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An integrated approach to understanding gut function and gut health of chickens

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Executive Summary

The Australian chicken meat industry maintains a high standard of bird health and performance efficiency by the in-feed addition of sub-therapeutic levels of antibiotics, coupled with stringent management procedures. The inclusion of in-feed antibiotics reduces the incidence of infectious diseases and improves bird growth. Due to fears of antibiotic resistance, the European Union have recently banned in-feed antibiotics, with consumer pressure also driving other countries toward reduced usage. The absence of in-feed antibiotics has led to an increased susceptibility to infectious diseases and has the potential to significantly reduce growth and performance in broilers. As such, the identification of natural alternatives to antibiotics is of importance to the industry in order to facilitate sustainable production of chicken meat without reliance on antibiotics. Current candidates for inclusion in poultry feed include prebiotics, probiotics, fatty acids, plant extracts and natural immunomodulatory compounds.

Within this ambitious project we aimed to improve our understanding of the complex interactions amongst nutrition, the commensal bacteria of the gastrointestinal tract, mucosal immunity, gut structure and function, intestinal gene expression and corresponding production efficiency and bird health. In order to achieve this aim we assessed the effects of in-feed antibiotics, and candidate antibiotic replacements in healthy birds and birds with *Clostridium perfringens*-induced necrotic enteritis.

In a series of metabolism experiments performed in healthy birds we observed that in-feed supplementation with prebiotics significantly influenced the intestinal microbial communities of broilers and altered a number of key immunological parameters. Omega-3 polyunsaturated fatty acid supplementation significantly influenced the fatty acid profiles of the plasma and breast tissue, and altered the intestinal mucosal immune system. In addition, the immunomodulatory compound lactoferrin had minor effects on the microbiota and immune system and appeared to influence mucin dynamics. These in-feed additives did not improve nor impair bird performance throughout these experiments. Our studies in healthy birds indicated that diet can indeed influence aspects of the intestinal environment; however, these diet-induced shifts may not necessarily lead to improved or impaired bird performance compared to control diets with and without in-feed antibiotics. Indeed, it appears that a high level of performance can be achieved under various microbial profiles and immune states. The optimal housing conditions provided in the research facility may have prevented these additives (including antibiotics) from imparting a growth-promoting effect; however, their capacity to influence the intestinal environment remained.

In a setting of necrotic enteritis, we observed that a medium chain fatty acid blend and a probiotic (*Lactobacillus johnsonii*) failed to replicate the growth- and health-promoting effects of antibiotics. The medium chain fatty acid blend did however decrease clostridial load and *L. johnsonii* decreased the incidence of intestinal lesions. These interesting findings suggest that perhaps other compounds/ organisms of this nature may be more successful in preventing necrotic enteritis, or indeed that strategically-selected combinations of additives may have the capacity to replicate antibiotic effects.

In addition to an assessment of the impact of antibiotic alternatives on bird performance and intestinal health, we performed a preliminary experiment to begin to characterise differentially expressed genes in the intestine of high and low performing birds. Our preliminary results indicate that there may indeed be a link between apparent metabolisable energy and gene expression. Genes involved in immunity and transport were among those differentially expressed between high and low performing birds. This study indicated that a more comprehensive assessment of the underlying differences in gene expression between high and low performing birds is required in order to characterise key genes and pathways responsible for performance differences in broilers.

In conclusion, we have demonstrated that in-feed additives can significantly influence a range of components which comprise the intestinal environment. Diet-induced shifts to the intestinal environment have the capacity to, but may not necessarily influence health status and performance as a high level of performance can be observed in birds with a range of microbial profiles and immune states. Further research into host-microbe crosstalk and the influence of nutrition on the intestinal environment is required, in addition to further screening of antibiotic alternatives, in sub-optimal housing conditions and in the setting of necrotic enteritis. It appears likely that one compound or organism will not have the capacity to completely replace antibiotic supplementation and a combination of strategically-selected feed additives will be required.

Importantly, the current project has successfully used a multi-disciplinary approach to investigate the influence of nutrition on the intestinal environment of chickens. This project has brought together experts in immunology, microbiology, molecular biology, genetics, nutrition and intestinal physiology to simultaneously assess the impact of feed additives on intestinal health and bird performance. This project highlights the capacity of the Australian Poultry CRC to fund ambitious, multi-institutional, multi-disciplinary projects which tackle key industry problems. The collaborative research team brought together within this project have the capacity to use and expand upon the techniques and database established in this project to further extend our knowledge of how the chicken intestine is influenced by nutrition, how different components of the intestinal environment interact with one another, and how this may influence bird performance and disease resistance.

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Introduction

Sub-therapeutic doses of antibiotics are used in poultry feed as a means to reduce the incidence of disease, and improve bird performance and production efficiency. The search for alternatives to in-feed antibiotics has intensified in recent years as consumer pressure for the withdrawal of antibiotics from poultry feed increases. The European Union banned the use of in-feed antibiotics in 2006 and the United States have decreased usage considerably as a result of voluntary reduction by producers and demands of restaurant chains (Singer and Hofacre 2006). Alternatives to in-feed antibiotics that have been investigated include prebiotics, probiotics, organic acids, enzymes and plant extracts (Dahiya et al. 2006). The majority of studies into these additives have produced conflicting or unconvincing evidence that their use has the potential to replace antibiotics without any loss of productivity or negative impacts on bird health. Therefore, further research is required to identify which products are most suitable for inclusion in poultry feed.

The overall performance of a broiler chicken can be influenced by many factors. Environmental factors including temperature (Leeson 1986) and humidity (Leeson 1986); management factors including stocking density (Feddes et al. 2002); the disease status of the flock (Van Immerseel et al. 2004); and nutritional and dietary factors such as grain type (Shakouri et al. 2008), energy value (Choct et al. 1999), and the addition of feed additives and growth promoters (Hooge 2004; Owens et al. 2008) will all influence bird performance. In addition to these factors, the means by which a bird partitions and handles available energy (Hughes 2001), maintains its immune system (Klasing 2007), and the establishment and maintenance of a favourable intestinal microbial community (Patterson and Burkholder 2003) will influence performance. Thus, when evaluating the influence of dietary additives on bird performance, an evaluation of the influence of nutrition on these parameters is needed.

The immune system

The natural immunity of a bird is critical for resistance to diseases which can cause mortality, or impair performance. Vaccines are available to supplement or boost the immune response to some key poultry-related diseases; however, this strategy can be expensive, and does not comprehensively cover all potential disease states and immune stimuli. Therefore, improvements in the natural immunity of the bird are important to prevent pathogenic diseases and limit productivity losses (Klasing 2007). An efficient means to manipulate bird immunity is via the diet. Diet can influence the immune system of the chicken either positively or adversely by numerous mechanisms, including; nourishment of the immune cells, nourishment of pathogens, modification of immune cell responses, protection, modification of the microbiota or microbial activity, and stimulation of the immune system (Klasing 2007). An overactive immune status can have a deleterious impact on bird growth and performance

(Klasing 2007); therefore a compromise is needed between delivering the nutrients required for optimal immunity, without negatively impacting upon bird performance. The nature of the immune response to different poultry diseases will vary, therefore, the delicate balance between optimal nutrition for immunity and productivity may indeed differ based on the most relevant diseases for a particular region.

Intestinal microbial communities

The intestinal microbiota of the broiler chicken contains over 600 different bacterial species at concentrations of up to 10^{11} colony forming units per gram of digesta (Apajalahti et al. 2004). The intestinal microbiota benefits the host by facilitating gut and immune development (Umesaki et al. 1999; Lu et al. 2003), preventing the colonisation of harmful bacteria by competitive exclusion (Nurmi et al. 1992) and the production of antimicrobial compounds (Brisbin et al. 2008a), increasing energy availability via the fermentation of indigestible carbohydrates (Jozefiak et al. 2004), and improving the availability and uptake of vitamins and minerals (Patterson and Burkholder 2003). The microbiota utilise a significant proportion of the nutrients derived from the feed (Apajalahti et al. 2004); therefore a healthy microbial balance must be maintained in order to ensure that host benefits outweigh this loss of nutrients. The maintenance of an optimal microbiota is important for sustained bird health; however, unfavourable microbial shifts can occur, which lead to pathogenic diseases and impaired performance.

Beneficial vs. harmful bacteria

Within the array of bacterial species present in the gastrointestinal tract, there are a myriad of species with different properties. Some of these bacteria are deemed to be more beneficial to the host due to these properties which include; pathogen exclusion, fat digestion, and non-starch polysaccharide degradation. Some species, particularly of the *Lactobacillus* and *Bifidobacterium* genera, have been identified as being extremely beneficial to the host (including avian and human). These bacteria have often been isolated and probiotic products developed in order to increase the relative concentrations of these strains within the microbiota. Recently the use of probiotics in poultry has gained increased attention as a natural growth promoter (Khan et al. 2007; Apata 2008). Probiotic-mediated improvements in bird performance (Khan et al. 2007) and immunity (Haghighi et al. 2006) have been documented previously; however, the reported effects of probiotics are inconsistent, and the optimal strains or combinations thereof, for a particular setting, have yet to be determined. Additionally, it is likely that the properties of these bacteria differ based on the presence or absence of other species within the intestine. As such, an improved understanding of the factors involved in the development of the gut microbiota from hatch and the modulation of the microbial composition(s) that facilitate optimal bird performance is required. Subsequently, dietary manipulation of the microbiota can be harnessed to facilitate the establishment of an optimal composition. An alternative dietary strategy to

probiotics has been the in-feed inclusion of prebiotics, which act to modify the existing microbiota and shift the microbial balance towards a higher proportion of beneficial bacteria (Patterson and Burkholder 2003).

Conversely, there are a number of bacterial species which have been associated with poultry diseases or loss of performance including; *Clostridium perfringens* (Dahiya et al. 2006; McDevitt et al. 2006) and *Escherichia coli* (Dziva and Stevens 2008); whilst others have been linked to food-borne illness in humans including *Salmonella* and *Campylobacter* (DuPont 2007). Dietary additives including single and multi-strain probiotics, prebiotics, organic acids and essential oils have been reported to reduce the levels of these bacteria within the gastrointestinal tract of poultry (Dahiya et al. 2006). These reductions have often been linked to improved bird performance (Xu et al. 2003), or reduced carcass yields of harmful bacteria (Solis de los Santos et al. 2008b).

Influence of diet on the microbiota and performance

A number of dietary factors can influence the microbial composition of birds. This includes grain type (Shakouri et al. 2008), fat levels (Knarreborg et al. 2002), feed form (Engberg et al. 2002), and the inclusion of additives such as prebiotics (Xu et al. 2003), organic acids (Chaveerach et al. 2004; Solis de Los Santos et al. 2008a; Solis de los Santos et al. 2008b) and feed enzymes (Bedford and Apajalahti 2001). A previously completed Australian Poultry CRC Project 03-3a[1] indicated that shifts in the intestinal microbiota caused by the addition of a feed additive (enzyme) can be linked to changes in bird performance (Torok et al. 2008).

Microbial activity

In addition to dietary manipulation of the intestinal microbial communities as a means to improve bird performance and immunity, another important consideration is the metabolic activity of the microbiota. Rehman and colleagues have demonstrated that particular in-feed additives (inulin), whilst not shifting the microbial profiles, can have a significant impact on the activity of the resident intestinal bacteria (Rehman et al. 2008). Changes to the production of short chain fatty acids (SCFA) can significantly alter the available energy and inturn influence bird performance; therefore, when evaluating the effects of a nutritional feed additive on the microbiota, one must consider not only the changes in composition, but shifts in metabolic activity.

[1] Australian Poultry CRC Project 03-3a: Application of advanced DNA-based tools for monitoring and managing the gut microflora of poultry. Project Leader: Dr Kathy Ophel-Keller.

Gut structure and function

Microarchitecture

The villi and crypts, which line the gastrointestinal epithelium, are crucial to optimal nutrient digestion and absorption (Jin et al. 1998; Sklan 2001). A healthy intestinal epithelium will facilitate optimal digestive and absorptive function by the secretion of digestive enzymes and the absorption of nutrients via the enterocytes which line the epithelium. Intestinal diseases of poultry can influence the intestinal microarchitecture (Gholamiandehkordi et al. 2007), and lead to sub-optimal bird performance due to reduced nutrient uptake. Numerous studies have demonstrated that dietary additives can influence villus/crypt architecture (Iji et al. 2001; Humphrey et al. 2002) and lead to an increased surface area for nutrient absorption.

Mucin composition

The gastrointestinal tract is lined with a mucus layer that is important for the protection of the intestinal epithelium (Smirnov et al. 2006). This mucus layer prevents physical damage to the absorptive cells of the intestine and also modulates the colonisation of intestinal bacteria (Smirnov et al. 2005). Mucin is a major component of the mucus layer and is produced by goblet cells (Friedman et al. 2003). The amount and sub-type of mucin produced can be influenced by bacterial interactions and nutritional factors (Smirnov et al. 2005; Smirnov et al. 2006); these mucin sub-types may inturn regulate bacterial adhesion (Lievin-Le Moal and Servin 2006). Therefore an understanding of the dynamics of mucin development and interactions with intestinal bacteria are important to the improvement of bird health and performance.

Intestinal gene expression

Intestinal gene expression underlies crucial biological systems which affect bird performance and immunity. A greater understanding of the regulation of genes involved in development, digestion, nutrient uptake, immunity and mucin production will facilitate the development of a means to manipulate these, via the diet, to improve bird productivity. Microarray technology provides the means to study the changes in gene expression of the entire genome simultaneously (Ramsay 1998; Schena et al. 1998). This technology can be harnessed to study the impact of nutrition on intestinal gene expression, known as nutrigenomics, and indicate which feed additives may improve or optimise key processes within the bird[2].

[2]Australian Poultry CRC Project 03-16a: Genomics-based technology for new health products. Project Leader: Dr Rob Moore.

The integrated picture

The capacity for nutrition to influence the immune system (Klasing 2007), microbial composition (Xu et al. 2003; Torok et al. 2008) and activity (Rehman et al. 2008), gut structure and function (Iji et al. 2001), mucin dynamics (Smirnov et al. 2005; Smirnov et al. 2006) and gene expression (Dawson 2006) is well established. It is also evident that changes in these parameters may inturn influence health status and performance, although the nature of these shifts in response to particular feed additives (including antibiotics, and natural alternatives) requires further elucidation. A further degree of complexity arises from the knowledge that significant crosstalk occurs within the intestinal environment. This facilitates the need to assess the integrated picture in order to understand and characterise the interactions amongst diet, the intestine and bird performance. For example, specific microbial species have been demonstrated to influence the immune system (Haghighi et al. 2006), intestinal structure (Chichlowski et al. 2007) and mucin distribution (Smirnov et al. 2005); whilst, mucin is known to modulate bacterial adherence (Lievin-Le Moal and Servin 2006) which may then subsequently modulate bird immunity. All of these facets appear inter-linked and changes in one will influence the rest of the intestinal milieu. Studying these aspects of the intestinal environment in isolation of one another will not provide an accurate indication of the impact of nutrition on the overall intestinal status of the birds. Reciprocal interactions occur amongst many aspects within the intestinal environment and shifts in one facet within the intestine will not occur in isolation of other components. With a greater understanding of the integrated picture, encompassing nutrition, the intestinal environment and performance, we can be more strategic in the design and formulation of diets to promote optimal bird health and performance.

Objectives

This project addressess one of the main objectives of the Australian Poultry CRC which is *sustained production of chicken meat without reliance on antibiotics*.

We aimed to provide an integrated picture of the complex interactions amongst nutrition, the commensal bacteria of the gastrointestinal tract, mucosal immunity, gut structure and function, intestinal gene expression, and corresponding production efficiency and bird health.

The following questions were addressed:-

- Can complex carbohydrates with purported prebiotic action promote the development of a beneficial gut microbiota and improve gut health?
- Can anti-inflammatory and immunomodulatory omega-3 polyunsaturated fatty acids (*n*-3 PUFA) improve the gut health of birds?

- What impact do immunomodulatory compounds have on the intestinal mucosal immune system, the intestinal microbiota and bird performance?
- How do beneficial bacteria in the gut, such as members of the *Lactobacillus* genus, respond to dietary, immunological, and physiological changes in the gut environment?
- Can natural alternatives to in-feed antibiotics elicit the same protection as antibiotics against necrotic enteritis?
- Will the identification of key genes through microarray analysis identify gene pathways that are important in optimal gut function, and will they represent potential targets for manipulation by gene silencing technology?

This project aimed to evaluate natural antibiotic alternatives and characterise the effects on bird performance. Importantly, we simultaneously evaluated the influence that antibiotics and natural alternatives had on the intestinal microbiota, microbial activity, the intestinal mucosal immune system, gut structure, and intestinal gene expression. This provided an overview of how these dietary additives influenced key performance-mediating processes. To our knowledge, a comprehensive study of this nature had not been performed previously. This process involved the assessment of healthy birds, and birds challenged with *Clostridium perfringens*.

This study will provide key information on potential antibiotic alternatives. In addition it will indicate if specific diet-induced shifts in the microbiota, immune system, gut structure and intestinal gene expression impacts on bird performance, either positively or negatively. This will provide critical information as to whether dietary manipulation of these key processes is achievable and how to optimally balance bird health, immunity and productivity.

Methodology

This unique project, to our knowledge, was the first to integrate assessment of bird performance (including apparent metabolisable energy) with a series of complex analyses including characterisation of gut microbial composition and activity, systemic and mucosal immune parameters, gut structure, intestinal mucin dynamics and tissue fatty acid composition. Throughout this project we have performed five separate experiments in order to assess the impact of candidate antibiotic replacements on these parameters in healthy birds (Chapters 1-4) and birds challenged with *C. perfringens* (Chapter 5).

We have employed a range of methodologies to perform these analyses, including; apparent metabolisable energy bioassays (Hughes et al. 2000), a *Clostridium perfringens* challenge experiment

(Kocher and Choct 2005), gut microbial profiling by terminal-restriction fragment length polymorphism analysis (T-RFLP) (Torok et al. 2008), and Lac PCR-denaturing gradient gel electrophoresis (Lac PCR-DGGE) (Walter et al. 2001), volatile fatty acid (VFA) analysis of caecal contents by gas chromatography (Storer et al. 1983), gene expression analysis by microarray (Crowley and Moore 2006), and plasma and tissue fatty acid composition by gas chromatography (Portolesi et al. 2007), in addition to a range of immunological assays outlined in Chapters 1 and 2.

Chapter 1. Scoping experiment; procedures for collection and transport of tissue samples for study of energy metabolism, immunology and gut microbial communities in chickens

Introduction

This ambitious project is primarily comprised of a series of small-scale individual bird metabolism studies, followed by intensive tissue collection in order to produce samples for analysis of energy metabolism, microbial communities, microbial activity, immune status, and gene expression. This difficult task is compounded by the geographical isolation of our collaborating research institutes, located in different Australian cities (South Australian Research and Development Institute, Adelaide; CSIRO Australian Animal Health Laboratory, Geelong; Australian National University, Canberra). As such, we must ensure that;

i) The tissue collection procedures are feasible within a relatively short time frame,

ii) Samples can be collected and transported to our collaborators in a timely manner,

iii) Samples arrive in a state that is suitable for use in the required assays.

Therefore, the primary purpose of this scoping experiment was to determine the adequacy of procedures used for collection and transport of tissue samples for study of bird-to-bird variation in energy metabolism, intestinal mucosal immunity, gene expression and gut microbial communities in healthy broiler chickens.

In this chapter we also aimed to assess how gender would impact on some of the key parameters to be assessed throughout this project (particularly performance and the immune response). This may influence the design of subsequent experiments within this project and indicate whether single or mixed-sex experiments should be performed.

Materials and Methods

Birds

Male and female Cobb 500 broiler chickens were obtained from the Baiada hatchery (Willaston, SA, Australia) and were raised in rearing pens in a temperature-controlled room until the commencement of the experimental period. All birds were given continual access to commercial starter crumbles

(Ridley Agriproducts, Steg 600 Starter, Murray Bridge, SA, Australia). All diets met or exceeded National Research Council guidelines for broiler chickens (NRC 1994). All procedures in this experiment and all subsequent metabolism experiments were approved by the Animal Ethics Committees of the Department of Primary Industries and Resources South Australia and the University of Adelaide. All procedures complied with the "Australian code of practice for the care and use of animals for scientific purposes" (Australian Agricultural Council, 1997) and "Australian model code of practice for the welfare of animals: Domestic Poultry" (Standing Committee on Agriculture and Resource Management, 1995).

Apparent metabolisable energy study

At 13 days post-hatch, a total of 48 chickens were transferred in pairs to 24 metabolism cages located in a temperature-controlled room initially kept between 25-27°C. Birds were placed in pairs for an initial three-day acclimation period to minimise stress associated with isolation. Birds continued to have free access to starter crumbles and water prior to, and during, the experimental period. Following the three-day acclimation period, birds were placed individually in 48 metabolism cages.

Apparent metabolisable energy (AME) values were determined in a classical seven-day AME study. Body weight was recorded at the beginning and end of the seven-day metabolism study period. The first three days enabled the chickens to adapt to solitary confinement in the metabolism cages. During the following four days, all excreta was collected daily and dried at 85°C. Moisture content of excreta voided over a 24 hour collection period was measured. Feed intake was recorded during the adaptation and collection phases of the study period. Dry matter (DM) contents of feed were measured. Gross energy values of dried excreta and feed were measured with a Parr isoperibol bomb calorimeter (Parr Instrument Company, Moline, IL, USA). AME values (in MJ/kg dry matter basis) were calculated as follows;

$$AME_{diet} = [(GE_{diet} \times g \text{ feed consumed}) - (GE_{excreta} \times g \text{ dry excreta})] / g \text{ feed consumed} / DM \text{ feed}$$

At day 25 post-hatch all birds were killed one at a time by intravenous injection of Lethobarb (0.5 ml/bird), and weighed. From all birds, one caecum and 3 cm sections of tissue and associated digesta from the midpoints of the duodenum, jejunum and ileum were collected and kept at 4^{o}C until frozen and stored at -20^{o}C. Samples were freeze-dried for bacterial DNA profiling of *Lactobacillus* species by the denaturing gradient gel electrophoresis (DGGE) technique.

In addition, from 24 birds, a 1 cm segment of duodenum and one caecal tonsil (CT) were washed with cold 95% ethanol and then placed in 2 ml of 95% ethanol in a 5 ml tube and kept at 4°C for

immunohistological analysis. Samples placed in 95% ethanol were transferred into 100% ethanol after 24 hours.

From the remaining 24 birds, both CTs were removed, washed in cold Dulbecco's Modified Eagle Medium (DMEM; Gibco®, Mount Waverly, Vic, Australia) supplemented with 1% penicillin/streptomycin and placed in a 5 ml tube containing 2 ml of DMEM supplemented with 1% penicillin/streptomycin and kept at 4°C for functional assays and cell phenotyping.

Denaturing gradient gel electrophoresis

Total nucleic acid was extracted using a modification (Torok et al. 2008) of a proprietary technique developed by the South Australian Research and Development Institute (Stirling et al. 2004). DGGE analysis was used to characterise *Lactobacillus* species within the intestine (Walter et al. 2001). The V3 region of the 16S rDNA was amplified from the total DNA using group-specific bacterial primers Lac1 and Lac2-GC (Lac PCR) and subjected to DGGE (Lac PCR-DGGE) by the method of Walter et al. (2001). PCR amplifications were conducted in a Cool Gradient Palm Cycler 9600 (Corbett Research, Sydney, NSW, Australia). The DGGE analysis was performed using the Bio-Rad DCode Universal Mutation Detection System (Hercules, CA, USA). Identification ladders for DGGE were prepared by combining the Lac PCR products prepared from DNA extracted from the reference *Lactobacillus* strains (*L. acidophilus* ATCC 4356, *L. aviarius* subsp. *aviarius* ATCC 43234, *L. crispatus* ATCC 33820, *L. gasseri* ATCC 33323, *L. johnsonii* ATCC 33200, *L. reuteri* ATCC 23272 and *L. salivarius* subsp. *salivarius* ATCC 11741). DGGs were stained with ethidium bromide and viewed by UV transillumination. The presence and absence of different *Lactobacillus* species was recorded.

Immunological assays

Isolation of leucocytes from caecal tonsil

Single cell leucocyte suspensions were obtained by dispersing CT tissue into the medium (DMEM) in a Petri dish, through a cell strainer (70 μm; BD Biosciences, MA, USA) using a piston from a 3 ml syringe. The cell suspension was then filtered through a fresh cell strainer, washed twice in DMEM and resuspended in DMEM containing 10% foetal calf serum (FCS). Cells were counted using a haemocytometer and concentration adjusted to 1 x 10^7/ml, then stored on ice until use.

Phenotypic characterisation of leucocytes by flow cytometry

Duplicate aliquots of leucocytes (2 x 10^5) were stained with 50 µl of a commercial antibody mixture (Southern Biotechnology Associates, Birmingham, AL, USA) in a 96-well plate. The B cell marker antibody mixture included; Bu1-biotin, IgM-spectral red (SPRD), IgA fluorescein-isothiocyanate (FITC) and IgG-phycoerythrin (PE). The lymphocyte marker antibody mixture was comprised of the conjugated antibodies; CD3-SPRD, CD4-FITC, and CD8-biotin. The final antibody mixture included antibodies to; Kul1-FITC, CD45-SPRD, and MHCII-PE. Cells were incubated with the antibody mixtures for 30 minutes at 4°C. Cells were then washed once with flow cytometry buffer (FB; PBS containing 0.1% NaN_3 and 2% foetal calf plasma) and 50 µl of streptavidin-APC (BD Pharmingen, San Diego, CA, USA) was added to the wells as required and plates were incubated at 4°C for 30 minutes. At the end of incubation, cells were washed once and resuspended in 200 µl of FB. A minimum of 30000 events were acquired and analysed using Cell Quest software on FACScaliber (BD, San Jose, CA, USA). Lymphocyte and monocyte gates were set according to the forward (FSC) and side scatter (SSC) properties of chicken leucocytes.

Phagocytosis

Phagocytosis was measured by the uptake of FITC-dextran (40 kDa; Sigma, Castle Hill, NSW, Australia) by leucocytes as previously described (Janardhana et al. 2007). Briefly 100 µl of FITC-dextran suspension at 1 mg/ml was dispensed into duplicate 96-well plates followed by 100 µl of leucocyte suspension containing 4 x 10^6 cells. The plates were sealed and incubated on a shaker at 4°C or at 37°C for 45 minutes. Following incubation, 200 µl of FB was added and the plates were centrifuged (500 g_{max}) for 5 minutes. The supernatant was discarded and 100 µl of trypan blue suspension at 100 µg/ml in PBS added for 3 minutes to quench any extracellular FITC-dextran. Cells were then washed twice and resuspended in 200 µl of FB and flow cytometric profiles acquired using Cell Quest (BD, San Jose, CA, USA). The background readings obtained at 4°C were deducted from the readings at 37°C to determine the percentage of cells performing active phagocytosis.

Lymphocyte responsiveness to Concanavilin A

Proliferative response of CT lymphocytes to Concanavilin A (Con A; Sigma, Castle Hill, NSW, Australia) was measured in triplicate by stimulating 10^6 cells in 100 µl suspension with 12.5 µg of Con A for 48 hours in 96-well plates. Cells cultured in medium only were used for background correction. Plates were incubated at 37°C in a humidified CO_2 incubator for 48 hours. ^{3}H-thymidine (0.5 µCi; Amersham Biosciences, Buckinghamshire, UK) was added to each well for the final 18 hours and cells were then harvested onto glass fibre filter mats using a cell harvester (Tomtec, Hamden, CT, USA).

Filters were placed in plastic pouches with 5 ml of scintillant and radioactivity (counts per minute; cpm) measured on a MicroBeta TriLux 1450 β counter (EG&G Wallac, Turku, Finland). Stimulation indices (SI) were expressed as cpm in Con A wells/cpm in background wells.

Statistical analyses

Performance data were analysed with SAS for Windows version 9.1 software package (SAS Institute Inc., Cary, NC, USA). Data were compared by analysis of variance using the General Linear Model (GLM) procedure with gender differences determined by Duncan's Multiple Range Test. Immunological data were compared by Student's T-test. DGGE profiles were compared using Dice's similarity coefficient (Dsc) based on the UPGMA (unweighted pair group methods using arithmetic averages) clustering algorithm using the BioNumerics software package (Applied Maths, Austin, TX, USA). For all comparisons, $p<0.05$ was considered significant.

Results

Apparent metabolisable energy study

At the commencement of the AME study period, mean body weight of male chickens was significantly greater than that of female birds ($p<0.05$; Table 1). During the AME period, body weight gain and feed intake were significantly greater in male birds compared to female birds ($p<0.05$). AME values were significantly greater in female birds compared to males birds ($p<0.05$), whilst no gender effects on feed conversion ratio or excreta moisture were observed ($p>0.05$).

Table 1. Performance parameters from chickens during the seven-day apparent metabolisable energy (AME) study period.

	BWS (g)	**BWG (g)**	**FI (g/bird/day)**	**FCR**	**AME (MJ/kg)**
Male	431 ± 8a	413 ± 8a	90.1 ± 1.7a	1.53 ± 0.01	13.52 ± 0.11a
Female	406 ± 5b	359 ± 6b	78.5 ± 1.0b	1.53 ± 0.01	13.85 ± 0.05b

Body weight start (BWS) and body weight gain (BWG) data are expressed as mean (g) ± SEM. Feed intake (FI) data are expressed as mean (g/bird/day) ± SEM. Feed conversion ratio (FCR) is expressed as feed intake/body weight gain during the AME period ± SEM. Apparent metabolisable energy (AME) is expressed as mean (MJ energy/kg dry matter basis) ± SEM. Values within a column that do not share a common letter are significantly different ($p<0.05$; n = 24/group).

Denaturing gradient gel electrophoresis

Clustering analysis indicated that there was no relationship between bird gender and *Lactobacillus* species profile (Figure 1).

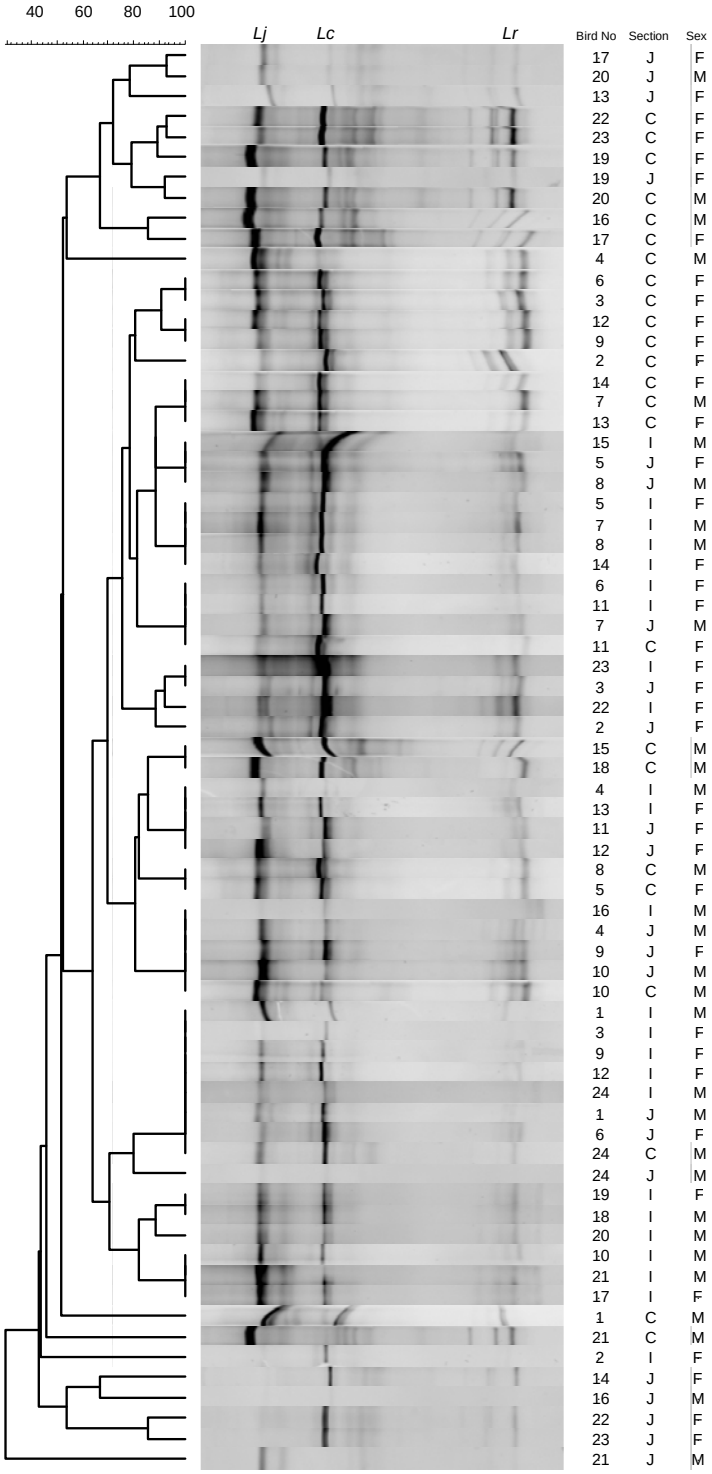


Figure 1. Lac PCR-DGGE profiles of jejunum (J), ileum (I), and caeca (C) from male and female birds. Lj, *L. johnsonii* ATCC 33200; Lr, *L. reuteri* ATCC 2327; Lc, *L. crispatus* ATCC 33820.

Immunological assays

Cell phenotypes

No significant differences were observed in B cell, T cell and macrophage markers in the caecal tonsils between male and female birds ($p>0.05$; Tables 2 and 3).

Table 2. T cell and macrophage markers.

	CD45$^+$	**CD3$^+$**	**CD4$^+$**	**CD8$^+$**	**KUL1$^+$**	**MHCII$^+$**
All	57.94 ± 2.04	39.48 ± 1.15	46.87 ± 3.69	46.50 ± 3.34	10.07 ± 1.03	4.78 ± 0.42
Female	57.85 ± 5.26	39.59 ± 1.76	50.78 ± 6.04	41.94 ± 5.91	11.32 ± 2.07	5.28 ± 0.95
Male	57.99 ± 1.76	39.43 ± 1.52	44.91 ± 4.70	48.78 ± 4.05	9.45 ± 1.16	4.54 ± 0.44

Data are expressed as percentage of positive cells ± SEM.

Table 3. B cell phenotypes.

	Bu1$^+$	**IgA$^+$**	**IgG$^+$**	**IgM$^+$**
All	29.25 ± 2.30	3.44 ± 0.58	3.13 ± 0.31	51.00 ± 1.14
Female	31.62 ± 4.95	3.06 ± 0.62	2.43 ± 0.32	52.33 ± 1.85
Male	28.06 ± 2.58	3.63 ± 0.84	3.47 ± 0.43	50.33 ± 1.50

Data are expressed as percentage of positive cells ± SEM.

Phagocytosis

No difference in caecal tonsil cell phagocytosis was observed between male and female birds ($p>0.05$; Figure 2).

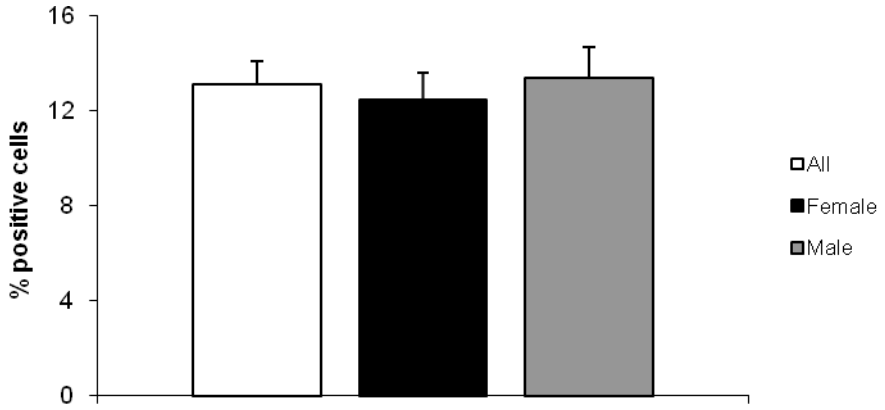


Figure 2. Phagocytosis of caecal tonsil cells. Data are expressed as mean (difference in percentage of FITC-dextran-positive cells at 37°C and 4°C) ± SEM.

