mean that the ability of the chicken to take up glucose is improved. It has also been seen that in ruminants INSR is involved in ruminal epithelial cell proliferation (Baldwin, 1999), and that insulin can regulate ruminal epithelial cell proliferation in combination with their receptors (Naeem *et al*., 2012).

6.5.1.5 Urea Cycle and Metabolism of Amino Groups

200g/t NCF was demonstrated to have an effect on a number of genes involved in the urea cycle and metabolism of amino groups. This pathway has gene interaction with the metabolism of protein, and amino acid synthesis and uptake. The enzyme carbamoyl phosphate synthase I (CPS-I) was down regulated in this pathway (-1.189 P= 0.0048). CPS-I is involved in the production of citrulline from ammonia. Citrulline is an important component in the arginine biosynthetic pathway. Arginine has many roles in the intestine, including protein synthesis, cell proliferation (Buga *et al*., 1998), polyamine synthesis (Wu *et al*., 2000), and maintenance of the epithelial barrier function (Mueller *et al*., 2000; Dekaney *et al*., 2003). Mueller *et al.* (2000) reported in rats that in small bowel transplantation experiments the application of L-arginine increased inflammation, which may mean that the down regulation of CPS-I could have led to a decrease in inflammation in the GI tract.

6.5.1.6 B cell signalling pathway

When investigating in further detail the pathways changed when NCF was supplemented at 400g/t, it was seen that the B cell signalling pathway was affected. Signals through the B cell antigen receptor are crucial to the development, survival and activation of B lymphocytes (Ingenuity Systems, 2013). This pathway has gene interaction with the development process of lymphocytes and immune response

(figure 6.11). One gene that was down regulated was spleen tyrosine kinase (Syk) (-1.257 P=0.0374). Syk function is through transmitting signals from mature antigen receptors, downstream of the pre-B cell antigen receptor in immature B cells, and therefore is involved in early steps of lymphoid development. It has been reported by Turner, *et al* (1995) Syk gene knockout mice have blocked B-cell development, resulting in a failure to produce mature B-cells. Therefore, the down regulation of Syk could suggest there will be a decrease in B cell development. Early growth response 1 (Egr-1) is another gene in this pathway that has been significantly down regulated by 400g/t NCF (-1.584 P=0.0210). Egr-1 is thought to be important in the development of thymocytes. T cell precursors are double-negative, which progress through a series of maturational steps to form double-positive thymocytes that express both CD4 and CD8, and then finally to mature single-positive thymocytes that express either CD4 or CD8. This development depends upon pre-T cell receptor signalling to engage a number of molecules. Egr are critical effectors of the signals that promote this developmental transition (Carter *et al*., 2007: Carleton *et al*., 2002). From the down regulation of genes in this pathway by 400g/t NCF it may mean that the immune system is being suppressed.

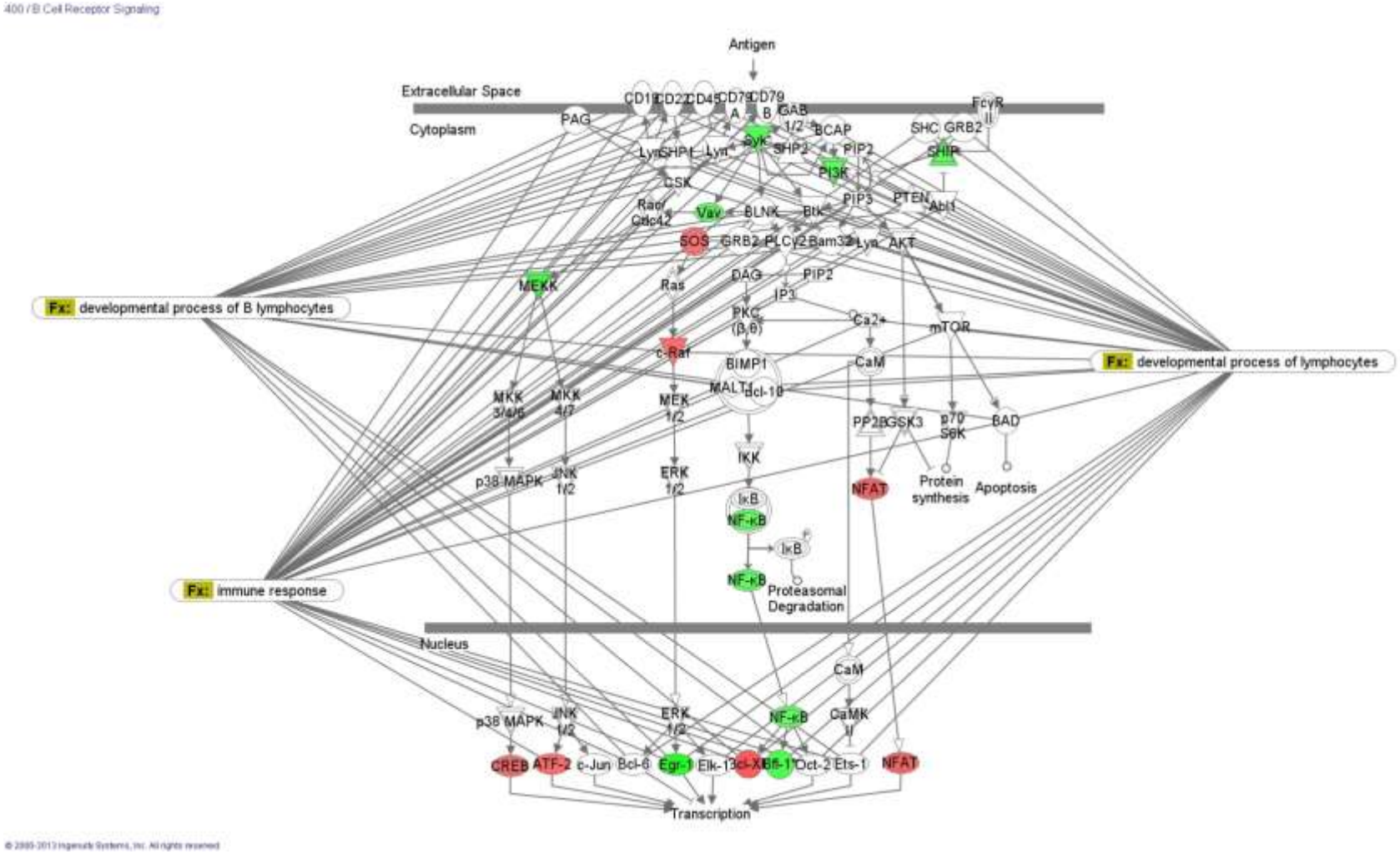

Figure 6.11. B cell signalling pathway showing gene interaction with the development process of lymphocytes and immune response.

6.5.1.7 Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes

The pathway regulating the expression of IL-2 in activated and anergic T lymphocytes was indicated to have been affected by 400g/t NCF, with a number of genes significantly affected having known functions in the immune response and development of lymphocytes. Nuclear factor of activated T-cells (NFATc3) was up regulated (1.266 P=0.0169) by 400g/t NCF compared to the control, and has been reported to control transcription of a number of genes, including those encoding a variety of cytokines when it is dephosphorylated and translocated into the nucleus (Bopp *et al*., 2005). Nuclear Factor Kappa-B1 (NF-kB1) was down regulated (1.134 P=0.0491) by 400g/t NCF. NF-kB is a pro-inflammatory factor. With the deletion of NF-kB1 in mice it has been reported that lipopolysaccharide (LPS) induces higher levels of the genes for IFN-b and IL-12 (p40) in macrophages. The transcription factor NF-kB plays a role in regulating innate immune gene expression by inhibiting lipopolysaccharide (LPS)-induced gene expression. Therefore, it is thought that NF-kB1 is an inhibitor of some immune-regulatory genes (Zhao *et al*., 2012), and the down regulation of this gene by NCF may have caused an increase in immune factors. In this pathway C-RAF was also up regulated (1.224 P=0.0327), which was similarly reported when feeding 200g/t NCF. As explained earlier, this may have an effect on inflammation and the tight junctions of the intestinal epithelial membrane.

T cell antigen receptors recognise and bind to major histocompatibility complex (MHC) associated antigenic peptides that are presented by antigen presenting cells. Binding leads to the activation of several downstream signalling pathways, including the activation of IL-2 promoter (Luo *et al*., 2011). Functions that are related to this pathway are the development of leukocytes and lymphocytes. One gene that was down regulated (1.368 P=0.0236) by 400g/t NCF is SLP-76, which is a leukocyte-

specific adapter molecule. It was reported that mice that were deficient in SLP-76 expression had no peripheral T cells as a result of an early block in thymopoiesis. Therefore, it is thought that the SLP-76 adapter protein is essential for thymocyte development and plays a vital role in translating signals mediated by pre-T cell receptors (Clements *et al*., 1998). The down regulation on SLP-76 may mean the NCF decreased the development of thymocytes. NFATc3 was up regulated (1.266 P=0.0169) and NF-kB1 down regulated which were discussed earlier, and are also important in this T cell antigen receptors signalling pathway.