Lymphocyte responsiveness to Con A

No significant difference was observed in the responsiveness of caecal tonsil lymphocytes to Con A stimulation between male and female birds ($p>0.05$; Figure 3).

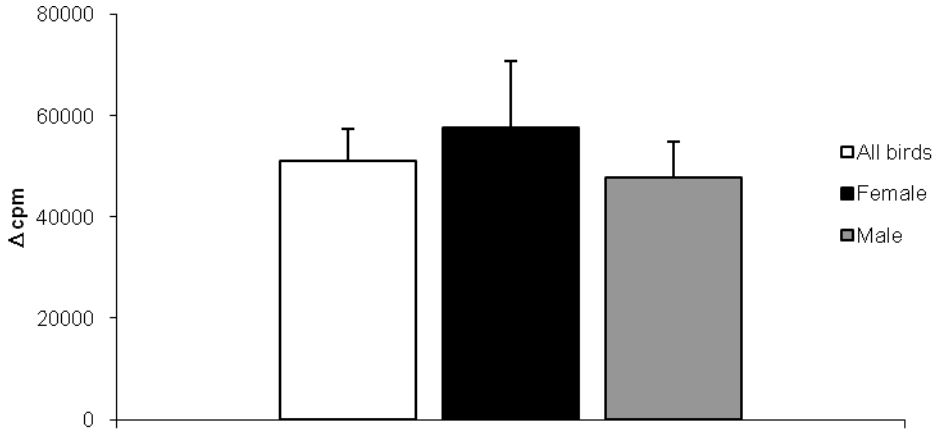


Figure 3. Lymphocyte proliferation in response to Con A. Data are expressed as change in counts per minute (Δ cpm) ± SEM.

Discussion

The apparent metabolisable energy bioassay can provide an in-depth assessment of bird energy metabolism (Annison et al. 1994; Choct et al. 1995). Previous studies have demonstrated considerable within-flock variation in the AME values of birds fed an identical diet (Hughes and Choct 1997). This indicates that individual birds partition and handle available energy with varying efficiency.

Additionally, a significant gender related AME difference was evident, with males consistently having a lower AME value (Hughes 2003). This is an interesting finding and indicated that whilst females had a greater level of available energy, significant amounts may have been lost to processes other than growth (Hughes 2003). The current study supports previous data as female chickens had a greater AME value compared to males. Significant differences in performance indicators such as body weight gain are also frequently observed between male and female broilers (Scheuermann et al. 2003; Ziaei et al. 2004; Lumpkins et al. 2008), a finding supported by the current study. Interestingly, the feed conversion ratios of male and female broilers were not significantly different indicating that whilst females consumed less food, and gained weight at a slower rate, their efficiency for conversion was comparable to males.

The intestinal microbiota is important for the development of the intestine, protection from pathogens, and immune modulation. This study indicated that there were no differences in the *Lactobacillus* species profiles between males and females. Previously, DGGE analysis has indicated that the overall intestinal microbial communities differ between male and female chickens (Lumpkins et al. 2008). In this scoping experiment we did not assess overall intestinal microbial communities (using T-RFLP) as the primary aim of this experiment was to ensure that techniques were adequate for future experiments within this project. The T-RFLP procedure has been comprehensively established within our research group, and therefore we did not wish to undergo this procedure for a study of this nature. Lumpkins and colleagues did not specifically assess the *Lactobacillus* profiles of these birds; therefore it is unclear whether the *Lactobacillus* species differed. Previously, gender differences in *Lactobacillus* species have been observed in Swiss Webster mice (Ge et al. 2006). Further research into this phenomenon is required in order to determine if there is a consistent difference in the microbiota of male and female birds, and whether any possible differences may contribute to growth or performance outcomes.

The immune system is important for the protection of the bird from numerous infectious diseases; however, the energy demands of an over-active immune system can reduce bird productivity (Klasing 2007). Therefore, a level of immunity which is a balance between adequate protection and energy demand is deemed optimal. In the current study we observed that there was no gender influence on the proportions of B cells, T cells and macrophages in the CT of broilers. Likewise, gender did not affect phagocytosis rates or lymphocyte responsiveness.

This preliminary experiment has indicated that there are detectable performance differences between male and female birds; however, based on the parameters assessed, the level of immunity between male and female birds appears to be comparable. As a result, for future experiments within this

project, we will focus solely on male broiler chickens in order to minimise the influence of bird gender on our results, particularly from a production standpoint.

Importantly, this study has indicated that our collection, packaging and transport procedures for live intestinal tissue are adequate for the necessary analyses to be performed at our collaborating research institutes. Therefore these procedures will be employed for future experiments within this project.

Chapter 2. Indigestible complex carbohydrates influence the gut microbial communities but do not affect broiler chicken performance

Introduction

Prebiotics are "non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria" (Patterson and Burkholder 2003). Prebiotic compounds including fructooligosaccharide (FOS), mannan-oligosaccharide (MOS), transgalactooligosaccharide (TOS), inulin and isomaltooligosaccharide (IMO) have been investigated in poultry (Spring et al. 2000; Xu et al. 2003; Chung and Day 2004; Biggs et al. 2007). The in-feed addition of FOS to broiler diets has been shown to increase levels of beneficial bacteria such as *Lactobacillus* and *Bifidobacteria* species and inhibit *E. coli* in the small intestine and caeca (Xu et al. 2003). FOS has also been reported to increase body weight gain, improve feed conversion ratio, stimulate digestive enzyme activity and increase ileal villus height (Xu et al. 2003). The addition of MOS to broiler diets has had varying results (Spring et al. 2000; Iji et al. 2001; Waldroup et al. 2003a; Waldroup et al. 2003b; Hooge 2004; Zaghini et al. 2005; Elmusharaf et al. 2006; Baurhoo et al. 2007; Biggs et al. 2007; Solis de los Santos et al. 2007). MOS increased jejunum villus height and *Lactobacillus* and *Bifidobacteria* species levels whilst reducing litter *E. coli* load (Baurhoo et al. 2007). MOS has also demonstrated the capacity to reduce the severity of mild coccidiosis infection (Hooge 2004), and decrease *Salmonella* numbers (Spring et al. 2000). Improvements in body weight, feed conversion ratio and reductions in mortality have also been reported in birds fed MOS, compared to feed without antibiotics (Hooge 2004). Indeed, the addition of MOS to broiler feed has been reported to promote a level of bird performance comparable to that of antibiotic-supplemented feed (Hooge 2004).

Prebiotics are a potential candidate for use in broiler feed as a natural alternative to in-feed antibiotics. In this chapter we compared the effects of dietary FOS and MOS supplementation on broiler growth and production and aimed to characterise the effects of these compounds on the intestinal microbiota, with particular focus on the *Lactobacillus* species. In addition we assessed the impact of prebiotics on the immune status of broilers.

Materials and Methods

Birds and diets

Male Cobb 500 broiler chickens (Baiada Hatchery, Willaston, SA, Australia) were raised in four floor pens in a temperature-controlled room. All birds were vaccinated with Eimeriavax 4m (Bioproperties Pty. Ltd., Glenorie, NSW, Australia) at day of placement, and at five and 11 days post-hatch. All experiments were approved by the Animal Ethics Committees of the University of Adelaide and the Department of Primary Industries and Resources South Australia.

All experimental diets were based on a commercial starter diet with no antibiotics or coccidiostats added (Table 4). The diets met or exceeded National Research Council guidelines for broiler chickens (NRC 1994). Mannanoligosaccharide (Bio-MOS®, Alltech Pty. Ltd., Nicholasville, KY, USA) was sourced from the cell wall of *Saccharomyces cerevisiae* and is comprised of mannan and glucan components. Fructooligosaccharide (oligofructose; Fibrulose® F97, Cosucra Group Warcoing, Warcoing, Belgium) was derived from chicory root and contained 97 ± 2% dietary fibre and 99.7% total carbohydrate. The FOS compound was comprised of fructose groups connected by $\beta_{2\text{-}1}$ bonds, predominantly terminating in a glucose sub-unit.

The four experimental diets were: i) Control (basal diet with no additives); ii) ZnB (basal diet + 50 ppm zinc bacitracin); iii) MOS (basal diet + 5 g/kg MOS); and iv) FOS (basal diet + 5 g/kg FOS; n = 12 birds/diet). Titanium dioxide (TiO_2; 5 g/kg) was added as a digestibility marker for the energy metabolism phase of the experiment (day 15-25). Birds had *ad libitum* access to water and experimental feed at all times.

Table 4. Experimental diets for chickens for the periods prior to (0-15 days) and during (15-25 day) the metabolism study[3].

Ingredient (g/kg)	Control	ZnB	MOS	FOS
	Period 0 – 15 days of age			
Starter crumble[1]	1000	999.7	995.0	995.0
Zinc bacitracin (50 ppm)	-	0.3	-	-
MOS	-	-	5.0	-
FOS	-	-	-	5.0
	Period 15 – 25 days of age			
Starter crumble[1]	995.0	994.7	990.0	990.0
Zinc bacitracin	-	0.3	-	-
MOS	-	-	5.0	-
FOS	-	-	-	5.0
Titanium dioxide	5.0	5.0	5.0	5.0

[1]Starter crumble comprises (in g/kg): wheat (638.9), hammer-milled wheat (8.0), oats (50.0), peas (60.0), meat meal (100.0), blood meal (10.7), tallow (10.0), solvent extracted soybean meal (93.7), ground oatmeal flour (16.7), monocalcium phosphate (3.75), dicalcium phosphate dehydrate (1.09), choline chloride 75% (0.5), L-lysine HCl (2.14), Alimet (2.57). Vitamin and mineral mixes exceeded NRC standards.

Apparent metabolisable energy study

At 13 days post-hatch, all birds (n = 48) were transferred to 24 metabolism cages in pairs for an initial three-day acclimation period to reduce stress associated with isolation. Following the acclimation period, birds were placed one per cage in 48 cages based on a randomised block design for measurement of the apparent metabolisable energy (AME) values of the diets.

At the start and end of the seven-day metabolism study period bird weight was recorded. During the final four days of the metabolism study period, total excreta were collected daily and dried overnight at 90^{o}C. Feed intake was recorded during the three-day adaptation period, and the four-day collection

[3] Tables 4, 6, 7 and 8, and Figure 4 are reproduced from Geier et al. Indigestible carbohydrates alter the intestinal microbiota but do not influence the performance of broiler chickens (2009). *Journal of Applied Microbiology* 106(5):1540-1548; with permission from Wiley-Blackwell Publishers.

period. Dry matter (DM) contents of feed sub-samples were measured and gross energy values of feeds (GE in MJ/kg), dried excreta, and freeze-dried ileal digesta were measured (Parr isoperibol bomb calorimeter; Parr Instrument Company, Moline, IL, USA). AME values (in MJ/kg dry matter basis) were calculated as follows;

AME_{diet} = [(GE_{diet} x g feed consumed) - ($GE_{excreta}$ x g dry excreta)] /g feed consumed /DM feed

Digesta was collected from the terminal ileum at day 25. Samples were kept at 4^{o}C until frozen then later freeze-dried for assessment of ileal digestible energy (IDE) by measurement of TiO_2 concentrations (Short et al. 1996). IDE was calculated as follows;

GE digestibility coefficient (GEDC) = 1 - ($GE_{ileal\ digesta}$/$DM_{ileal\ digesta}$/$Marker_{ileal\ digesta}$)/(GE_{diet}/DM_{diet}/$Marker_{feed}$)

then

IDE (in MJ/kg dry matter basis) = (GEDC x GE_{diet})/DM feed

Sample collection

At the completion of the metabolism study (25 days post-hatch), blood was collected from 24 birds (n = 6 birds/treatment) by venipuncture of the brachial vein and stored in lithium heparin coated tubes at 4°C until centrifuged (1000 *g*) and plasma separated and frozen. All birds were then killed by pentobarbitone injection (0.5 ml/kg live weight; Lethobarb, Virbac Australia Pty Ltd, Milperra, NSW, Australia). One caecum and 3 cm sections of tissue and digesta from the midpoints of the jejunum and ileum were collected and stored at 4^{o}C until frozen and later freeze-dried for microbial profiling. The other caecum was removed and contents placed in a 5 ml tube, acidified (0.5 M H_2SO_4; 0.5 ml/g caecal content), and stored at -18°C for VFA analysis. In addition, from 24 birds, a 1 cm segment of duodenum and one caecal tonsil (CT) were washed with cold 95% ethanol and then placed in 2 ml of 95% ethanol in a 5 ml tube and kept at 4°C for immunohistological analysis. Samples placed in 95% ethanol were transferred into 100% ethanol after 24 hours. From the remaining 24 birds, both CTs were removed, washed in cold Dulbecco's Modified Eagle Medium (DMEM; Gibco®, Mount Waverly, Vic, Australia) supplemented with 1% penicillin/streptomycin and placed in a 5 ml tube containing 2 ml of DMEM supplemented with 1% penicillin/streptomycin and kept at 4°C for functional assays and cell phenotyping.

Microbial profiling

Sample preparation

Total nucleic acid was extracted from freeze-dried samples using a modification (Torok et al. 2008) of a proprietary technique developed by the South Australian Research and Development Institute (Stirling et al. 2004). These samples were sub-divided into two aliquots with one used to profile the overall gut bacterial communities by terminal-restriction fragment length polymorphism (T-RFLP), and the other to characterise the *Lactobacillus* species present by denaturing gradient gel electrophoresis (Lac PCR-DGGE).

Terminal-restriction fragment length polymorphism

T-RFLP analysis was performed following the previously described technique (Torok et al. 2008). Briefly, bacterial rDNA was amplified with universal 16S primers 27F (Lane 1991) and 907R (Muyzer et al. 1995). PCR reactions were performed in duplicate using a MJ Research PTC-225 Peltier thermal cycler (GeneWorks, Adelaide, SA, Australia). The specificity of PCR products were determined by gel electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining. PCR product (200 ng) was digested with 2 U *Msp*I (Genesearch, Arundel, Queensland, Australia) in recommended enzyme buffer. The length of the fluorescently labelled terminal restriction fragments (T-RFs) were determined by comparison with an internal standard in duplicate following separation by capillary electrophoresis (ABI 3700 automated DNA sequencer; Applied Biosystems, Scoresby, Vic, Australia). Data were analysed using GeneScan 3.7 software (Applied Biosystems). Identification of operational taxonomic units (OTU) was performed as described previously (Torok et al. 2008).

Denaturing gradient gel electrophoresis

Characterisation of *Lactobacillus* species was performed using the Lac PCR-DGGE technique as described previously in Chapter 1 and by Walter et al. (2001). Analysis was performed on individual samples from the jejunum, ileum and caecum of each bird, and from pooled jejunal, ileal and caecal samples from all birds within a treatment group.

Volatile fatty acid analysis

The VFA composition of chicken caecal contents were determined by a modification of the method of Storer et al. (1983). After thawing, samples were sub-sampled (2 ml) and VFAs extracted with an equal quantity of deionised water containing hexanoic acid as an internal standard (IS). Approximately 2 ml was removed and vortexed before centrifugation at 3000 rpm for 10 minutes. A sample (500 µl)

of the supernatant was removed and placed in a small (50 ml) ground glass fitting flask (with side-arm) prior to distillation. The contents and the flask were frozen in liquid nitrogen and the flask connected via an elbow, with aperture, to a collecting tube. The system was evacuated under high vacuum via the flask side arm then the unit closed. Water and VFAs were then removed from the flask by sublimation into a collecting tube placed in liquid nitrogen. After the contents of the flask had dried (usually within 30 minutes), the condensed fluids in the collecting tube were removed and stored in a sealed vial at -18°C prior to analysis (Storer et al. 1983).

Individual fatty acids were identified and quantified by gas liquid chromatography using a Hewlett-Packard chromatograph (5890A II) fitted with a 50 m capillary column (BP20, SGE, Melbourne, Vic, Australia). Hydrogen was used as the carrier gas (head pressure 30 kPa). The injector was held at 250°C and the flame ionisation detector held at 350°C. The instrument was run isothermally at 130°C. Fatty acids were identified by comparison with the retention times of known standards (Matreya, Pleasant Gap, PA, USA). Fatty acids were quantified by use of the IS (hexanoic acid) using appropriated response factors. Results were expressed as molar percentages of the total present, and concentration (μmol/ml).

Immunological assays

Isolation of leucocytes from caecal tonsil

Leucocyte isolation was carried out as described in Chapter 1.

Phenotypic characterization of leucocytes by flow cytometry

Phenotypic analysis of leucocytes was performed as described in Chapter 1.

Phagocytosis

Phagocytosis was measured as described in Chapter 1.

Lymphocyte responsiveness to Con A

The proliferative response of lymphocytes to Con A was assessed as outlined in Chapter 1.

Plasma immunoglobulin titres

Titres of immunoglobulin (Ig) isotypes were determined by enzyme-linked immunosorbent assay (ELISA). 96-well microtitre plates were coated with a suspension (100 µl) of goat anti-chicken IgM (Bethyl A-30-102A, Montgomery, TX, USA), goat anti-chicken IgG (Bethyl A-30-104A) or mouse anti-chicken IgA (Southern Biotech, 8330-01, Birmingham, AL, USA) at a concentration of 1 µg/ml in carbonate buffer on a shaker for 2 hours at room temperature and then overnight at 4°C. The coating was then discarded and plates were washed once with PBS + 0.05% tween. Wells were blocked with 150 µl of Power Block™ (BioGenex, San Ramon, CA, USA) and plates incubated on a shaker at room temperature for one hour. The blocking agent was discarded and then 100 µl of serially diluted (3x) plasma samples were added. Samples were diluted in PBS + 5% foetal bovine plasma and incubated on a shaker. Plates were then washed three times with PBS/tween. Goat anti-chicken IgG (H+L)-HRP (100 µl of a 1:2000 suspension) was added and incubated. After one hour, TMB substrate (100 µl) was added and the reaction stopped after 10 minutes by the addition of 0.5 M H_2SO_4. Optical density (OD) was determined using a Titertek Multiscan Plus (Titertek Multiscan Plus, ICN Flow, Finland). The end point OD was determined by the average OD of diluent well + 3 x standard deviation. Titres were calculated as the reciprocal of the dilution at which the OD was ≥ the end point OD.

Mucosal IgA

The frequency of IgA-secreting plasma cells at the mucosal surface of the duodenum was determined as previously described (Muir et al. 2000; Biggs et al. 2007). Briefly, duodenal sections were fixed in cold ethanol and embedded in paraffin. Sections (3-6 µm) were cut onto gelatinised slides, dried and stored at 4°C. Sections were de-paraffinised, rehydrated and stained with anti-chicken IgA-FITC (Southern Biotechnology Associates, Birmingham, AL, USA) for one hour, rinsed in PBS and counter-stained with Evans blue. Sections were examined using an Olympus inverted microscope (IX71; Olympus Australia, Mt. Waverley, Vic, Australia) and Olympus DP70 high resolution colour camera (Olympus Australia). IgA^+ cells were quantified using image analysis software (AnalySIS®, Soft Imaging System GmbH, Munster, Germany). The number of IgA^+ cells was counted from the base of the lamina propria to the tip of the villi. A minimum of five images were counted for each bird.

Cytokine expression

Total RNA was prepared from CT tissue using a total RNA isolation kit (Cartagen, Seattle, WA, USA) according to the manufacturer's instructions. Extracted RNA was subjected to DNase treatment using DNase 1 (Sigma-Aldrich, Castle Hill, NSW, Australia). The DNase-treated RNA was then reverse transcribed to complimentary DNA (cDNA) using a reverse transcription kit (Promega, Madison, WI,

USA). Primer and probe sets for chicken GAPDH, IL-6, IFN-γ and IL-10 are outlined in Table 5. Primers were designed using the Primer Express software program (PE Applied Biosystems, Foster City, CA, USA). Expression of cytokine genes in treatment groups were quantified relative to the control samples using $2^{-\Delta\Delta Ct}$ method as described previously (Karpala et al. 2008).

Table 5. Primers and probes for quantitative RT-PCR analysis of chicken cytokine mRNA.

Cytokine	Forward and reverse primers	Probe
ChIL-6	CCTGGCGGCCACGAT CGAGTCTGGGATGACCACTTC	CAGATGGTGATAAATCC
ChIFN-γ	GGCGTGAAGAAGGTGAAAGATATCA GCTTTGCGCTGGATTCTCAAG	CAAGCTCCCGATGAACG
ChIL-10	GCCTGAAGGCGACGATTC TGATGGCTTTGCTCCTCTTCTC	CTGTCACCGCTTCTTC
GAPDH	CCCCAATGTCTCTGTTGTTGAC CAGCCTTCACTACCCTCTTGAT	CTTGGCTGGTTTCTCC

chIL-6, chicken interleukin-6; chIFN-γ, chicken interferon-γ; chIL-10, chicken interleukin-10; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Statistical analyses

Performance data were analysed using the SAS for Windows version 9.1 software package (SAS Institute Inc., Cary, NC, USA). An analysis of variance was performed using the General Linear Model (GLM) procedure with differences among diets determined by Duncan's Multiple Range Test. Immunological data and volatile fatty acid profiles were compared using a non-parametric Kruskal-Wallis test.

Comparisons of intestinal microbial communities among diets (determined by T-RFLP) and identification of bacterial species accounting for the observed differences were done using the PRIMER-6 software package (PRIMER-E Ltd., Plymouth, UK). Individual T-RFLP and Lac PCR-DGGE data were analysed following the procedures outlined previously (Torok et al. 2008). Bray-Curtis measures of similarity (Bray and Curtis 1957) were determined to identify similarities between the microbial profiles of individual birds based on the T-RFLP-generated OTU data following standardization and fourth root transformation. Bray-Curtis measures of similarity were also used to analyse the presence/absence data for *Lactobacillus* profiling (Lac PCR-DGGE), as scored against the reference *Lactobacillus* strains. One-way analysis of similarity (ANOSIM) (Clarke 1993) was used to indicate whether bacterial profiles differed significantly amongst diets for T-RFLP and Lac PCR-DGGE generated data. Similarity percentages analyses (SIMPER) (Clarke 1993) were used to identify

the *Lactobacillus* species which contributed to any observed dissimilarity amongst dietary treatments. Pooled Lac PCR-DGGE profiles were also compared by Dice's similarity coefficient (D_{SC}) based on the UPGMA (unweighted pair group methods using arithmetic averages) clustering algorithm using the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium). $p<0.05$ was considered significant for all statistical comparisons.

Results

Apparent metabolisable energy study

No diet-induced differences were observed among birds for any performance parameters (body weight gain, feed intake, feed conversion ratio, apparent metabolisable energy and ileal digestible energy; $p>0.05$; Table 6).

Table 6. Bird performance parameters during the seven-day apparent metabolisable energy (AME) study period.

	BWS (g)	**BWG (g)**	**FI (g/bird/day)**	**FCR**	**AME (MJ/kg)**	**IDE (MJ/kg)**
Control	412 ± 8	465 ± 11	103 ± 2.3	1.56 ± 0.02	13.66 ± 0.11	13.64 ± 0.29
ZnB	410 ± 9	431 ± 14	98 ± 1.5	1.60 ± 0.05	13.63 ± 0.14	13.87 ± 0.37
MOS	404 ± 9	443 ± 14	101 ± 2.7	1.60 ± 0.03	13.58 ± 0.14	14.00 ± 0.47
FOS	423 ± 11	450 ± 17	101 ± 2.9	1.58 ± 0.03	13.91 ± 0.07	14.02 ± 0.31

Body weight start (BWS) and body weight gain (BWG) data are expressed as mean (g) ± SEM. Feed intake (FI) data are expressed as mean (g/bird/day) ± SEM. Feed conversion ratio (FCR) is expressed as feed intake/body weight gain during the AME period ± SEM. Apparent metabolisable energy (AME) is expressed as mean (MJ energy/kg dry matter basis) ± SEM. Ileal digestible energy (IDE) is expressed as mean (MJ energy/kg dry matter basis) ± SEM. No significant differences were observed among treatment groups ($p>0.05$; n = 12 birds/treatment). Due to insufficient sample, n = 10 for ileal digestibility for MOS-treated birds.

Microbial profiles

Terminal-restriction fragment length polymorphism

Ileal bacterial communities differed in ZnB-fed birds compared to all other diets ($p \leq 0.05$; Table 7). FOS-fed birds also had a significantly different microbial profile compared to chickens fed the Control diet ($p<0.05$), whilst bordering on a significantly different profile compared to MOS-fed chickens ($p<0.06$). Caecal microbial communities of MOS-fed chickens were significantly different to that of chickens fed ZnB and FOS diets ($p<0.05$).

Table 7. One-way ANOSIM of overall ileal and caecal microbial communities associated with diet.

Ileum		**Control**	**ZnB**	**MOS**	**FOS**
	Control	-	**0.107**	**0.018**	**0.139**
	ZnB	*0.051*	-	**0.142**	**0.239**
	MOS	*0.321*	*0.019*	-	**0.092**
	FOS	*0.011*	*0.003*	*0.057*	-
Caecum		**Control**	**ZnB**	**MOS**	**FOS**
	Control	-	**0.074**	**0.065**	**-0.07**
	ZnB	*0.108*	-	**0.161**	**0.083**
	MOS	*0.129*	*0.009*	-	**0.167**
	FOS	*0.916*	*0.107*	*0.009*	-

Data are expressed as the R-statistic (bold), with significance level (p) in italics. The R-statistic value describes the extent of similarity between each pair in the ANOSIM, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between groups. For all analyses, p less than or equal to 0.05 was considered significant. For ileal microbial communities, the global R-value was 0.119 at a significance level of 0.002. For caecal microbial communities the global R-value was 0.08 at a significance level of 0.022.

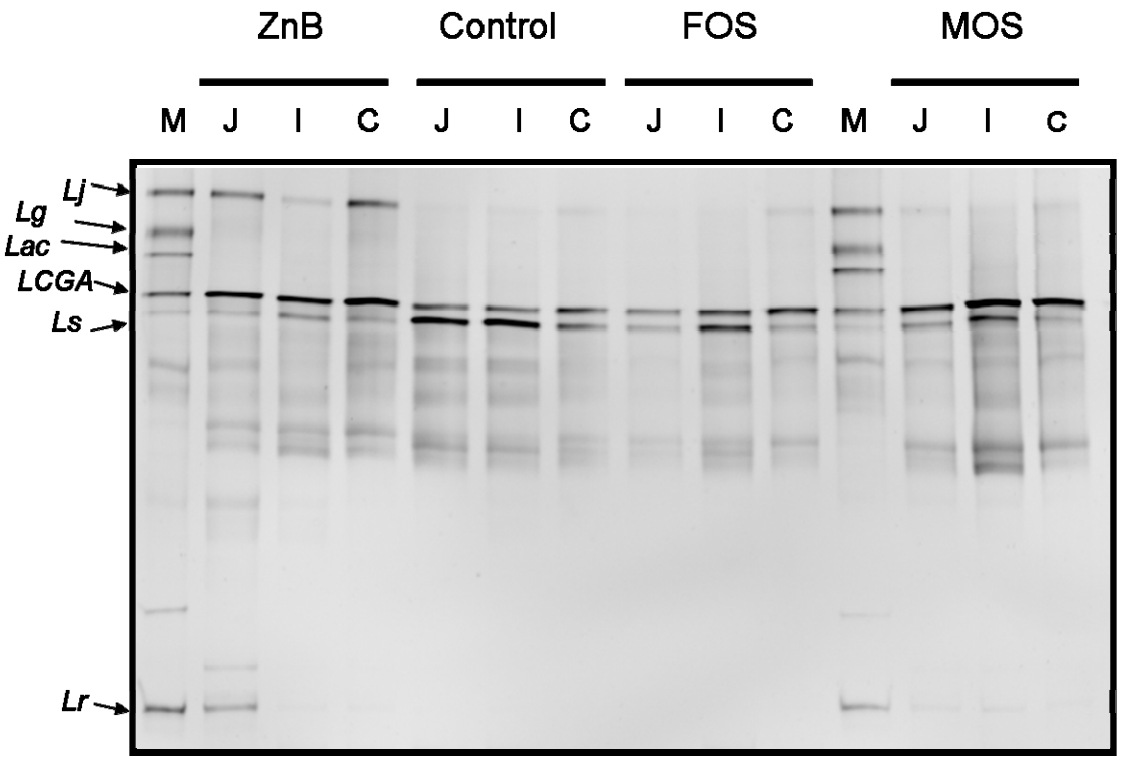
Denaturing gradient gel electrophoresis

Lac PCR-DGGE profiles of pooled samples clustered into three main groups (Figures 4a and 4b). All clusters contained *L. salivarius* and *L. crispatus*, *L. gallinarum* and/or *L. amylovorous*. The latter three species belong to the Group A acidophilus taxonomic group, which cannot be distinguished using Lac PCR-DGGE (Guan et al. 2003), and will be referred to here collectively as LCGA. The consensus profiles of all intestinal sections (jejunum, ileum and caecum) from birds fed FOS and Control diets clustered together. These clusters were comprised of *L. salivarius* and LCGA. The profiles from the jejunum, ileum and caecum of MOS-fed birds clustered separately due to the presence of *L. reuteri*.

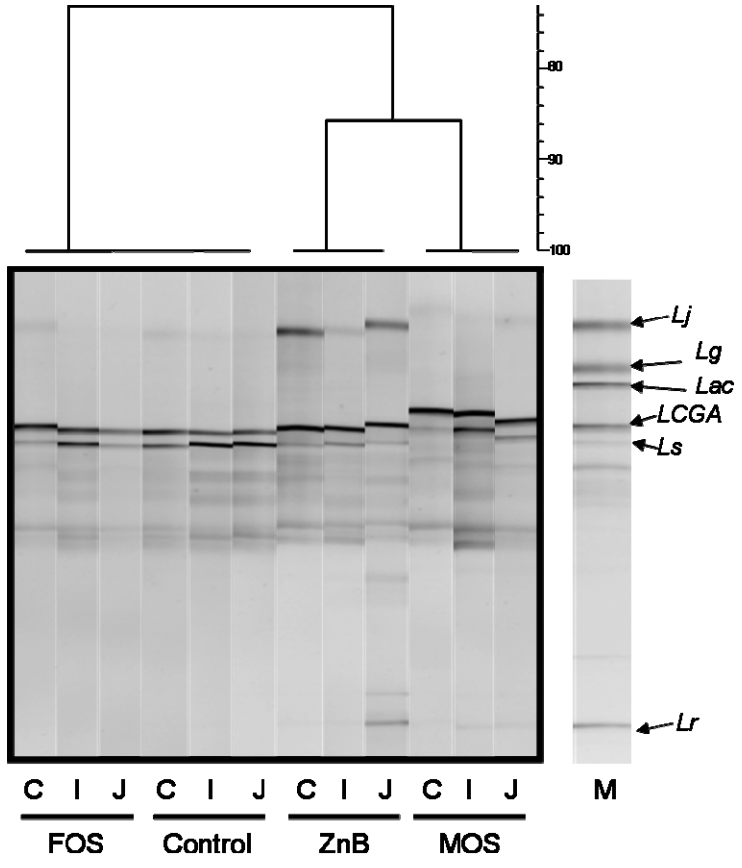
Consensus profiles of ZnB-fed birds were characterised by the presence of *L. reuteri* and *L. johnsonii*. ZnB-fed birds that had detectable levels of *L. johnsonii* also possessed a band of comparable intensity to that of LCGA and *L. salivarius* (Figures 4a and 4c). This was not observed in the majority of birds fed MOS where *L. johnsonii* was a minor contributor (Figures 4a and 4c, data not shown). In ZnB and MOS-fed birds, *L. reuteri* was a minor contributor to the overall *Lactobacillus* profile, as was the case in the jejunum of ZnB-fed chickens (Figures 4a and 4c).

The ANOSIM of the *Lactobacillus* profiles of individual birds (Table 8) closely reflected the clustering of the pooled profiles. *Lactobacillus* profiles in the jejunum of Control and MOS-fed birds were significantly different to one another ($p<0.05$, Table 8), with a strong trend toward a difference between birds fed the ZnB and MOS diets ($p<0.06$). *Lactobacillus* profiles in the ileum differed between MOS and FOS-fed birds ($p<0.05$; Table 8). Additionally, there was an observable trend toward a difference between birds fed the Control and ZnB diets ($p<0.06$). The caecal *Lactobacillus* profiles did not significantly differ amongst dietary treatments ($p>0.05$). SIMPER analysis indicated that *L. johnsonii* was a significant contributor to the ileal *Lactobacillus* shifts between Control and ZnB-fed chickens and that *L. johnsonii* was predominantly associated with ZnB-fed birds. *L. johnsonii* was detected in 2/12 birds fed the Control diet and 7/12 birds fed ZnB. SIMPER analysis indicated that no other specific *Lactobacillus* species significantly contributed to the observed shifts in the profiles amongst dietary treatments.

a)


ZnB
Control
FOS
MOS
M J I C J I C J I C M J I C
Lj
Lg
Lac
LCGA
Ls
Lr

b)


80
90
100
Lj
Lg
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LCGA
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C I J C I J C I J C I J M
FOS
Control
ZnB
MOS

c)

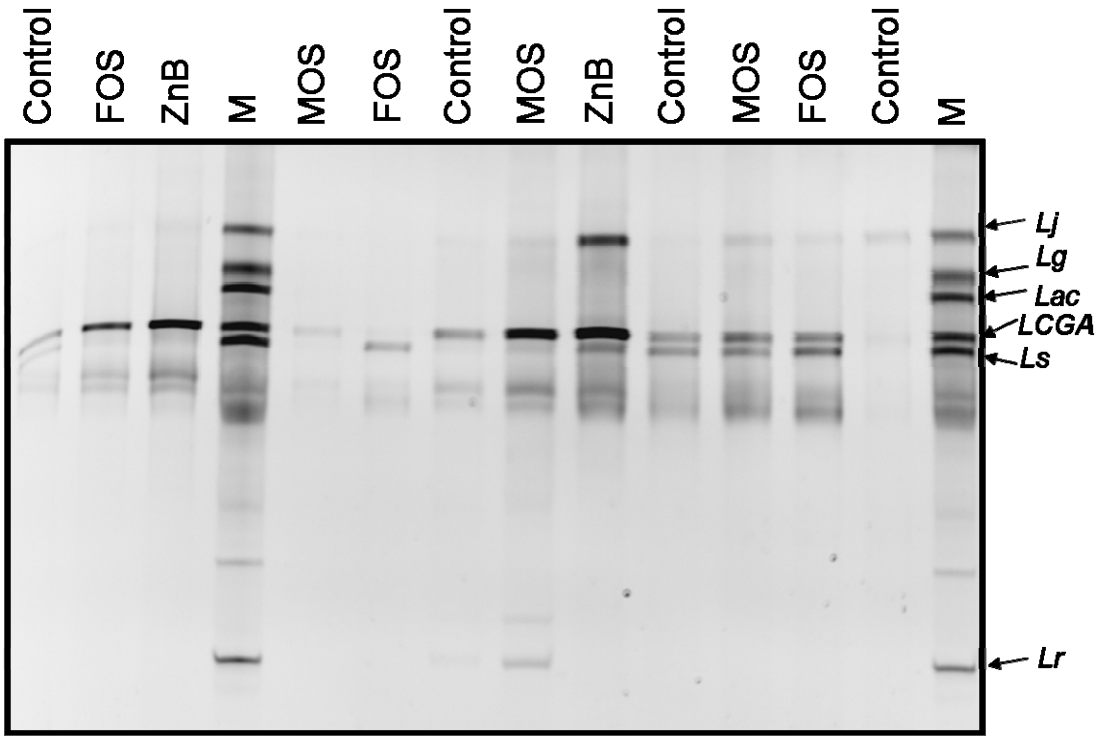


Figure 4. Lac PCR-DGGE profiles of pooled samples for jejunum (J), ileum (I), and caeca (C) of each diet (Figure 4a) and analysis using Dice's Similarity (Dsc; Figure 4b). Figure 4c contains a representative Lac PCR-DGGE of individual samples (caecal). The marker lane (M) contains the identification ladder comprised of Lac PCR products from the following reference strains: *Lj, L. johnsonii* ATCC 33200; *Lg*, *L. gasseri* ATCC 33323; *Lac*, *L. acidophilus* ATCC 4356; *Ls, L. salivarius* subsp. *salivarius* ATCC 11741; and *Lr, L. reuteri* ATCC 23272. LCGA marks the migration of *L. crispatus, L. gallinarum* and *L. amylovorous* species, and is represented by *L. crispatus* ATCC 33820.

Table 8. One-way ANOSIM of jejunal, ileal and caecal *Lactobacillus* species associated with diet.

Jejunum		**Control**	**ZnB**	**MOS**	**FOS**
	Control	-	**0.027**	**0.101**	**0.049**
	ZnB	*0.226*	-	**0.096**	**-0.036**
	MOS	*0.019*	*0.058*	-	**0.074**
	FOS	*0.142*	*0.693*	*0.093*	-
Ileum		**Control**	**ZnB**	**MOS**	**FOS**
	Control	-	**0.082**	**0.043**	**0.050**
	ZnB	*0.058*	-	**0.009**	**-0.061**
	MOS	*0.187*	*0.074*	-	**0.123**
	FOS	*0.132*	*0.980*	*0.045*	-
Caecum		**Control**	**ZnB**	**MOS**	**FOS**
	Control	-	**0.103**	**-0.001**	**0.008**
	ZnB	*0.061*	-	**0.052**	**0.031**
	MOS	*0.479*	*0.145*	-	**-0.023**
	FOS	*0.451*	*0.235*	*0.568*	-

Data are expressed as the R-statistic (bold), with significance level (p) in italics. The R-statistic value describes the extent of similarity between each pair in the ANOSIM, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between groups. For all analyses, p less than or equal to 0.05 was considered significant. For jejunal *Lactobacillus* communities the global R-value was 0.066 at a significance level of 0.028. For ileal *Lactobacillus* communities, the global R-value was 0.057 at a significance level of 0.035. For caecal *Lactobacillus* communities the global R-value was 0.025 at a significance level of 0.196.

Volatile fatty acid concentrations

No significant differences in volatile fatty acid levels were observed amongst the experimental treatments ($p>0.05$; Table 9).

Table 9. Volatile fatty acid concentrations of caecal contents.

	Acetic	Propionic	Butyric	Iso-butyric	Valeric	Iso-valeric	Unknown	Total
Control	13.05 ± 4.21	2.62 ± 0.75	2.31 ± 0.99	0.08 ± 0.08	0.04 ± 0.04	0.03 ± 0.03	0.05 ± 0.05	18.18 ± 5.86
ZnB	12.51 ± 1.54	8.48 ± 3.08	1.35 ± 0.20	0.0 ± 0.0	0.07 ± 0.04	0.0 ± 0.0	0.29 ± 0.14	22.70 ± 3.88
MOS	11.29 ± 2.98	3.33 ± 0.52	1.67 ± 0.60	0.07 ± 0.07	0.12 ± 0.04	0.11 ± +0.06	0.29 ± 0.14	16.88 ± 4.10
FOS	18.99 ± 1.64	6.42 ± 2.46	2.51 ± 0.39	0.03 ± 0.03	0.11 ± 0.06	0.03 ± 0.03	0.11 ± 0.11	28.20 ± 3.61

Data are expressed as mean μMol/ml ± SEM (n = 6 birds/treatment).

Immunological assays

Cell phenotypes

No significant differences were observed in T cell and macrophage proportions amongst dietary treatments ($p>0.05$, Table 10).

Table 10. Phenotypic analysis of T cells and macrophages in caecal tonsil of broiler chickens.

	$CD45^+$	$CD3^+$	$CD4^+$	$CD8^+$	$KUL1^+$	$MHCII^+$
Control	91.28 ± 1.38	44.56 ± 2.84	48.16 ± 2.60	6.94 ± 2.09	17.88 ± 3.43	10.93 ± 2.00
ZnB	90.17 ± 3.36	50.25 ± 1.63	46.25 ± 1.58	7.12 ± 3.86	13.44 ± 1.54	13.33 ± 2.18
MOS	84.47 ± 7.37	49.17 ± 2.63	48.04 ± 3.92	7.13 ± 2.31	14.35 ± 4.39	6.55 ± 1.37
FOS	89.60 ± 1.92	47.55 ± 8.55	49.78 ± 3.31	5.62 ± 2.94	18.20 ± 3.80	11.46 ± 3.21

Data are expressed as percentage of positive cells ± SEM (n = 6 birds/treatment).

The proportions of $Bu1^+$ cells in birds fed ZnB, FOS and MOS were significantly lower than that of Control-fed birds ($p<0.05$, Table 11). No significant differences in the proportions of IgA^+, IgG^+ and IgM^+ cells were observed amongst dietary treatments ($p>0.05$).

Table 11. Phenotypic analysis of B cells in the caecal tonsils of broiler chickens.

	Bu1$^+$	IgA$^+$	IgG$^+$	IgM$^+$
Control	26.10 ± 1.70a	1.63 ± 0.42	5.33 ± 1.14	28.68 ± 3.30
ZnB	18.27 ± 1.53b	0.82 ± 0.23	4.69 ± 0.43	20.86 ± 1.66
MOS	19.89 ± 2.09b	1.06 ± 0.53	4.35 ± 0.79	22.74 ± 3.44
FOS	20.57 ± 2.11b	0.98 ± 0.19	4.44 ± 0.87	29.05 ± 3.22

Data are expressed as percentage of positive cells ± SEM (n = 6 birds/treatment). Values within a column that do not share a common letter are statistically different ($p<0.05$).

Phagocytosis

No differences in the proportion of functional phagocytes in the caecal tonsils were observed amongst dietary treatments ($p>0.05$; Figure 5).

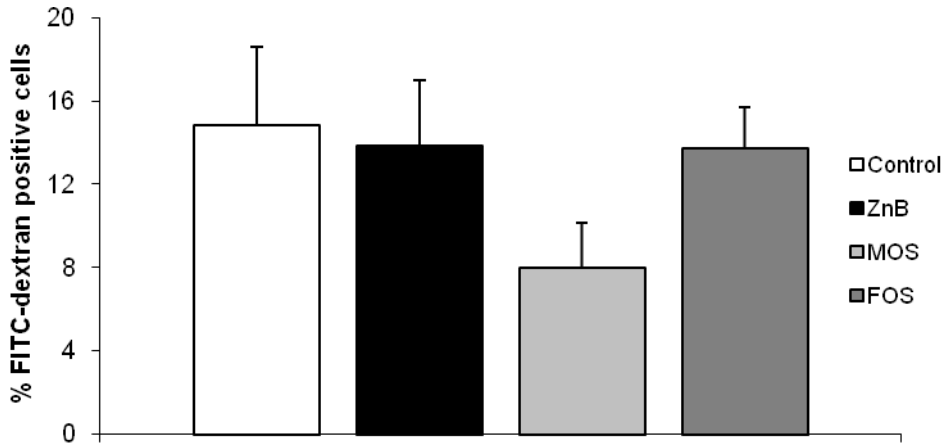


Figure 5. Phagocytosis of caecal tonsil cells. Data are expressed as mean percentage of FITC-dextran-positive cells ± SEM (n = 6 birds/treatment).

Lymphocyte responsiveness to Con A

No significant differences were observed in the proliferative capacity of lymphocytes against Con A ($p>0.05$; Figure 6). There was a trend toward a decrease in proliferative capacity of lymphocytes from ZnB, FOS and MOS-treated birds compared to Control-fed chickens, however no statistically significant differences were observed ($p>0.05$).

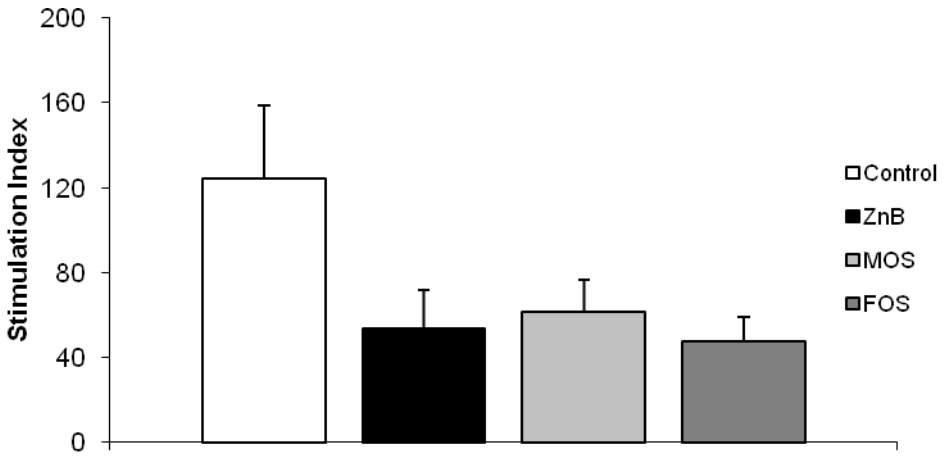
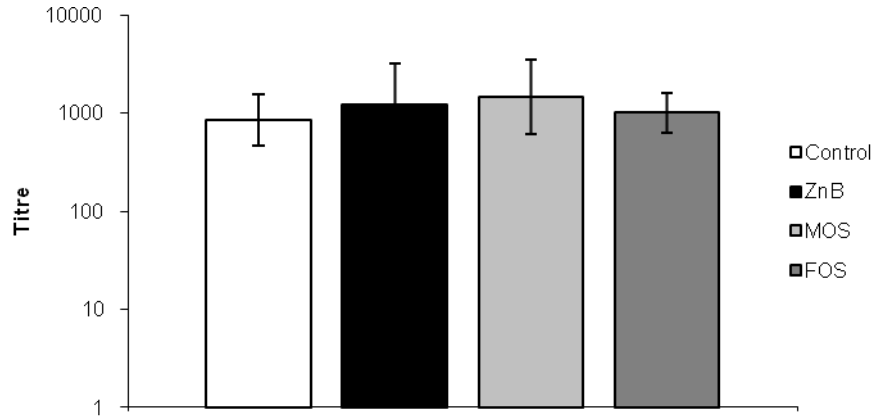


Figure 6. Lymphocyte proliferation in response to Con A. Data are expressed as change in counts per minute (Δ cpm) ± SEM (n = 6 birds/treatment).

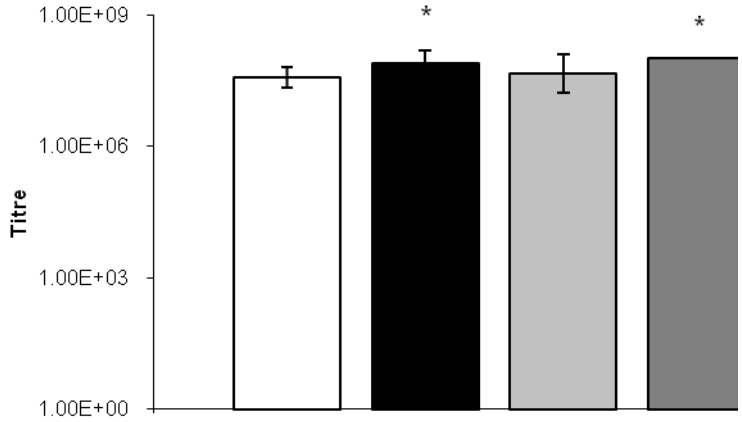
Plasma immunoglobulin titres

No significant differences in plasma IgA antibody titres were observed amongst dietary treatments ($p>0.05$, Figure 7a). ZnB and FOS-treated birds had a significantly greater antibody titre of IgM and IgG compared to Control-fed birds ($p<0.05$, Figures 7b and 7c). No other differences in IgM and IgG titres were observed amongst dietary treatments ($p>0.05$).

a) IgA



b) IgM



c) IgG

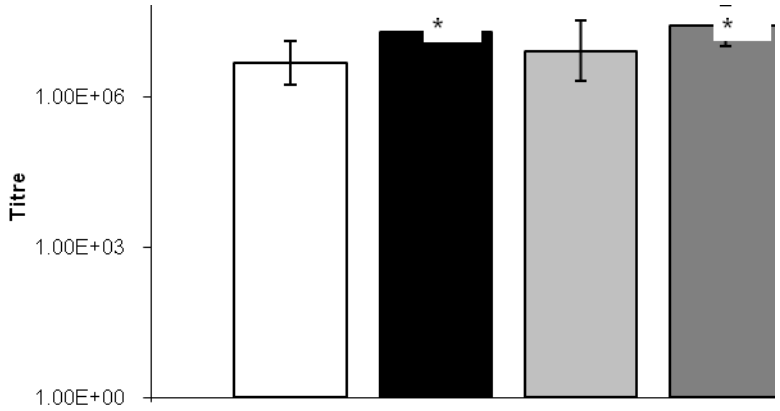


Figure 7. Plasma immunoglobulin titres. a) IgA, b) IgM, c) IgG. Data are expressed as geometric mean ± confidence interval (n = 6 birds/treatment). * indicates a statistically significant difference compared to Control diet ($p<0.05$).

Mucosal IgA

No significant differences in duodenal mucosal IgA^+ cell proportions were observed amongst treatment groups ($p>0.05$, Figure 8).

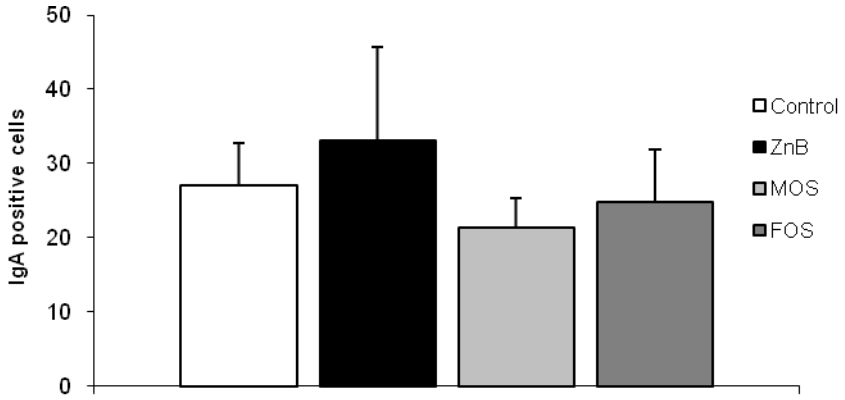


Figure 8. IgA^+ cells in the duodenal mucosa. Data are expressed as mean number of IgA^+ cells ± SEM (n = 6 birds/treatment).

Cytokine expression

No differences in the mRNA expression of IL-6, IFN-γ or IL-10 were observed amongst dietary treatments ($p>0.05$, Figure 9).

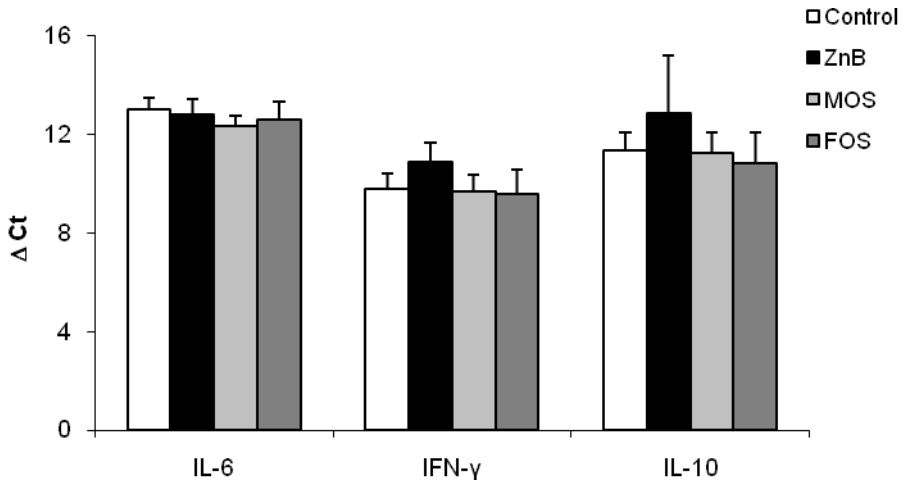


Figure 9. mRNA expression of IL-6, IFN-γ and IL-10 in the CT determined using qRT-PCR. Data are expressed as mean (Δ Ct) ± SEM.

Discussion

The in-feed supplementation of broiler diets with the prebiotic compounds MOS or FOS significantly altered the intestinal microbial communities of the bird compared to ZnB. However, supplementation with prebiotics also appeared to influence a number of immune parameters in a similar fashion to antibiotic diet (ZnB). Interestingly, these observed shifts in immune status and intestinal microbiota did not influence broiler growth and performance efficiency, positively or negatively, compared to basal starter diet with and without ZnB.

Prebiotics have been reported to beneficially affect the microbiota, improve nutrient utilisation and enhance the immune system (Patterson and Burkholder 2003; Biggs et al. 2007; Huang et al. 2007). MOS has demonstrated the capacity to increase intestinal villus height (Iji et al. 2001; Baurhoo et al. 2007), enzyme activities (Iji et al. 2001), and levels of bacteria such as lactobacilli and bifidobacteria, whilst decreasing *E. coli* (Baurhoo et al. 2007). In-feed supplementation with MOS has also been reported to improve body weight gain (Hooge et al. 2003). A meta-analysis by Hooge (2004) encompassing 44 trials indicated that MOS supplementation improved body weight, feed efficiency and reduced mortality compared to diets with no additives; with improvements comparable to the effects of antibiotics. However, a number of individual studies have demonstrated that MOS failed to convey a growth promoting effect (Iji et al. 2001; Baurhoo et al. 2007; Solis de los Santos et al. 2007), a finding that was reflected in the current study.

The in-feed supplementation of broiler diets with FOS (5 g/kg) did not improve performance. Previously, improved body weight gain and feed conversion have been reported following the addition of FOS (4 g/kg) to broiler diets, whilst 2 and 8 g/kg had little effect (Xu et al. 2003). Improvements in the activities of small intestinal amylase, total proteases, an increase in lactobacilli and bifidobacteria and a decrease in *E. coli* compared to control-fed birds were also reported in birds fed 4 g/kg FOS (Xu et al. 2003). In the current study, whilst FOS did not affect performance, it did influence the overall intestinal microbiota. Hooge (2004) stated that when little environmental stress is placed on broiler chickens, a lack of a positive response to antibiotics or alternative growth promotants is often observed. This may have been evident in the current study, as MOS, FOS and ZnB did not improve the performance of broilers. Factors including broiler strain, housing type, litter age, feed composition and stocking density may underlie the highly variable responses to in-feed additives, reported in the literature. These compounds may only lead to health- and performance-enhancing improvements when birds are exposed to sub-optimal conditions; this may not have occurred in the current study. Importantly, it should also be noted that neither FOS nor MOS conveyed any negative effects on performance or bird health in the current study.

Torok and colleagues (2008) have reported a relationship between the overall intestinal microbial communities of broiler chickens and energy metabolism. In the current study, the microbial shifts observed, including changes in the profiles of *Lactobacillus* species, did not affect the energy metabolism of the bird, nor any performance parameters. This finding indicates that a high level of performance can be observed in birds with a range of microbial compositions. The nature and extent of any shifts to the intestinal microbiota, including the genera and species involved, may determine whether bird performance is affected.

The overall intestinal microbial profiles of ZnB-fed birds were significantly different to MOS and FOS-fed birds in the ileum, and MOS-fed birds in the caecum; indicating that these additives influence the microbiota by different means. Interestingly, whilst both prebiotic compounds altered the microbiota, the profiles of birds fed FOS were distinctly different to those of MOS-fed birds, as were the *Lactobacillus* species profiles in the ileum. This indicated that whilst both FOS and MOS influence the intestinal bacteria, they likely act in a different manner and influence a diverse array of organisms. A greater understanding of how different prebiotic compounds affect the intestinal microbial communities of broilers, and which microbial shifts promote improved bird health will be critical in the selection of the most promising prebiotics to be included in feed as an alternative to antibiotics. As the intestinal microbiota has previously been linked with bird performance (Torok et al. 2008), microbial manipulation to establish a community conducive to optimal performance will be a major consideration when designing prebiotic-based animal feed.

Feed supplementation with ZnB led to a significant shift in the overall microbial profile in the ileum of broilers compared to an antibiotic-free diet. Previously, the use of in-feed antibiotics has been demonstrated to influence the microbiota, including *Lactobacillus* species (Engberg et al. 2000; Knarreborg et al. 2002; Wise and Siragusa 2007). In the current study we found that ZnB-containing diet caused a shift in the microbial communities of the ileum compared to ZnB-free diet, but did not have a significant effect in the caecum. This is supported by Wise and Siragusa (2007) who reported that the ileal microbial communities were most susceptible to antibiotics.

T-RFLP and Lac PCR-DGGE were used in the current study to provide a detailed and accurate snapshot of the overall intestinal microbiota. A recent study by Torok et al. (2008) which successfully linked overall shifts in the intestinal microbiota of broilers with changes in energy metabolism highlighted the potential benefits of using T-RFLP technology for studies of this nature. In addition, the isolation and sequencing of T-RFLP-derived OTUs which contribute to shifts in the microbiota between treatments may be used to identify indicator organisms linked to performance. In the current study, Lac PCR-DGGE was used to profile the *Lactobacillus* genus of broilers by indicating the presence and absence of specific *Lactobacillus* species. The identification of *L. johnsonii* and *L.*

reuteri as key organisms responsible for the differences in *Lactobacillus* profiles between treatments highlights the value of this technique in studies of this nature. Lac PCR-DGGE analysis indicated the consistent presence of the LCGA and *L. salivarius* regardless of the overall changes in the microbiota; which supports previous reports of the autochthonous nature of these species in the chicken gastrointestinal tract (Knarreborg et al. 2002; Lan et al. 2002; Guan et al. 2003; Guban et al. 2006; Gong et al. 2007; Souza et al. 2007). The power of simultaneously using T-RFLP and Lac PCR-DGGE is emphasised in the current study, as T-RFLP provided an overview of the entire microbial community of a gut section, whilst Lac PCR-DGGE produced a detailed profile of a specific genus of interest, in this instance, *Lactobacillus*.

The use of intestinal microbial profiling and an improved understanding of how microbial shifts influence bird performance are critical to the strategic selection of feed additives, such as prebiotics, to replace antibiotics. Investigations must focus not only on the microbial profile within the intestine however; but also the metabolic activity of these bacteria. Rehman et al. (2008) demonstrated that the prebiotic compound inulin did not affect the microbiota, but did in fact influence the metabolic activity of these bacteria. Interestingly, in the current study despite the shifts in the microbiota, no changes in the volatile fatty acid profiles were observed. This indicates that different microbial profiles can sustain a similar level of microbial activity, as suggested by volatile fatty acid profile.

The caecal tonsils are the major lymphoid organ in the gut. CTs respond to antigens entering the caeca and are influenced by changes to the microenvironment (Sasai et al. 2000). In the current study we analysed the phenotypic and functional characteristics of components within the CT, including macrophages, B cells and T cells. In addition, the frequency of IgA-secreting cells in the intestine were assessed, as they play a critical role in microbial defence (Macpherson and Slack 2007). We observed that prebiotics, particularly FOS, influenced the functional and phenotypic expression of immune cells, in the CT. FOS also influenced the systemic response, as indicated by plasma immunoglobulin titres. We also observed a general trend of immunosuppression, at functional (low proliferative response), and phenotypic (low B cell percentage) levels in FOS, MOS and ZnB-treated birds. The mechanisms underlying these findings may however, be distinctly different amongst treatment groups.

The immune changes reported in the current study may have been mediated by prebiotic and antibiotic-induced microbial shifts, which may have in turn contributed to an apparent suppression of *in vitro* lymphocyte proliferation via dendritic cells (Braat et al. 2004). Crosstalk between bacteria, immune cell toll like receptors within the CT, and intestinal epithelial cells may cause an attenuated immune response via NF-κB inhibition (Kelly et al. 2004; Yilmaz et al. 2005; Brisbin et al. 2008a; Brisbin et al. 2008b). Further investigation into the interactions between nutrition, the immune system and the microbiota is indicated.

The observed FOS-induced reduction in B cell proportion may be localised to the CT as plasma IgM and IgG antibody titres were significantly higher relative to birds fed the Control diet. The specificity of these antibody titres has not been determined; however, they were not against IBV vaccine (data not shown). FOS-induced shifts in the microbial profiles may have contributed to the observed increase in antibody titres as there are reports of bacteria-mediated enhancement of systemic natural antibody levels (Haghighi et al. 2006). Similarly to FOS, MOS and ZnB were found to decrease CT B cell proportions; however, in contrast to FOS-fed birds, no systemic effects on immunoglobulin levels were detected in MOS-fed birds. Birds fed ZnB however, also displayed elevated plasma levels of IgM and IgG.

The current study indicated that similar to FOS, MOS may also have an immunosuppressive effect on CT lymphocyte proliferation, as a strong trend toward a decrease was observed. Immunosuppressive effects of mannan on human lymphocytes have been reported previously (Nelson et al. 1984; Muchmore et al. 1990; Nelson et al. 1991). Mannan has been found to promote immunosuppression both directly, by blocking T and B cell receptors, and indirectly, by inducing prostaglandin E2 (PGE2) production by monocytes or antigen presenting cells. This in turn depresses the T cell proliferative response to mitogens and inhibits the generation of B cell colonies in spleen and lymph nodes (Coates et al. 1955; Jelinek and Lipsky 1985). In chickens, MOS may function by similar means in order to reduce the frequency of competent Con A-responsive cells and inhibit the generation of CT B cells. However, similarly to the observed effects in FOS-treated birds, the decrease in CT B cell proportions in birds fed MOS was not reflected in the percentages IgM^+, IgG^+ or IgA^+ cells in the CT, nor in the plasma. This is in contrast to previous reports of enhanced plasma antibody titres (Shashidhara and Devegowda 2003). Furthermore there was no variation in the frequency of IgA^+ cells in the duodenum. Interestingly, no diet-induced differences were observed in pro-inflammatory (IFN-γ, IL-6) and anti-inflammatory (IL-10) cytokine mRNA expression in the CT, suggesting that differential cytokine expression may not play a role in the observed changes in immune parameters.

The immunological effects of FOS and MOS observed in the current study closely resembled that of ZnB, including apparent low proliferative responses to Con A and low proportions of $Bu1^+$ cells. Despite the similar responses, it is likely that these compounds are functioning by distinctly different pathways. The physiological implications of the localised immune suppression on overall immunocompetence have yet to be elucidated and further investigation is necessary. We have demonstrated that prebiotic compounds can influence the gut-associated lymphoid tissue (GALT), both functionally and phenotypically. We hypothesise that these prebiotic-mediated immunological changes may at least partially be a result of direct interaction between prebiotics and gut immune cells, and may also be due to an indirect action of prebiotic-mediated alterations in the intestinal microbiota

and subsequent alterations in microbial-derived products which interact with immune cells. Interestingly however, whilst prebiotics appeared to influence the immune system in a similar fashion to ZnB, the effects of these compounds on the intestinal microbiota were quite distinct to one another.

The current study has demonstrated that whilst both MOS and FOS significantly influenced the intestinal microbiota, *Lactobacillus* species, and immune status to varying degrees, these compounds did not alter broiler performance. We postulate that subsequent effects of diet-induced microbial shifts on bird performance may be determined by the nature and extent of the shift, and the species involved. A high level of bird performance may indeed be supported by a range of microbial compositions. Likewise, we have demonstrated that diet-induced shifts in the immune status of the birds can occur, without influencing bird performance, either positively, or negatively. The poultry industry will benefit from the identification of bacterial species and microbial compositions which promote improved bird performance, and the development of feeding regimes that can facilitate the development and maintenance of an optimal microbiota. Additionally, the characterisation of an optimal immune status for a balance between health and performance, and the development of diets that can facilitate an optimal immune status will advantage the poultry industry. As the benefits of a healthy intestinal microbiota are well documented, it appears that the use of prebiotics in poultry warrants further investigation. It may be the case however that whilst prebiotics alone cannot replace in-feed antibiotics, they may form part of a combination of compounds which act to improve performance via reinforcement of the intestinal microbiota and bird immunity.

Chapter 3. In-feed omega-3 polyunsaturated fatty acid supplementation increases tissue omega-3 incorporation and influences mucosal immunity

Introduction

Omega-3 polyunsaturated fatty acids (*n*-3 PUFA) are well documented for their health-promoting effects (Calder 2006). Consumption of *n*-3 PUFA has demonstrated therapeutic potential in a range of human inflammatory conditions including rheumatoid arthritis (Kremer 2000), inflammatory bowel disease (Belluzzi 2002), and asthma (Woods et al. 2002), with *n*-3 PUFA believed to significantly reduce the risk of coronary heart disease (Harris et al. 2008). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the two *n*-3 PUFAs with the greatest reported anti-inflammatory capacity. The most common source of *n*-3 PUFA is fish oil, which is particularly high in the two most potent *n*-3 fats, EPA and DHA.

In poultry, diets with added *n*-3 PUFA in the form of fish oil have been found to improve bird growth and performance efficiency (Lopez-Ferrer et al. 2001; Schreiner et al. 2005), reduce the inflammatory response (Korver and Klasing 1997), and alter immune parameters including antibody production (Korver and Klasing 1997) and lymphocyte proportion and responsiveness (Wang et al. 2000). As such, the anti-inflammatory and immunomodulatory properties of *n*-3 PUFA indicate potential use as an alternative to in-feed antibiotics in order to dampen the inflammatory response and increase bird growth.

In this chapter we aimed to compare the effects of in-feed fish oil supplementation on broiler growth and performance efficiency, fatty acid composition of breast tissue, bird immune status and the intestinal microbial communities, with particular focus on *Lactobacillus* species.

Materials and Methods

Birds and diets

Male Cobb 500 broiler chickens (Baiada Hatchery, Willaston, SA, Australia) were raised in four floor pens in a temperature-controlled room. All birds were vaccinated with Eimeriavax 4m (Bioproperties Pty Ltd, Glenorie, NSW, Australia) at placement, and at five and 11 days post-hatch. All procedures were approved by the Animal Ethics Committees of the University of Adelaide and the Department of Primary Industries and Resources South Australia.

All experimental diets were based on a commercial starter diet (Ridley Agriproducts Pty Ltd, Murray Bridge, SA, Australia) with no added antibiotics or coccidiostats (Table 12). Diets met or exceeded National Research Council guidelines for broiler chickens (NRC 1994). SALmate™ (Feedworks, Romsey, Vic, Australia) which is comprised of 42% fish oil and 58% starch (DHA: 40.5 g/kg, EPA: 36.0 g/kg, total *n*-3 PUFA: 113.0 g/kg; Table 13) was used as the source of *n*-3 PUFA. The four experimental diets were; i) Control (standard diet with no additives), ii) ZnB (standard diet + 50 ppm zinc bacitracin), iii) 2% SALmate™ (standard diet + 2% wt/wt SALmate™) and iv) 5% SALmate™ (standard diet + 5% wt/wt SALmate™; Table 14). Twelve birds were fed each experimental diet. Titanium dioxide (5 g/kg) was added as a digestibility marker during the energy metabolism phase of the experiment as described in Chapter 2. Birds had continual access to water and experimental feed.

Table 12. Experimental diets for chickens prior to (0-15 days) and during (15-25 days) the apparent metabolisable energy study.

Ingredient (g/kg)	Control	ZnB	2% SALmate™	5% SALmate™
		Period 0 – 15 days of age		
Starter crumble[1]	950.0	949.7	950.0	950.0
Zinc bacitracin (50 ppm active ingredient)	-	0.3	-	-
SALmate™	-	-	20.0	50.0
Tallow	21.0	21.0	12.6	-
Milled wheat	29.0	29.0	17.4	-
		Period 15 – 25 days of age		
Starter crumble[1]	945.0	944.7	945.0	945.0
Zinc bacitracin	-	0.3	-	-
SALmate™	-	-	20.0	50.0
Tallow	21.0	21.0	12.6	-
Milled wheat	29.0	29.0	17.4	-
Titanium dioxide	5.0	5.0	5.0	5.0

[1] Starter crumble comprises (in g/kg): wheat (638.9), hammer-milled wheat (8.0), oats (50.0), peas (60.0), meat meal (100.0), blood meal (10.7), tallow (10.0), solvent extracted soybean meal (93.7), ground oatmeal flour (16.7), monocalcium phosphate (3.75), dicalcium phosphate dehydrate (1.09), choline chloride 75% (0.5), L-lysine HCl (2.14), Alimet (2.57). Vitamin and mineral mixes exceeded NRC standards.

Table 13. Fatty acid composition of SALmate™ and the percentage contribution of fatty acids to experimental diets.

Fatty acid (FA)	SALmate™	SALmate™ 2% in diet (% FA added to diet)	SALmate™ 5% in diet (% FA added to diet)
C14:0	2.00	0.04	0.100
C16:0	5.70	0.114	0.285
C16:1	2.90	0.058	0.145
C18:0	1.25	0.025	0.063
C18:1	8.90	0.178	0.445
C18:2 (*n*-6)	2.25	0.045	0.113
C18:3 (*n*-3)	2.45	0.049	0.123
C20:1	2.90	0.058	0.145
C20:5 (*n*-3)	3.60	0.072	0.180
C22:1	3.10	0.062	0.155
C22:5 (*n*-3)	1.20	0.024	0.060
C22:6 (*n*-3)	4.05	0.081	0.203

Data are expressed as % contribution of fatty acid (FA) to total diet.

Table 14. Fatty acid analysis of experimental diets.

	Control	ZnB	2% SALmate™	5% SALmate™
C16:0	21.26	21.21	20.78	19.74
C18:0	11.90	11.93	10.45	7.86
Total Sats	36.40	36.39	34.88	31.69
Total Trans	2.01	2.04	1.73	1.48
C18:1 (*n*-9)	29.04	29.18	27.81	25.38
Total *n*-9	30.02	30.14	29.55	28.21
Total *n*-7	3.96	3.94	4.75	5.72
C18:2 (*n*-6)	24.26	23.92	23.56	24.59
C18:3 (*n*-6)	0.06	0.05	0.05	0.06
C20:2 (*n*-6)	0.02	0.05	0.03	0.03
C20:3 (*n*-6)	0.01	0.01	0.01	0.01
C20:4 (*n*-6)	0.22	0.22	0.24	0.28
C22:2 (*n*-6)	0.02	0.02	0.02	0.02
C22:4 (*n*-6)	0.05	0.05	0.05	0.05
C22:5 (*n*-6)	0.01	0.01	0.03	0.06
Total *n*-6	24.64	24.32	23.99	25.11
C16:2 (*n*-3)	0.04	0.04	0.05	0.07
C18:3 (*n*-3)	1.73	1.73	1.66	1.65
C18:4 (*n*-3)	0.24	0.24	0.21	0.14
C20:3 (*n*-3)	0.02	0.02	0.03	0.02
C20:5 (*n*-3)	0.05	0.05	0.63	1.39
C22:5 (*n*-3)	0.05	0.10	0.29	0.56
C22:6 (*n*-3)	-	0.09	0.69	1.49
Total *n*-3	2.14	2.27	3.56	5.31

Data are expressed as percentage distribution of fatty acid in the experimental diet.

Apparent metabolisable energy study

The apparent metabolisable energy study and tissue collection procedures were carried out as described in Chapter 2.

Polyunsaturated fatty acid analysis of plasma and breast tissue

The fatty acid profiles were assessed in the plasma and breast tissue of 24 birds (n = 6/treatment). Prior to kill, blood was collected by venipuncture of the brachial vein and stored in lithium heparin tubes at 4^{o}C until centrifuged (1000 *g*) and the plasma separated and frozen. At kill, a 5 g sample of breast muscle was collected and stored at 4^{o}C until frozen at -20^{o}C.

Fatty acid methyl esters (FAMEs) were separated and quantified on a Hewlett-Packard 6890 gas chromatograph with a 50 m capillary column (0.32 mm internal diameter; SGE, Vic, Australia) coated with 70% cyanopropyl polysilphenylene-siloxane (BPX-70; 0.25 μm film thickness) fitted with a flame ionisation detector. Helium was the carrier gas (split-ratio 20:1). The injector temperature was 250°C and the detector temperature was 300°C. Initial oven temperature was set at 140°C, and programmed to increase to 220°C at 5°C per minute and held for up to 3 minutes. Fatty acids were identified based on the retention time of standards obtained from Nucheck Prep Inc. (Elysian, MN, USA) using Chemstation software (Agilent Technologies, Santa Clara, CA, USA).

Microbial profiling

Sample collection and preparation

Sample collection, freeze-drying and total nucleic acid extraction for T-RFLP and Lac PCR-DGGE was performed according to the techniques described in Chapter 2.

Terminal-restriction fragment length polymorphism

T-RFLP analysis was performed according to the techniques outlined in Chapter 2 and by Torok et al. (2008).