6.5.1.8 p38 Mitogen-activated protein kinase (MAPK) signalling pathway

400g/t NCF also had an effect on the p38 Mitogen-activated protein kinase (MAPK) signalling pathway (Figure 6.12), which is activated by a range of environmental stresses and inflammatory cytokines. The activation of p38MAPKs results in the downstream phosphorylation of several protein kinases termed MAPK-activated protein kinases (MKs). MAPK signal transduction pathway plays a significant role in the recruitment of leukocytes to sites of inflammation, and a p38MAPK inhibitor would lead to a reduction in the production of inflammatory mediators (Herlaar and Brown 1999). Therefore, the supplementation of NCF may affect the immune system though this pathway. The gene encoding for TRAF-interacting protein with forkhead-associated domain (TIFA) was up regulated (1.143 P=0.0190) by 400g/t NCF. It has been reported that TIFA mediates IL-1 signalling and may have a function in innate immunity (Takatsuna *et al*., 2003). TIFA is also thought to play a role in the activation of macrophages by lipopolysaccharides (LPS), which are mediated by Toll-like receptor 4 (TLR4). TLRs transmit signals when going through the recruitment of adaptor proteins including tumor necrosis factor-associated factor

6 (TRAF6), which mediates the activation of I_kB kinase (IKK). TIFA has been shown to bind to TRAF6 and activate IKK (Minoda *et al*., 2006).

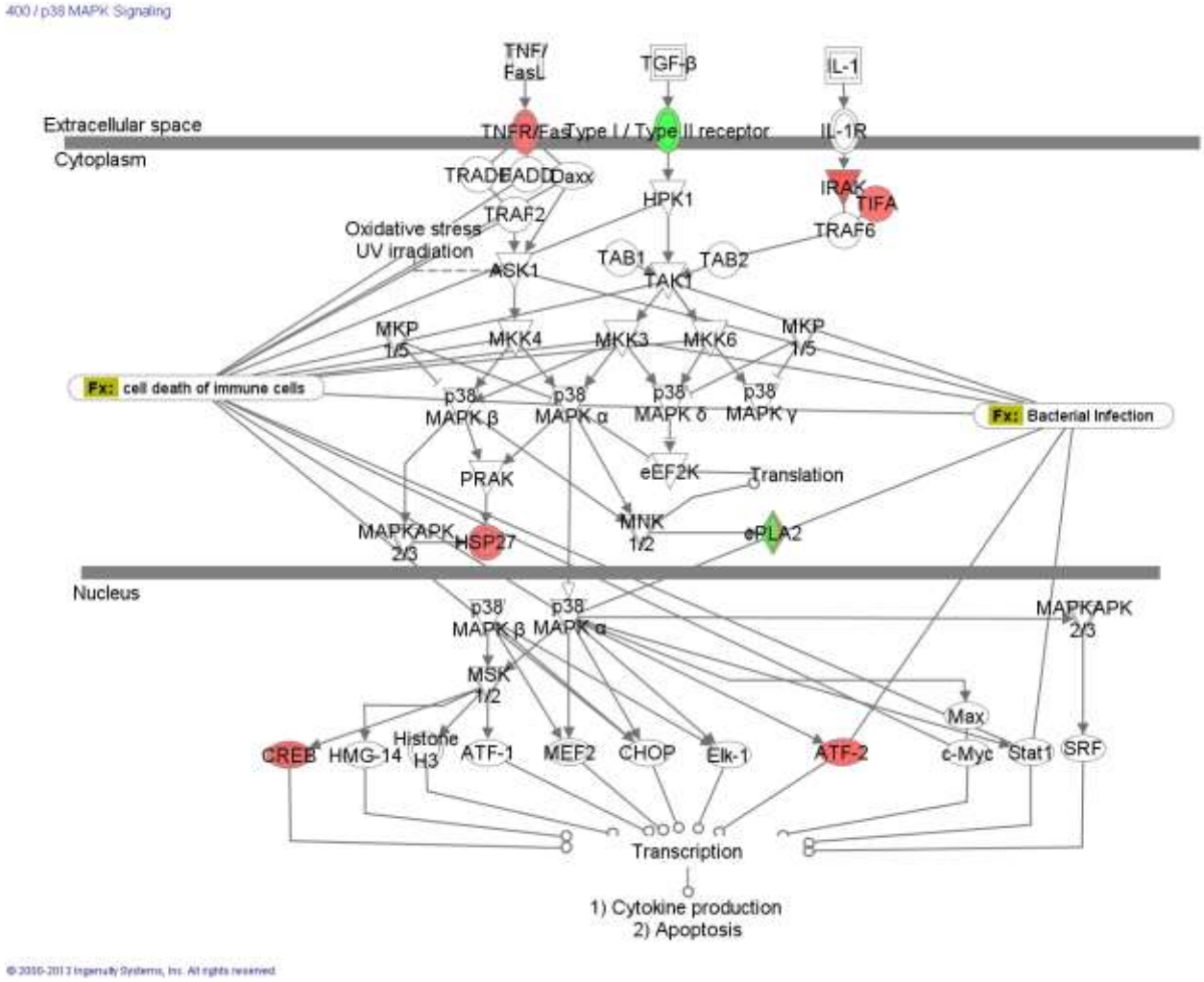

Figure 6.12. p38 Mitogen-activated protein kinase (MAPK) signalling pathway showing gene interactions with the protein kinase cascade.

6.5.1.9 G-Protein Coupled Receptor Signalling

The G-Protein Coupled Receptor Signalling canonical pathway was affected by 400g/t NCF. G proteins are key in trans-membrane signalling by coupling a multitude of receptors to enzymes, channel proteins and other effector molecules. Phosphodiesterase 7A (PDE7A) is a gene involved in the g-protein coupled receptor signalling pathway, which was down regulated (-1.222 P=0.0029) by 400g/t NCF. PDE7A is thought to have a vital role in T cell activation, as a study showed that when PDE7A expression was blocked it inhibited T cell proliferation and IL-2 production (Yang *et al*., 2003). Therefore, the down regulation of PDE7A may mean that NCF suppresses the immune system; however, there is little data about the effects of PDE7A, and more research is needed.

400g/t also up regulated a number of genes involved in the Gs-coupled receptor which regulates human G protein alpha s (Gαs). Kveberg *et al*., (2002) reported that Gαs mediates movement/chemotaxis of human natural killer cells through chemokine ligand 5 (RANTES) and sphingosine-1-phosphate. Natural killer cells play an important role in the early defence against viruses and are cytotoxic lymphocytes. Therefore, the up regulation of the Gs-coupled receptor may have a knock- on effect on the movement of natural killer cells and their cytotoxic abilities.

6.5.1.10 MIF-mediated Glucocorticoid Regulation

MIF-mediated Glucocorticoid Regulation was also seen to be affected by 400g/t of NCF compared to the control. Glucocorticoids inhibit production of several cytokines, enzymes and cell surface molecules essential for immune function and the inflammatory process (Almawi and Melemedjian, 2002). The gene Nuclear factor-kB1 (NK-$_K$B1) was down regulated (1.134 P=0.0491) by 400g/t NCF. In this

pathway, NK-$_K$B1 activation leads to transient up regulation of a number of genes encoding cytokines, growth factors, immunomodulatory molecules, apoptosis related genes and others (Blackwell *et al.*, 2009) As you can see from Figure 6.13, this pathway is also linked to Inducible nitric oxide synthase (iNOS) signalling. iNOS is an enzyme that produces nitric oxide (NO). Macrophages when stimulated with microbial components or cytokine produce iNOS. NO has antimicrobial functions and therefore has an influential role in immune responses (Bogdan, 2001; Blanchette *et al*., 2003; Bogdan *et al*., 2000; MacMicking *et al*., 1997; Cheeseman *et al*., 2008).