Denaturing gradient gel electrophoresis

Lactobacillus species were analysed using Lac PCR-DGGE as described previously in Chapters 1 and 2, and by Walter et al. (2001).

Volatile fatty acid analysis

Volatile fatty acids were analysed using the techniques outlined in Chapter 2, and by Storer et al. (1983).

Immunological assays

Isolation of leucocytes from caecal tonsil

Leucocyte isolation was carried out as described in Chapter 1.

Phenotypic characterisation of leucocytes by flow cytometry

Phenotypic analysis of leucocytes was performed as described in Chapter 1.

Phagocytosis

Phagocytosis was measured as described in Chapter 1.

Lymphocyte responsiveness to Con A

Due to contamination of the caecal tonsils during transport, analysis of lymphocyte proliferation was not performed for this experiment.

Plasma immunoglobulin titres

Plasma immunoglobulin titres were determined using the techniques described in Chapter 2.

Mucosal IgA

The frequency of IgA-secreting plasma cells at the mucosal surface of the duodenum was determined using the procedures outlined in Chapter 2.

Cytokine expression

Cytokine expression was determined using the techniques described in Chapter 2.

Statistical analyses

Performance data were analysed using the SAS for Windows version 9.1 software package (SAS Institute Inc., Cary, NC, USA). Data were compared by analysis of variance using the General Linear Model (GLM) procedure with differences amongst diets determined by Duncan's Multiple Range Test. Fatty acid levels, volatile fatty acid profiles and immunological data were compared by non-parametric Kruskal-Wallis test. Microbial data were compared using the techniques described in Chapter 2. For all comparisons, $p<0.05$ was considered significant.

Results

Apparent metabolisable energy study

Throughout the seven-day energy metabolism study period, no differences in body weight gain, feed intake, feed conversion ratio or ileal digestible energy were observed amongst treatments ($p>0.05$, Table 15). The AME values of ZnB-fed birds were significantly greater than all other dietary treatments ($p<0.05$; Table 15). AME values did not differ significantly amongst birds fed Control, 2% and 5% SALmate™ diets ($p>0.05$).

Table 15. Performance parameters from chickens during the apparent metabolisable energy period.

	BWS (g)	BWG (g)	FI (g/bird/day)	FCR	AME (MJ/kg)	IDE (MJ/kg)
Control	417 ± 16	473 ± 16	103 ± 2.9	1.53 ± 0.03	13.95 ± 0.20b	13.68 ± 0.52 (n = 11)
ZnB	412 ± 8	460 ± 18	100 ± 3.0	1.54 ± 0.04	14.53 ± 0.14a	14.50 ± 0.28 (n = 7)
2% SALmate™	429 ± 14	491 ± 17	106 ± 3.1	1.51 ± 0.02	13.79 ± 0.21b	14.23 ± 0.44 (n = 10)
5% SALmate™	454 ± 13	486 ± 15	106 ± 3.0	1.53 ± 0.02	13.92 ± 0.12b	14.23 ± 0.40 (n = 11)

Body weight start (BWS) and body weight gain (BWG) data are expressed as mean (g) ± SEM. Feed intake (FI) data are expressed as mean (g/bird/day) ± SEM. Feed conversion ratio (FCR) is expressed as feed intake/body weight gain during the AME period ± SEM. Apparent metabolisable energy (AME) is expressed as mean (MJ energy/kg dry matter basis) ± SEM. Ileal digestible energy (IDE) is expressed as mean (MJ energy/kg dry matter basis) ± SEM. Values within a column which do not share a common letter are significantly different ($p<0.05$; n = 12 birds/treatment for Control and 5% SALmate™, and n = 11 birds/treatment for ZnB and 2% SALmate™). Note; due to insufficient

sample available from some birds, ileal digestible energy values were calculated from different number of birds per treatment group).

Plasma and tissue polyunsaturated fatty acid levels

The plasma fatty acid profiles were significantly influenced by dietary treatment (Table 16). Plasma EPA, DHA, DPA and total *n*-3 PUFA levels were significantly elevated in birds fed 2% SALmate™ and 5% SALmate™ diets compared to ZnB and Control-fed birds ($p<0.05$). Additionally, the 5% SALmate™ diet promoted greater levels of these compounds compared to the 2% SALmate™ diet ($p<0.05$). Birds fed the 5% SALmate™ diet had significantly lower plasma *n*-6 PUFA levels compared to birds fed all other diets ($p<0.05$). Total saturated fatty acids levels were elevated significantly in 2% SALmate™ and 5% SALmate™-fed chickens compared to birds fed ZnB and Control diets, with 5% SALmate™-fed chickens also exhibiting a level of saturated fatty acid greater than that of birds fed the 2% SALmate™ diet ($p<0.05$). Total monounsaturated and trans fatty acids in the plasma were lower in 2% SALmate™ and 5% SALmate™-fed chickens compared to ZnB and Control-fed birds ($p<0.05$), with birds fed 5% SALmate™ also displaying lower trans fat levels compared to birds fed 2% SALmate™ ($p<0.05$).

Breast fatty acid composition was also significantly affected by dietary inclusion of *n*-3 PUFA (Table 17). EPA, DHA, DPA and total *n*-3 PUFA levels in breast tissue were significantly elevated in 2% SALmate™ and 5% SALmate™-fed chickens compared to ZnB and Control diets ($p<0.05$). Furthermore, chickens fed 5% SALmate™ had a greater DHA incorporation into the breast tissue compared to chickens fed the 2% SALmate™ diet ($p<0.05$). Levels of *n*-6 PUFA in the breast tissue were not affected ($p>0.05$). Total trans fatty acids were lower in the 5% SALmate™-fed chickens compared to birds fed all other diets ($p<0.05$).

Table 16. Fatty acid distribution in the plasma of broiler chickens.

Fatty Acid	Control	ZnB	2% SALmate™	5% SALmate™
C16:0	19.92 ± 0.29a	19.31 ± 0.69a	20.77 ± 0.33a	23.27 ± 0.31b
C18:0	20.62 ± 0.39a	21.39 ± 0.91a	20.71 ± 0.33a	19.04 ± 0.28b
Total Sats	42.35 ± 0.23a	42.54 ± 0.19a	43.50 ± 0.19b	44.38 ± 0.23c
Total Trans	0.79 ± 0.02a	0.75 ± 0.02a	0.68 ± 0.02b	0.53 ± 0.03c
Total Monos	22.50 ± 0.81a	23.06 ± 1.07ab	19.34 ± 1.20b	17.36 ± 1.14b
C18:1 (*n*-9)	17.98 ± 0.85a	17.58 ± 0.92a	14.76 ± 0.88b	13.68 ± 1.18b
Total *n*-9	21.38 ± 0.68a	20.68 ± 0.83a	17.36 ± 1.03b	16.16 ± 1.42b
Total *n*-7	3.13 ± 0.41ab	4.04 ± 0.24a	3.30 ± 0.36ab	2.37 ± 0.32b
C18:2 (*n*-6)	22.92 ± 0.37a	22.51 ± 0.16a	22.32 ± 0.53a	19.09 ± 0.46b
C18:3 (*n*-6)	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.05 ± 0.00
C20:2 (*n*-6)	0.55 ± 0.01a	0.63 ± 0.05a	0.52 ± 0.03ab	0.42 ± 0.04b
C20:3 (*n*-6)	1.23 ± 0.06	1.17 ± 0.05	1.10 ± 0.04	1.07 ± 0.07
C20:4 (*n*-6)	4.24 ± 0.36	4.00 ± 0.62	4.36 ± 0.54	5.68 ± 0.63
C22:2 (*n*-6)	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C22:4 (*n*-6)	-	-	-	0.01 ± 0.00
C22:5 (*n*-6)	0.28 ± 0.03	0.27 ± 0.04	0.22 ± 0.03	0.24 ± 0.02
Total *n*-6	29.28 ± 0.45a	28.64 ± 0.75ab	28.59 ± 0.76a	26.58 ± 0.56b
C16:2 (*n*-3)	0.10 ± 0.00a	0.11 ± 0.01a	0.10 ± 0.01ab	0.08 ± 0.00b
C18:3 (*n*-3)	0.25 ± 0.00a	0.25 ± 0.01a	0.23 ± 0.00b	0.19 ± 0.01c
C18:4 (*n*-3)	-	-	-	0.01 ± 0.01
C20:3 (*n*-3)	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.01
C20:5 (*n*-3)	0.52 ± 0.03a	0.54 ± 0.04a	1.36 ± 0.14b	2.09 ± 0.13c
C22:5 (*n*-3)	0.49 ± 0.02a	0.52 ± 0.08a	0.89 ± 0.12b	1.37 ± 0.16c
C22:6 (*n*-3)	1.30 ± 0.11a	1.47 ± 0.18a	3.66 ± 0.47b	5.96 ± 0.73c
Total *n*-3	2.69 ± 0.14a	2.93 ± 0.29a	6.28 ± 0.69b	9.72 ± 0.95c

Data are expressed as mean (% distribution) ± SEM. Values within a row with a different letter are significantly different ($p<0.05$).

Table 17. Fatty acid distribution in breast tissue of broiler chickens.

Fatty Acid	Control	ZnB	2% SALmate™	5% SALmate™
C16:0	21.82 ± 0.28	21.19 ± 0.36	21.94 ± 0.45	22.56 ± 0.45
C18:0	7.78 ± 0.28	8.84 ± 0.27	8.31 ± 0.34	8.36 ± 0.26
Total Sats	34.34 ± 0.49	35.62 ± 0.39	35.67 ± 0.46	35.99 ± 0.35
Total Trans	0.83 ± 0.10a	0.76 ± 0.09a	0.76 ± 0.09a	0.49 ± 0.05b
Total Monos	46.73 ± 1.08	43.72 ± 0.67	42.84 ± 1.22	43.02 ± 1.27
C18:1 (*n*-9)	32.40 ± 0.92	30.37 ± 0.48	30.13 ± 0.68	30.68 ± 0.85
Total *n*-9	35.75 ± 1.01	34.09 ± 0.39	33.07 ± 0.69	33.54 ± 0.87
Total *n*-7	11.47 ± 0.70	10.52 ± 0.43	10.17 ± 0.58	9.78 ± 0.41
C18:2 (*n*-6)	10.76 ± 0.49	11.34 ± 0.30	10.80 ± 0.38	10.66 ± 0.44
C18:3 (*n*-6)	0.15 ± 0.01a	0.15 ± 0.02a	0.11 ± 0.02ab	0.11 ± 0.01b
C20:2 (*n*-6)	0.26 ± 0.02	0.32 ± 0.02	0.30 ± 0.02	0.27 ± 0.02
C20:3 (*n*-6)	0.85 ± 0.04	1.14 ± 0.14	0.93 ± 0.07	0.76 ± 0.07
C20:4 (*n*-6)	1.73 ± 0.10	2.09 ± 0.16	1.90 ± 0.14	1.64 ± 0.19
C22:2 (*n*-6)	0.07 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
C22:4 (*n*-6)	0.01 ± 0.01	0.01 ± 0.01	-	-
C22:5 (*n*-6)	-	-	-	-
Total *n*-6	13.83 ± 0.56	15.08 ± 0.58	14.08 ± 0.58	13.47 ± 0.67
C16:2 (*n*-3)	0.31 ± 0.15	0.25 ± 0.14	0.34 ± 0.08	0.19 ± 0.08
C18:3 (*n*-3)	0.55 ± 0.02b	0.46 ± 0.02a	0.49 ± 0.03ab	0.50 ± 0.02ab
C18:4 (*n*-3)	0.21 ± 0.09	0.17 ± 0.09	0.25 ± 0.07	0.11 ± 0.05
C20:3 (*n*-3)	0.06 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
C20:5 (*n*-3)	0.54 ± 0.04a	0.60 ± 0.03a	1.17 ± 0.11b	1.37 ± 0.14b
C22:5 (*n*-3)	0.51 ± 0.04a	0.64 ± 0.04a	1.05 ± 0.09b	1.23 ± 0.15b
C22:6 (*n*-3)	0.71 ± 0.06b	0.97 ± 0.07a	2.10 ± 0.18c	2.55 ± 0.27d
Total *n*-3	2.88 ± 0.30a	3.15 ± 0.27a	5.46 ± 0.41b	5.98 ± 0.60b

Data are expressed as mean (% distribution) ± SEM. Values within a row with a different letter are significantly different ($p<0.05$).

Microbial profiles

Terminal-restriction fragment length polymorphism

No significant differences were observed in the overall microbial communities in the ileum and caecum of birds fed the four experimental diets ($p>0.05$; Table 18).

Table 18. One-way analysis of similarities (ANOSIM) for ileal and caecal microbial communities associated with diet.

Ileum	**Control**	**ZnB**	**2% SALmate™**	**5% SALmate™**
Control	-	**0.05**	**-0.032**	**0.004**
ZnB	*0.184*	-	**-0.068**	**0.013**
2% SALmate™	*0.610*	*0.896*	-	**-0.059**
5% SALmate™	*0.348*	*0.334*	*0.827*	-
Caecum	**Control**	**ZnB**	**2% SALmate™**	**5% SALmate™**
Control	-	**0.052**	**0.110**	**0.001**
ZnB	*0.197*	-	**0.112**	**-0.063**
2% SALmate™	*0.056*	*0.062*	-	**-0.042**
5% SALmate™	*0.467*	*0.855*	*0.747*	-

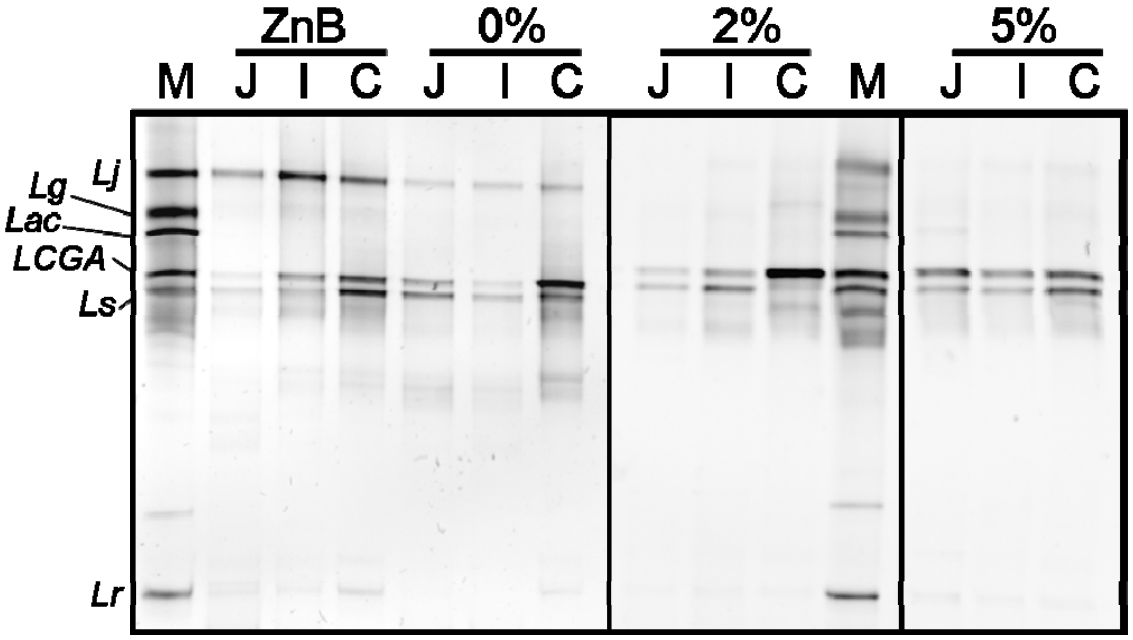
Data are expressed as the R-statistic (bold), with significance level in italics. For all analyses a significance level of 0.05 was considered significant. For ileal microbial communities, the global R-value was -0.012 at a significance level of 0.587 which is not considered significant. For caecal microbial communities the global R-value was 0.027 at a significance level of 0.233 which is not considered significant.

Denaturing gradient gel electrophoresis

Lactobacillus species were analysed by comparing pooled (Figures 5a and 5b) and individual (Table 19) Lac PCR-DGGE profiles. The pooled *Lactobacillus* profiles in the jejunum and ileum of 2% SALmate™ and 5% SALmate™-fed birds, and the caecal profile of 5% SALmate™-fed birds, clustered together and consisted of *L. salivarius* and LCGA. The consensus caecal *Lactobacillus* profile of 2% SALmate™-fed chickens was characterised by a band which co-migrated with LCGA. The pooled profiles of all intestinal sections of birds fed the ZnB and Control diets contained *L. johnsonii*, *L. salivarius* and/or LCGA.

No differences in the presence and absence of *Lactobacillus* species were detected in the jejunum or ileum ($p>0.05$; Table 19). Individual caecal *Lactobacillus* profiles of Control and 2% SALmate™-fed birds were significantly different ($p<0.05$). Bray-Curtis similarity analysis suggested that *L. johnsonii* contributed significantly to the dissimilarity between birds fed Control and 2% SALmate™ diets, with *L. johnsonii* detected in 8/12 Control-fed birds and only 2/12 in 2% SALmate™-fed birds.

a)



b)

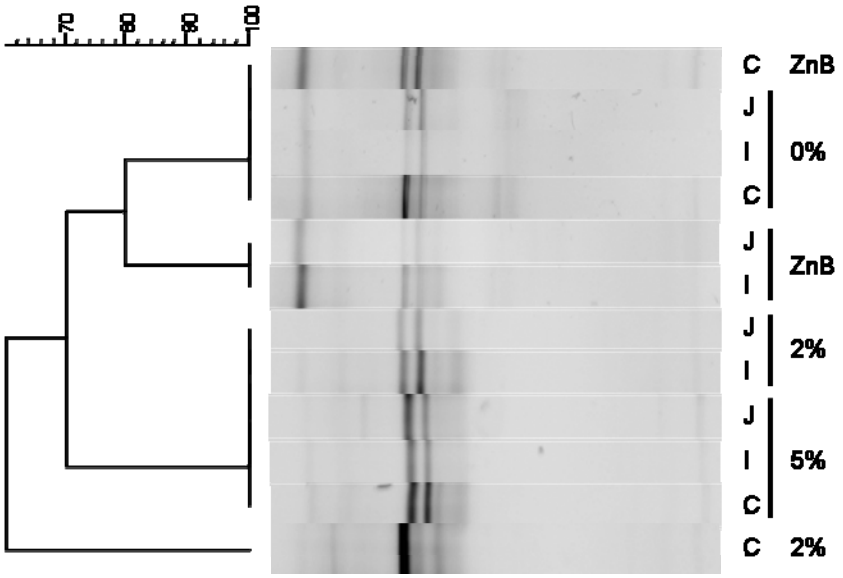


Figure 10. Lac PCR-DGGE profiles of pooled samples for jejunum (J), ileum (I), and caeca (C) of each diet (Figure 5a) and analysis using Dice's Similarity (Dsc; Figure 5b). The marker lane (M) contains the identification ladder comprised of Lac PCR products from the following reference strains: Lj, *L. johnsonii* ATCC 33200; Lg, *L. gasseri* ATCC 33323; Lac, *L. acidophilus* ATCC 4356; Ls, *L. salivarius* subsp. *salivarius* ATCC 11741; and Lr, *L. reuteri* ATCC 23272. LCGA marks the migration of *L. crispatus*, *L. gallinarum* and *L. amylovorous* species, and is represented by *L. crispatus* ATCC 33820. ZnB, zinc bacitracin; 0%, Control; 2%, 2% SALmate™; 5%, 5% SALmate™.

Table 19. One-way ANOSIM of jejunal, ileal and caecal *Lactobacillus* species associated with diets.

Jejunum	**Control**	**ZnB**	**2% SALmate™**	**5% SALmate™**
Control	-	**-0.035**	**-0.05**	**0.01**
ZnB	*0.776*	-	**-0.06**	**0.092**
2% SALmate™	*0.794*	*1.00*	-	**0.112**
5% SALmate™	*0.329*	*0.069*	*0.056*	-
Ileum	**Control**	**ZnB**	**2% SALmate™**	**5% SALmate™**
Control	-	**0.052**	**-0.035**	**0.047**
ZnB	*0.171*	-	**0.003**	**0.083**
2% SALmate™	*0.710*	*0.378*	-	**0.039**
5% SALmate™	*0.101*	*0.058*	*0.155*	-
Caecum	**Control**	**ZnB**	**2% SALmate™**	**5% SALmate™**
Control	-	**0.090**	**0.204**	**0.049**
ZnB	*0.150*	-	**0.089**	**0.036**
2% SALmate™	*0.007*	*0.119*	-	**0.017**
5% SALmate™	*0.140*	*0.263*	*0.318*	-

Data are expressed as the R-statistic (bold), with significance level (p) in italics. The R-statistic value indicates the extent of similarity between each pair in the ANOSIM. Values approaching unity indicate that the two groups are entirely separate and a zero value indicates that there is no difference between groups. For all analyses, $p<0.05$ was considered significant. For jejunal microbial communities the global R-value was 0.004 at a significance level of 0.372 which is not significant. For ileal microbial communities, the global R-value was 0.034 at a significance level of 0.097 which is not significant. For caecal microbial communities the global R-value was 0.088 at a significance level of 0.036.

Volatile fatty acid concentrations

Acetic acid levels were significantly greater in Control-fed birds compared to birds fed 5% SALmate™ ($p<0.05$, Table 20). Total levels of volatile fatty acids were greater in the Control-fed birds compared to birds fed ZnB and 5% SALmate™ ($p<0.05$).

Table 20. Volatile fatty acid concentrations of caecal contents.

	Acetic	Propionic	Butyric	Iso-butyric	Valeric	Iso-valeric	Unknown	Total
Control	13.31 ± 2.94a	3.14 ± 0.46	2.77 ± 0.90	0.0 ± 0.0	0.37 ± 0.06	0.09 ± 0.06	0.0 ± 0.0	19.68 ± 2.99a
ZnB	7.95 ± 1.38ab	1.79 ± 0.38	1.54 ± 0.38	0.0 ± 0.0	0.20 ± 0.10	0.05 ± 0.05	0.0 ± 0.0	11.53 ± 1.65b
2% SALmate	9.96 ± 2.75ab	3.24 ± 0.80	2.34 ± 0.82	0.0 ± 0.0	0.28 ± 0.08	0.11 ± 0.08	0.32 ± 0.14	16.26 ± 2.86ab
5% SALmate	7.03 ± 1.60b	2.28 ± 0.65	2.23 ± 0.85	0.0 ± 0.0	0.34 ± 0.10	0.27 ± 0.17	0.06 ± 0.06	12.22 ± 1.71b

Data are expressed as mean μMol/ml (± SEM). n = 6 for Control and 5% SALmate™ diets. n = 5 for ZnB and 2% SALmate™ diets.

Immunological assays

Cell phenotypes

Chickens fed 2% SALmate™-containing diet had a significantly lower proportion of $MHCII^+$ cells compared to ZnB-fed birds ($p<0.05$, Table 21), with a trend towards a decrease in $CD45^+$ cells. No differences were observed in the proportions of $CD3^+$, $CD4^+$, $CD8^+$ and $KUL1^+$ cells amongst dietary treatments ($p>0.05$).

Table 21. Phenotypic analysis of T cells and macrophages in caecal tonsils of broiler chickens.

	$CD45^+$	$CD3^+$	$CD4^+$	$CD8^+$	$KUL1^+$	$MHCII^+$
Control	75.70 ± 3.65	28.86 ± 2.23	42.22 ± 5.51	19.27 ± 2.85	1.86 ± 0.46	73.22 ± 3.51ab
ZnB	87.96 ± 8.49	35.87 ± 3.68	51.93 ± 3.19	16.80 ± 1.47	1.09 ± 0.23	86.54 ± 6.93b
2% SALmate	67.51 ± 3.93	31.22 ± 2.61	36.22 ± 5.00	23.52 ± 3.03	2.12 ± 0.67	63.58 ± 4.73a
5% SALmate	71.06 ± 3.72	26.25 ± 2.30	41.01 ± 5.13	14.38 ± 2.68	1.81 ± 0.46	73.60 ± 3.10ab

Data are expressed as percentage of positive cells ± SEM (n = 6 birds/treatment). Values within a column that do not share a common letter are significantly different ($p<0.05$).

The proportions of IgA^+ cells in the caecal tonsils of birds fed 2% and 5% SALmate™ diets were significantly greater compared to birds fed Control and ZnB diets ($p<0.05$, Table 22). No differences were observed in the proportions of $Bu1^+$, IgG^+ and IgM^+ cells amongst dietary treatments ($p>0.05$).

Table 22. Phenotypic analysis of B cells in caecal tonsils of broiler chickens.

	Bu1$^+$	IgA$^+$	IgG$^+$	IgM$^+$
Control	49.2 ± 3.9	6.2 ± 0.8a	6.7 ± 2.8	49.1 ± 3.6
ZnB	49.7 ± 4.7	7.9 ± 1.7a	6.0 ± 1.8	52.3 ± 4.5
2% SALmate™	48.6 ± 3.9	14.2 ± 2.1b	7.5 ± 1.5	51.6 ± 2.4
5% SALmate™	51.7 ± 4.8	13.4 ± 4.1b	6.9 ± 1.2	53.7 ± 4.1

Data are expressed as percentage of positive cells ± SEM (n = 6 birds/treatment). Values within a column that do not share a common letter are significantly different ($p<0.05$).

Phagocytosis

No significant differences in phagocytic activity were observed amongst treatment groups ($p>0.05$, Figure 11).

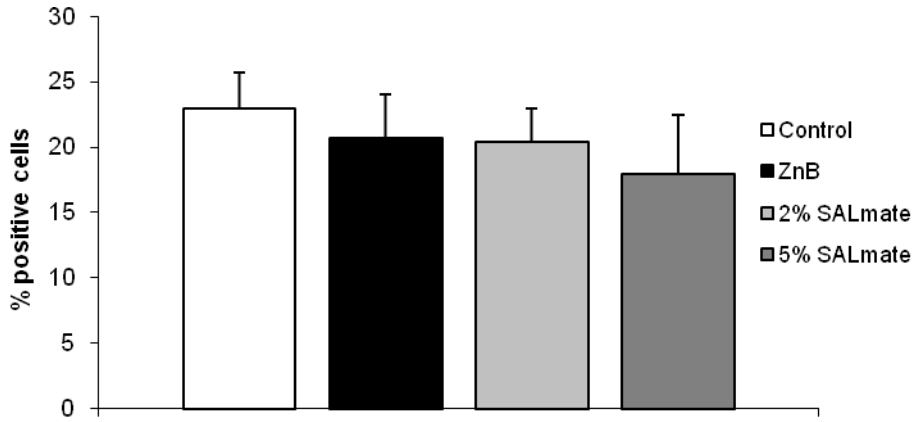
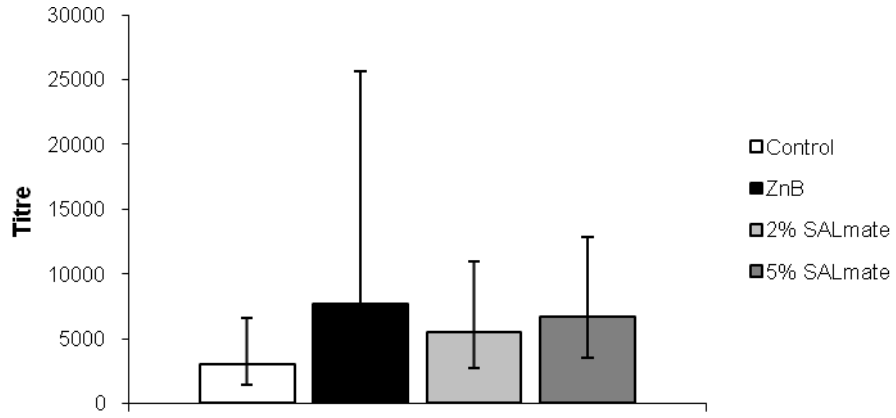


Figure 11. Phagocytosis of caecal tonsil cells. Data are expressed as mean percentage of FITC-dextran-positive cells ± SEM (n = 6 birds/treatment).

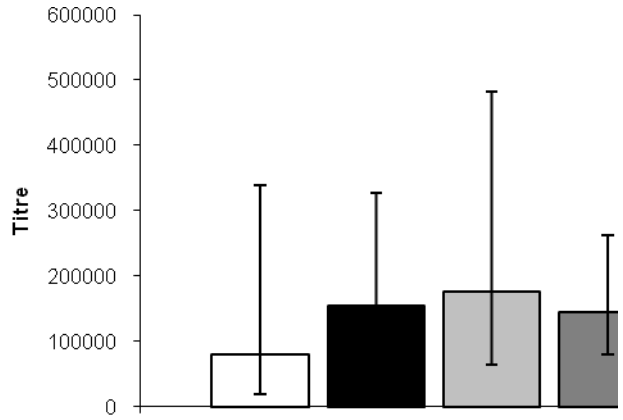
Plasma immunoglobulin titres

No significant differences in plasma antibody titres of IgA, IgM and IgG were observed amongst treatment groups ($p>0.05$, Figures 12a, b and c).

a) IgA



b) IgM



c) IgG

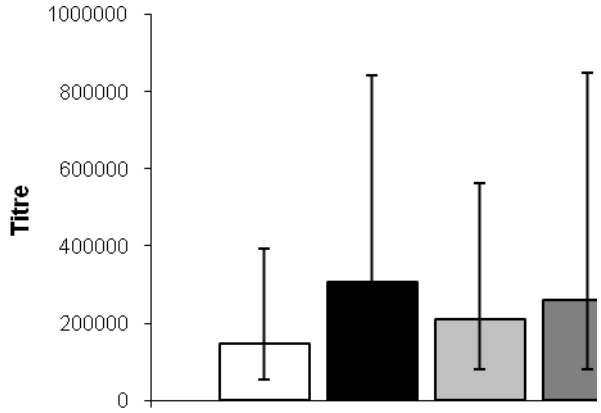


Figure 12. Plasma immunoglobulin titres. a) IgA, b) IgM, c) IgG. Data are expressed as geometric mean ± confidence interval (n = 6 birds/treatment).

Mucosal IgA

The proportions of mucosal IgA^+ cells in the duodenum were not significantly different amongst dietary treatments ($p>0.05$, Figure 13).

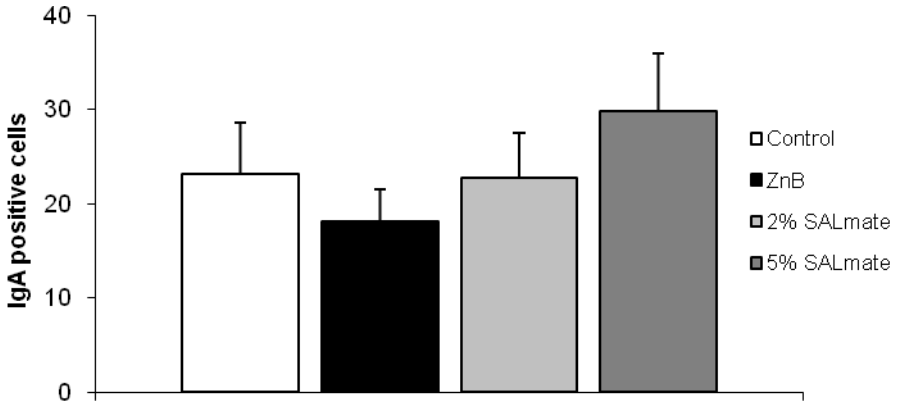


Figure 13. IgA^+ cells in the duodenal mucosa. Data are expressed as mean number of IgA^+ cells ± SEM (n = 6 birds/treatment).

Cytokine expression

The mRNA expression of IL-6, IFN-γ and IL-10 were not significantly different amongst treatment groups ($p>0.05$, Figure 14).

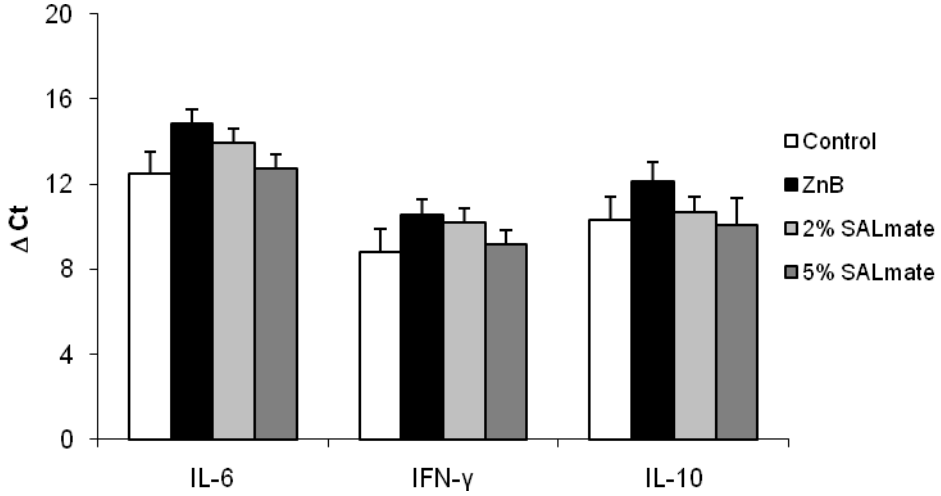


Figure 14. mRNA expression of IL-6, IFN-γ and IL-10 in the caecal tonsils determined using qRT-PCR. Data are expressed as mean (Δ Ct) ± SEM.

Discussion

In this chapter we report that the in-feed supplementation of broiler feed with SALmate™ (an *n*-3 PUFA source) significantly increased plasma and breast *n*-3 levels. Omega-3 PUFA did not influence the overall microbial communities; however, a minor change in the intestinal *Lactobacillus* profile was observed. Significant *n*-3 PUFA-induced shifts in caecal tonsil T and B cell proportions were also evident. The addition of *n*-3 PUFA, however, did not alter broiler performance compared to control diets with and without antibiotics.

The lack of an *n*-3 PUFA-induced improvement in broiler performance in the current study is in contrast to previous reports of the growth-promoting effects of fish oil products. Fish oil at inclusion rates of 2% (+ 6% tallow) and 4% (+ 4% tallow) has been found to increase body weight gain compared to 8% tallow (Lopez-Ferrer et al. 2001). Similar fish oil-induced increases in body weight gain have been reported in other studies (Schreiner et al. 2005). The lack of fish oil-induced performance improvements in the current study may be due to variations in fish oil source and fat composition, dietary fat levels and diet composition.

The inclusion of zinc bacitracin improved the AME values of birds but did not improve key performance indicators such as body weight gain and feed conversion. The dietary inclusion of ZnB, and other in-feed antibiotics, has produced contrasting results in previous studies. ZnB has been reported to increase broiler weight and feed intake, but not improve feed conversion (Abdulrahim et al. 1999), whilst Engberg and colleagues have shown that ZnB improved weight gain whilst not affecting feed efficiency (Engberg et al. 2000). In contrast, Teo and Tan recently reported that ZnB did not improve weight gain or feed intake but significantly lowered feed conversion (Teo and Tan 2007). Our study has indicated that whilst there was an increase in the AME of the birds, this improvement failed to translate into improved growth or production efficiency. Previously, Hooge has suggested that birds under minimal stress may not respond to dietary inclusion of antibiotics (Hooge 2004). The current study, coupled with the results of Chapter 2, partially support this theory. Together, these studies indicate that factors including bird strain, housing conditions, feed composition and type of antibiotic can influence the effects that an in-feed antibiotic can have on the performance of broiler chickens.

The incorporation of dietary-derived *n*-3 PUFA into the tissue of broiler chickens has been documented previously (Hulan et al. 1988; Lopez-Ferrer et al. 2001; Schreiner et al. 2005). Omega-3 PUFA has been comprehensively demonstrated to play an important role in human health; however, human consumption of foods high in *n*-3 (such as fish) is well below recommended levels (Kris-Etherton et al. 2000). The current study supports previous findings as dietary inclusion of SALmate™ significantly elevated EPA, DHA and DPA levels, all highly potent *n*-3 fats. It should be noted

however that the inclusion of high levels of certain fish-derived *n*-3 PUFA may decrease the sensory quality of chicken meat (Schreiner et al. 2005). Therefore, whilst increasing the *n*-3 PUFA in chicken meat may prove beneficial, one must ensure that the sensory quality is not compromised. This was not assessed in the current project.

In addition to the reported SALmate™-induced increases in plasma and breast *n*-3 levels, we observed alterations in the levels of a number of other fatty acids. These shifts were largely isolated to the plasma and were not seen in the breast tissue. However, in the breast tissue, SALmate™ did decrease trans fatty acids; a finding reflected in the plasma. Trans fatty acids are associated with increased inflammation in humans and can increase the likelihood of cardiovascular disease and diabetes (Mozaffarian et al. 2006). The decrease in trans fat is likely to be a result of the lower levels of trans fatty acids in the SALmate™-containing diets. Interestingly, no differences were observed in breast *n*-6 PUFA associated with increased *n*-3 PUFA intake; this is most likely due to the SALmate™ diets having comparable *n*-6 PUFA levels to the control diets. Whilst *n*-6 PUFA levels remained similar, the decrease in *n*-6:*n*-3 PUFA ratio, associated with elevated *n*-3 PUFA levels may be of benefit as these fatty acids compete with one another in key metabolic pathways which can lead to the production of pro-inflammatory compounds (Calder, 2006).

Previously, a small number of studies have reported *n*-3 PUFA-mediated effects on the intestinal microbiota. Fish oil has been demonstrated to decrease bacteroides and increase bifidobacteria in rats (Hekmatdoost et al. 2008). In addition, Kankaanpaa and colleagues have reported that DHA significantly decreased the *in vitro* growth of *Lactobacillus casei* Shirota and reduced the adhesion of *L. casei* Shirota, *Lactobacillus* GG and *L. bulgaricus* (Kankaanpaa et al. 2001); indicating that *n*-3 PUFA may have the capacity to influence the intestinal microbiota *in vivo*. Similar *in vitro* reports of the growth inhibitory properties of *n*-3 PUFA toward bacterial isolates have been reported previously (Thompson and Spiller 1995). The current study indicated that the inclusion of SALmate™ did not significantly influence the overall intestinal microbiota. Minor changes were observed in the *Lactobacillus* species profiles of the caeca between chickens in the Control and 2% SALmate™ treatment groups; however, this was not evident in the 5% SALmate™-fed birds; indicating an inconsistent effect of *n*-3 PUFA. Previously, *n*-3 PUFA have been shown to influence cell membrane composition, adhesion characteristics and growth of lactobacilli (Kankaanpaa et al. 2001; Kankaanpaa et al. 2004); this may influence their capacity to colonise the intestine. These shifts in the *Lactobacillus* species profiles did not convey any performance-enhancing effects in the current study. Concurrent T-RFLP and Lac PCR-DGGE has indicated that *n*-3 PUFA had a minimal effect on the intestinal microbiota of broiler chickens in the current study. This finding, coupled with the microbial results from Chapter 2, demonstrate that different feed additives can have varying effects on the microbiota, but may not necessarily influence bird performance. As expected, dietary supplementation

with prebiotic compounds had a more pronounced effect on the microbiota compared to the addition of *n*-3 PUFA.

Interestingly, the current study indicated that there was little difference in the intestinal microbial profiles between Control and ZnB-fed birds; whereas in Chapter 2, in-feed ZnB significantly altered the overall bacterial communities in the ileum compared to the Control diet. Additionally, numerous studies have indicated that in-feed antibiotics can influence the intestinal bacteria (Engberg et al. 2000; Knarreborg et al. 2002; Wise and Siragusa 2007). These conflicting results in two experiments within the same project, held within six months of each other, may suggest significant flock variation in susceptibility of the intestinal microbial communities to in-feed antibiotics. Together, these studies indicate that more research into the intestinal microbiota of the chicken and the influence of dietary components on microbial composition is needed.

Acetate and total volatile fatty acid production were greater in Control birds compared to birds fed ZnB and 5% SALmate™. This difference in VFA production between controls and ZnB-fed birds was not observed in Chapter 2. In neither study, the caecal microbial communities differed significantly between Control and ZnB-fed birds. These studies highlight the fact that similar microbial compositions can have differing levels of activity, and may indeed influence the bird to varying extents. The inconsistencies in the metabolic activities of the microbiota indicate another level of complexity in understanding the microbial communities of the intestine, and using dietary manipulations to consistently impart a desired effect.

The immunoregulatory and anti-inflammatory properties of *n*-3 PUFA in animals and humans are well documented (Alexander 1998; Calder and Grimble 2002; Shaikh and Edidin 2006). *n*-3 PUFA have been demonstrated to alter immune cell membrane fatty acid composition by competing with, and replacing arachidonic acid. Increased membrane *n*-3 fatty acid proportions may influence membrane fluidity, production of cell signalling molecules, expression of membrane proteins, and eicosanoid sub-type synthesis, producing derivatives which are less potent than those derived from arachidonic acid. Previous studies have described *n*-3 PUFA-induced changes in the expression of various cell surface molecules (Sanderson et al. 1997; Calder 2007). In the current study significantly lower proportions of $MHCII^+$ cells were observed in *n*-3 PUFA-treated birds, with a strong trend toward lower $CD45^+$ cells. Interestingly, the PUFA-induced decrease in $MHCII^+$ cells was only observed in 2% SALmate™-treated birds, not birds fed the higher level (5%), possibly indicating that the dosage is critical in mediating these observed effects. Previously, Sanderson and colleagues (1997) described fish oil-induced decreases in MHCII expression in rats, which is consistent with the results obtained from chickens in the current study. This lower proportion of $MHCII^+$ antigen presenting cells may facilitate decreased antigen presentation to $CD4^+$ T cells and thus, lower T helper cell activation. This

may also be reflected in the trend towards a decrease in the proportion of $CD45^+$ cells, as CD45 is a signal transduction molecule critical for T cell activation (Ledbetter et al. 1993; Hermiston et al. 2003). Overall, the low proportions of these phenotypes may contribute to low immune responsiveness in the CT. Interestingly, CT IgM^+ and IgG^+ cell proportions were maintained whilst IgA^+ cell proportion was significantly increased in the CT of PUFA-treated birds. However, circulating IgM, IgG and IgA were not significantly influenced. Previously, fish oil-induced increases in circulating plasma IgA, and IgA levels in intestinal flushings have been reported in rats (Lin et al. 2007). Overall, the results of the current study indicate that whilst *n*-3 PUFA may suppress the cells contributing to CT cell mediated immunity in broilers; it does not appear to influence the humoral arm of acquired immunity. To further characterise the potential benefits of *n*-3 PUFA to the immune system, a study involving an immunological challenge is required.

The immune status of birds fed Control and ZnB diets in the current study was consistent with that observed in Chapter 2, indicating a repeatable effect of these diets on the immune status of the birds. The only observed differences in the responses of birds between the two studies were a significantly lower $Bu1^+$ cell proportion in ZnB-fed birds in the current study, which was not observed in Chapter 2, and increased plasma IgM and IgG titres in birds fed ZnB compared to Control in Chapter 2, which was not evident in Chapter 3.

In summary, *n*-3 PUFA increased the incorporation of EPA, DHA and DPA in the breast tissue and influenced immune cell proportions in the CT; however *n*-3 PUFA had little impact on the intestinal microbiota, and did not influence bird performance. Omega-3 PUFA may be beneficial as a natural feed additive with immunomodulatory effects in broilers and human health-promoting effects due to increased *n*-3 levels of chicken meat. However, it appears unlikely that *n*-3 PUFA supplementation alone could replicate the effects of in-feed antibiotics.

Chapter 4. The impact of lactoferrin on performance, mucosal immunity, intestinal microarchitecture and gut microbial composition in broiler chickens

Introduction

Lactoferrin (Lf) is an 80kD iron-binding glycoprotein from the transferrin family, derived from a number of sources including exocrine secretions, neutrophils and blood (Fischer et al. 2006; Pan et al. 2007). Lactoferrin has been investigated as a treatment for a range of infectious and inflammatory conditions in both humans and animals, largely due to its immunoregulatory, antibacterial, antifungal and antiviral activities (Fischer et al. 2006; Pan et al. 2007). Dietary supplementation with lactoferrin has been shown to improve performance of agricultural animals including poultry (Humphrey et al. 2002) and pigs (Wang et al. 2006).

The current study investigated the potential for Lf to influence the intestinal microbiota, mucin composition, intestinal microarchitecture and immune status. Additionally, we examined the impact of any Lf-induced changes on broiler growth and performance. Previous studies in this project have indicated a lack of a performance effect amongst dietary treatments (including ZnB vs. Control) during the AME period. Therefore, as part of this chapter we also performed a small-scale growth experiment, in addition to an AME bioassay, in order to study the effects of these dietary additives under conditions more similar to industry practice.

Materials and Methods

Birds, management and diet

Male Cobb 500 broiler chickens (Baiada Hatchery, Willaston, SA, Australia) were raised in rearing pens in a temperature-controlled room. All diets were based on a commercial starter diet (Ridley Agriproducts Pty Ltd, Murray Bridge, SA, Australia) without any added antibiotics or coccidiostats (Table 23). All diets used in this study met or exceeded the National Research Council guidelines for broiler chickens (NRC 1994). All procedures were approved by the Animal Ethics Committees of the University of Adelaide and Primary Industries and Resources South Australia.

The four experimental groups were; Control (basic diet with no additives), ZnB (basic diet + 50 ppm zinc bacitracin), Lf 250 mg/kg (basic diet with 250 mg/kg Lf; bovine lactoferrin, Australia's Own Pty Ltd, The Entrance, NSW, Australia), and Lf 500 mg/kg (basic diet with 500 mg/kg Lf; Table 23). All

birds received these experimental diets from hatch. Titanium dioxide (5 g/kg) was added to the basal diet as a digestibility marker for the energy metabolism phase of the experiment. All birds had free access to feed and water at all times throughout the experiment.

Table 23. Experimental diets for chickens prior to (0-15 days), and during (15-25 days) the apparent metabolisable energy (AME) study.

Ingredient (g/kg)	Control	ZnB	Lf 250 mg/kg	Lf 500 mg/kg
		Period 0 – 15 days of age		
Starter crumble[1]	1000	999.7	999.75	999.5
Zinc bacitracin (50 ppm active ingredient)	-	0.3	-	-
Lactoferrin	-	-	0.25	0.5
		Period 15 – 25 days of age		
Starter crumble[1]	995	994.7	994.75	994.5
Zinc bacitracin	-	0.3	-	-
Lactoferrin	-	-	0.25	0.5
Titanium dioxide	5.0	5.0	5.0	5.0

[1] Starter crumble comprises (in g/kg): wheat (638.9), hammer-milled wheat (8.0), oats (50.0), peas (60.0), meat meal (100.0), blood meal (10.7), tallow (10.0), solvent extracted soybean meal (93.7), ground oatmeal flour (16.7), monocalcium phosphate (3.75), dicalcium phosphate dehydrate (1.09), choline chloride 75% (0.5), L-lysine HCl (2.14), Alimet (2.57). Vitamin and mineral mixes exceeded NRC standards.

Growth study

Newly-hatched chickens (240) were placed into 24 pens (n = 10 birds/pen), with each dietary treatment replicated six times in a randomised block design. Body weight and feed intake were recorded at placement and on days 7, 14, 21, 28, 35 and at the end of the trial period (day 39). Body weight gain, feed intake and feed conversion ratios were calculated for each period, and for the overall duration of the experiment. At day 23 post-hatch, 96 birds (n = 24 birds/treatment group) were removed from the pens and transferred into single bird metabolism cages for the apparent metabolisable energy study. To reduce any stress associated with isolation, the birds were placed in pairs for a three-day acclimation period, at which time all birds were placed in individual cages.

Apparent metabolisable energy study

The apparent metabolisable energy values of the diets were determined in a seven-day period between days 25-32 post-hatch. The first three-day period allowed time for acclimation of birds to isolation. Body weight was recorded at the beginning and end of the seven-day period. Feed intake was measured during the initial three-day acclimation period and over the subsequent four-day period. During the four-day period, excreta was collected daily and dried at 90°C for determination of excreta moisture content. Dry matter (DM) contents of samples of feeds were measured. Gross energy (GE in MJ/kg) values of feed, dried excreta, and freeze-dried ileal digesta were measured by bomb calorimetery (Parr Instrument Company, Moline, IL, USA). The AME values (in MJ/kg dry matter basis) were calculated as follows;

AME_{diet} = [(GE_{diet} x g feed consumed) - ($GE_{excreta}$ x g dry excreta)] /g feed consumed /DM feed

At 32 days post-hatch, blood was collected from 48 birds (n = 12 birds/treatment) by venipuncture of the brachial vein and transferred into lithium heparin-coated vacutainers, then kept at 4^{o}C until centrifuged (1000 *g*) and plasma collected and frozen for immunological assays. At 36 days post-hatch all birds were killed. One bird each from the Control and 250 mg/kg Lf groups were culled during the experiment due to poor health. From all birds, one caecum and a 3 cm section of tissue and associated digesta from the midpoint of the ileum was collected and stored at 4^{o}C until frozen. Samples were freeze-dried for characterisation of bacterial communities by the T-RFLP and Lac PCR-DGGE techniques. Both CTs and the spleen were collected, washed with cold PBS, and placed into sterile 5 ml tubes containing 3 ml Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin for cell phenotyping by flow cytometry (n = 12 birds/treatment). Additionally, one caecum was collected, its contents emptied into a 5 ml tube and H_2SO_4 (0.5 M) was added at a rate of 0.5 ml per 1 g of caecal content for analysis of VFAs (n = 12 birds/treatment). Segments (1 cm) of the intestine from the duodenum, jejunum and ileum were collected into 10% buffered formalin for histological analyses and mucin staining (n = 12 birds/treatment). Further segments (1 cm) were collected from the duodenum, jejunum and ileum, rinsed in PBS and stored in a 5 ml tube containing RNAlater for gene expression analysis (Chapter 6). From all birds, the remaining digesta in the terminal ileum was collected and kept at 4^{o}C until frozen, then later freeze-dried for assessment of ileal digestibility by measurement of titanium dioxide concentrations. Ileal digestible energy (IDE) was measured by the technique described in Chapter 2.

Histology and mucin analysis

Following a 24 hour fixation in 10% buffered formalin; segments were stored in 70% ethanol and then routinely processed and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin

and eosin for quantitative analysis of crypt depth and villus height. For all measurements, 20 villi and crypts were selected at random from each bird. Image analysis was performed using an Olympus light microscope and digital camera (Tokyo, Japan) and the AnalySIS image analysis program (AnalySIS 5, Olympus, Tokyo, Japan).

The periodic acid Schiff (PAS)/alcian blue (AB) staining technique was used to visualise goblet cells containing mucins with neutral (PAS) and acidic (AB) glycoproteins. Briefly, sections (4 μm) were de-paraffinised in histolene for 10 minutes, then re-hydrated in 100%, 80%, 30% and 0% ethanol for 2 minutes each. Slides were stained with periodic acid (Sigma, Castle Hill, NSW, Australia) for 20 minutes. Sections were then rinsed with running water for 3 minutes and subsequently stained with Schiff's reagent (Sigma) for 20 minutes. Excess Schiff's reagent was removed with running water and then sections were counter-stained with Alcian blue pH 2.5 (Sigma) for 30 minutes. Sections were rapidly immersed in 3% acetic acid twice and rinsed with running water. Sections were then dehydrated with graded ethanol and histolene. Cells were identified as being either; PAS^+ (pink), indicative of mucins comprising neutral sugars, AB^+ (blue), indicative of mucins comprising acidic sugars, or PAS^+AB^+ (purple/mixture) indicative of intermediate mucins which produce both acidic and neutral sugars. Data were expressed as number of positive cells/crypt area (mm^2). Ten villi were selected at random for analysis for each bird.

Microbial profiling

Terminal-restriction fragment length polymorphism

T-RFLP analysis was performed on samples from the jejunum and caecum following the technique described previously in Chapter 2, and by Torok et al. (2008).

Denaturing gradient gel electrophoresis

Characterisation of *Lactobacillus* species was performed using the Lac PCR-DGGE technique based on methods outlined in Chapters 1 and 2, and by Walter et al. (2001).

Volatile fatty acid analysis

Volatile fatty acids were analysed based on the techniques described in Chapter 2.

Immunological analysis

Isolation of leucocytes from caecal tonsil and spleen

Caecal tonsil and spleen were harvested, immediately placed in ice-cold FACS buffer (PBS containing 2% FCS and 0.1% sodium azide, NaN_3), and processed for surface marker staining. Briefly, cells from CT and spleen were separated by passing through a 70 μm nylon cell strainer to make single cell suspensions. The sieved spleen cell suspension was then layered over Lymphoprep (Axis-Shield) in a ratio of 3:4 and centrifuged at 1100 *g* for 15 minutes to separate red blood cells from leucocytes. The leucocyte layer was removed and washed by centrifugation (400 *g* for 5 minutes) in FACS buffer. The supernatant was discarded and the leucocyte pellet re-suspended in FACS buffer at 2~3 x 10^6 cells/ml.

Following mouse anti-chicken monoclonal antibodies against avian cell surface antigens were used; CD3 (clone CT-3), CD4 (clone CT-4), CD8α (clone 3-298), MHC I (clone F21-2) MHC II (clone 2G11), Bu-1a (clone 21-1A4), CD44 (AV6), CD45 (LT40), TCRγδ (clone TCR1), TCRαβ/Vb1 (clone TCR2), TCRαβ/Vb2 (clone TCR3) and mouse anti-chicken monocyte/macrophage (clone KUL01) (Southern Biotech, Birmingham AL, USA). Streptavidin-allophycocyanin (BD Pharmingen, San Diego, CA, USA) was used to detect the biotin-conjugated antibody.

Phenotypic characterisation of leucocytes by flow cytometry

T cell populations from CT and spleen were stained with different cell markers and analysed by flow cytometer. Briefly, 2 x 10^5 cells were incubated with the appropriate monoclonal antibodies at 4°C for 20 minutes washed twice in FACS buffer. Where required, a secondary antibody incubation step was performed in FACS buffer as above. All samples were analysed on a FACScalibur flow cytometer (Becton Dickinson, Sunnyvale, CA, USA), with a minimum of 5 × 10^4 cells analysed for each sample. Analysis of data was performed using CELLquest software (Becton Dickinson, Sunnyvale, CA, USA).

Plasma immunoglobulin titres

Plasma immunoglobulin titres were determined using the procedures outlined in Chapter 2.

Statistical analyses

Performance data from the growth study and AME study were analysed with the SAS for Windows version 9.1 software package (SAS Institute Inc., Cary, NC, USA). Analysis of variance using the General Linear Model (GLM) procedure was used to compare parameters with differences between

diets determined by Duncan's Multiple Range Test. Goblet cell numbers, immunological data and volatile fatty acid profiles were compared by Kruskal-Wallis ANOVA. Comparison of intestinal microbial communities (T-RFLP and Lac PCR-DGGE) was performed using the techniques described in Chapter 2. DGGE profiles were compared qualitatively using Dice's similarity coefficient (Dsc) based on the UPGMA (unweighted pair group methods using arithmetic averages) clustering algorithm using the BioNumerics software package (Applied Maths, Austin, TX, USA). For qualitative comparisons, band search filters, optimisation and position tolerance of 10%, 1% and 1%, respectively, were used.

Results

Growth study

At the commencement of the trial, newly-hatched chickens allocated to the ZnB group had a lower body weight compared to the Lf 250 mg/kg and Lf 500 mg/kg fed birds ($p<0.05$; Table 24). Body weights at days 7, 14, 21, 23, 28, 35, and at the completion of the trial (day 39) were not significantly different amongst any treatment group at any time point ($p>0.05$; Table 24 and data not shown). Additionally, body weight gain, feed intake and feed conversion ratio did not differ amongst any treatment group at any stage of the trial period ($p>0.05$).

Table 24. Performance parameters from chickens prior to removal for metabolism experiment.

	BW day 0 (g)	**BW day 21 (g)**	**BWG (g)**	**FI (g/bird)**	**FCR**
Control	46.9 ± 0.5ab	792 ± 18	745 ± 18	1229 ± 46	1.65 ± 0.03
ZnB	46.2 ± 0.3a	794 ± 15	748 ± 15	1242 ± 32	1.66 ± 0.04
Lf 250mg/kg	47.7 ± 0.6b	783 ± 19	735 ± 19	1159 ± 39	1.58 ± 0.04
Lf 500mg/kg	48.3 ± 0.2b	793 ± 7	744 ± 7	1197 ± 34	1.61 ± 0.04

Body weight (BW) and body weight gain (BWG) data are expressed as mean (g) ± SEM. Feed intake (FI) data are expressed as mean (g/bird/day) ± SEM. Feed conversion ratio (FCR) is expressed as feed intake/body weight gain during the AME period ± SEM. Values within a column that do not share a common letter a significantly different ($p<0.05$)

Apparent metabolisable energy study

At the commencement of the seven-day AME study, no body weight differences were observed amongst treatment groups ($p>0.05$; Table 25). During the AME period, no differences in body weight

gain, feed intake, feed conversion ratio, AME or ileal digestible energy values were observed amongst treatment groups ($p>0.05$; Table 25).

Table 25. Performance parameters from chickens following the seven-day apparent metabolisable energy (AME) study period.

	BWS (g)	BWG (g)	FI (g/bird/day)	FCR	AME (MJ/kg)	IDE (MJ/kg)
Control	1110 ± 21	641 ± 12	153 ± 2.3	1.68 ± 0.02	14.02 ± 0.10	12.95 ± 0.36
ZnB	1104 ± 23	643 ± 15	153 ± 2.3	1.68 ± 0.03	13.99 ± 0.10	12.99 ± 0.37
Lf 250 mg/kg	1092 ± 25	645 ± 13	157 ± 2.9	1.70 ± 0.02	14.33 ± 0.13	13.58 ± 0.36
Lf 500 mg/kg	1137 ± 21	655 ± 12	157 ± 2.4	1.69 ± 0.02	14.03 ± 0.11	12.86 ± 0.43

Body weight start (BWS) and body weight gain (BWG) data are expressed as mean (g) ± SEM. Feed intake (FI) data are expressed as mean (g/bird/day) ± SEM. Feed conversion ratio (FCR) is expressed as feed intake/body weight gain during the AME period ± SEM. Apparent metabolisable energy (AME) is expressed as mean (MJ energy/kg dry matter basis) ± SEM. Ileal digestible energy (IDE) is expressed as mean (MJ energy/kg dry matter basis) ± SEM.

Morphometric analyses and goblet cell mucin compositions

Villus height, crypt depth and villus to crypt ratio were determined in the ileum. No significant differences were observed for any parameter amongst treatment groups ($p>0.05$; Table 26). The proportions of goblet cells producing acidic, intermediate or neutral mucins were also not significantly different amongst treatments ($p>0.05$; Table 26); however, there was an observable trend toward an increase in acidic mucin producing goblet cells in lactoferrin-treated birds ($p<0.1$).

Table 26. Ileum morphometry and goblet cell analysis.

	Morphometry		Goblet Cell Type		
	Villus Height (μm)	**Crypt Depth (μm)**	**Acidic**	**Intermediate**	**Neutral**
Control	773 ± 22	171 ± 4	252 ± 24	821 ± 46	207 ± 25
ZnB	796 ± 32	174 ± 5	266 ± 30	881 ± 88	177 ± 25
Lf 250 mg/kg	795 ± 33	172 ± 9	329 ± 44	922 ± 109	171 ± 34
Lf 500 mg/kg	814 ± 39	170 ± 8	392 ± 57	919 ± 56	135 ± 29

Ileum villus height and crypt depth data are expressed as mean (μm) ± SEM. Acidic, intermediate and neutral mucin producing goblet cells are expressed as mean number of cells/epithelial area (mm^2).

Microbial profiles

Terminal-restriction fragment length polymorphism analysis

Overall intestinal bacterial communities in the ileum and caecum were characterised by T-RFLP analysis. No significant changes in the microbial profiles of the ileum were observed amongst treatment groups ($p>0.05$; Table 27). In the caecum, birds fed ZnB had a significantly different bacterial profile compared to Control-fed birds, and birds fed Lf 250 mg/kg ($p<0.05$; Table 27). In addition, significantly different caecal microbial profiles were observed between birds fed Lf 250 mg/kg and Lf 500 mg/kg ($p<0.05$).

Table 27. One-way analysis of similarities (ANOSIM) of ileal and caecal microbial communities.

Ileum	Control	ZnB	Lf 250 mg/kg	Lf 500 mg/kg
Control	-	**-0.02**	**-0.004**	**-0.036**
ZnB	*0.72*	-	**0.008**	**0.001**
Lf 250 mg/kg	*0.48*	*0.31*	-	**-0.004**
Lf 500 mg/kg	*0.94*	*0.42*	*0.44*	-
Caecum	**Control**	**ZnB**	**Lf 250 mg/kg**	**Lf 500 mg/kg**
Control	-	**0.069**	**0.001**	**0.005**
ZnB	*0.03*	-	**0.099**	**0.027**
Lf 250 mg/kg	*0.42*	*0.006*	-	**0.058**
Lf 500 mg/kg	*0.33*	*0.17*	*0.05*	-

Data are expressed as the R-statistic (bold), with significance level in italics. For all analyses a significance level of 0.05 was considered significant. For ileal microbial communities the global R-value was -0.009 at a significance level of 0.067 which is not significant. For caecal microbial communities the global R-value was 0.043 at a significance level of 0.02 which is considered significant.

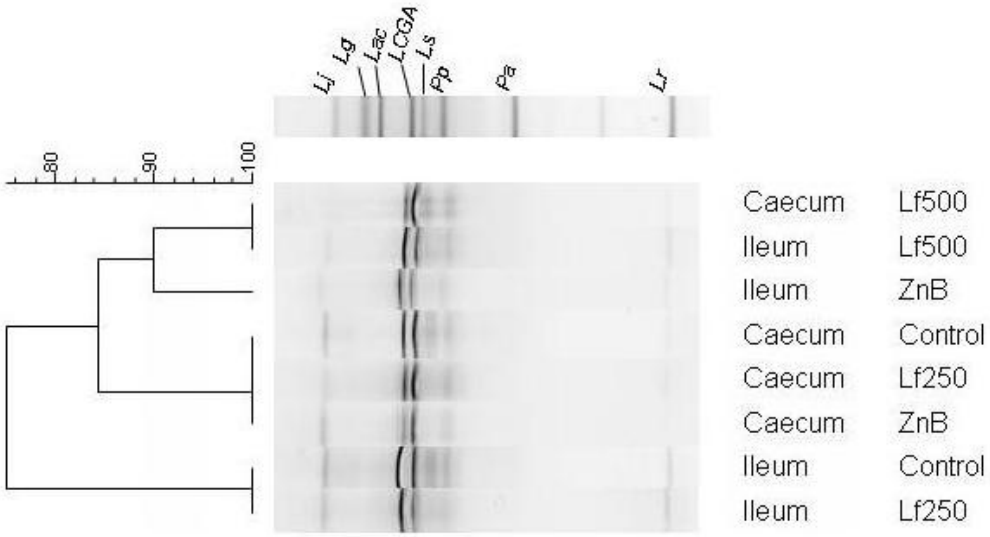
Denaturing gradient gel electrophoresis

The Lac PCR-DGGE profiles from the pooled samples were grouped into three clusters (Figure 15a) that all contained LCGA and *L. salivarius.* The clusters containing the pooled caecal samples from Control, Lf 250 mg/kg and ZnB-fed birds and ileal samples from Control and Lf 250 mg/kg fed birds also contained detectable *L. johnsonii* bands. *L. reuteri* was detected in the pooled samples from the ileum of Control and Lf 250 mg/kg fed birds. The relative intensity of the LCGA and *L. salivarius* samples suggested that these species dominated the *Lactobacillus* microflora in all diets.

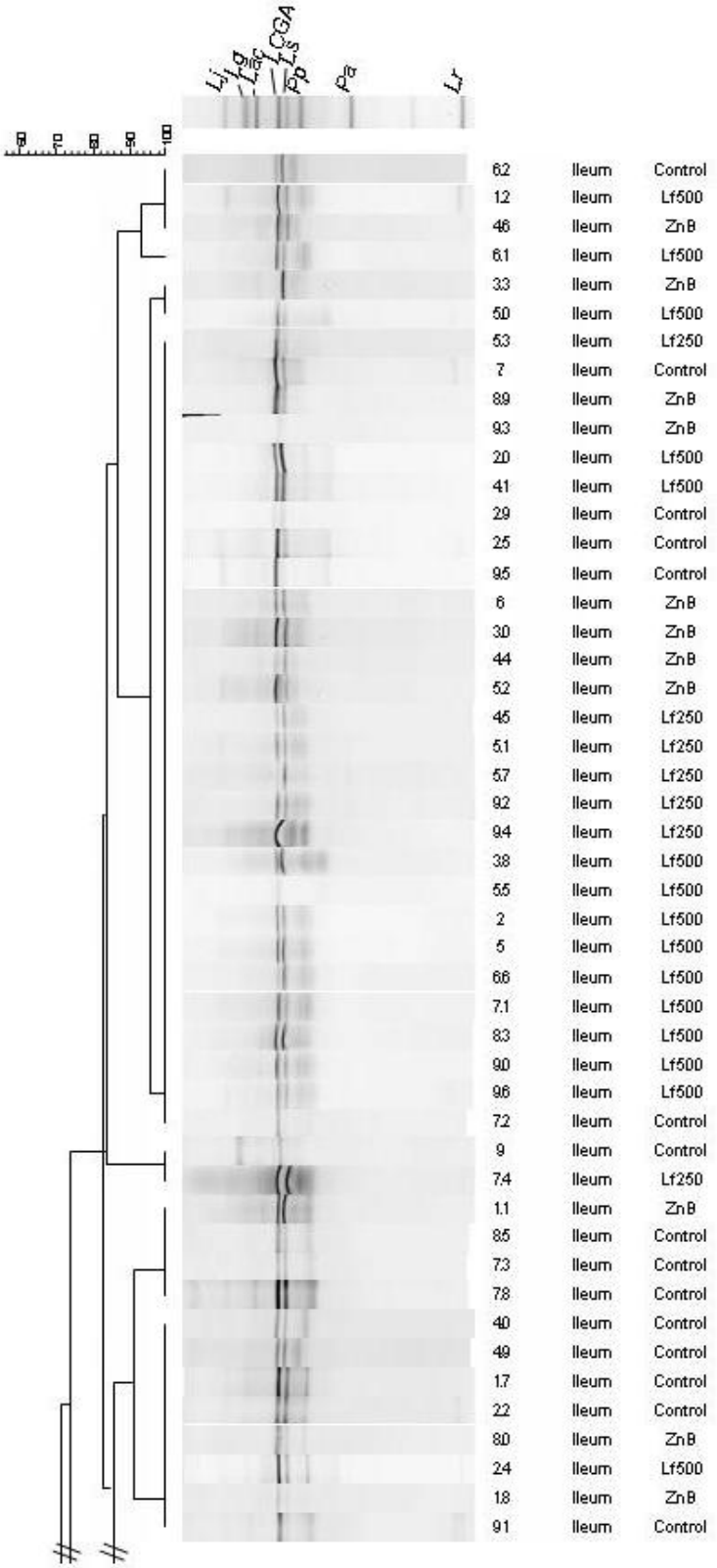
In general, the Lac DGGE profiles of individual ileal and caecal samples were comprised of three different profile types (Figure 15b and c). Approximately half of the samples were characterised by a very similar, simple profile comprised of LCGA and *L. salivarius* bands consistent with the pooled samples. A number of these profiles also contained low intensity diffuse bands either migrating to a similar position as *P. pentosaceous* and/or migrating between *P. pentosaceous* and *P. acidilactici.* Birds from all diets were represented in these simple profiles with the majority of Control birds being represented (16/24 ileal and caecal samples). Relatively few birds contained detectable *L. johnsonii* and/or *L. reuteri* bands (less than 22 and 20 caecal and ileal profiles out of 94 birds, respectively), and the remaining profiles were dominated by LCGA or *L. salivarius* with few other bands present. Birds from all four diets were also represented in these two profile types. Future in-depth analysis of the

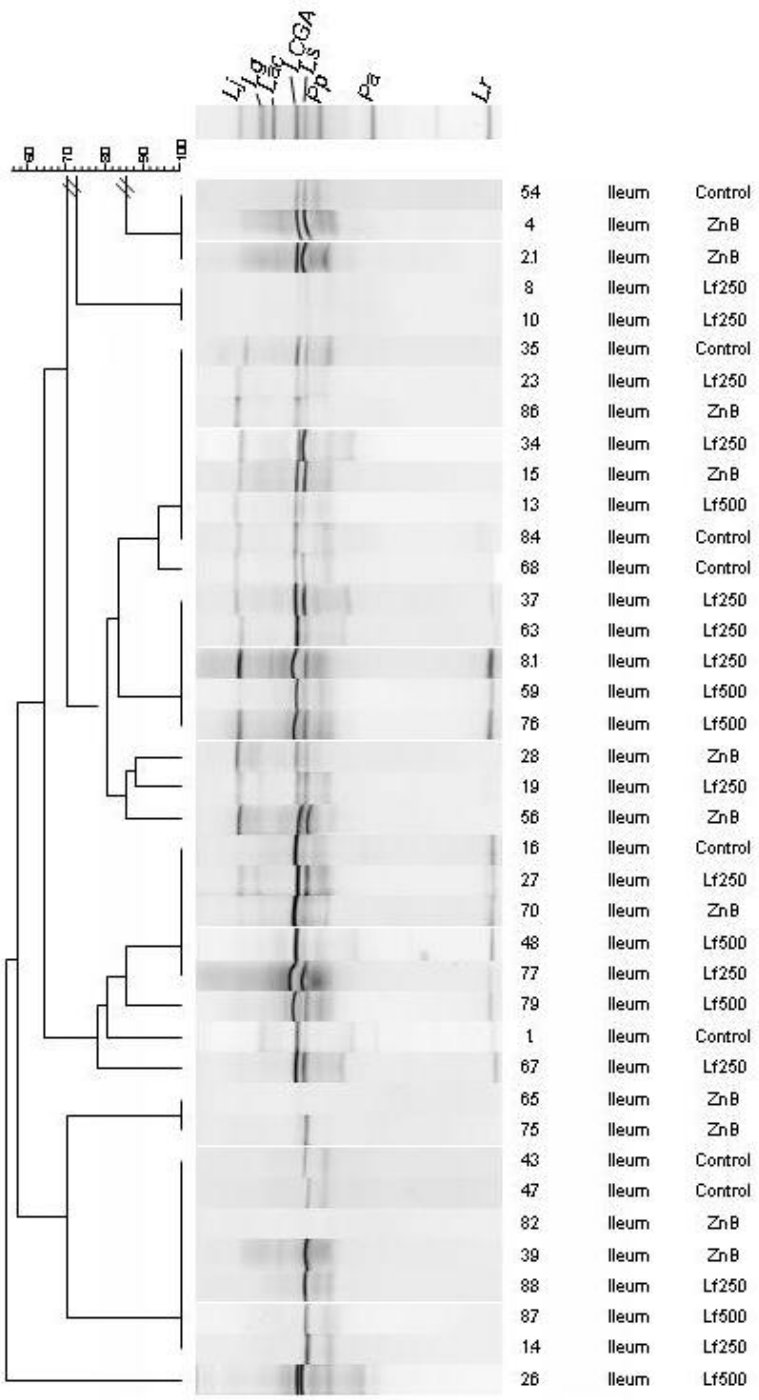
profiles of individual birds may determine if these variable species and bands are related to dietary treatment.

a)



b)


Lj_LG
Lac_LGA
Lp_LS
Pa
Lr
100
80
60
40
20
0
62 Ileum Control
12 Ileum Lf500
46 Ileum ZnB
61 Ileum Lf500
33 Ileum ZnB
50 Ileum Lf500
53 Ileum Lf250
7 Ileum Control
89 Ileum ZnB
93 Ileum ZnB
20 Ileum Lf500
41 Ileum Lf500
29 Ileum Control
25 Ileum Control
95 Ileum Control
6 Ileum ZnB
30 Ileum ZnB
44 Ileum ZnB
52 Ileum ZnB
45 Ileum Lf250
51 Ileum Lf250
57 Ileum Lf250
92 Ileum Lf250
94 Ileum Lf250
38 Ileum Lf500
55 Ileum Lf500
2 Ileum Lf500
5 Ileum Lf500
66 Ileum Lf500
71 Ileum Lf500
83 Ileum Lf500
90 Ileum Lf500
96 Ileum Lf500
72 Ileum Control
9 Ileum Control
74 Ileum Lf250
11 Ileum ZnB
85 Ileum Control
73 Ileum Control
78 Ileum Control
40 Ileum Control
49 Ileum Control
17 Ileum Control
22 Ileum Control
80 Ileum ZnB
24 Ileum Lf500
18 Ileum ZnB
91 Ileum Control