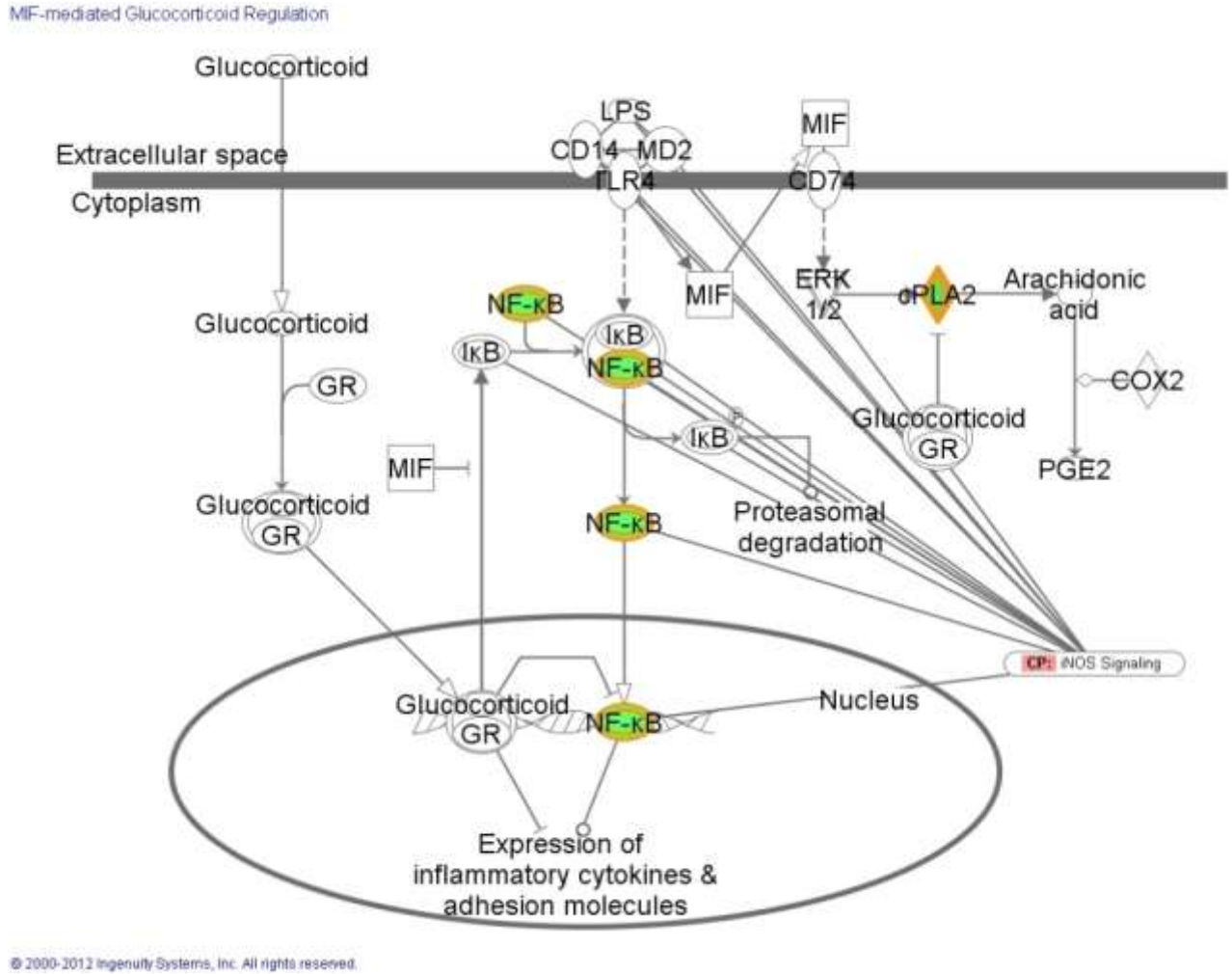

Figure 6.13. MIF-mediated Glucocorticoid Regulation pathway with interactions with iNOS signalling.

6.5.1.11 Glyoxylate and Dicarboxylate Metabolism

The Glyoxylate and Dicarboxylate Metabolism canonical pathway was affected by 800g/t NCF. In this pathway Aconitase 2 (ACO2) was down regulated (1.089 P=0.0241). ACO2 is an enzyme that catalyses the inter-conversion of citrate to isocitrate via cis-aconitate in the second step of the TCA cycle (Wang *et al*., 2013). The down regulation of ACO2 may mean there is a decreased ability for citrate to be changed into isocitrate, which may affect the energy produced by the TCA cycle. 4-hydroxy-2-oxoglutarate aldolase (HOGA) is also an enzyme in this pathway, which was up regulated (1.123 P=0.0099). It breaks down 4-hydroxyproline into glyoxylate and pyruvate, which can be then broken down into glycine, oxalate and glycolate (Riedel *et al*., 2011). The up regulation of HOGA suggest the 800g/t is having an effect on the metabolism of 4-hydroxyproline and glyoxylate, and may be feeding pyruvate into the TCA cycle.

800g/t NCF in the Glyoxylate and Dicarboxylate Metabolism canonical pathway down regulated (1.162 P=0.0497) methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) which encodes for three enzymes; methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase and formate–tetrahydrofolate ligase (Figure 6.14). MTHFD1 is key in folate metabolism (Krajinovic *et al*., 2004). The B-vitamin folate is essential for many biochemical reactions and metabolic functions through providing methyl groups in several reactions (McCann *et al*., 2004). One of these carbon transfer reactions is involved in purine synthesis (Selhub *et al*., 2009). Therefore, the down regulation of MTHFD1 may affect a number of metabolic functions in the bird.

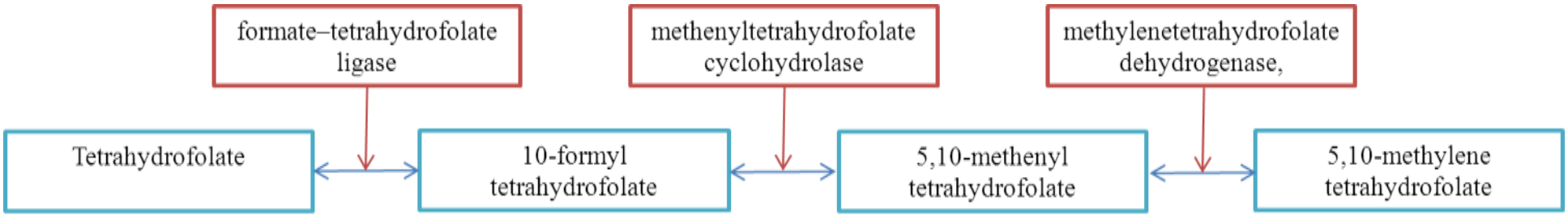

Figure 6.14. Part of the folate metabolism pathway showing three enzymes: methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase and formate–tetrahydrofolate ligase, encoded by methylenetetrahydrofolate dehydrogenase 1 (MTHFD1).

6.5.1.12 Linoleic Acid Metabolism

The linoleic acid metabolism pathway was affected by 800g/t NCF compared to the control. Phospholipases A2, group X (PLA2G10) was down regulated (1.143 P=0.0029). Phospholipases A2 are enzymes that release fatty acids from the second carbon group of glycerol. This particular phospholipase specifically recognizes acyl bond at the sn-2 (phospholipase A_2) position of phospholipids and releases arachidonic acid and lysophospholipids. There are numerous links of PLA2 with function in the body which include the immune system and phospholipid digestion and metabolism (Dennis 1994). PLA2 is thought to be the first step in the eicosanoid cascade and the generation of pro-inflammatory intermediates, including prostaglandins and leukotrienes (Sandercock and Mitchell, 2004). Therefore, the down regulation of PLA2 may mean the production of pro-inflammatory intermediates through the eicosanoid cascade was reduced.

The gene encoding for fatty acid desaturase 1 (FADS1) in the linoleic acid metabolism pathway was up regulated (1.143 P=0.0220), which is a member of the linoleoyl-CoA desaturase (Gene View: FADS1). The enzymes FADS2 and FADS1 are important in the production of arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3), from linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), as shown in Figure 6.15 (Das 2006; Arbo *et al*., 2011). Increased fatty acid desaturase activity in erythrocytes due to high n-6 to n-3 ratios, is suggested to promote endogenous production of pro-inflammatory arachidonic acid (Martinelli *et al*., 2008; Arbo *et al*., 2011). Therefore, the up regulation of FADS1 when feeding 800g/t may increase the levels of the pro-inflammatory arachidonic acid in broilers.

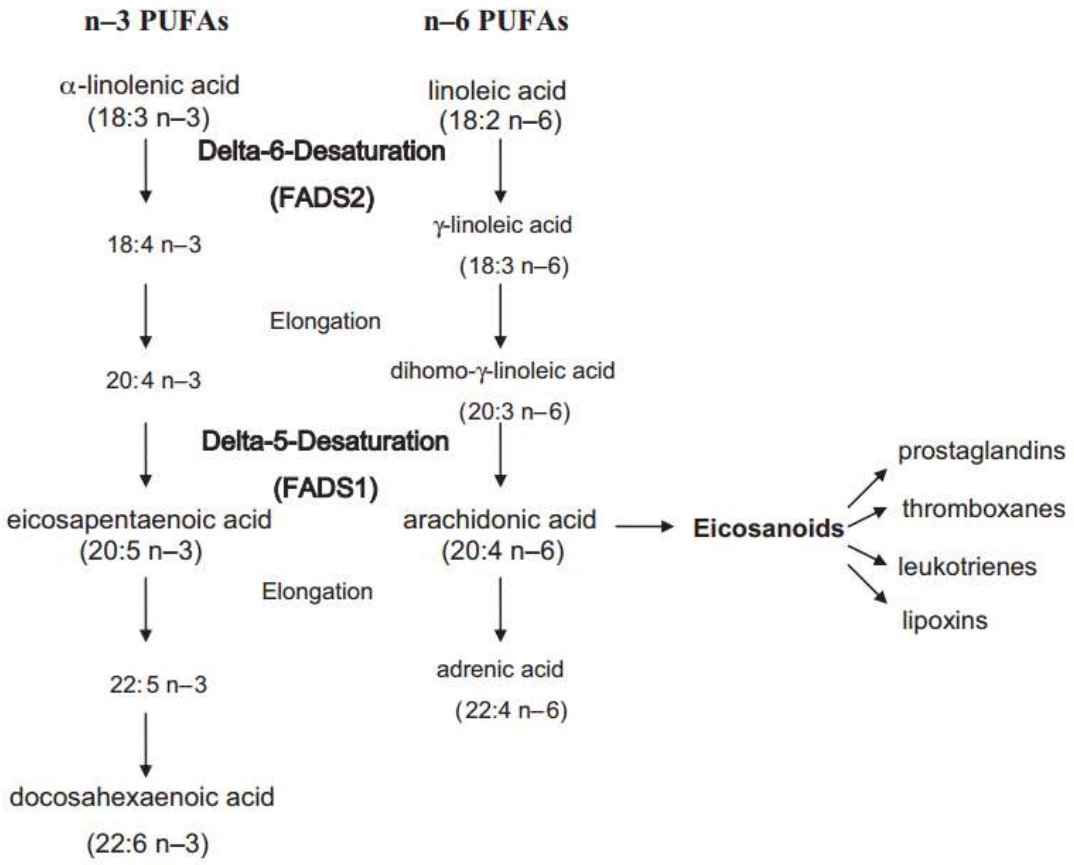

Figure 6.15 The n-3 and n-6 fatty acid metabolism pathways (Martinelli *et al*., 2008).

6.5.1.13 G-Protein Coupled Receptor Signalling

Like 400g/t NCF, the G-protein coupled receptor signalling canonical pathway was also affected by 800g/t NCF. However, 400g/t NCF up regulated a number of genes involved in the Gs-coupled receptor, whereas 800g/t up regulated DRD1 and HRH2 and down regulated CNR1 and MC2R. The effect of 800g/t NCF on the Gs-coupled receptor, may as with 400g/t NCF effected Gas and the movement of natural killer cells and their cytotoxic abilities, as reported earlier. The Gi coupled receptor was also affected by 800g/t NCF, with a number of genes up or down regulated. The Gi coupled receptor regulates G-alpha i/o (Gαi/o). The inhibition of G-alpha i/o in humans has been reported to decrease production of nitric oxide in cultured endothelial cells (eNOS)

(McGuane *et al*., 2011). It has been reported that endogenous eNOS plays an important role in angiogenesis in vitro and in vivo (Ziche *et al*., 1994). Murohara *et al*., (1999) demonstrated that endogenous eNOS contributes to endothelial cell migration *in vitro*. Phosphodiesterase (PDE) 5A and 11A are genes involved in the g-protein coupled receptor signalling pathway, which were up regulated by 800g/t of NCF (1.380 P = 0.005; 1.144 P=0.010 respectively). PDE11A catalyses the production of adenosine monophosphate (AMP) from cyclic AMP (Fawcett *et al*., 2000). It has been reported that an increase in cyclic AMP can result in a decreased cytokine response of immune cells and decreased inflammatory response in organisms (Ernst *et al*., 2010; Lin *et al*., 2011). This could suggest that an up regulation of PDE with NCF supplementation may result in a decrease in cyclic AMP, and an increase in immune and inflammation response.

6.5.2 Immunoglobulin

The interaction observed between NCF and Ca But on IgG levels in the bird at day 21, indicated that the sole supplementations of Ca But did not alter IgG levels from the control, where as NCF on it only and in combination with Ca But decreased IgG levels. This may be due to NCF having an immune modulating effect on the bird (Lyons and Bourne, 1995; Kelly, 2004; Ewing, 2008; Vondruskova et al., 2010), which is not a mode of action seen with Ca But supplementation. However, when looking at the treatment effect at 21 days on IgG, there was a trend but not a significant difference. No significant effect were observed when feeding NCF, Ca But and Na But had on broiler immunoglobulin levels in serum at days 7, 14, 21 or 42. This illustrates that any effect that NCF or Butyrate has on the immune system of the bird is not through changing the

concentrations of immunoglobulin. However this lack of effect may be due to the variability between replicates, which has been reported by other authors (Janardhara *et al.*, 2009; Kim *et al.*, 2011). The lack of effect of the supplements on the levels of IgG, IgM, and IgA in blood serum was also found in a number of studies when feeding MOS in broilers (Midilli *et al*., 2008; Janardhana *et al*., 2009; Kim *et al*., 2011), horse (Gurbuz *et al*., 2010) and pigs (White *et al*., 2002). This may be due to the lack of immune challenge or physiological stress on the birds. As with this study, all of these studies had no specific immune challenge on the animal. In contrast, Castillo *et al*, (2008) also reported no significant effect on IgG, IgM and IgA serum even with temperature stressing the pigs. In addition, Sauerwein *et al*, (2007) found that pigs fed yeast cell wall extracts had higher levels of IgA in their serum without any challenge being applied. Therefore, published data is variable and it is difficult to draw conclusions from.

It has been seen that butyrate can affect inflammation (Hammer *et al.*, 2008), which may mean that butyrate is having an effect on the immune system. This study found that the IgG level in birds fed Na But at 21 days was very close to being significantly higher than the other diets ($P = 0.058$). This implies that Na But is causing an increase in the activity of the immune system. The increase in IgG also coincided with a decrease in performance, indicating that the immune system could have been using more energy, and therefore less was available for growth. However, as suggested in chapter 5, this may have also been caused by a diet manufacturing issue, as the growth was negatively effect to an extent that is very unlikely to be caused by a licenced feed product. White *et al* (2002) found that when feeding a dried yeast product in combination with an organic

acid (citric acid), the serum IgG levels increased over the control in pigs but yeast on its own did not, suggesting that the organic acid may be having an effect on IgG levels. This may help to explain the trend seen in this trial when feeding butyrate.

The supplements in this study showed no effect on levels of immunoglobulins. However, the supplements might have changed the specificity of the immunoglobulins, which was not measured in this project. Shashidhara *et al* (2003) found that broiler breeders fed MOS have a higher antibody response against infectious bursal disease virus (IBDV), Olivera *et al* (2009) also found this in broilers in the fourth and fifth weeks, as well as reporting increased antibody responses against Newcastle disease virus (NDV) at three, four and five ($P<0.03$) weeks of age was higher with MOS. This could suggest that MOS products are more effective at stimulating the humoral immune responses against specific vaccine viruses, rather than unspecific immunoglobulin levels.

Figures 6.7, 6.8 and 6.9 show the immunoglobulin levels in serum of broiler over time. Levels of IgA increased over the birds life. Sauerwein *et al* (2007) also reported this in pigs. By contrast, IgM increased from 7 to 14 days, then decreased at 21 days and then at 42 increased to its highest level. IgG decreased from 7 to 14 days, but then increased to its highest level at 21 days and slightly decreased at 42 days. A similar pattern in serum levels of IgG were seen in pigs where levels decreased and then increased (Sauerwein *et al*., 2007).

6.5.3 Digestive enzymes

It was seen that feeding NCF, Ca But and Na But either alone or in combination had no

significant effect on the broiler digestive enzymes, amylase, chymotrypsin and trypsin in either the duodenum or jejunum at any time during the trial period. This suggests that NCF, Ca But and Na But does not affect digestibility of the bird though increasing activity of these particular enzymes. However this lack of effect may be due to the variability between replicates, which has been reported by other authors (Matur *et al.*, 2010) This was also reported when feeding saccharomyces cerevisiae extract in broilers (Matur *et al*., 2010). However, in contrast it has been reported that broilers fed MOS have increased specific activities of other enzymes (maltase, leucine aminopeptidase and alkaline phospatase) in the jejunum (Iji *et al*., 2001), as well as amylase and chymotrypsin activity in the pancreas (Matur *et al*., 2010).