Lj-g
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54 Ileum Control
4 Ileum ZnB
21 Ileum ZnB
8 Ileum Lf250
10 Ileum Lf250
35 Ileum Control
23 Ileum Lf250
86 Ileum ZnB
34 Ileum Lf250
15 Ileum ZnB
13 Ileum Lf500
84 Ileum Control
68 Ileum Control
37 Ileum Lf250
63 Ileum Lf250
81 Ileum Lf250
59 Ileum Lf500
76 Ileum Lf500
28 Ileum ZnB
19 Ileum Lf250
56 Ileum ZnB
16 Ileum Control
27 Ileum Lf250
70 Ileum ZnB
48 Ileum Lf500
77 Ileum Lf250
79 Ileum Lf500
1 Ileum Control
67 Ileum Lf250
65 Ileum ZnB
75 Ileum ZnB
43 Ileum Control
47 Ileum Control
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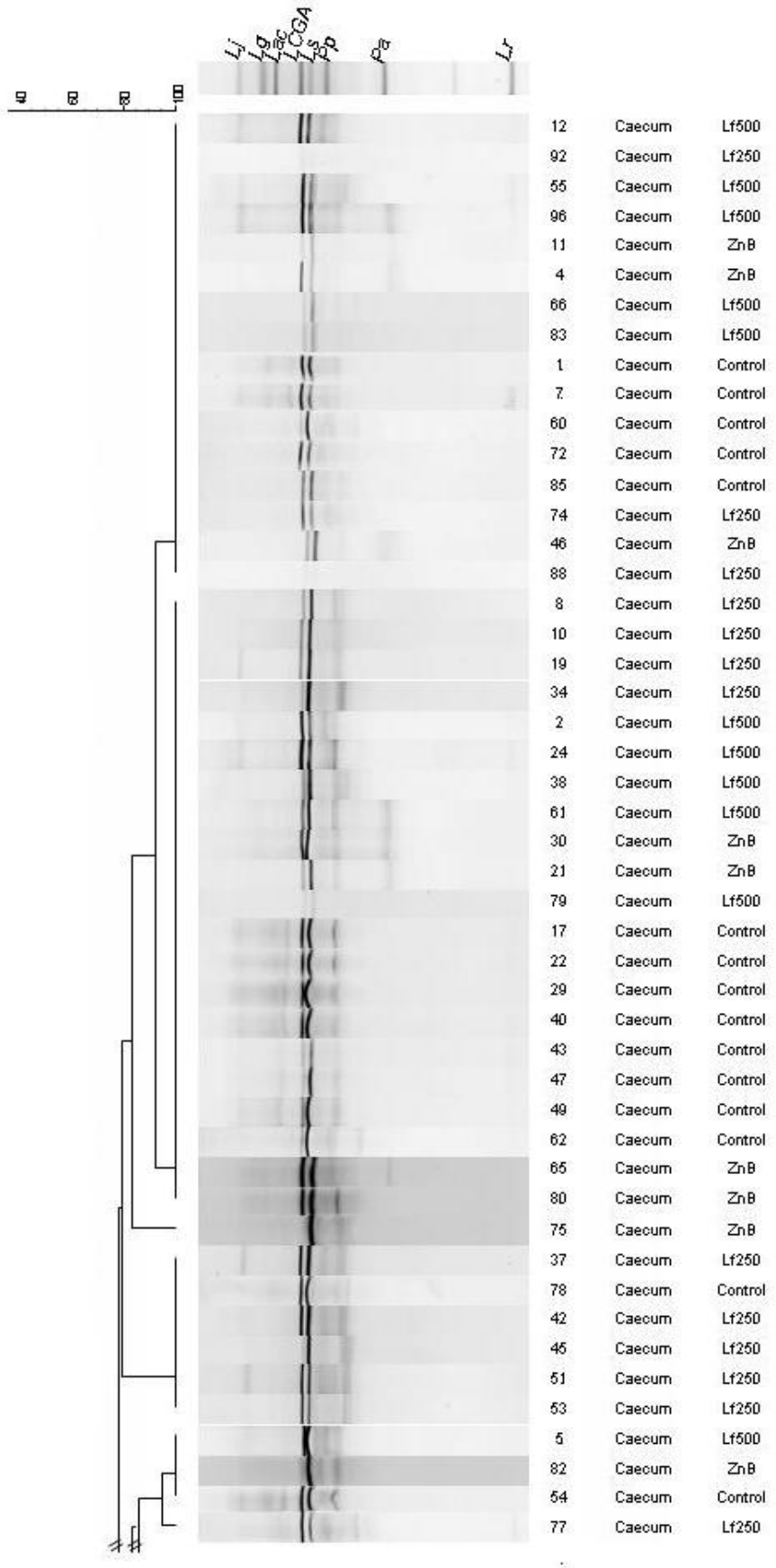
39 Ileum ZnB
88 Ileum Lf250
87 Ileum Lf500
14 Ileum Lf250
26 Ileum Lf500

c)


Lj
Lg
LacCGA
Ls
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12 Caecum Lf500
92 Caecum Lf250
55 Caecum Lf500
96 Caecum Lf500
11 Caecum ZnB
4 Caecum ZnB
66 Caecum Lf500
83 Caecum Lf500
1 Caecum Control
7 Caecum Control
60 Caecum Control
72 Caecum Control
85 Caecum Control
74 Caecum Lf250
46 Caecum ZnB
88 Caecum Lf250
8 Caecum Lf250
10 Caecum Lf250
19 Caecum Lf250
34 Caecum Lf250
2 Caecum Lf500
24 Caecum Lf500
38 Caecum Lf500
61 Caecum Lf500
30 Caecum ZnB
21 Caecum ZnB
79 Caecum Lf500
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22 Caecum Control
29 Caecum Control
40 Caecum Control
43 Caecum Control
47 Caecum Control
49 Caecum Control
62 Caecum Control
65 Caecum ZnB
80 Caecum ZnB
75 Caecum ZnB
37 Caecum Lf250
78 Caecum Control
42 Caecum Lf250
45 Caecum Lf250
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53 Caecum Lf250
5 Caecum Lf500
82 Caecum ZnB
54 Caecum Control
77 Caecum Lf250

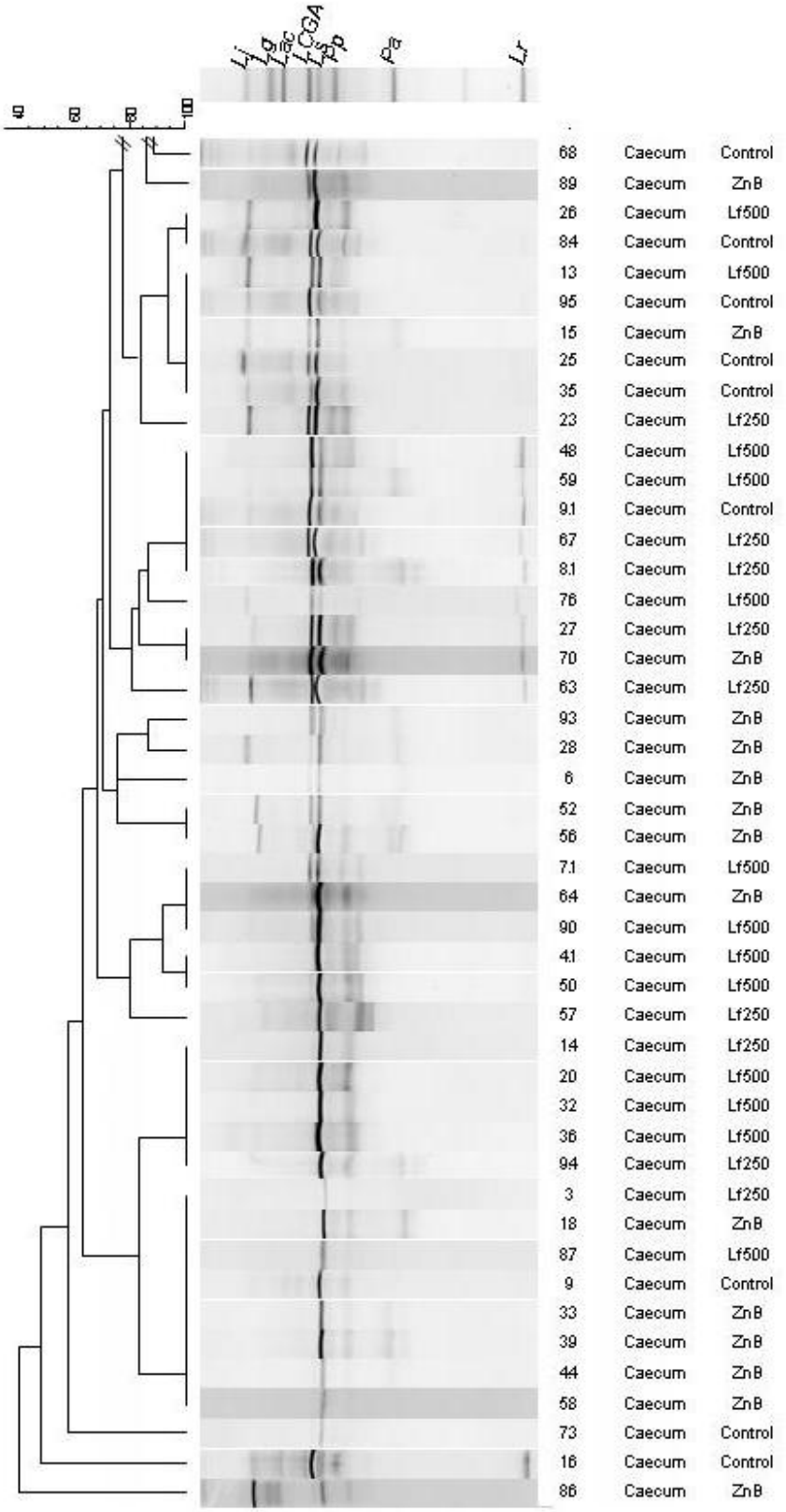

Li
Lg
Lac
LCGA
Lsp
Pa
Lr
68 Caecum Control
89 Caecum ZnB
26 Caecum Lf500
84 Caecum Control
13 Caecum Lf500
95 Caecum Control
15 Caecum ZnB
25 Caecum Control
35 Caecum Control
23 Caecum Lf250
48 Caecum Lf500
59 Caecum Lf500
91 Caecum Control
67 Caecum Lf250
81 Caecum Lf250
76 Caecum Lf500
27 Caecum Lf250
70 Caecum ZnB
63 Caecum Lf250
93 Caecum ZnB
28 Caecum ZnB
6 Caecum ZnB
52 Caecum ZnB
56 Caecum ZnB
71 Caecum Lf500
64 Caecum ZnB
90 Caecum Lf500
41 Caecum Lf500
50 Caecum Lf500
57 Caecum Lf250
14 Caecum Lf250
20 Caecum Lf500
32 Caecum Lf500
36 Caecum Lf500
94 Caecum Lf250
3 Caecum Lf250
18 Caecum ZnB
87 Caecum Lf500
9 Caecum Control
33 Caecum ZnB
39 Caecum ZnB
44 Caecum ZnB
58 Caecum ZnB
73 Caecum Control
16 Caecum Control
86 Caecum ZnB

Figure 15. Lac PCR-DGGE profiles of pooled (a) and individual samples from the ileum (b) and caecum (c) using Dice's Similarity indicated by the scale at the top left. The marker lane at the top of the figure contains the identification ladder comprised of Lac PCR products from the following reference strains: *Lj, L. johnsonii* ATCC 33200; *Lg*, *L. gasseri* ATCC 33323; *Lac*, *L. acidophilus* ATCC 4356; *Ls, L. salivarius* subsp. *salivarius* ATCC 11741; *Pp*, *Pediococcus pentosaceous* ATCC 43200; *Pa*, *Pediococcus acidilactici* ATCC 8042; and *Lr, L. reuteri* ATCC 23272. *LCGA* marks the migration of *L. crispatus, L. gallinarum* and *L. amylovorous* species, and is represented by *L. crispatus* ATCC 33820.

Volatile fatty acid concentrations

No differences were observed in the concentrations of volatile fatty acids amongst treatment groups ($p>0.05$; Table 28). The molar percentages of propionic acid were significantly lower in birds fed Lf 250 mg/kg compared to ZnB-fed birds and birds fed Lf 500 mg/kg ($p<0.05$; data not shown).

Table 28. Volatile fatty acid concentrations of caecal contents.

	Acetic	Propionic	Butyric	Iso-butyric	Valeric	Iso-valeric	Iso-caproic	Total
Control	78.68 ± 14.92	6.93 ± 2.02	18.73 ± 3.66	0.38 ± 0.27	1.28 ± 0.32	0.60 ± 0.22	0.02 ± 0.02	106.63 ± 19.88
ZnB	92.05 ± 12.06	6.53 ± 0.94	25.56 ± 3.54	0.0 ± 0.0	1.54 ± 0.17	0.42 ± 0.18	0.0 ± 0.0	126.09 ± 16.11
Lf 250 mg/kg	109.28 ± 9.85	6.04 ± 1.51	29.97 ± 3.69	0.47 ± 0.31	1.79 ± 0.31	0.38 ± 0.26	0.06 ± 0.06	147.98 ± 14.10
Lf 500 mg/kg	91.25 ± 11.65	8.45 ± 1.23	25.53 ± 3.97	0.0 ± 0.0	2.29 ± 0.36	0.81 ± 0.22	0.0 ± 0.0	128.33 ± 16.20

Data are expressed as mean (μMol/ml) ± SEM.

Immunological analysis

Cell phenotypes

Phenotypic analysis of spleen lymphocytes indicated that birds fed the Control diet had a lower proportion of $CD8^+$ cells compared to birds fed ZnB or lactoferrin at either dose ($p<0.05$; Table 29). In the CT, birds fed lactoferrin had a higher proportion of $CD4^+$ cells compared to Control-fed birds ($p<0.05$; Table 30).

Table 29. Phenotypic analysis of spleen cells.

	TCR1	TCR2	TCR3	MHCI	MHCII	Kul-1	CD3	CD4	CD8	CD44	CD45	Bu1
Control	17.7 ± 1.7	19.5 ± 1.5	0.9 ± 0.1	88.4 ± 1.3	54.7 ± 2.9	50.8 ± 2.3	40.4 ± 1.7	9.2 ± 0.4	19.3 ± 0.7a	71.0 ± 2.7	84.2 ± 1.5	45.3 ± 2.1
ZnB	19.6 ± 1.4	19.8 ± 1.1	1.1 ± 0.1	86.0 ± 1.7	54.9 ± 2.8	49.3 ± 1.7	44.0 ± 1.5	9.8 ± 0.6	22.5 ± 0.9b	73.7 ± 1.7	83.1 ± 1.3	43.4 ± 1.5
Lf 250 mg/kg	17.7 ± 0.9	20.3 ± 1.5	1.0 ± 0.1	87.5 ± 1.0	55.9 ± 2.0	50.4 ± 1.6	44.3 ± 2.0	9.6 ± 0.6	24.0 ± 1.4b	76.6 ± 1.5	84.7 ± 1.6	41.4 ± 1.7
Lf 500 mg/kg	16.7 ± 1.7	20.0 ± 1.6	1.6 ± 0.7	82.5 ± 2.6	56.5 ± 1.5	48.2 ± 1.8	43.7 ± 1.5	9.6 ± 0.7	23.8 ± 1.0b	75.0 ± 2.3	83.2 ± 2.8	45.5 ± 1.4

Data are expressed as percentage of positive cells ± SEM (n = 12 birds/treatment; n = 11 birds for Lf 500 mg/kg). Values within a column that do not share a common letter are significantly different ($p<0.05$).

Table 30. Phenotypic analysis of caecal tonsil cells.

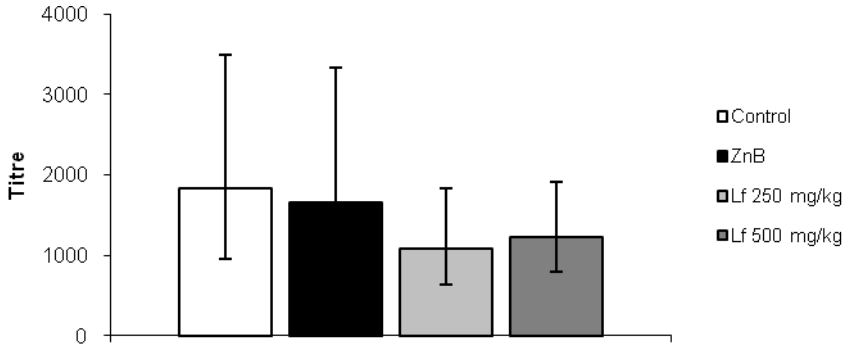
	TCR1	TCR2	TCR3	MHCI	MHCII	Kul-1	CD3	CD4	CD8	CD44	CD45	Bu1
Control	8.4 ± 0.8	24.4 ± 1.2	1.8 ± 0.2	64.9 ± 3.1	33.9 ± 1.5	37.7 ± 2.4	32.1 ± 1.7	15.7 ± 0.7a	16.5 ± 1.2	59.8 ± 3.5	64.1 ± 2.5	35.6 ± 1.4
ZnB	7.6 ± 0.7	24.3 ± 1.2	1.8 ± 0.2	63.6 ± 1.7	32.8 ± 2.0	33.7 ± 2.9	36.7 ± 0.9	17.5 ± 0.6ab	19.7 ± 1.2	62.0 ± 3.3	65.0 ± 3.1	38.8 ± 1.8
Lf 250 mg/kg	7.8 ± 0.5	25.1 ± 1.2	1.8 ± 0.3	62.5 ± 2.2	31.2 ± 1.8	35.3 ± 2.1	37.0 ± 2.2	18.6 ± 1.1b	20.1 ± 1.4	61.9 ± 2.7	66.6 ± 2.7	36.4 ± 1.2
Lf 500 mg/kg	7.9 ± 0.4	23.1 ± 1.2	2.1 ± 0.2	64.7 ± 1.4	30.8 ± 1.4	39.3 ± 2.8	36.3 ± 1.2	19.6 ± 1.2b	19.6 ± 1.1	60.3 ± 2.0	61.7 ± 2.5	37.2 ± 1.5

Data are expressed as percentage of positive cells ± SEM (n = 12 birds/treatment; n = 11 birds for Lf 500 mg/kg). Values within a column that do not share a common letter are significantly different ($p<0.05$).

Plasma immunoglobulin titres

No significant differences were observed in plasma IgA, IgM or IgG titres amongst treatment groups ($p>0.05$; Figure 16).

a)



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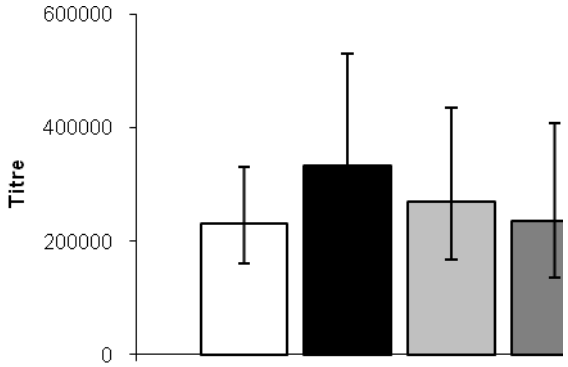

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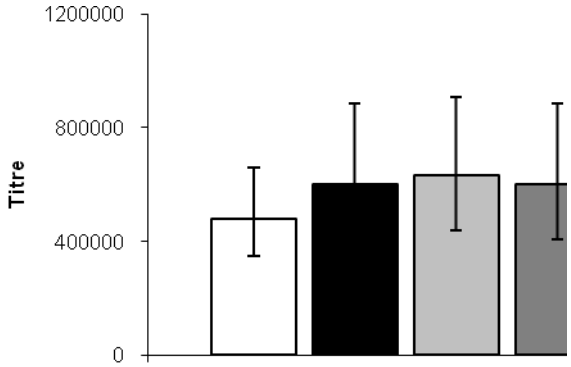


Figure 16. Plasma immunoglobulin titres. a) IgA, b) IgM, c) IgG. Data are expressed as geometric mean ± confidence interval (n = 12 birds/treatment, n = 11 for Lf 500 mg/kg).

Discussion

In the current study, involving a small-scale growth experiment and an AME bioassay, no growth-promoting effects of Lf were evident. Previously, Lf has been shown to improve growth performance and feed efficiency of weaned pigs (Wang et al. 2006); whilst Shan and colleagues, reported a trend towards improved growth in weanling pigs that did not reach statistical significance (Shan et al. 2007). In calves however, Lf administration has been reported to have no effect on growth (English et al. 2007), whilst in broilers, dietary inclusion of Lf (125 mg/kg) was found to have no effect on growth or feed efficiency (Humphrey et al. 2002). This result is consistent with the findings of the current study which assessed the higher doses of 250 and 500 mg/kg. Humphrey and colleagues demonstrated that Lf in conjunction with lysozyme did however impart positive effects on feed efficiency. As reported in Chapters 2 and 3, the dietary inclusion of ZnB did not influence growth and performance parameters in the current study. Again, this may indicate that the absence of environmental stressors may be the cause for the lack of antibiotic-induced effect on bird growth and performance despite the extra exposure to litter in the growth study and the older age of the birds.

The villus/crypt architecture of the ileum was not influenced by dietary inclusion of Lf. This is consistent with Humphrey and colleagues (2002) who reported that Lf alone had no influence on villus height and crypt depth. A combination of Lf and lysozyme was found to increase villus height in the duodenum, but did not affect the ileum (Humphrey et al. 2002), which was the intestinal segment assessed in the current study. In pigs, Lf supplementation (1 g/kg) increased the villus height of the mid-intestine and decreased crypt depth (Wang et al. 2006). These findings, coupled with the improved growth performance of pigs in the same study, may indeed indicate a species specific effect of lactoferrin that is not evident in poultry; conversely, the higher dose of Lf employed by Wang and colleagues (1 g/kg) compared to poultry studies (125-500 mg/kg) may account for these conflicting results.

The composition of intestinal mucin can influence gut development (Uni et al. 2003; Forder et al. 2007), which may inturn lead to performance differences. Mucin is also important in the regulation of bacterial colonisation within the intestine (Lievin-Le Moal and Servin 2006). The current study demonstrated that Lf did not have a significant effect on goblet cell sub-type distribution; however, a trend was observed towards an increase in acidic mucin-producing goblet cells in Lf-fed birds. The biological relevance of such a shift is not yet understood. Previous studies have demonstrated that dietary manipulation (Smirnov et al. 2005), bacterial exposure (Forder et al. 2007) and management factors (Uni et al. 2003) can influence mucin composition and goblet cell distribution (Smirnov et al. 2005). A much greater understanding of the relationship between mucin and the intestinal microbiota is required, in addition to a greater knowledge of how different mucin compositions can promote

changes in the microbiota and subsequently, how, or if, these changes can influence bird immune status, health and performance.

Previous experiments within this project have indicated that dietary manipulations (particularly prebiotics and antibiotics) have the capacity to influence the overall intestinal bacterial communities present in broiler chickens (Chapters 2 and 3). In the current study we observed that Lf supplementation did not significantly influence the intestinal microbial communities in the ileum compared to Control and ZnB diets. In the caecum however, Lf, at an inclusion level of 250 mg/kg, significantly altered the overall bacterial communities compared to ZnB-fed birds. The same effect was not observed at a dose rate of 500 mg/kg. Interestingly, the caecal microbial profiles of birds fed 250 mg/kg Lf and 500 mg/kg Lf were significantly different to one another. Neither Lf dosage promoted a significantly different microbiota compared to the Control-fed birds. Lactoferrin has been reported to possess antibacterial activity (Pan et al. 2007); which may underlie the observed shifts in the composition of the microbiota.

The extent of the similarity between the microbial populations of Control and ZnB-fed birds has differed throughout the current project. In Chapter 2 we observed a significantly different profile in the ileum of ZnB-fed birds compared to Control birds, with no discernable differences in the caecum; whilst in Chapter 3 no differences were seen in either gut section between these two treatments. In the current study, no differences were observed in the ileum; however, a significant difference was found in the caecum. This indicates that the antibiotic-induced effects on the microbiota are not consistent and may be influenced by external factors including the batch of birds, feed and environmental conditions including litter (although as many factors as possible were controlled during experimentation). These inconsistencies will complicate future attempts to i) identify an optimal microbiota for health and performance, and ii) devise a means to consistently facilitate the development of an optimal microbiota via dietary manipulation.

Analysis of the *Lactobacillus* profiles of individual birds did not indicate any apparent relationships between diets and the presence or absence of *Lactobacillus* species. Further multivariate statistical analysis, as described by Torok et al. (2008) and employed in Chapters 2 and 3, may however identify further relationships. Analysis of pooled samples from all birds within a dietary treatment indicated minor differences largely based on detection of *L. johnsonii* and/or *L. reuteri*. The ileal *Lactobacillus* profiles of birds fed Control and Lf 250 mg/kg appeared similar and contained both of these species, whilst the profiles of birds fed ZnB or Lf 500 mg/kg were relatively similar and simpler. Less delineation was apparent in the caecum. It is important to note that the detection levels of these two species are based on analysis by the Bionumerics program and are not quantitative. In contrast, using traditional culture-based techniques, Wang and colleauges indicated that dietary Lf supplementation

increased total *Lactobacillus* levels in the colon of pigs (Wang et al. 2007b); whilst Lf has also demonstrated the capacity to improve *Lactobacillus* growth *in vitro* (Kim et al. 2004). In the current study we did not attempt to quantify *Lactobacillus* levels and have focussed solely on the presence and absence of particular species.

As has been consistently reported throughout this project, the observed changes in the intestinal microbial populations have not significantly impacted upon VFA production, one indicator of microbial activity. In the current study, no changes in the concentrations of VFAs were observed, with only a shift in the percentage of propionic acid observed in birds fed 250 mg/kg compared to 500 mg/kg and ZnB-fed birds. These interesting findings support the results reported in Chapter 2 that indicate a range of microbial populations may accommodate a similar pattern of microbial activity.

Previously, Lf administration has been reported to modulate the immune system by a number of means including; decreased pro-inflammatory cytokine production (Legrand et al. 2005), increased anti-inflammatory cytokine production (Legrand et al. 2005), increased Ig production (Wang et al. 2007a), regulation of Th1 and Th2 balance (Fischer et al. 2006), and stimulation of phagocytosis (Shan et al. 2007). In the current study, Lf supplementation was found to increase the proportions of $CD8^+$ cells in the spleen and $CD4^+$ cells in the CT. These findings partially support previous data describing increased numbers of $CD8^+$ cells in the colon of mice (Wang et al. 2000), and $CD4^+$ and $CD8^+$ cells in the spleen of mice (Kuhara et al. 2000). Together, these studies suggest that Lf may influence T cell activation in poultry. Previously, a Lf-induced increase in serum IgM, IgA and IgG has been reported in piglets fed 1 g/kg Lf (Shan et al. 2007). The lack of a similar response to Lf in the current study may be related to dosage or host species.

In conclusion, supplementation of broiler feed with the immunomodulatory compound, lactoferrin, had no effect, either positively or negatively, on broiler growth, metabolism or performance. Lf influenced spleen and CT T cell proportions compared to additive-free diet and the overall microbial communities compared to ZnB-containing diet, with no significant effects on microbial activity, intestinal microarchitecture and goblet cell distribution. Previous reports indicate that the beneficial properties of Lf may be advantageous to the poultry industry, as an antibiotic alternative, when birds are faced with a pathogenic challenge; however no improvements on the performance of healthy birds are evident. Future studies into the effects of Lf in sub-optimal conditions and in the presence of an immune stimulus are indicated.

Chapter 5. Assessment of antibiotic alternatives in a model of necrotic enteritis

Introduction

Necrotic enteritis (NE), caused by the bacterium *Clostridium perfringens*, is one of the world's most prominent poultry diseases (Van Immerseel et al. 2004). The economic impact of NE on the world-wide poultry industry is estimated at over $1 billion per annum (www.canadianpoultrymag.com). Necrotic enteritis is typified by intestinal lesions, diarrhoea, impaired digestive function, reduced nutrient absorption, and decreased feed intake (Van Immerseel et al. 2004; Gholamiandehkordi et al. 2007). In its acute form, NE can cause significant flock mortality over a period of several days. Sub-clinical necrotic enteritis can significantly impair bird performance.

Currently in Australia, NE is controlled by the in-feed supplementation of antibiotics such as zinc bacitracin. The European Union have enforced a ban on the use of in-feed antibiotics, and consumer pressure in other regions may force similar restrictions on antibiotics use. Therefore, alternative strategies for the control of NE are needed which can limit the health and economic impact of the disease. Suggested alternatives to in-feed antibiotics include probiotics, prebiotics and organic acids (Dahiya et al. 2006).

The aims of this study were to assess the capacity for a medium chain fatty acid (MCFA) product (Selacid® Green Growth), and a candidate probiotic organism, *Lactobacillus johnsonii*, to reduce the severity of NE in broiler chickens. In addition, we aimed to assess the effects of these compounds on the intestinal microbiota and microbial activity.

Materials and Methods

Birds, management and diet

This study was performed at the University of New England (Armidale, NSW, Australia). 1200 male Cobb 500 broiler chickens (Baiada Hatchery, Kootingal, NSW, Australia) were raised in floor pens in a temperature-controlled room. All procedures were approved by the Animal Ethics Committee of the University of New England and complied with the "Australian code of practice for the care and use of animals for scientific purposes" (Australian Agricultural Council, 1997) and the "Australian model code of practice for the welfare of animals: Domestic Poultry" (Standing Committee on Agriculture

and Resource Management, 1995). All birds were vaccinated against Marek's disease, infectious bronchitis and Newcastle disease.

All diets were based on a standard commercial starter diet without any added antibiotics or coccidiostats (Table 31; Ridley Agriproducts, Tamworth, NSW, Australia). All diets met or exceeded National Research Council guidelines for broiler chickens (NRC 1994). The six experimental groups were; Unchallenged (standard diet with no additives), Challenged (standard diet with no additives), ZnB (standard diet + 50 ppm zinc bacitracin), Selacid (standard diet + 2 kg/Mt Selacid® Green Growth), Vehicle (standard diet with no additives) and *L. johnsonii* (standard diet with no additives n = 25 birds/pen, n = 8 pens/treatment). All groups except for the Unchallenged group were challenged with *C. perfringens* to induce NE. Birds in the Unchallenged group were separated from challenged birds within the holding facility, in order to minimise the likelihood of cross-contamination. The Vehicle and *L. johnsonii* groups were gavaged with PBS (vehicle), or *L. johnsonii*. In this study, the concentration of the candidate probiotic, *L. johnsonii*, was at least 10^9 cfu/ml in PBS, without bacterial extracellular products. Each chick in the *L. johnsonii* treatment group was orally administered 0.5 ml of the highly concentrated culture solution using a crop needle on day 1, and 1 ml on days 3, 7 and 12.

Table 31. Basal diets for necrotic enteritis challenge experiment.

Component	1-3 week (Starter)	4-6 week (Finisher)
Basal concentrate	g/kg	g/kg
Wheat	262.0	214.0
Sorghum	350.25	400.2
Mung beans	100.0	100.0
Tallow in mixer	32.5	34.0
Sunflower meal	-	25.0
Canola meal	60.0	60.0
Cottonseed meal	-	50.0
Soybean meal	157.0	81.5
Limestone B10	15.5	16.0
Kynofos/Biofos MDCP	11.5	11.0
Salt	1.75	1.5
Sodium bicarbonate	2.0	2.0
Choline chloride 75%	0.6	0.6
DL-Methionine	2.1	1.3
L-Lysine scale 3	2.1	0.4
L-Threonine	0.2	-
Vitamin and mineral premix[1]	2.5	2.5
Calculated chemical composition		
ME, MJ/Kg	12.26	12.39
Crude protein	200.02	190.00
Crude fibre	35.17	43.14
Crude fat	52.16	54.47
Lysine	11.49	8.98
Methionine + Cysteine	8.32	7.37
Calcium	9.73	9.79
Available phosphorous	6.50	6.71
Sodium	1.62	1.65
Chloride	2.19	1.75

[1]Vitamin and mineral premix (Ridley Agriproducts Pty Ltd., Tamworth, NSW, Australia) contained the following minerals in mg/kg of diet: vitamin A (as *all-trans* retinol), 12,000 IU; cholecalciferol, 3,500 IU; vitamin E (as d-α-tocopherol), 44.7 IU; vitamin B12, 0.2 mg; biotin, 0.1 mg; niacin, 50 mg; vitamin K3, 2 mg; pantothenic acid, 12 mg; folic acid, 2 mg; thiamine, 2 mg; riboflavin, 6 mg;

pyridoxine hydrochloride, 5 mg; D-calcium pantothenate, 12 mg; Mn, 80 mg; Fe, 60 mg; Cu, 8 mg; I, 1mg; Co, 0.3 mg; and Mo, 1 mg.

Necrotic enteritis challenge procedure

The necrotic enteritis challenge was performed in accordance to the procedures outlined previously (Kocher and Choct 2005).

Feed

Upon arrival at the facility until day 7, birds were fed starter diets with their corresponding dietary additive included. Between days 8-15 inclusive (prior to inoculation with *C. perfringens*), all birds were fed a high-protein diet based on 50% (w/w) fish meal (with corresponding dietary additives included). After day 15, the original starter diets were returned until day 21. The starter feeds were replaced by finisher feed on day 21 and remaining birds were kept until day 28.

Eimeria inoculation

On day 9, all birds (except unchallenged controls) were given a suspension of 2,500 oocysts of *Eimeria acervulina*, *E. maxima* and *E. tenella* in 1 ml PBS (Bioproperties Pty Ltd, Glenorie, NSW, Australia). The three species of *Eimeria* had been purified by serial passages through 3-week-old *Eimeria*-free chickens, and the sporulated oocysts were stored in 2% (w/v) potassium dichromate at 10°C before inoculation. Birds in the Unchallenged group received sterile PBS in place of *Eimeria*.

Clostridium perfringens inoculation

A primary poultry isolate of *C. perfringens* type A was obtained from CSIRO (AAHL, Geelong, Vic, Australia), and maintained in thioglycollate broth (USP alternative, Oxoid, Hampshire, UK) with 30% (v/v) glycerol at -20°C. The challenge inocula was prepared fresh by growing the bacterium overnight at 39°C in 1000 ml of thioglycollate broth with added starch (10 g/L) and casitone (5 g/L). The stock culture of *C. perfringens* was sub-cultured earlier in cooked meat media (Oxoid) and thioglycollate broth. On day 15, birds in challenged groups were individually inoculated with 1 ml of *C. perfringens* suspended in thioglycollate broth at a concentration of 3.5 x 10^8 cfu/ml. Further gavages that were planned on days 16 and 17 were abandoned due to confirmed NE-related mortality at day 16, indicative of a successful challenge. Birds in the Unchallenged group received 1 ml of sterile thioglycollate broth. Unchallenged birds were always serviced first to reduce the likelihood of cross-contamination. In addition, staff entering the Unchallenged pens wore disposable over-boots or clean boots washed in a disinfectant bath kept adjacent to the pen door. Feed was withdrawn from all pens for 3 hours on all inoculation days, prior to commencement of inoculation.

Lesion scoring

On days 15 and 18 two chickens per pen were randomly selected for lesion scoring, clostridia enumeration and tissue collection. The small intestine from each bird was incised longitudinally and examined for evidence of gross necrotic lesions. Small intestinal lesions were scored according to the previously described criteria (Prescott et al. 1978).

Clostridia enumeration

Clostridia enumeration was performed on days 15 and 18. Enumeration was carried out on digesta collected from the two birds per pen that were selected for lesion scoring. Fresh digesta samples (1 g) from the ileum and caeca were transferred into 15 ml MacCartney bottles containing 10 ml of anaerobic broth. The suspension was homogenised for 2 minutes in CO_2-flushed plastic bags using a bag mixer (Interscience, St. Norm, France) and serially diluted in 10-fold increments in anaerobic broth as described previously (Miller and Wolin 1974). A sub-sample of the homogenised suspension (1 ml) was then transferred into 9 ml of anaerobic broth and serially diluted from 10^{-1} to 10^{-5} (ileal samples) or 10^{-1} to 10^{-6} (caecal samples). From the last three diluted samples, 0.1 ml was plated on the appropriate medium (10 ml) for enumeration of microbial populations. All plates were incubated in an anaerobic cabinet (Model SJ-3, Kalter Pty. Ltd., Edwardstown, SA, Australia) and bacterial number counted using a colony counter (Selby, Model SCC100, Biolab Australia, Sydney, NSW, Australia).