As the main site of digestion in the bird is the small intestine, most enzyme secretion occurs into the proventriculus or duodenum. While it might be anticipated from the literature that the activity of enzymes would be higher in the duodenum than the jejunum; it was seen in the current study that amylase, chymotrypsin and trypsin levels were higher in the jejunum than the duodenum. This may be due to the selection of tissue taken from the duodenum being before the pancreatic ducts, which enter the later portion of the ascending branch of the duodenum (Larbier and Leclercq, 1992). This may mean that the enzyme activity level measured in the duodenum represented residual activity resulting from anti-peristalsis of the small intestine moving digesta and enzyme anteriorly in the intestinal tract, rather than a true representation of maximum enzyme activity in that section of intestine. The increased levels of amylase in the jejunum in comparison to the duodenum are consistent with previous reports that most carbohydrate digestion occurs in the jejunum (Osman 1982). Another reason trypsin

activity may be higher in the jejunum rather than the duodenum is that trypsin is produced by the pancreas in an inactive form, such as trypsinogen, which is secreted in to the duodenum and then activated by enterkinase in the GI tract. Therefore, higher levels in the jejunum may be due to more trypsin being in its active form. It has been reported that the trypsin activity increases from hatch to 15 days of age (Leeson and Summers 2001). This was also seen in this trial.

6.6 CONCLUSION

NCF in this project had no profound effect on the digestive enzyme activity or serum immunoglobulin level measured in this trial, which demonstrates that NCF is not having its effects though these measures, however may be still having an effect on the immune system or digestive capabiltys of the bird but through other mechanism not measured in this projects. It was seen in this chapter that the mechanism underlying the supplementation of NCF may change depending on the inclusion level. 200g/t NCF altered immune and metabolism genes, 400g/t NCF altered mostly immune genes, whereas 800g/t altered mostly metabolism genes. From the alterations seen in the gene expression, when feeding NCF it is indicated that one of NCF main mechanisms is through increasing the immune defences of the bird. This emphasizes that performance improvements are more likely to be observed with some level of challenge placed on the bird.

CHAPTER 7: GENERAL DISCUSSION

7.1 INTRODUCTION

The recognised risk of antibiotic-resistant bacteria is a global priority (Roe and Pillai, 2003) and has put increasing pressure on non-EU countries to decrease their use of antibiotic growth promoters, and EU countries to reduce their therapeutic antibiotic usage in poultry. Therefore there is a requirement for a viable feed supplement, which will improve g`ut health, immunity and bird performance, whilst reducing the proposed risk to human health. A newly refined natural carbohydrate fraction (NCF) from the cell wall of *Saccharomyces cerevisiae* yeast was produced, with suggested prebiotic properties that could improve gut health, immunity and bird performance. The aim of this research was to quantify the efficacy of this feed supplement (alone and in conjunction with other supplements) in improving gut health, immunity and bird performance, as well as to identifying possible modes of action. From these aims, the overarching hypothesis of this project is that NCF improves bird performane through improving gut health. The first posed mechanism in support of this hypothesis is that NCF increases the gut surface area, thereby increasing digestive capacity. The second posed mechanism is that NCF improves immune defence against pathogens by decreasing damage from adverse inflammation. The following general discussion evaluates the findings from the individual trials presented in chapters 3, 4, 5, and 6 and also collectively reviews the observations drawn. The effect of NCF on gut health, immunity and bird performance, as well as the potential variability in feed supplements and between trial variation are also discussed.

7.2 OVERALL BIRD PERFORMANCE

When evaluating bird performance across the whole project, it was observed that feeding NCF to broilers resulted in highly variable performance between trials. When a commercial stocking density was implemented in Trial 1, improved FCR in starter and grower feeding phases were seen with NCF supplementation but there was no effect on performance when stocking density was reduced in subsequent trials. From this, the effects of yeast cell wall derived supplements on bird performance are stronger if the birds are under some level of challenge, which has also been previously reported in broilers and pigs (Nolet, *et al*., 2007; Castillo, *et al*. 2008; Che, *et al*. 2012). It was noted that, when comparing body weight performance of control birds in Trial 1 against the control birds in Trials 2 and 3, birds were 400g lighter on day 42, which supports the suggnestion that birds in trial 1 were under more physiological stress that bird in Trial 2 and 3.

The performance data in Trial 1 indicated an inconsistent response to NCF supplementation level suggesting more than one mechanism behind the observed response to NCF supplementation due to the cubic response at 14 day. Variability with supplementation level of NCF in modulation of gene expression relating to immunity and metabolism may be a factor in observed performance effects. Another mechanism behind the improvement in FCR in the starter and grower phase could be NCF supporting improved development of the gut and defence systems, such as immunoglobulin and mucin production.

7.3 POSSIBLE ROLE OF IMMUNITY AND METABOLISM GENE EXPRESSION IN MECHANISMS BEHIND BIRD PERFORMANCE.

The following two sections relate the improvements in performance observed in Trial 1 to the detected alterations in gene expression of the immune system and metabolism of the birds. Whilst NCF inclusion without doubt affects expression of immune associated genes, the specific effects vary with rate of NCF inclusion. Studies of gene expression result in large and complex data sets making it difficult to define overall effects. The expression of immune and metabolic genes (Table 7.1) provides an overall interpretation of the data which is subsequently discussed in more detail.

Table 7.1 Interpreted summary of overall NCF effects on the alteration of gene expression.

Inclusion	Immune gene expression	Metabolic gene expression
200g/t NCF	Substantial	Moderate
400g/t NCF	Substantial	None
800g/t NCF	Small	Substantial

7.3.1 Immunity

In Trial 1, the consistent improvement seen in the body weights of birds fed NCF at 200g/t is partially explained by changes in expression of specific genes relating to immune function of the birds. It was observed that birds fed 200g/t NCF had down regulated CPS-I, resulting in a decrease in inflammation in the GI tract (Mueller *et al*., 2000). In addition, the up regulation of Act1 decreased numbers of B cells (Qian *et al*.,

2004). These suppressions of the immune system decrease the energy requirement of the immune system, leading to the improved body weights recorded. In contrast, the down regulation of Isopentenyl-diphosphate Delta-isomerase would have increased activation of γδ T cells. This increases the readiness of the immune function of birds fed 200g/t NCF, resulting in the bird being able to overcome the pathogen more quickly. When birds are presented with a challenge this readiness reduces loss of growth and feed conversion efficiency as the pathogen is overcome before colonisation and substantial damage to the gut can occur. This is supported by Trial 1, where the use of commercial stocking density placed the birds under a level of challenge and the birds fed 200g/t NCF maintained their production level greater than birds fed the control.

Similarly to birds fed 200g/t NCF, feeding 400g/t NCF to broilers in Trial 1 resulted in a mixed alteration of the immune system, with some pathways up regulated and others down regulated. However, where 200g/t NCF led to an overall improvement in performance, the suppressive and stimulatory effect of 400g/t NCF on differing immune pathways appear to have annulled each other. The down regulation of Syk by 400g/t NCF decreased B cell development (Turner, *et al* 1995), and the down regulation of Egr-1, PDE7A and SLP-76 would have resulted in the suppression of the immune system leading to a decreased energy need for the immune system (Clements *et al*., 1998; Yang *et al*., 2003; Carter *et al*., 2007). However 400g/t NCF also increased immune factors through the down regulation of NF-kB1 and up regulated of TIFA. Overall, it was demonstrated that feeding 400g/t NCF had no effect on body weights of the birds, as the opposing alterations in the gene expression of the immune system resulted no net change in the overall energy required by the immune system.

800g/t NCF did not have as profound an effect on the immune system gene expression as the lower NCF inclusion rates. In Trial 1, improvements in body weights of birds fed NCF at 800g/t were observed but the effects of 800g/t NCF on the alterations in gene expression suggest an *increase* in energy required for the immune system. Based on the immune system gene expression findings alone, a decrease in body weight compared with the control was anticipated. However, as Trial 1 presented a challenging environment this stimulation of the immune system allowed the bird to defend itself faster, resulting in a decrease in the loss of production caused by pathogenic challenge. The improvements seen in body weights when feeding 800g/t NCF are likely to be due to this inclusion level having a greater effect on the alterations of expression of genes involved in the metabolism of the birds.

7.3.2 Metabolism

Both birds fed 200g/t and 800g/t showed alteration in expression of metabolic genes, which correlated with improvements in performance. In contrast, feeding 400g/t to birds resulted in neither alteration in metabolic gene expression or performance improvement.

When investigating the effects of the varying levels of NCF, it was demonstrated that 200g/t altered expression of genes involved in the metabolism of carbohydrates and protein, homeostasis of D-glycogen, the transport of fatty acids and amino acid synthesis and uptake. Feeding 200g/t NCF up regulated HNF-1α and INSR, resulting in increased ability of the chicken to take up glucose (Kim *et al*., 2001). Therefore one possible mechanism behind the improved performance may be through increased availability of glucose available for growth.

Trial 1 improvements in body weights were also demonstrated in birds fed NCF at 800g/t, but not to the same extent as 200g/t. However, 800g/t NCF affected the gene expression of more energy related metabolic pathways than 200g/t NCF fed birds. This suggests that at commercial stocking densities a balance between up regulation of immune and metabolic genes is required for optimum performance. Supplementation at 800g/t NCF had profound effects on expression of not only the enzyme involved in the TCA cycle but also on glyoxylate and dicarboxylate metabolism pathways. This may affect the energy produced by the TCA cycle, and therefore the amount of energy available for growth. The additional effects of 800g/t NCF on the expression of enzymes involved in folate metabolism, linoleic acid metabolism and phospholipid digestion and metabolism may have also resulted in the bird being more efficient.