Mortality and culling

All birds were examined twice daily. Dead chickens were immediately collected for post-mortem analysis and body weight was recorded.

Bird performance

Live weight and feed consumption were recorded on days 0, 9, 14, 21 and 28. Body weight gain and feed conversion ratios were calculated. Adjusted body weight gain within a particular period was defined as;

((live weight + weight of dead birds)/(number of live birds + number of dead birds))-weight at day 0

Adjusted FCR within a period was defined as;

Feed consumed/(total live weight + total dead weight-weight at day 0)

Tissue collection

On day 15, from two birds per pen, a 3 cm segment of the jejunum and ileum (including associated digesta), and one caecum was collected from the mid-points of the respective sections and placed in a 70 ml jar and kept at 4°C until later frozen for microbial profiling.

On day 18, 3 birds in each pen (n = 24 birds/treatment) were killed for tissue collection. From pens 1-24 (n = 12 birds/treatment), the contents of one caecum were collected and H_2SO_4 (0.5 M) was added at a rate of 0.5 ml per 1 g of caecal content, and stored at 4°C until later frozen for volatile fatty acid analysis. A segment (1 cm) of duodenum and jejunum were washed with 95% ethanol and then stored in 3 ml of 95% ethanol and kept at 4°C for immunohistochemistry. From pens 25-48, a segment (1 cm) of duodenum, jejunum and ileum was collected in 10% buffered formalin for 24 hours and then transferred to 70% ethanol, for histological analysis. Segments (1 cm) of duodenum, jejunum and ileum were also collected and stored in RNAlater for gene expression analysis. From all birds (pens 1-48; n = 24 birds/treatment), one caecum, and segments (3 cm) of tissue and associated digesta were collected from the jejunum and ileum and stored at 4°C until later frozen for microbial profiling.

Microbial profiling

Terminal-restriction fragment length polymorphism

T-RFLP analysis was performed following the techniques outlined in Chapter 2, and described previously (Torok et al. 2008).

Denaturing gradient gel electrophoresis

Characterisation of *Lactobacillus* species was performed using the Lac PCR-DGGE technique based on methods outlined in Chapters 1 and 2, and by Walter et al. (2001).

Volatile fatty acid analysis

Volatile fatty acids were analysed as described in Chapter 2.

Statistical analyses

Performance data were analysed with the SAS for Windows version 9.1 software package (SAS Institute Inc., Cary, NC, USA). Analysis of variance using the General Linear Model (GLM) procedure was used to compare parameters with differences between diets determined by Duncan's

Multiple Range Test. Intestinal lesion scores, clostridia levels, mortality/culling and volatile fatty acid profiles were analysed by Kruskal-Wallis ANOVA. Comparison of intestinal microbial communities (T-RFLP) was performed using the techniques described in Chapter 2. DGGE profiles were compared qualitatively using Dice's similarity coefficient based on the UPGMA (unweighted pair group methods using arithmetic averages) clustering algorithm using the BioNumerics software package (Applied Maths, Austin, TX, USA). For qualitative comparisons, band search filters, optimisation and position tolerance of 10%, 1% and 1%, respectively, were used.

Results

Bird performance

At day 9, prior to *C. perfringens* challenge, birds treated with *L. johnsonii* had a significantly greater body weight compared to birds fed basal diet (Unchallenged and Challenged control groups; $p<0.05$; Table 32). When body weight was adjusted to incorporate mortalities, birds in the *L. johnsonii* group had a greater body weight compared only to birds fed basal diet that were to be challenged (Challenged control group). No differences in body weight gain were observed at day 14 ($p>0.05$). At days 21 and 28, birds fed ZnB had the greatest body weights compared to all other challenged groups ($p<0.05$). The mean body weight of ZnB-fed birds was comparable to birds that were not challenged with *C. perfringens* ($p>0.05$). No differences in body weight were observed amongst the other challenged groups ($p>0.05$).

Feed conversion ratios between 0-21 days and 0-28 days were significantly lower in birds fed ZnB compared to other challenged birds ($p<0.05$; Table 33). FCR values for ZnB-fed birds were comparable to unchallenged control birds ($p>0.05$). When FCR was adjusted to incorporate bird mortality, birds fed ZnB again had a lower FCR compared to other challenged groups in the periods between days 0-21 and 0-28 ($p<0.05$). Birds fed Selacid had a significantly lower adjusted FCR compared to the Challenged group between days 0-21 and 0-28 ($p<0.05$). In the period between days 0-28, birds treated with *L. johnsonii* also had a lower adjusted FCR compared to birds in the Challenged group ($p<0.05$). No differences were observed between birds fed Selacid and *L. johnsonii* throughout the experimental period ($p>0.05$).

Table 32. Bird body weight.

	Live weight					Adjusted weight			
	Day 0	**Day 9**	**Day 14**	**Day 21**	**Day 28**	**Day 9**	**Day 14**	**Day 21**	**Day 28**
Unchallenged	37.2 ± 0.17	196 ± 1bc	380 ± 4	800 ± 10a	1374 ± 17a	195 ± 2ab	375 ± 5	715 ± 12a	1371 ± 16a
Challenged	37.8 ± 0.36	192 ± 5c	368 ± 6	552 ± 26b	1008 ± 46b	189 ± 5b	365 ± 8	444 ± 16b	1008 ± 46b
ZnB	37.6 ± 0.27	201 ± 4abc	386 ± 5	817 ± 12a	1420 ± 14a	197 ± 6ab	384 ± 5	722 ± 12a	1385 ± 24a
Selacid	37.4 ± 0.23	205 ± 2ab	380 ± 4	567 ± 20b	1048 ± 30b	202 ± 3a	375 ± 5	458 ± 11b	1033 ± 32b
Vehicle	37.2 ± 0.11	201 ± 1abc	378 ± 5	580 ± 14b	1082 ± 15b	198 ± 1ab	377 ± 5	453 ± 17b	1082 ± 15b
L. johnsonii	37.9 ± 0.28	208 ± 2a	383 ± 5	574 ± 13b	1065 ± 21b	205 ± 2a	381 ± 5	455 ± 15b	1054 ± 23b

Live weight and adjusted body weight are expressed as mean (g) ± SEM. For all data, n = 8 pens per treatment. Values within a column that do not share a common letter are significantly different ($p<0.05$).

Table 33. Bird feed conversion ratio.

	Live FCR				Adjusted FCR			
	D 0-9	**D 0-14**	**D 0-21**	**D 0-28**	**D 0-9**	**D 0-14**	**D 0-21**	**D 0-28**
Unchallenged	1.32 ± 0.04	1.21 ± 0.03	1.76 ± 0.05b	1.61 ± 0.02b	1.32 ± 0.05	1.18 ± 0.03ab	1.29 ± 0.01c	1.41 ± 0.02cd
Challenged	1.51 ± 0.10	1.26 ± 0.05	4.08 ± 0.51a	2.59 ± 0.27a	1.46 ± 0.09	1.25 ± 0.05a	1.58 ± 0.06a	1.66 ± 0.08a
ZnB	1.34 ± 0.08	1.13 ± 0.02	1.75 ± 0.04b	1.62 ± 0.04b	1.30 ± 0.05	1.12 ± 0.02b	1.25 ± 0.01c	1.37 ± 0.01d
Selacid	1.29 ± 0.06	1.19 ± 0.01	3.29 ± 0.25a	2.19 ± 0.10a	1.27 ± 0.05	1.16 ± 0.01b	1.47 ± 0.02b	1.51 ± 0.01bc
Vehicle	1.37 ± 0.08	1.21 ± 0.05	3.98 ± 0.40a	2.35 ± 0.14a	1.34 ± 0.07	1.20 ± 0.04ab	1.56 ± 0.04ab	1.59 ± 0.04ab
L. johnsonii	1.25 ± 0.04	1.17 ± 0.02	3.85 ± 0.40a	2.28 ± 0.12a	1.23 ± 0.04	1.16 ± 0.01b	1.53 ± 0.03ab	1.51 ± 0.03bc

Live feed conversion ratio (FCR) and adjusted FCR are expressed as mean ± SEM. For all data, n = 8 pens per treatment. Values within a column that do not share a common letter are significantly different ($p<0.05$).

Bird mortality and culls

No significant differences in bird mortality were observed amongst groups in the first 14 days post-hatch ($p>0.05$, Table 34). In the period following *C. perfringens* challenge (day 14-21), bird mortality was significantly elevated in Challenged, Selacid, Vehicle and *L. johnsonii*-treated birds compared to Unchallenged birds and birds fed ZnB ($p<0.05$). No differences in bird mortality were observed in the period between days 21 and 28 ($p>0.05$).

Table 34. Bird mortality throughout the experimental period.

	Mortality and Culls			
	Day 0-9	**Day 9-14**	**Day 14-21**	**Day 21-28**
Unchallenged	0.5	2.5	5.5a	1.0
Challenged	3.0	1.0	36.0b	0.0
ZnB	3.0	0.5	7.5a	2.5
Selacid	1.5	3.0	30.5b	1.0
Vehicle	2.0	1.0	35.5b	0.0
L. johnsonii	2.0	1.5	35.5b	1.0

Percentage mortality for all birds within a treatment for each time period. Values with a column that do not share a common letter are significantly different ($p<0.05$).

Clostridia enumeration

No differences in clostridia numbers were observed amongst treatment groups at day 15 prior to gavage with *C. perfringens* ($p>0.05$; Table 35). At day 18, birds in the Challenged group had greater clostridia levels compared to birds fed ZnB and Selacid ($p<0.05$). Birds fed ZnB had significantly lower clostridia levels compared to birds in the Vehicle control group. Clostridia levels were also reduced in birds fed Selacid compared to birds in the Vehicle control and *L. johnsonii* groups ($p<0.05$).

Table 35. Clostridia enumeration in the jejunum at day 15 (prior to Clostridium perfringens gavage), and at day 18.

	Clostridia enumeration	
	Day 15	**Day 18**
Unchallenged	3.50 ± 0.12	3.69 ± 0.28ab
Challenged	3.81 ± 0.28	4.23 ± 0.35a
ZnB	3.60 ± 0.20	3.45 ± 0.32b
Selacid	5.14 ± 0.69	3.42 ± 0.16b
Vehicle control	3.71 ± 0.43	4.16 ± 0.22a
L. johnsonii	3.47 ± 0.24	4.44 ± 0.35a

Data are expressed as mean (log concentration) ± SEM. Values within a column that do not share a common letter are significantly different ($p<0.05$).

Lesion scores

Lesion scoring was performed on the duodenum, jejunum and ileum. No lesions were observed in the ileum of any bird at day 15 or day 18 (data not shown). At day 15, challenged controls, vehicle controls and birds treated with Selacid and *L. johnsonii* had elevated lesion scores compared to unchallenged controls ($p<0.05$, Table 36). ZnB-treated birds had a significantly lower lesion score compared to challenged control birds, vehicle controls, *L. johnsonii* and Selacid-treated birds ($p<0.05$) with a comparable lesion score to unchallenged birds ($p>0.05$). Additionally, in the duodenum at day 15, *L. johnsonii*-treated birds had a significantly lower lesion score compared to birds in the Selacid and Vehicle control groups ($p<0.05$). On day 18, duodenum lesion scores were significantly greater in challenged controls, vehicle controls and Selacid-fed birds compared to unchallenged controls ($p<0.05$). ZnB-fed birds had significantly lower duodenum lesion scores compared to challenged controls, vehicle controls and Selacid-fed birds whilst having a lesion score comparable to unchallenged birds ($p>0.05$). *L. johnsonii*-treated birds had a lower duodenum lesion score compared to challenged and vehicle control birds, and birds fed Selacid ($p<0.05$). In the jejunum, day 18 lesion scores were significantly greater in challenged and vehicle controls and Selacid-fed birds compared to unchallenged controls ($p<0.05$). ZnB-fed birds had a significantly lower lesion score compared to challenged and vehicle controls, and Selacid-fed birds ($p<0.05$) with a score comparable to unchallenged control birds ($p>0.05$).

Table 36. Lesion scoring in the duodenum and jejunum of chickens at day 15 and day 18.

	Lesion score			
	Day 15		**Day 18**	
	Duodenum	**Jejunum**	**Duodenum**	**Jejunum**
Unchallenged	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
Challenged	0.84 ± 0.20bc	0.31 ± 0.14b	0.56 ± 0.17b	0.75 ± 0.27b
ZnB	0.0 ± 0.0a	0.0 ± 0.0a	0.06 ± 0.04a	0.0 ± 0.0a
Selacid	1.13 ± 0.17b	0.78 ± 0.27b	0.28 ± 0.10b	0.50 ± 0.18b
Vehicle	1.13 ± 0.15b	0.59 ± 0.20b	0.53 ± 0.17b	0.38 ± 0.10b
L. johnsonii	0.38 ± 0.17c	0.28 ± 0.13b	0.09 ± 0.09a	0.19 ± 0.11a

Data are expressed as mean (score) ± SEM. Values within a column that do not share a common letter are significantly different.

Microbial profiles

Terminal-restriction fragment length polymorphism

Overall intestinal bacterial communities were characterised in the jejunum by T-RFLP. Unchallenged control birds had a significantly different profile compared to Challenged birds ($p<0.05$; Table 37), indicating that the challenge procedure had a direct impact on the overall microbiota. Unchallenged birds also displayed a significantly different microbial profile compared to challenged birds fed ZnB, Selacid and Vehicle control ($p<0.05$), with a strong trend toward a difference compared to *L. johnsonii*-treated birds ($p<0.1$). The overall microbial communities of challenged control birds were significantly different compared to ZnB and Vehicle-treated birds ($p<0.05$); however, they were not significantly different compared to Selacid and *L. johnsonii*-treated birds ($p>0.05$). Birds fed ZnB had a significantly different microbial composition compared to all other treatment groups ($p<0.001$). Birds fed Selacid, Vehicle and *L. johnsonii* were not significantly different to one another; however there appeared to be a trend toward a difference between Selacid and Vehicle-treated birds ($p<0.1$).

Table 37. One-way analysis of similarities (ANOSIM) for jejunal microbial communities associated with diet.

Jejunum	Unchallenged	Challenged	ZnB	Selacid	Vehicle	*L. johnsonii*
Unchallenged	-	**0.134**	**0.404**	**0.132**	**0.127**	**0.066**
Challenged	*0.012*	-	**0.299**	**0.004**	**0.103**	**-0.01**
ZnB	*<0.001*	*<0.001*	-	**0.457**	**0.546**	**0.374**
Selacid	*0.014*	*0.388*	*<0.001*	-	**0.060**	**-0.005**
Vehicle	*0.021*	*0.024*	*<0.001*	*0.084*	-	**-0.008**
L. johnsonii	*0.078*	*0.543*	*<0.001*	*0.483*	*0.513*	-

Data are expressed as the R-statistic (bold), with significance level in italics. For all analyses a significance level of 0.05 was considered significant. For jejunal microbial communities the global R-value was 0.178 at a significance level of 0.0001 which is considered significant.

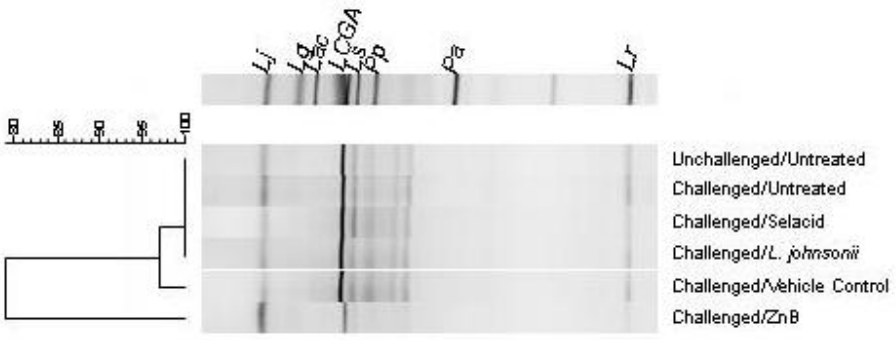
Denaturing gradient gel electrophoresis

The Lac PCR-DGGE profiles from pooled samples were grouped into three clusters (Figure 17a) that all contained *L. johnsonii,* LCGA, and *L. salivarius.* The two clusters containing Unchallenged/Untreated, Challenged/Untreated, Challenged/Selacid, Challenged/*L. johnsonii* and Challenged/Vehicle control also contained *L. reuteri*, which was below the band detection limit in the pooled Challenged/ZnB sample. The relative high intensity of the LCGA band in the first two clusters suggested that these species were the main contributors to the *Lactobacillus* microflora in birds fed

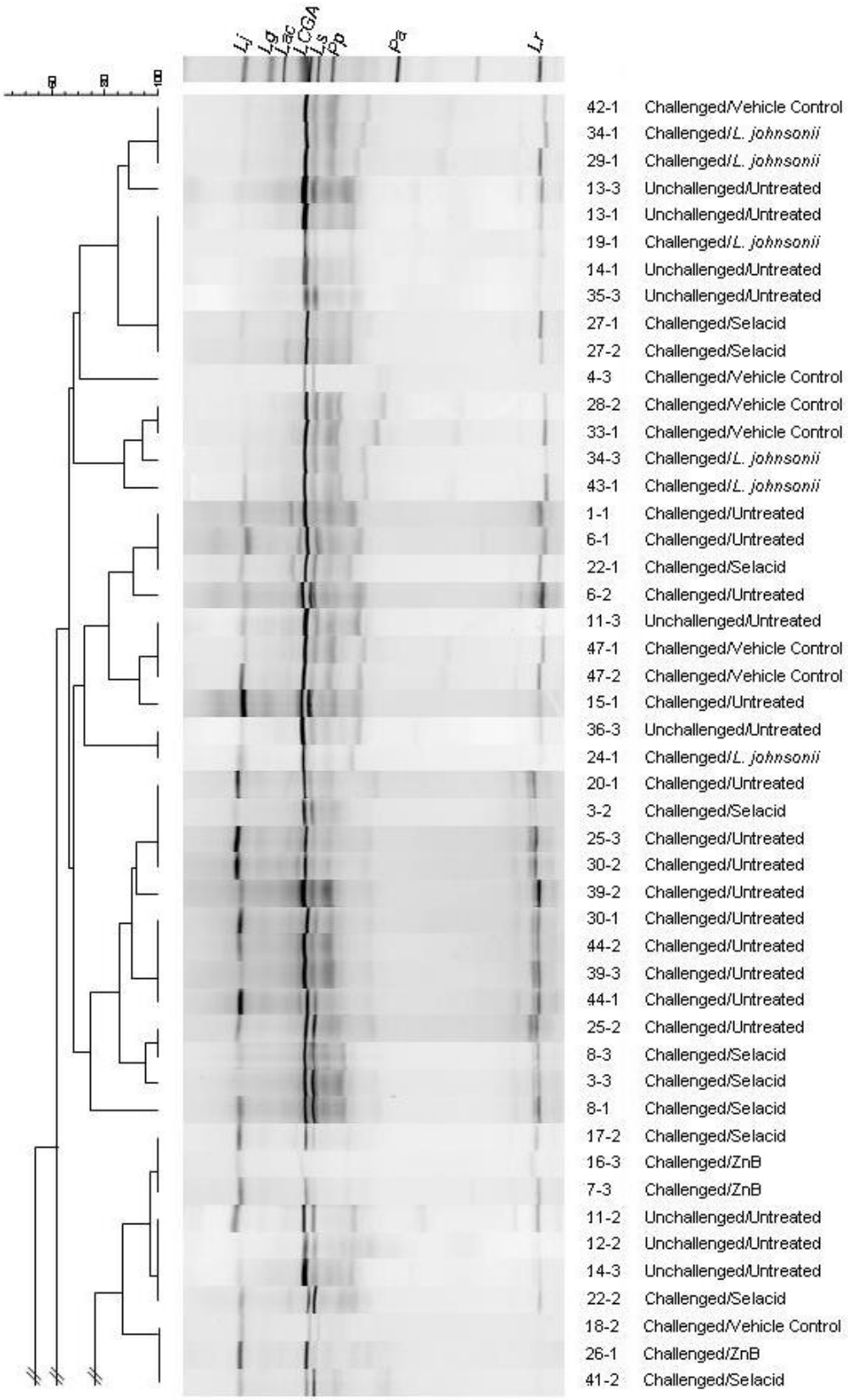
these diets. In comparison, the intensity of the bands corresponding to *L. johnsonii*, *L. reuteri*, and *L. salivarius* suggested that they were minor contributors. Additional low intensity and diffuse bands that migrated with *P. acidilactici*, and between *P. acidilactici* and *P. pentosaceous* were also present in these two clusters but were below the band detection limit in the pooled Challenged/ZnB sample. The presence and identity of these and other bands (see below) will be examined further in the future.

Consistent with the pooled data, qualitative comparison of the *Lactobacillus* species of individual birds using Bionumerics analysis indicated that almost every bird appeared to harbour LCGA (Figure 17b). The presence of LCGA in all birds is consistent with findings reported in Chapters 2 and 3. The detection of bands corresponding to *L. johnsonii, L. salivarius, L. reuteri*, as well as the multiple bands between *P. acidilactici* and *P. pentosaceous* varied. In a small number of birds, additional bands were also detected migrating along with *L. reuteri*. For the most part, the detection of these other species was not obviously associated with diet apart from clusters of Challenged/Untreated (9/16 birds clustered together with three Challenged/Selacid birds) and Challenged/ZnB (7/16 birds clustered with 100% identity together with two birds fed Challenged/*L. johnsonii*). Visual comparison of *Lactobacillus* profiles within diets confirmed variability in the microflora with the least variability observed among birds fed the ZnB diet (Figure 17b and data not shown). Interestingly, the *L. johnsonii* band was detected in only half of the birds fed the Challenged/*L. johnsonii* diet, yet was present in 16/16 of ZnB-fed birds, and 13/16 birds in the Challenged/Untreated group. This species was not commonly detected (<7 birds) in the other three diets. Future in-depth statistical analysis of the profiles of the individual birds is required to determine if these variable species and bands are significantly related to diet and treatment.

a)



b)


Lj
Lg
Lac
LCGA
Ls
Pp
Pa
Lr
42-1 Challenged/Vehicle Control
34-1 Challenged/L. johnsonii
29-1 Challenged/L. johnsonii
13-3 Unchallenged/Untreated
13-1 Unchallenged/Untreated
19-1 Challenged/L. johnsonii
14-1 Unchallenged/Untreated
35-3 Unchallenged/Untreated
27-1 Challenged/Selacid
27-2 Challenged/Selacid
4-3 Challenged/Vehicle Control
28-2 Challenged/Vehicle Control
33-1 Challenged/Vehicle Control
34-3 Challenged/L. johnsonii
43-1 Challenged/L. johnsonii
1-1 Challenged/Untreated
6-1 Challenged/Untreated
22-1 Challenged/Selacid
6-2 Challenged/Untreated
11-3 Unchallenged/Untreated
47-1 Challenged/Vehicle Control
47-2 Challenged/Vehicle Control
15-1 Challenged/Untreated
36-3 Unchallenged/Untreated
24-1 Challenged/L. johnsonii
20-1 Challenged/Untreated
3-2 Challenged/Selacid
25-3 Challenged/Untreated
30-2 Challenged/Untreated
39-2 Challenged/Untreated
30-1 Challenged/Untreated
44-2 Challenged/Untreated
39-3 Challenged/Untreated
44-1 Challenged/Untreated
25-2 Challenged/Untreated
8-3 Challenged/Selacid
3-3 Challenged/Selacid
8-1 Challenged/Selacid
17-2 Challenged/Selacid
16-3 Challenged/ZnB
7-3 Challenged/ZnB
11-2 Unchallenged/Untreated
12-2 Unchallenged/Untreated
14-3 Unchallenged/Untreated
22-2 Challenged/Selacid
18-2 Challenged/Vehicle Control
26-1 Challenged/ZnB
41-2 Challenged/Selacid

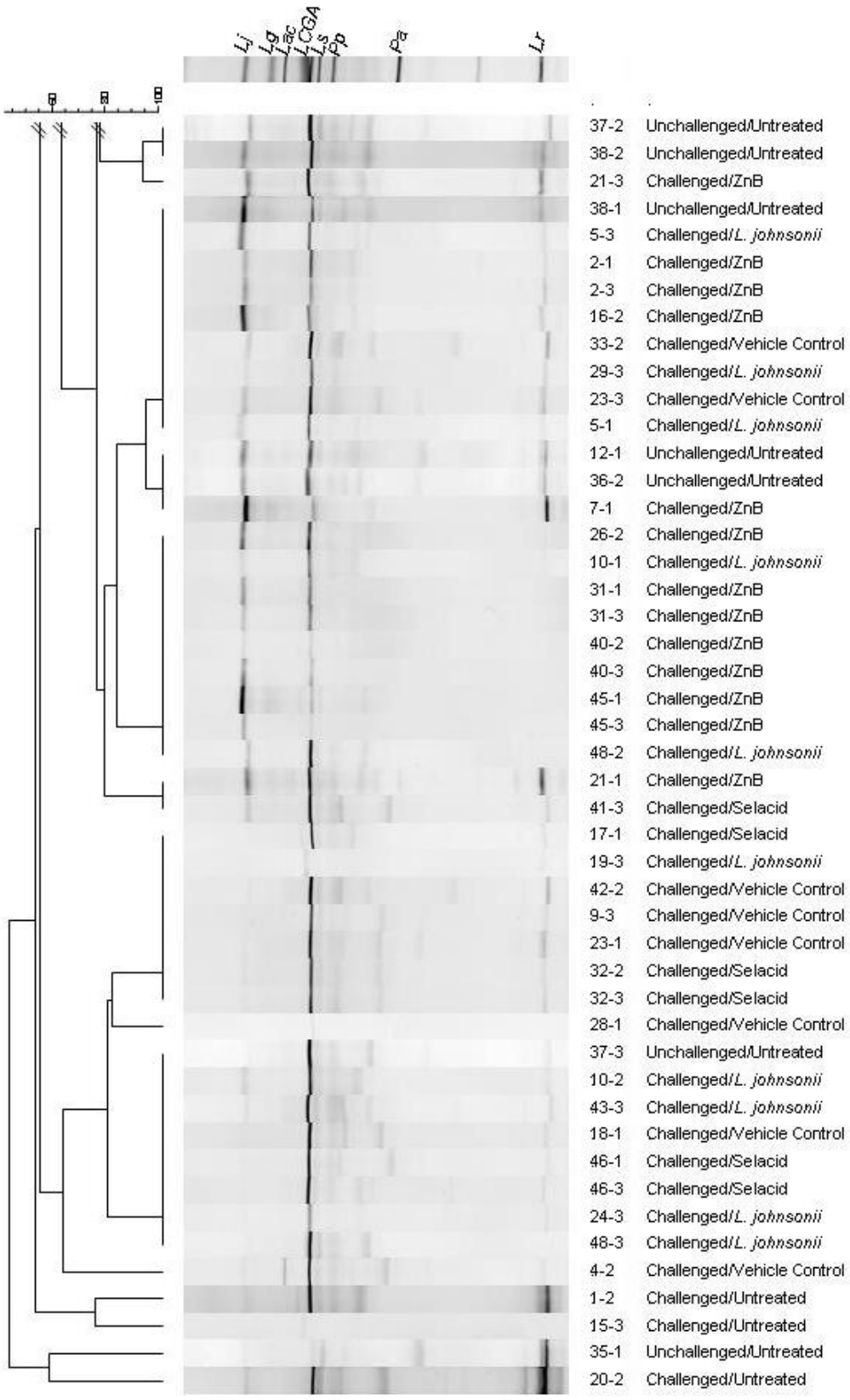

Lj
Lg
Lac
LCGA
Ls
Pp
Pa
Lr
37-2 Unchallenged/Untreated
38-2 Unchallenged/Untreated
21-3 Challenged/ZnB
38-1 Unchallenged/Untreated
5-3 Challenged/L. johnsonii
2-1 Challenged/ZnB
2-3 Challenged/ZnB
16-2 Challenged/ZnB
33-2 Challenged/Vehicle Control
29-3 Challenged/L. johnsonii
23-3 Challenged/Vehicle Control
5-1 Challenged/L. johnsonii
12-1 Unchallenged/Untreated
36-2 Unchallenged/Untreated
7-1 Challenged/ZnB
26-2 Challenged/ZnB
10-1 Challenged/L. johnsonii
31-1 Challenged/ZnB
31-3 Challenged/ZnB
40-2 Challenged/ZnB
40-3 Challenged/ZnB
45-1 Challenged/ZnB
45-3 Challenged/ZnB
48-2 Challenged/L. johnsonii
21-1 Challenged/ZnB
41-3 Challenged/Selacid
17-1 Challenged/Selacid
19-3 Challenged/L. johnsonii
42-2 Challenged/Vehicle Control
9-3 Challenged/Vehicle Control
23-1 Challenged/Vehicle Control
32-2 Challenged/Selacid
32-3 Challenged/Selacid
28-1 Challenged/Vehicle Control
37-3 Unchallenged/Untreated
10-2 Challenged/L. johnsonii
43-3 Challenged/L. johnsonii
18-1 Challenged/Vehicle Control
46-1 Challenged/Selacid
46-3 Challenged/Selacid
24-3 Challenged/L. johnsonii
48-3 Challenged/L. johnsonii
4-2 Challenged/Vehicle Control
1-2 Challenged/Untreated
15-3 Challenged/Untreated
35-1 Unchallenged/Untreated
20-2 Challenged/Untreated

Figure 17. Lac PCR-DGGE profiles of pooled (a) and individual (b) jejunal samples using Dice's Similarity indicated by the scale at the top left. The marker lane at the top of the figure contains the identification ladder comprised of Lac PCR products from the following reference strains: *Lj*, *L. johnsonii* ATCC 33200; *Lg*, *L. gasseri* ATCC 33323; *Lac*, *L. acidophilus* ATCC 4356; *Ls*, *L. salivarius* subsp. *salivarius* ATCC 11741; *Pp*, *Pediococcus pentosaceous* ATCC 43200; *Pa*, *Pediococcus acidilactici* ATCC 8042; and *Lr*, *L. reuteri* ATCC 23272. *LCGA* marks the migration of *L. crispatus*, *L. gallinarum* and *L. amylovorous* species, and is represented by *L. crispatus* ATCC 33820. One sample from the Challenged/Vehicle control group was excluded as the sampe did not amplify using the Lac PCR technique.

Volatile fatty acid concentrations

Isovaleric acid levels were significantly greater in challenged control birds and birds treated with Selacid, Vehicle and *L. johnsonii*, compared to unchallenged controls and ZnB-fed birds ($p<0.05$; Table 38). The concentrations of all other measured VFAs were not significantly different amongst treatments ($p>0.05$). The molar percentages of propionic acid were greater in challenged controls and Selacid-fed birds compared to unchallenged control birds ($p<0.05$; data not shown). Birds fed ZnB had a significantly lower molar percentage of propionic acid compared to challenged control birds ($p<0.05$). The molar percentages of isovaleric acid were greater in challenged controls, and birds fed Selacid, Vehicle and *L. johnsonii* compared to unchallenged control birds ($p<0.05$; data not shown). Birds fed ZnB also had a lower molar percentage of isovaleric acid compared to Vehicle and *L. johnsonii*-treated birds ($p<0.05$).

Table 38. Volatile fatty acid concentrations of caecal contents.

	Acetic	Propionic	Butyric	Iso-butyric	Valeric	Iso-valeric	Iso-caproic	Total
Unchallenged	34.39 ± 7.75	1.54 ± 0.41	7.94 ± 2.00	0.0 ± 0.0	0.21 ± 0.09	0.05 ± 0.05a	0.0 ± 0.0	44.12 ± 10.11
Challenged	30.53 ± 6.48	3.93 ± 0.99	9.71 ± 1.61	0.47 ± 0.26	0.54 ± 0.13	0.93 ± 0.28b	0.11 ± 0.08	46.23 ± 8.45
ZnB	34.75 ± 5.18	1.59 ± 0.28	9.01 ± 1.30	0.0 ± 0.0	0.33 ± 0.08	0.22 ± 0.10a	0.07 ± 0.07	45.97 ± 6.57
Selacid	45.70 ± 10.60	2.94 ± 0.73	14.78 ± 3.54	0.12 ± 0.12	0.48 ± 0.15	0.67 ± 0.21b	0.0 ± 0.0	64.70 ± 14.03
Vehicle	29.70 ± 6.42	1.92 ± 0.47	9.58 ± 2.28	0.38 ± 0.17	0.27 ± 0.10	0.77 ± 0.20b	0.0 ± 0.0	42.62 ± 8.80
L. johnsonii	35.92 ± 9.72	2.42 ± 0.53	12.06 ± 2.58	0.47 ± 0.22	0.41 ± 0.13	0.99 ± 0.22b	0.0 ± 0.0	52.28 ± 12.42

Data are expressed as mean (μMol/ml) ± SEM. Values within a column that do not share a common letter are significantly different ($p<0.05$).

Discussion

In the current study, the two antibiotic alternatives, Selacid (a MCFA product) and *L. johnsonii*, failed to prevent the onset and reduce the severity of NE in broiler chickens. In-feed addition of ZnB however, successfully maintained bird weight and FCR, limited NE-induced bird mortality, prevented the development of intestinal lesions and reduced intestinal clostridia load. The microbial communities in the jejunum of ZnB-fed birds were significantly different to all other treatment groups. The *Lactobacillus* species profiles also appeared to differ in ZnB-fed birds compared to all other diets. Treatment with *L. johnsonii* appeared to improve intestinal lesion scores; however this did not translate into improvements in mortality, body weight or FCR. Likewise, Selacid treatment successfully decreased intestinal clostridia load; however, this did not translate into performance improvements or reduced bird mortality.

Selacid is a proprietary blend of MCFAs and organic acids. Previously, MCFA compounds have demonstrated antimicrobial activity against poultry pathogens including *E. coli* (Marounek et al. 2003), *Salmonella* sp. (Van Immerseel et al. 2006), *Campylobacter jejuni* (Solis de los Santos et al.

2008b) and *C. perfringens* (Skrivanova et al. 2005). The mechanism of action is not completely understood; however, undissociated MCFAs are believed to enter the bacterial cell and dissociate in the cytoplasm, causing metabolic uncoupling and cell death (Skrivanova et al. 2005). Interestingly, in the current study we observed a Selacid-induced decrease in intestinal clostridia levels, which is consistent with the reported activity of MCFA in previous studies (Skrivanova et al. 2005); however, this decrease did not translate to an improvement in broiler performance and survival. This indicates that Selacid may not decrease *C. perfringens* levels post-challenge in a timely manner, and/or that *C. perfringens* levels may be elevated for a sufficient period to elicit damage. Alternatively, as total clostridia were enumerated, not specific *C. perfringens* levels, it may be the case that Selacid is lowering total clostridia but not *C. perfringens* specifically. Further investigation into the direct effects of Selacid on *C. perfringens* load is required; future sequencing of OTUs may allow us to monitor *C. perfringens* using T-RFLP. This study indicated that MCFA supplementation of broiler diets can successfully decrease clostridia load but not prevent the onset of NE, nor reduce gut damage in surviving chickens.

The use of probiotic and other bacterial strains to prevent *C. perfringens* colonisation and NE in broilers has demonstrated some potential in previous studies. A number of reports have demonstrated the *in vitro* capacity for bacterial strains to prevent *C. perfringens* growth, including *Bacillus subtilus* PB6 (Teo and Tan 2005) and *Lactobacillus* sp. (Olnood et al. 2007). However, few bacterial strains have successfully demonstrated the capacity to prevent the development of NE *in vivo*. *L. johnsonii* FI9785 has been reported to prevent *C. perfringens* colonisation in pathogen-free broilers (La Ragione et al. 2004). Interestingly, the *L. johnsonii* strain used in the current study did not demonstrate any anti-clostridial properties. This may be related to the observation that less than half of the birds administered this strain had detectable *L. johnsonii* based on Lac-DGGE analysis, perhaps suggesting that this strain does not effectively colonise and persist in the gastrointestinal tract during challenge. This finding, coupled with the results of La Ragione et al. (2004) may indicate strain specificity of *L. johnsonii* against *C. perfringens*.