It is also likely that NCF is having an indirect metabolic effect on performance through increased bacterial butyrate production. Butyric acid is produced in the chicken GI tract by bifidobacteria (Rolfe, 2000) and used as an energy source by host epithelial cells (Lawhon *et al*., 2002; Guilloteau *et al*., 2010). There is consensus that MOS increases the population of bifidobacteria in the GI tract (Baurhoo *et al*., 2007; Jung *et al*., 2008; Jacob and Parsons, 2009), therefore NCF is likely to increase levels of butyric acid in the GI tract and thus increase energy available to the bird for growth through this mechanism.

Trial 1 demonstrated that supplementing diets with NCF at varying levels affects gene expression and performance differently. The best performance was seen when NCF was included in the diet at 200g/t, where observed alterations in immune genes were substantial and metabolism genes were moderate. Moderate immune and substantial

metabolic alterations of genes were also observed alongside improved performance in birds fed 800g/t NCF, but performance improvements of 800g/t NCF were not as profound as in birds fed 200g/t NCF. From this it can be concluded that alterations in gene expression of both the immune system and metabolism are required for optimum growth. These alterations were achieved using NCF at the dietary supplementation level of 200g/t, suggesting this is the optimum level for commercial application.

7.4 INTESTINAL HEALTH AND RELATED GENE EXPRESSION

Villus height, crypt depth, goblet cells and intestinal weight and lengths were measured in this project as indicators of gut health as the main proposed mode of action for this feed supplement is to irreversibly bind to pathogenic bacteria in the GI tract, preventing them from damaging the gut (Spring, *et al.,* 2000). The mechanism behind this mode of action is that, by preventing colonisation of the gut by pathogenic bacteria, they are unable to secrete toxins that cause damage to the epithelial cells (Yason *et al.,* 1987). Although 200g/t NCF indicated down regulation of CPS-I thereby effecting proliferation, differentiation, and migration of intestinal epithelial cells (Wu *et al.,* 2000), Trials 1, 2 and 3 showed no significant effects of NCF on the gut health of the birds through villus height or crypt depth. However there was a cubic response of duodenum length to body weight ratio to NCF ROI, which mirrored the response of several performance paramenters, suggesting duodenum development might have some involvement in the possible mechanistic differences occurring at 200g/t and 800g/t NCF inclusion.

It was observed that, at a gene expression level, NCF may yet have improved the integrity of the gut: birds fed 200g/t and 400g/t NCF had up regulated C-RAF, which has been reported to increase level of tight junction proteins and improve barrier function of the intestine (Zimmerman *et al.,* 2011). This suggests that NCF may have improved intestinal health but through a different mechanism, which was not identified by measuring villus height and crypt depth.

The effect of NCF and other yeast cell wall supplements on parameters representing gut health are reported to be variable (Zhang *et al.,* 2005; Sohail *et al*., 2012). This could be due to differences between the environmental conditions of the trial: birds in studies with a pathogenic challenge seem to benefit from yeast cell wall products more than those without a challenge. An animal with no pathogenic infection of the GI tract, that is fed a supplement modifying the microflora population or immune system, will gain little benefit from a supplement, because there is no impediment to growth potential caused by excessive sloughing of the villi.

In the first trial the dose response of jejunum goblet cell area was cubic, with 800g/t of NCF significantly increased the area of jejunum goblet cells. Whereas the dose response of the goblet cell area per 165 μm of villi was linear, with a trend for increasing goblet cell area per 165 μm of villi with increasing NCF. This suggesting that an effect on the mucin profile of broilers in the jejunum is more likely at high dosage of NCF, however there were no mucin specific genes up or down regulated by NCF when looking at the gene expression data. Trial 3 showed no significant difference when feeding NCF in goblet cell number or area, but whether this is due to the younger bird (one week post hatch) or the lower NCF inclusion level (200g/t) cannot be determined from this trial.

Published data on goblet cell area is very limited, but Brummer, *et al.* (2010) also found that the area of goblet cells increased with supplementation of MOS. Insight into the mechanisms behind this goblet cell response would be highly beneficial to understanding the effect of MOS supplementation on gut health. When investigating the effects of butyrate salts in the third trial, goblet cells measurements were not significantly affected, but there was a trend toward increasing goblet cell area per 165µm of villi when feeding calcium butyrate. It has been also reported by Nahrling, *et al.* (2010) that butyrate in piglets increased MUC2 expression, which is key in mucin production. Therefore the observed trend in Trial 1 towards increased mucin production at 800g/t NCF may be due to NCF increasing levels of butyrate in the GI tract by increasing the population of butyrate-producing bacteria. However there appear to be no published studies on the effects of butyrate salts on goblet cells in broilers, and one paper that found that feeding sodium butyrate had no effect on goblet cell number in pigs (Manzanilla *et al.*, 2006).

An increase in goblet cell area is thought to show that the storage capacity of the goblet cell for mucin has increased (Smirnov, *et al.*, 2005). An increase in mucin storage means that the bird is more capable of forming a protective layer on the villi, thereby helping protect the intestine from damage caused by enteropathogens if there was a challenge from pathogenic bacteria (Smirnov, *et al.*, 2006; Brümmer, *et al.*, 2010). Previous studies have suggested that MOS affects mucin production through direct crosstalk between beneficial intestinal microbes and goblet cells (Mack, *et al.*, 1999; Freitas, *et al.*, 2003; Smirnov, *et al.*, 2005; Uni and Smirnov 2006; Chee, 2008). However, the current study suggests an indirect mechanism via MOS or NCF increasing

levels of butyrate producing bacteria, which then leads to the butyrate affecting mucin production of the bird.

7.5 DIGESTIVE ENZYMES AND RELATED GENE EXPRESSION

A recently posed mode of action behind the effects of MOS in improving performance is through increased digestive enzyme activity (Iji *et al*. 2001; Matur *et al*. 2010). Therefore activities of three key digestive enzymes, amylase, chymotrypsin and trypsin, were measured in Trial 1 and 3. In Trial 1 and 3 it was observed that NCF did not significantly affect the activity of these digestive enzymes. Whether this result is a truly meaningful contribution is difficult to determine, as published values for activity of these enzymes in poultry vary by orders of magnitude (Matur *et al*. 2010; Kadhim, *et al*., 2011), suggesting that producing reliable data from these assays is challenging. However the differing values derived from birds of differing ages suggest the assay is capable of identifying differences. Whilst amylase activity was not affected by NCF supplementation, microarray demonstrated that NCF at 200g/t may have increased the uptake of glucose by up regulating the gene encoding the insulin receptor (Kim *et al*., 2001). Therefore, the increase in performance seen with 200g/t NCF supplementation may be attributed to an increased ability to uptake glucose, rather than an increase in the glucose monomers available for absorption through increased digestive enzyme activity.

7.6 NATURAL CARBOHYDRATE FRACTION LEVEL

In trial two it was shown that NCF has little or no effect on the birds performance or gut health. This was initially thought due to the lack of challenge on the birds from the

environment, resulting in all birds already performing to their optimum in the trial, meaning that NCF could not improve performance or gut health. However when the levels of NCF were analysed in the diets the measured levels did not reflect the formulated linear increase in NCF. When the assay was repeated, inconsistencies between runs and laboratories were revealed, therefore the usefulness of data from this trial was very limited. The diets were analysed three times, twice at the Bioscience Centre in Dunboyne, Ireland and once at The Bioscience Centre in Lexington, USA. All of the assays performed showed unexpected results that were inconsistent. This could be due to a number of factors: firstly the assay may not reliable, secondly there is a factor in the feed interacting with the NCF or assay, thirdly there was a problem with the activity of the NCF and finally the mix of NCF in the feed was not homogenous. Due to the variability of measured NCF in the diet between the two laboratories, in combination with no clear pattern of effects on performance or gut health in this trial, it was decided that no further analysis would be performed on this trial.

7.7 SODIUM AND CALCIUM BUTYRATE

The main focus of the project was to investigate the mechanisms underlying any effect of supplementing broiler diets with NCF, but the final phase of the project (Trial 3) investigated any interaction upon feeding NCF in combination with another type of gut health supplement; calcium butyrate or sodium butyrate. Overall Trial 3 showed that no effects of NCF, butyrate salts or their combination on the performance or gut health of young broilers. This supports the hypotheses of Trial 3, that feeding NCF and butyrate salts exert their effects on performance via similar modes of action and would therefore not show an additive effect when fed in combination. The lack of response to any

treatment in Trial 3 is likely to be due to the lack of environmental challenge being placed on the birds. Ideally this trial would have included a pathogenic challenge but this was not possible due to the lack of Animals (Scientific Procedures) Act 1986 Home Office licence (required for this type of trial) at Nottingham Trent University poultry facility. In addition, the design of Trial 2 and 3 (weekly sampling days) constantly reduced stocking density compared to commercial levels, thereby further decreasing any challenge experienced by the birds in these trials. This was due to the requirement to cull birds each week for the sample, which allows for the investigation into any mechanisms occurring in young birds following NCF supplementation.

7.8 CONCLUSION

The aim of this project was to investigate and clarify some of the mechanisms behind the observed effects of gut health supplements in broilers. This project indicated that NCF instigates some of its effects through alteration to the immune and metabolic systems of the bird at a gene expression level. Furthermore the project provides evidence that some of the immune adaptations are borne out through alteration to mucin production by goblet cells in the GI tract. Trial 1 showed that the mechanism underlying the effects of NCF supplementation change depending on the inclusion level: 200g/t NCF altered immune and metabolism genes, 400g/t NCF altered mostly immune genes, whereas 800g/t altered mostly metabolism genes. Histological and enzymatic investigations did not provide any clear mechanism for how metabolic alterations at gene expression level resulted in the improved performance related to NCF supplementation. From the alterations seen in the gene expression and mucin capacity

when feeding NCF it is indicated that NCF is increasing immune defences to prevent growth impedance, rather than any metabolic, growth promoting effect. This emphasises that performance improvements are more likely to be observed with some level of challenge placed on the bird.

This indicates that feed supplements such as prebiotics may be fed as a type of insurance policy, as they increase the readiness of the immune system to overcome pathogenic challenge. However if there is little risk of pathogenic infection, the cost of adding the supplement to the feed, combined with potential decrease in production efficiency due to repartitioning of energy from growth to immune readiness, may outweigh its benefits. However in modern production systems, it is unlikely that the birds will be free from the risk of pathogenic infection. This project demonstrated that the effects of the supplementation of NCF are variable, suggesting that the optimum level of inclusion of NCF may vary depending on the bird age and environment, as well as the activity of NCF.

7.9 FUTURE DIRECTION

The clearest deduction from this study is that immense complexity lies within the mechanisms underlying the effects of successful gut health feed supplements. This project has scrutinised a number of possible modes of actions for NCF effects on broilers, however a number of avenues remain unexplored.

7.9.1 Quantities of mannan and β-glucan within and between different yeast cell wall products

The consistency of a feed supplement is vital for the industry to ensure reproducible results can be achieved in similar situation. Investigation in to the variability of mannan and β-glucan within and between a yeast cell wall feed supplement, would clarify whether the variation between trials using the same or similar products is due to the product or the environmental conditions. Presently available yeast cell wall products are generally claimed to either contain mannan oligosaccharides, β-glucans or both. It would be interesting to measure levels of both mannan oligosaccharides and β-glucans in commercial products to quantify the variability between brands and batches in their active contents.

7.9.2 Levels of immune cells within the bird

Altered expression of genes affecting B and T cell development in the bird was consistent throughout all NCF treatment groups. Further investigation in the levels of B and T lymphocytes in the blood of birds fed NCF may help to understand the effect of NCF on the adaptive immune system. Additionally, such an investigation would increase understanding of how NCF may protect the performance of a bird when there is a pathogenic challenge.

7.9.3 The effects of sodium butyrate and calcium butyrate on gene expression

The observed effects of NCF on bird performance and gene expression may be through increasing the population of butyrate-producing bacteria in GI tract. Therefore it would

be interesting to investigate the effect of feeding a butyrate feed supplement on the gene expression of the birds. Comparing alterations of feeding butyrate on gene expression to alterations when feeding NCF to birds may expose the underlying mechanism behind butyrate and NCF supplementation.

7.9.4 Comparative trials

There are many types of feed supplements presently used in the poultry industry, which are thought to improve intestinal health and performance of broilers. These included similar prebiotics, probiotics and organics acids. It would be valuable to investigate several other supplements produced from yeast cell walls in a single trial alongside the NCF to deepen the understanding of how similar feed supplements affect birds under identical environmental conditions.

7.9.5 Implementation of a health challenge on the birds

When feeding any supplement claiming to improve bird health (whether intestinal health, leg health or immunity), the bird needs to be performing sub-optimally to see a profound treatment effect. If the bird is in optimum health there will be no apparent effect of the supplement on the bird, unless the supplement is delivering growth promoting effect rather than health promoting effects. To gain the greatest insight and understanding, future trials on health promoting supplements should contain a challenge, and therefore be carried out under a Animals (Scientific Procedures) Act 1986 Home Office licence. The challenge could be in the form of a dietary, pathogenic or environmental challenge.

7.9.6 Use of prebiotics after therapeutic antimicrobial treatment

Although the uses of antimicrobials as growth promoters are banned in the EU, the poultry industry still extensively uses antimicrobials as treatment for a disease outbreak. Using antimicrobials in broilers decreases the total number of bacteria in the GI tract, including beneficial gram positive bacteria (Gunal *et al*., 2006). Following treatment with antibiotics, the entire profile of bacteria in the GI tract alters. Although there is a decrease in total numbers of bacteria, the *proportion* of harmful bacteria increases (Apajalahti and Kettunen, 2013). This means that, after treatment with antibiotics, the initial bacterial population in the GI tract is potentially harmful. It would therefore be interesting to investigate the effects of a prebiotic immediately after using an antibiotic in the diet to see the resulting bacteria population growth in the GI tract.

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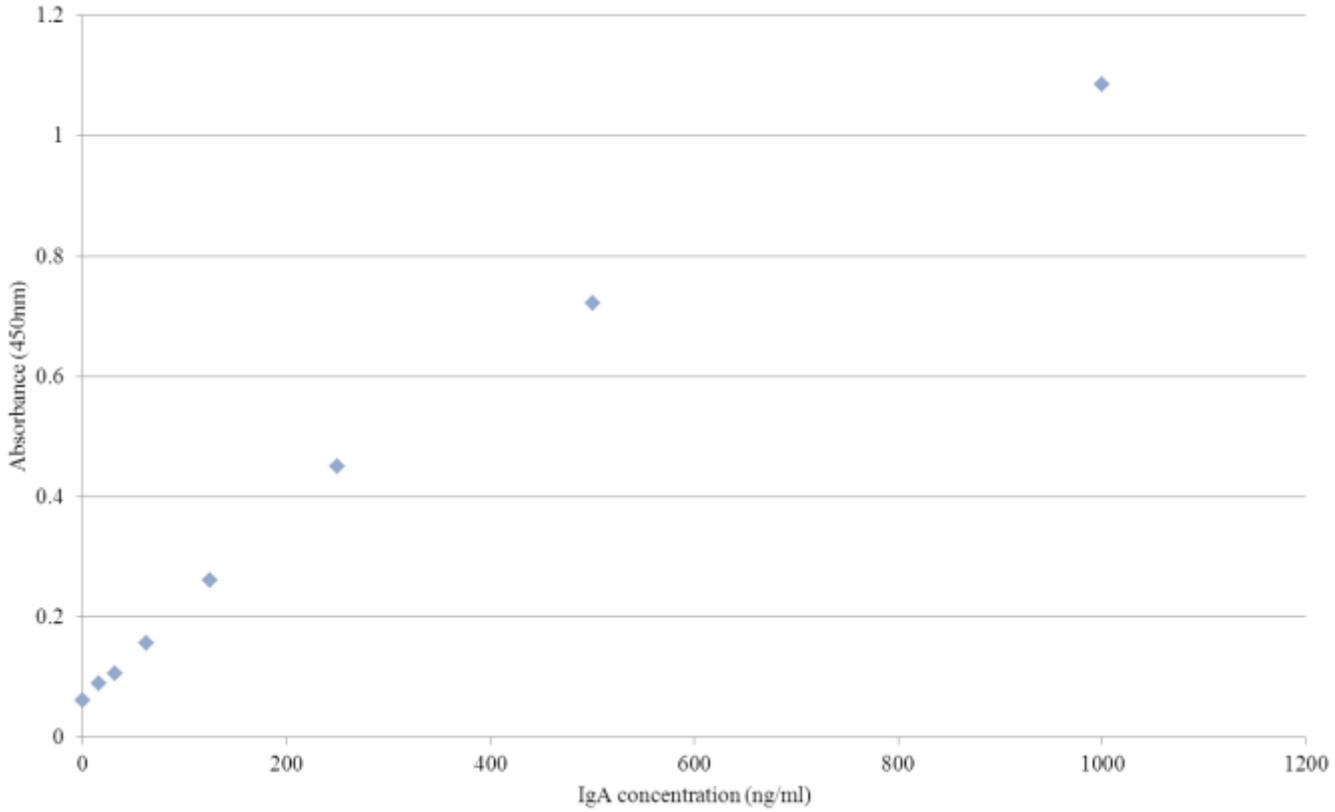
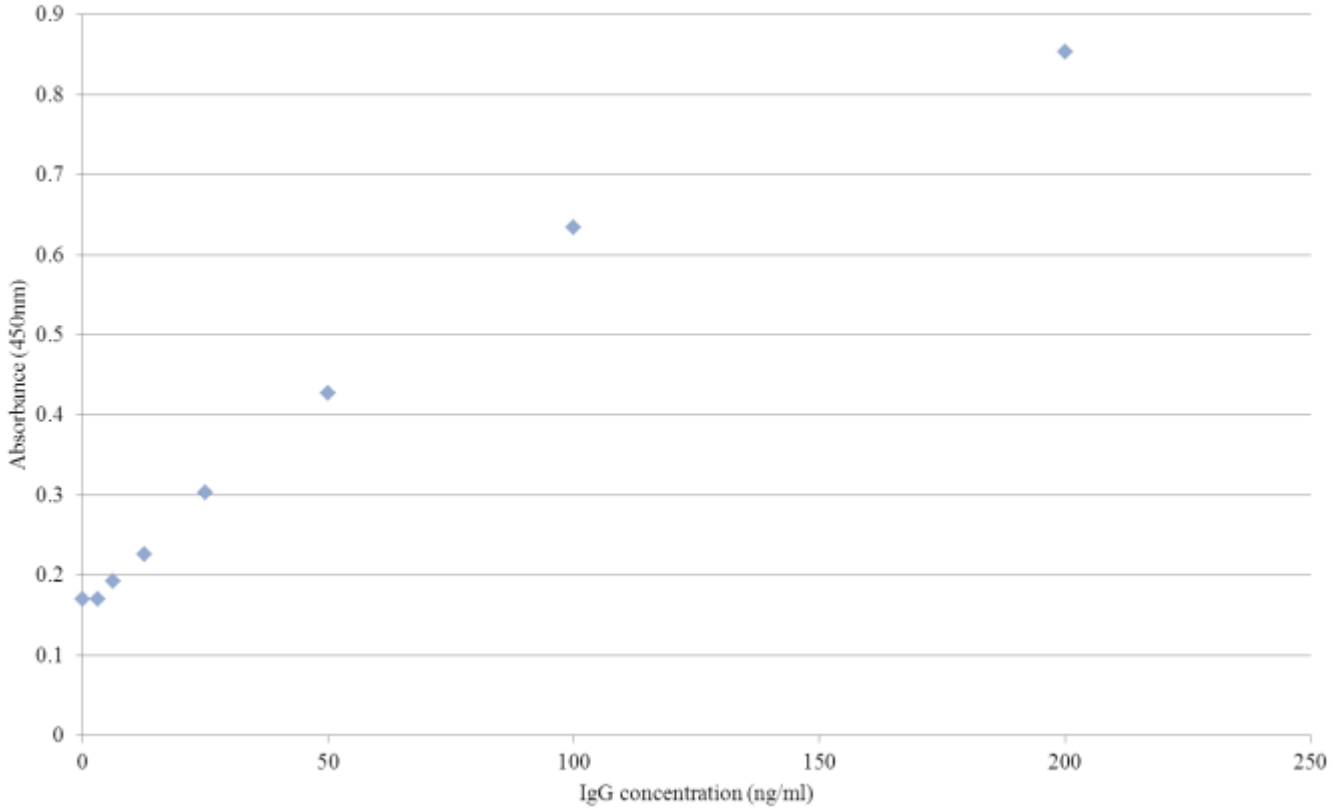
APPENDIX 1



Immunoglobulin-A typical standard curves

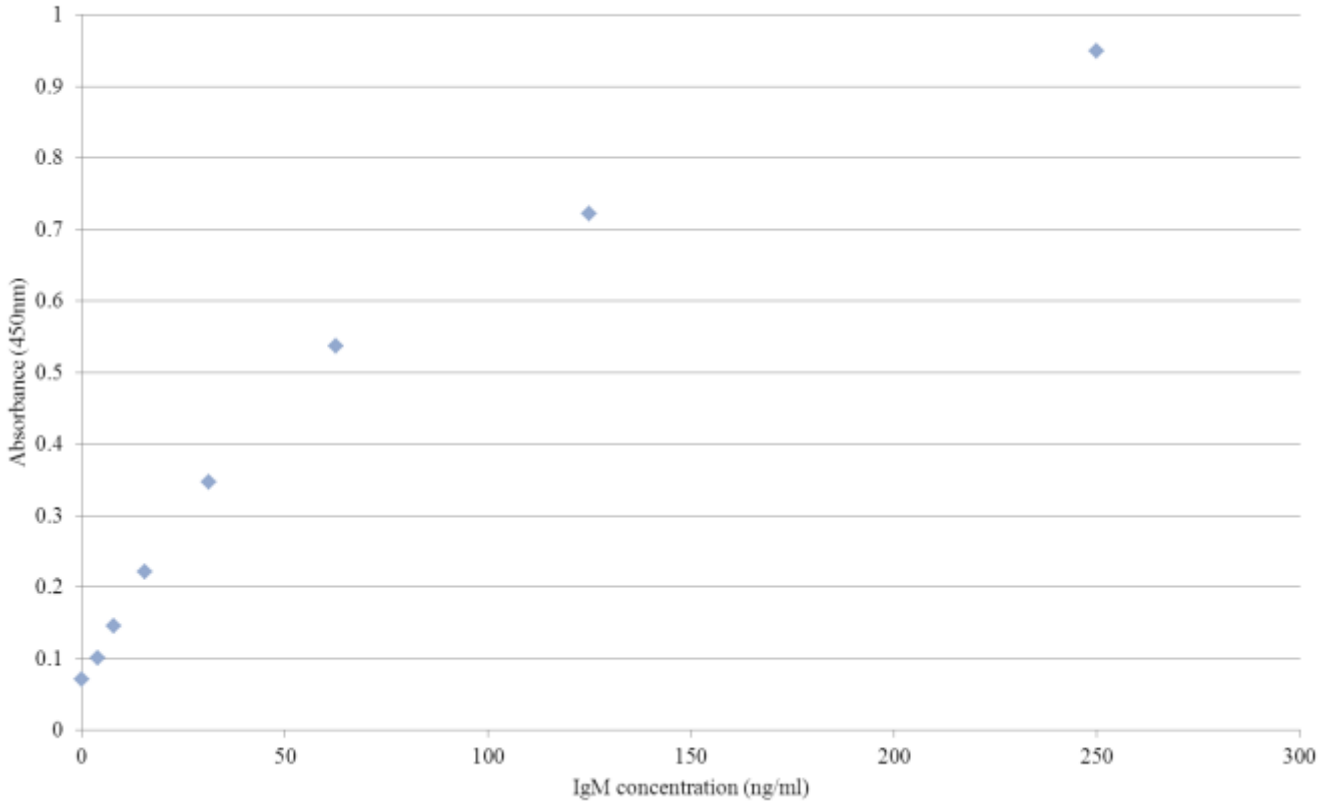
APPENDIX 2:



Immunoglobulin-G typical standard curves

270

APPENDIX 3:



Immunoglobulin-M typical standard curves

271

APPENDIX 4:

Procedure for measuring levels of IgM, IgA and IgG in serum using an enzyme linked immunosorbentassay (ELISA) micro titre plate for the detection of chicken IgM, IgA or IgG in serum

1. Add 100µl of standard or sample to well. - Note: Run each standard or sample in duplicate.
2. Cover plate and incubate at room temperature (20-25□C) for 1 hour.
3. Wash plate FOUR times.
4. Add 100 µl of Chicken IgA/G/M Detection Antibody to each well.
5. Cover plate and incubate at room temperature for 1 hour.
6. Wash plate FOUR times.
7. Add 100 µl of HRP Solution A to each well.
8. Cover plate and incubate at room temperature for 30 minutes.
9. Wash plate FOUR times.
10. Add 100 µl of TMB Substrate Solution to each well.
11. Develop the plate in the dark at room temperature for 30 minutes.
12. Stop reaction by adding 100 µl of Stop Solution to each well.
13. Measure absorbance on a plate reader at 450 nm

HRP -strepavidin-conjugated horseradish peroxidase

TMB - 3,3',5,5'-tetramethylbenzidine

APPENDIX 5:

Attribute	Supposed levels		
	NCF 200g/t	NCF 400g/t	NCF 800g/t
Starter	338	531	989
Grower	360	487	818
Finisher	316	483	847

APPENDIX 6:

Proximate Analysis

	Trial 1			Trial 2			Trial 3	
Component %	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower
Protien	20.11	18.74	17.89	22.30	22.40	17.60	18.60	16.80
Ether extract	6.23	6.50	6.51	3.90	5.03	3.40	6.93	7.39
Ash	6.69	6.39	5.31	10.70	9.10	10.40	9.20	10.20
Dry matter	87.83	87.30	87.15	86.20	89.20	86.20	88.80	89.10