In the current study we observed an *L. johnsonii*-mediated decrease in intestinal lesion scores compared to challenged controls. Previously, the capacity for probiotic bacteria to protect the intestine from lesions has been well documented in rats (Llopis et al. 2005). The mechanisms behind this protection may include a reduced inflammatory response, encompassing decreased pro-inflammatory cytokine production. The observed improvements in intestinal lesion scores in the current study however failed to translate into improved performance and reduced mortality, indicating a lack of a beneficial effect of the candidate probiotic strain.

The overall microbial profiles in the jejuna of unchallenged control birds were significantly different to birds challenged with *C. perfringens*, indicating that challenged birds displayed shifts in the levels of a range of intestinal microbes, not solely *C. perfringens*. This shift in the overall microbial communities could be a downstream effect as *C. perfringens* may influence other commensal bacteria. It should be noted that the intestinal microbiota of all birds may have been influenced by the feeding of a 50% fishmeal diet as part of the challenge procedure. High levels of protein are likely to influence the bacterial communities of the intestine (Dahiya et al. 2006; McDevitt et al. 2006). In the current study, any fishmeal-induced shift would be consistent across all birds and therefore does not compromise our interpretation; however, further studies in birds fed diets that are more representative of the Australian poultry industry may be required in order to completely and accurately understand the role of the microbiota in NE.

Interestingly, ZnB-treated birds displayed a significantly different jejunal microbial profile compared to all other groups, including unchallenged and challenged control birds. The mechanisms underlying the protective capacity of ZnB in this study are not known, but a direct antimicrobial effect on *C. perfringens* is likely to be involved. In addition to decreasing *C. perfringens* levels, a direct antimicrobial effect of ZnB against *C. perfringens* may have prevented further *C. perfringens*-induced shifts in other microbial species. It is also likely that ZnB directly influenced an array of other bacterial species; this shift in microbial composition may have subsequently prevented *C. perfringens* colonisation by competitive exclusion. Interestingly, the *Lactobacillus* microflora was the simplest and most consistent in birds fed ZnB compared to the other diets and, of particular note, every bird harboured *L. johnsonii* (and LCGA). Whether *L. johnsonii* or other components of the microflora contributed to competitive exclusion remains to be determined but does suggest that native strains of this species are able to persist under challenge and potentially supports the strain-specific phenomenon mentioned previously. The ZnB-mediated microbial shifts, may have also altered host-microbe crosstalk, and thus contributed to improved bird immunity and altered mucin dynamics. This may have facilitated an increased capacity for the birds to prevent *C. perfringens* colonisation and maintain a high level of performance. In the current study, traditional culture techniques were used to quantify clostridia levels; however, this technique did not discriminate between *Clostridium* species. This limits these interpretations, as a number of non-pathogenic commensal *Clostridia* have been identified, and may potentially have beneficial properties (Lu et al. 2008). Interestingly, it was observed that ZnB and Selacid had the capacity to reduce clostridial load (as indicated by traditional techniques); however, only ZnB demonstrated the capacity to shift the overall microbiota (as indicated by T-RFLP). This indicated that Selacid did not have the capacity to shift the overall microbiota and that Selacid-induced shifts in clostridia were not sufficient to influence the overall microbial profile. Sequencing of T-RFLP-derived OTUs will facilitate the identification of *C. perfringens* and allow specific monitoring of this species. Further sequencing of T-RFLP-derived OTUs will provide information as to the nature

of any other species responsible for shifts in the microbial communities amongst groups. This may facilitate the identification of commensal species which assist in protection from *C. perfringens*, and subsequently may be associated with improved bird performance under NE challenge conditions. Future research should focus on the discovery of natural feed additives that facilitate the colonisation of these beneficial commensal organisms and limit the growth of *C. perfringens* and other harmful bacteria.

As was observed with Selacid, *L. johnsonii* treatment failed to influence the overall microbiota compared to challenged control birds. This may indicate that the particular *L. johnsonii* strain, given at an early stage of the experiment by oral gavage, may not have colonised the intestine long-term which is supported by the Lac DGGE data which indicated that only half of the birds had detectable levels of *L. johnsonii*. Alternatively it may suggest that the increase in *L. johnsonii* was not large enough to influence the overall microbial profile and that any interactions between *L. johnsonii* and other organisms did not change the existing microbiota to a detectable extent. Further identification at the strain level would be required to indicate if the *L. johnsonii* detected in the remaining birds was indeed the gavage strain, or another strain.

In contrast to the observed effects on the overall microbiota, Lac PCR-DGGE analysis indicated that there appeared to be little difference in the *Lactobacillus* profiles of unchallenged and challenged birds with the *Lactobacillus* profiles in the jejuna of individual birds being quite variable. In contrast, the *Lactobacillus* microflora in birds fed ZnB was different to the other birds, demonstrated the least variation, and the highest prevalence of *L. johnsonii* (16/16 birds). This may indicate that these potential classes of antibiotic alternatives may not have the capacity to impart reproducible effects on the microbiota to the extent of current antibiotic-based feeding regimens. Our observation that necrotic enteritis challenge did not influence the presence and absence of *Lactobacillus* species in the jejunum is supported by recent quantitative results (Mikkelsen et al. 2009). It should be noted that the ileum is considered the region of the gastrointestinal tract that harbours the greatest proportion of lactobacilli (Wise and Siragusa 2007) although it is thought that this region is "seeded" by the *Lactobacillus* microflora in the proximal regions of the gut. Treatment and diet-induced effects on *Lactobacillus* profiles may have been more pronounced in this region; however, due to time and budget constraints, characterisation of only one gut region was possible in this study. The observation of higher lesion scores in the jejunum led to its selection as the most important gut section for microbial profiling by T-RFLP and DGGE. Future analysis of other gut regions may be possible. It should be noted that the Lac PCR-DGGE analysis is not quantitative and that changes in actual levels of these species may have been influenced by these treatments.

Interestingly, treatment with *L. johnsonii* and Selacid improved bird weight in the first nine days post-hatch, indicating potential benefits in the early post-hatch period. These benefits however were no longer evident at day 14, indicating that the early improvements did not translate into any long-term production benefits. Previously, inconsistent growth-promoting properties of probiotics have been reported in broilers, with some strains improving weight gain (Khan et al. 2007; Apata 2008), whilst others demonstrate no growth improvements (O'Dea et al. 2006; Timmerman et al. 2006). Few recent studies have investigated the impact of MCFA supplementation on broiler growth in healthy birds, with the majority of studies focussed on their capacity to prevent pathogenic infection or improve carcass quality (Solis de Los Santos et al. 2008a; Solis de los Santos et al. 2008b). These studies often do not report the effects of MCFAs on growth in the period prior to pathogen challenge. Zheng and colleagues however, recently reported a MCFA-induced decrease in body weight gain, believed to be due to a decreased feed intake which may result from palatability issues associated with the additives (Zheng et al. 2006). These results are supported by a numerical decrease in body weight gain observed in Australian Poultry CRC Project 03-3b[4]. These findings indicate that diets containing MCFA must be carefully formulated in order to not decrease palatability associated with high doses of MCFA. In the current study, feed intake in the Selacid group was not decreased compared to other experimental diets (including *L. johnsonii*, Vehicle and Challenged groups), indicating no compounding palatability issues. The current results, coupled with previous reports indicate that factors including probiotic strain, MCFA type, bird strain, environmental conditions, feed and the underlying intestinal microbiota may influence the capacity for probiotics and MCFA to affect bird performance. In the current study, an in-depth analysis, including intestinal microbial profiling, was not performed on birds at this early stage; therefore, the mechanism of action underlying the observed growth improvements is unknown. Regardless of the mechanism of action, the possible *L. johnsonii*- and Selacid-induced benefits could not be maintained in the presence of NE challenge, suggesting that they are not suitable alternatives to ZnB.

In summary, in-feed supplementation with *L. johnsonii* or Selacid did not successfully prevent the onset of NE in broiler chickens, whilst conventional treatment with ZnB prevented the development of NE and maintained bird performance during the challenge period. *C. perfringens* challenge appeared to significantly alter the microbiota of broilers, and ZnB influenced the intestinal microbial communities differently compared to all other treatments. The potential role of probiotics and MCFAs in the prevention of NE remains unclear; however, the observations that MCFA supplementation decreased intestinal clostridial load and *L. johnsonii* decreased lesion scores indicate that compounds/organisms of this nature may indeed have the capacity to reduce the severity of NE. Further research is needed to indicate whether specific MCFA combinations are capable of preventing

[4] Australian Poultry CRC Project 03-3b: Development and validation of cell and tissue assays to assess nutrient uptake and digestive function in poultry. Project Leader: Dr. C. Smyl.

NE. Likewise, other probiotic compounds, or combinations of probiotic strains, may have prophylactic properties; therefore, further screening of candidate probiotics may reveal a probiotic with the capacity to prevent NE.

Chapter 6: Intestinal gene expression analysis in high and low AME birds

Introduction

Throughout this project specific aspects of the chicken's intestinal biology have been measured, in particular a number of immune parameters have been assessed. Such targeted investigation can provide important insights into specific aspects of the bird's response to different feeding regimes. The number of such specific assays that can be performed is limited and requires of us the ability to choose assays that are likely to be informative. It would be useful to be able to take a more global approach to the analysis of the underlying biological changes. An alternative approach to understanding the physiological changes occurring in chickens, following the application of different feeding regimes, is to study changes in gene expression.

Gene expression underlies the manifestation of phenotype seen in the structure and function of cells, tissues and organisms. Therefore, a study of gene expression patterns and changes under different conditions has the potential to inform us about underlying physiological processes. The flow of information from the genome through to the functional organism is complex and so the study of gene transcription is certainly not capable of giving a complete picture of the forces at work in shaping phenotype as many post transcription processes (e.g. translational control, protein modification, etc.) are also very important. Despite these limitations gene expression analysis is potentially a very powerful tool for understanding the biological processes that are occurring in any given sample. It has the great advantage of giving a global view of the processes that are occurring. This means that we don't have to choose specific assays and have the possibility of measuring change not only in systems that we would suspect may be changing but also detecting alterations in systems that were previously unsuspected of involvement.

Microarray technologies provide an approach to the characterisation of gene expression patterns. By using a microarray populated with probes for all known chicken genes it is possible to build up a comprehensive picture of the global gene transcription occurring in a particular sample. A comparison of microarray results for different samples can give a picture of differences in gene expression and hence provide some insight into the changes in biological processes that are active in each sample. It is anticipated that an enhanced knowledge of the regulation of genes involved in gut immunity, function, and nutrient uptake, as well as interaction with the intestinal microbiota, will better equip nutritionists to improve bird productivity.

We aimed to investigate how our microarray capability could be used to elucidate genes whose products may be responsible for a chicken's ability to efficiently utilise nutrients available in a diet. Throughout this project a series of animal trials were performed and we were constantly on the lookout for specific samples from these trials that would be most suitable for microarray analysis. The ideal samples would be from treatment groups that showed clear biological differences, particularly with regard to the efficiency of use of different diets and bird performance. None of the trials described in the first four chapters of this report produced whole treatment groups of birds that were different in performance to the control groups. However, careful analysis of the individual bird performance data from the trial described in Chapter 4 revealed groups of birds that were of interest to subject to microarray analysis. We chose to analyse birds, from across the groups that had extreme apparent metabolisable energy (AME) values.

Within any group of chickens it is common to see large differences in individual bird performance despite all efforts to supply common and constant environmental and feeding conditions. It appears that some birds have a head start or natural advantage in terms of efficient digestion. There may be many reasons why bird performance can vary so much; for example, it could be caused by the bird's underlying genetics, by differences in feed induced early gut maturation, earlier access to feed post-hatch, differences in resident microbiota, or by stochastic variations in biochemical processes. Whatever the underlying causes the variation in bird performance is a burden for the chicken producer and it is desirable, from a commercial perspective, to minimise such variation. We aimed to apply microarray analysis to compare gene expression in birds with high and low AME values to investigate possible correlations between specific gene expression patterns and bird performance.

It is important to note that this microarray experiment is a scoping experiment designed to investigate the feasibility of establishing linkages between AME values and gene expression. It is by no means an exhaustive or comprehensive study. The bird trials were not designed with this analysis in mind so sample numbers and the range of AME values are not ideal but are sufficient to test our hypothesis that correlations can be drawn.

Materials and Methods

Sample collection and experimental design

Two microarray experiments were conducted using duodenum samples collected from the animal trials listed in Chapter 4 of this report. Experiment 1 involved birds fed Lf whilst Experiment 2 involved birds fed the Control diet. Tissue samples were from the mid-point of the duodenum and had

been stored in RNAlater (Qiagen Pty Ltd, Vic, Australia) to stabilise the RNA content of each sample. Samples were chosen according to their AME values and separated into two groups, high and low AME respectively. Details of the samples used and their AME values are listed below (Table 39).

Table 39. Details of samples used in microarray experiments 1 and 2.

	Sample number	AME value	High/Low AME value
Experiment 1	50	12.9103	Low
	94	13.0323	Low
	76	12.9688	Low
	61	13.3642	Low
	79	13.7786	Low
	51	14.6162	High
	55	14.6559	High
	59	14.9914	High
	53	15.1747	High
	77	15.5324	High
Experiment 2	54	13.0343	Low
	68	13.3134	Low
	62	13.8729	Low
	72	13.997	Low
	84	14.0381	Low
	85	14.1974	High
	78	14.2248	High
	91	14.2844	High
	95	14.6801	High
	49	15.0232	High

Each sample was independently hybridised to a microarray. The gene expression profiles of high and low AME groups were compared within each experiment to obtain the overall differential gene expression patterns.

Microarray design and hybridisation

Total RNA was isolated from each duodenal sample using the Meridian total RNA isolation kit (Cartagen, San Carlos, CA, USA). Five micrograms of total RNA was reverse transcribed into cDNA and in-directly labelled with Cy3 using the ULS cDNA Synthesis and Labelling Kit (Kreatech Technologies, Amsterdam, The Netherlands). Labelled probes were concentrated using Microcon

Ultracel YM-30 columns (Amicon Bioseparations) and the qualtity of each sample was verified by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Whole genome chicken arrays were pre-hybridised at 42°C for 45 m in pre-hybridisation buffer (25% (v/v) formamide, 5 x SSC, 0.1% (w/v) SDS, 10 mg/ml salmon testes DNA) then incubated with labelled probe in hybridisation solution (25% (v/v) formamide, 5 x SSC, 0.1% (w/v) SDS, 25% (v/v) KREAblock) for 16 h at 42°C. Post hybridisation all arrays were washed once in (2 x SSC, 0.1% (w/v) SDS) for 5 min, once in (0.1 x SSC, 0.1% SDS) for 10 min and three times in 0.1% SSC, each for 1 min.

The whole genome chicken microarray was printed locally at the CSIRO-AAHL based Australian Centre for Poultry Immunogenomics with a MicroGrid II spotting robot using a set of 20,460 long oligos (65-75nt) designed at the Roslin Institute based upon chicken Ensembl gene transcripts and other genomic information supplied by various research groups around the world (http://www.ark-genomics.org/microarrays/byspecies/chicken).

Microarray analysis

Post hybridisation all arrays were scanned and gene expression signals captured using an ArrayWORX (Applied Precision, Issaquah, WA, USA) fluorescent scanner. Quality control measures were applied to each array. All arrays were background corrected and all spot intensities less than two standard deviations of the background were given a set value of 0. Each array was globally normalised; the output from each array was multiplied by a normalisation factor such that the total signal intensities of all arrays were equivalent. Global normalisation was used as it ensures that the measured intensities are comparable across all slides. This sort of normalisation allows comparison of all arrays without biases derived from what we expect the expression patterns to be. Subsequent statistical tests were carried out using Genespring 7.2 (Silicon Genetics, Santa Clara, CA, USA) to determine all genes 2-fold differentially regulated ($p = 0.05$). Gene ontologies were assigned using GO annotation and mapped to their top-level entry.

Results

Experiment 1

A total of 430 genes were 2-fold differentially expressed when comparing the high and low AME groups at a p-value of 0.05. Some genes were more highly expressed in the high AME group and other genes were more highly expressed in the low AME group. These differentially expressed genes included a number of immune and transport genes. Some of the genes are detailed in Table 40.

Table 40. Selected genes differentially expressed between high and low AME groups in microarray experiment one. Fold change values above 2 indicate higher expression in the high AME group whereas values below 0.5 indicate higher expression in the low AME group.

Gene ID	Fold Change	p-value	Description
RIGG18871	11.52	0.0406	aminopeptidase
RIGG04470	5.052	0.0163	vesicle amine transport protein 1
RIGG19369	4.111	0.0407	phospholipid-transporting ATPase IH
RIGG01084	3.764	0.0281	Carbonyl reductase 1
RIGG20043	3.691	0.0434	Gallus gallus beta-defensin 1
RIGG08267	3.116	0.0243	Sulfate transporter
RIGG19207	2.632	0.0482	membrane glycoprotein M6
RIGG19458	2.132	0.0396	repressor of molybdate ABC transporter genes
RIGG00333	2.114	0.0298	vacuolar proton pump, subunit 2
RIGG12736	2.098	0.0331	Carbonic anhydrase 7
RIGG12109	0.49	0.0125	PCTP-like protein
RIGG00643	0.484	0.02	Cationic amino acid transporter-4
RIGG08187	0.483	0.0384	Long-chain-fatty-acid--CoA ligase 3
RIGG18740	0.452	0.0366	Adipocyte fatty acid binding protein
RIGG05954	0.447	0.0406	phospholipid-transporting ATP
RIGG17290	0.439	0.0366	solute carrier family 39 (zinc transporter)
RIGG20050	0.421	0.0299	Gallus gallus interleukin-1beta
RIGG10528	0.393	0.0166	Fatty acid binding protein 6 (bile acid-binding protein)
RIGG08818	0.388	0.034	Uroporphyrinogen decarboxylase
RIGG20034	0.264	0.0339	Gallus gallus interleukin 8
RIGG16419	0.0339	0.0425	Interleukin-8 precursor (Embryo fibroblast protein 1)

Comparison of experiment 1 and 2

A total of 867 genes were differentially expressed by 2-fold or greater between the high and low AME groups in experiment 2. Thirty-nine of the genes found to be differentially regulated in experiment 2 were also regulated in experiment 1 (Figure 18). Of the 39 genes common to both experiments several are known to be involved in immune and digestive processes. Several of these common genes are listed in Table 41.

Table 41. Selected genes differentially expressed between high and low AME groups in both microarray experiment one and two.

Gene ID	Fold Change	Description
RIGG12230	19.21	leucine zipper protein 2
RIGG18055	9.304	Gallus gallus L-type voltage-gated calcium channel alpha1D subunit
RIGG18784	7.581	similar to CD8 alpha chain
RIGG01084	3.764	Carbonyl reductase 1 (CBR1)
RIGG16500	2.21	FAT tumor suppressor homolog 4
RIGG19458	2.132	repressor of molybdate ABC transporter genes
RIGG10044	0.462	G protein-coupled receptor
RIGG02546	0.445	Mannan-binding lectin serine peptidase 2 (MASP2)
RIGG03009	0.381	Regulator of G-protein signaling protein GAIP
RIGG11230	0.378	similar to islet cell autoantigen 512
RIGG09730	0.353	Gallus gallus tubulin, beta 6
RIGG09940	0.318	MHC Rfp-Y class I alpha chain
RIGG06518	0.318	Peroxisome assembly protein 10
RIGG20034	0.264	Gallus gallus interleukin 8 (IL8)
RIGG19688	0.194	Gallus gallus beta-globin (HBB)
RIGG17103	0.0355	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin)

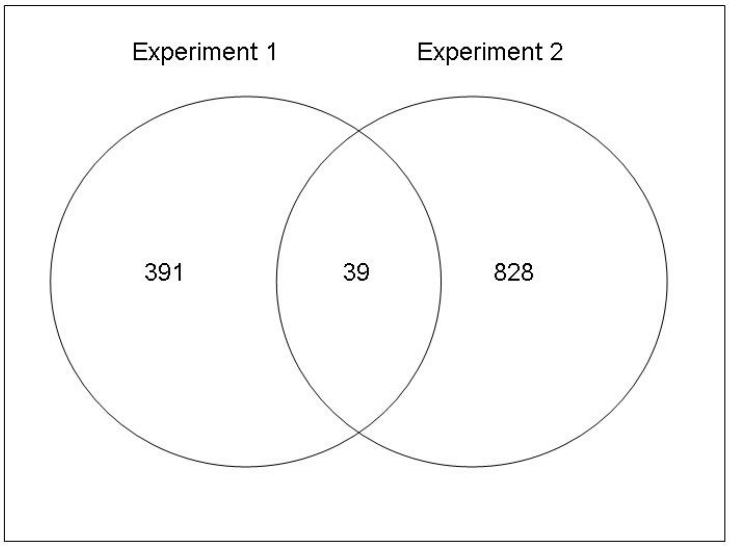


Figure 18. Venn diagram displaying the number of 2-fold differentially expressed genes that are common between experiment one and two (shown in overlap of the two circles).

Discussion

Apparent metabolisable energy provides a measurement of bird energy metabolism. Previous studies have identified considerable differences in AME values between birds fed an identical diet (Hughes and Choct 1997). This indicates that individual birds handle available energy with varying efficiency. The preliminary microarray experiment reported here has indicated that there are detectable gene expression differences between high and low AME birds. These results suggest that it may be possible to correlate AME values with gene expression. The identification of key genes responsible for the variation in these AME values would provide an invaluable tool for future bird breeding programs. Furthermore it may be possible to use this gene expression information to identify cellular processes that are active in birds fed particular diets and hence assist in tailoring feeding regimes to get optimum performance from birds. To achieve these goals a much more extensive study would be required using much larger sample groups and multiple independent experiments to define particular gene subsets that are most informative and reliably correlated to AME values.

While this experiment was solely done to investigate the possibility of correlating differences in individual bird performance and gene expression, it has identified a number of key immune genes that are of interest. Many of these immune genes may influence a bird's ability to maintain a healthy gut. This is of vital importance to the poultry industry especially with the possibility of eradication of in feed antibiotics. Thus future nutrigenomics studies could focus on the role of immune genes in individual bird performance. Throughout the trials carried out for this project particular elements of the birds immune system have been analysed; this gene expression work points to other specific immune assays that could be performed in order to confirm some of the differences that have been identified, for example it would be interesting to directly assay for interleukin-8 and interleukin-1beta activity and other elements of the innate immune system such as beta-defensin 1. The microarray results also suggest that the activity of certain enzymes and transporter molecules may be important in determining efficient energy use and so it would be of use to directly assay for such enzymes (e.g. aminopeptidase, carbonyl reductase, etc.).

One of the key challenges in fully using the output of these microarray experiments is to be able to fully integrate the knowledge regarding gene expression into a coherent model of cellular biochemistry. Ideally what is required is that every gene in the chicken genome is understood in terms of the function of the protein that is encoded and that that protein function can be mapped to metabolic pathways and roles within the cell. Unfortunately, at the present time, the function of at least a third of the genes in the chicken genome are completely unknown and for many of the genes of supposed known function those functions have not been accurately mapped to particular biochemical pathways.

This assignment of genes to pathways/functions within the cell is known as gene ontology and is a key task being undertaken by the scientific community around the world. Future progress in this area, and integration of the knowledge into the analysis of the connection of gene expression and underlying cellular physiology, is required in order to maximise the analytical power of these experiments.

Overall this initial experiment suggests that there is a link between AME values and gene expression. What we cannot tell at present are the causal links between AME and gene expression. Which of the particular genes identified actually play an intimate role in the efficient use of nutrients and which are responding simply as bystanders, reacting to other things that are happening in the gut? These results provide the groundwork for future nutrigenomics work, which could identify key genes and pathways responsible for the considerable differences in AME values between seemingly identical birds.

Discussion of Results

The aim of this project was to improve our understanding of the complex interactions amongst nutrition, the commensal bacteria of the gastrointestinal tract, mucosal immunity, gut structure and function, intestinal gene expression, and corresponding production efficiency and bird health. To achieve this aim, we have performed a series of bird metabolism studies, a small-scale growth study and a necrotic enteritis challenge experiment in order to assess the effects of antibiotics and candidate antibiotic alternatives on the intestinal environment and subsequent bird performance. A recurring finding throughout the project was that no antibiotic alternative studied led to any observable growth promoting effect in broiler chickens. Indeed, a frequently used in-feed antibiotic, zinc bacitracin, also failed to impart a growth promoting effect in experiments involving healthy birds. This is not an uncommon phenomenon, as Hooge (2004) commented that when little stress is placed on broilers, performance is often similar between diets with and without added antibiotics. As discussed in the preceding chapters, this lack of an antibiotic effect may have been due to the absence of an environmental stimuli or "dirty", sub-optimal conditions that would elicit an antibiotic effect. These factors could include stocking density, litter quality and age, water and feed quality and environmental conditions such as temperature and humidity. It was possible that the transfer of birds from litter to metabolism cages at a relatively young age (13 days) may have prevented some of these factors from influencing bird performance; therefore, changes were made to the experimental protocol in Chapter 4 in order to increase the likelihood of performance differences (including increased bird age prior to transfer to metabolism cages and the inclusion of a small-scale growth study where birds were housed on litter in pens for the duration of the trial). These alterations failed to elicit the expected performance differences. Further studies of this nature should focus on developing a repeatable model to ensure performance differences between antibiotic and additive-free control birds. This may involve re-using litter, increasing stocking density to be comparable with industry practices, or relaxing our stringent cleaning procedures between batches of birds, a technique previously employed to promote performance differences (Humphrey et al. 2002). However, even when these conditions were maintained by Humphrey and colleagues, one out of two studies failed to induce an antibiotic-induced improvement in feed efficiency, with neither study producing observable differences in body weight gain between birds fed antibiotic and control diets (Humphrey et al. 2002). One further possible explanation for the lack of antibiotic effect may be associated with the specific antibiotic used; zinc bacitracin. ZnB has been used extensively in the industry in Australia and has been used in our research facility at SARDI-PPPI for over 10 years; with the potential for resistance to develop over this period. A variable response of different flocks of birds to ZnB (and potentially other antibiotics) has been highlighted in this study as the microbiota of Control and ZnB-fed birds were similar to one another in certain studies (Chapter 3), but significantly different in other studies (Chapters 2 and 4).

Interestingly however, despite the lack of performance effects, it was observed in Chapters 2-4 that antibiotics and antibiotic alternatives had significant effects on a range of parameters associated with the intestinal health of the bird, including the microbiota, microbial activity and the mucosal immune system. In the absence of subsequent performance differences, a thorough understanding of the overall effects of these changes is difficult; however, it is obvious from these studies that particular compounds may possess the capacity to shift the intestinal environment in a favourable manner. For example, prebiotics were observed to influence key immunological parameters in a similar manner to ZnB. This may indicate that prebiotics could be a substitute for at least some of the key properties of antibiotics. Importantly, it must be noted that none of the additives that were assessed impaired performance in healthy birds.

Due to the observed lack of performance differences in Chapters 2-4 we undertook to expand this project to incorporate a study in birds with an infectious challenge that would promote detectable performance differences. We selected necrotic enteritis as it is a major poultry disease in Australia and world-wide; its incidence expected to increase with the withdrawal of in-feed antibiotics (McDevitt et al. 2006). Additionally, in-feed antibiotic supplementation with ZnB is known to be extremely successful in preventing the onset of NE, therefore it provided an ideal industry relevant model to screen the capacity for candidate alternatives to replicate this effect. Indeed, in this study, performed at the University of New England, we found that ZnB successfully prevented the onset of NE in broilers; however, supplementation with MCFA and a candidate probiotic organism (*L. johnsonii*) had no protective effects, indicating that they are not likely alternatives for use in poultry.

Whilst these studies indicate that MCFA and this particular *L. johnsonii* strain are not suitable replacements for antibiotics, these findings do not rule out the likelihood that other MCFA mixtures and probiotic organisms may have protective effects. Likewise, other potential classes of antibiotic alternatives, including prebiotics, *n*-3 fats, immunomodulatory compounds and plant extracts may be of benefit to broiler health and performance. It may indeed be the case that some compounds/organisms are more suited to improving growth and performance efficiency in healthy birds but cannot prevent outbreaks of NE or other infectious diseases; whilst others may prevent NE but have little effect on growth promotion in healthy birds. It is quite likely that no one individual compound or organism can completely replace the effects of antibiotics, and that strategically-selected combinations may be a more promising avenue of investigation. For example, a MCFA compound may be selected for its capacity to decrease pathogen load, whilst a prebiotic may be selected to maintain overall microbial health and stimulate the growth and activity of beneficial commensal bacteria; in turn modulating the mucosal immune system via host-microbe crosstalk and contributing to a healthy intestinal environment. This introduces another layer of complexity however, as different compounds may act synergistically or antagonistically *in vivo*.

This project ambitiously attempted to provide an integrated picture of the intestinal environment in response to various feed additives in both healthy birds, and those challenged with *C. perfringens*. This task has produced a significant body of data that indicates the extent of the influence that feed additives can impart upon key components of intestinal health.

Microbiota

A healthy intestinal microbiota is critical for the optimal performance of the bird as increased pathogen levels have been demonstrated to underlie conditions which limit performance, such as NE (Jia et al. 2009). Throughout this project we have employed T-RFLP to characterise the overall microbial communities, and Lac PCR-DGGE to focus more specifically on the *Lactobacillus* genus. We observed that the in-feed supplementation with ZnB significantly altered the intestinal microbial communities of the ileum in Chapter 2, had no affect in Chapter 3, and influenced the caecal communities in Chapter 4. The inconsistent effects of ZnB amongst these three studies may indicate that the microbiota of different flocks of birds may respond variably to antibiotics. These findings further complicate studies involving intestinal microbial profiling, as feed additives may not have consistent, repeatable effects. Further studies involving the assessment of identical diets and feed additives over a series of trials are needed in order to determine the variability of the microbial response and whether dietary manipulation will have the capacity to consistently influence the microbial communities in order to promote the development of a desirable microbiota.

In a series of experiments performed during this project we observed differential effects of feed additives on the microbiota. Prebiotics appeared to influence the intestinal microbial profiles to an extent, whilst *n*-3 PUFA only appeared to have a minimal effect on the *Lactobacillus* genus, with lactoferrin influencing the microbiota compared to ZnB. No microbial shifts led to a significant improvement or worsening of bird performance. This indicates that a range of different microbial compositions can facilitate a relatively high level of bird performance. Interestingly, it was often observed that the feed additives assessed in the current project shifted the microbiota compared to ZnB; however, the profiles were comparable to the control diet with no additives. This indeed may indicate that some of the observed differences may have been more related to the effects of ZnB rather than that of the feed additive. This trend was observed in studies involving prebiotics and lactoferrin. Indeed, when searching for compounds which can influence the microbiota in a positive manner for bird health and performance one must consider comparisons with antibiotic-containing and antibiotic-free diets, and more must be known as to which microbial profiles are most conducive to a high level of bird performance.

The intestinal microbiota is highly complex and a greater understanding of the importance of the microbiota for bird health is needed. Further microbial profiling may indeed identify particular organisms which are related to high and low performance or provide an overview of which microbial profiles are conducive to efficient bird production. The highly complex nature of the microbiota and interactions amongst bacterial species will complicate this process. Additionally, it should be determined if dietary manipulation can consistently facilitate the development of a particular microbial profile over time and in different environmental conditions.

***Lactobacillus* species**

Throughout this project we maintained a specific focus on the *Lactobacillus* species profiles of broiler chickens. Lactobacilli naturally reside in the chicken gastrointestinal tract and are considered "autochthonous", with various species found in the gut flora of birds raised under different conditions in different locations. Members of this genus may therefore represent "healthy marker species". Furthermore, a variety of beneficial properties of lactobacilli have been reported in poultry (Patterson and Burkholder 2003; La Ragione et al. 2004; Apata 2008), and other hosts including human (Ljungh and Wadstrom 2006).

Consistent with the literature, lactobacilli were identified in birds in all the experiments although the species represented varied. Of particular note, *L. crispatus*, *L. gallinarum* and/or *L. crispatus* were found in nearly every bird in all experiments, while the presence of *L. johnsonii*, *L. salivarius*, and/or *L. reuteri* varied. Sophisticated statistical analyses, previously used by Torok et al. (2008), were utilised to characterise the *Lactobacillus* species profiles of individual birds across dietary treatments. This approach was used to explore the relationship between *Lactobacillus*, dietary manipulation, and improved performance. Significant differences in *Lactobacillus* microflora were noted in Chapter 2 and 3 where *L. johnsonii* was the main contributor to ileal shifts in Control and ZnB-fed birds, and also a significant contributor to caecal differences between Control and 2% SALmate^{TM}, respectively. Differences in the prevalence of this species were also noted in Chapter 5, and statistical procedures will be applied to the results described in the final two chapters to determine if there are significant differences. Over the course of the experiments, it is important to note that lactobacilli were present regardless of significant shifts in the total microflora, the presence of antibiotics, and differences in diet.

Analysis of the results, however, did not link lactobacilli to other aspects investigated. In Chapter 1 we reported that gender did not appear to influence the *Lactobacillus* species profile in the intestine. While we observed that feed additives can influence the *Lactobacillus* species profiles of broilers to varying extents in Chapters 2-5, none of these changes were associated with improved bird performance. Lactobacilli are known to convey numerous beneficial properties to the host, and as

mentioned earlier, it may be the case in the current project that the optimal conditions for birds provided in Chapters 1-4 may have prevented any shifts in the microbiota from having an effect on performance, either positively or negatively.

A *Lactobacillus johnsonii* strain was administered as a potential probiotic in Chapter 5, in the setting of a necrotic enteritis challenge. This particular strain could not counteract the severity of *C. perfringens*-induced damage. Background or native *L. johnsonii* were present in ZnB and challenged birds, and other *Lactobacillus* species were present indicating that they can persist during a *C. perfringens* challenge. Further investigation is therefore warranted into the use of *Lactobacillus* species, and other bacteria, such as *Bifidobacteria*, as probiotics to improve the growth and performance of broilers, and protect from infectious diseases. In addition, the use of multi-strain and multi-species combinations should be investigated further and may be more likely to counteract *C. perfringens in vivo.* Further studies are also needed to assess the effect of dietary manipulation on endogenous lactobacilli in sub-optimal conditions and during NE challenge in order to assess whether lactobacilli (or other microbes) can be linked to improved performance. Indeed, the identification of commensal organisms linked to high levels of performance may lead to the isolation of ideal candidates for new probiotics.

Microbial activity

As was the case with intestinal microbial profiling, the microbial activity within broilers was quite different across studies. In Chapters 2 and 4 we reported no effects of the in-feed additives on microbial activity, assessed by VFA production, despite shifts in microbial profiles. In Chapter 3 however, despite no effects on the overall bacterial communities, changes to the levels of VFAs were observed. This indicated that there may be variability not only in microbial profiles, but also in the metabolic activities of the microbiota. Assessment of microbial activity by measuring VFA production can be difficult as multiple species can produce the similar VFA profiles. Future studies in this area should expand the investigation into other determinants of microbial activity and also begin to characterise the shifts in microbial gene expression.

Immunity

In contrast to the microbial communities, the immune responses to ZnB appeared more consistent throughout the project. Zinc bacitracin did not affect caecal tonsil cytokine expression and phagocytosis in Chapters 2 and 3, indicating a consistent effect; likewise caecal tonsil T and B cell phenotypes were predominantly consistent with only the proportion of $Bu1^+$ cells different amongst studies. Plasma IgA responses to ZnB were also consistent across studies; however IgG and IgM responses differed. Interestingly, we observed that prebiotic administration had a similar effect to ZnB on key immune parameters including plasma Ig levels and B cell phenotypes, whilst appearing to also

influence lymphocyte proliferation. This suggests an additional role of prebiotics on immune status, to complement the well documented effects on the intestinal microbiota. Whether the prebiotic-induced microbial shifts led to the observed immune modulation via host-microbe crosstalk, or whether direct prebiotic-immune cell interaction is involved requires further investigation. Studies into the effects of MOS, FOS, and other prebiotics in a disease challenge environment and in sub-optimal conditions are indicated, in order to determine if they can successfully and consistently improve bird performance. In addition to prebiotics, compounds with known immunomodulatory properties, such as *n*-3 PUFA and lactoferrin, influenced key immune parameters. Future studies into the effects of these additives in the presence of an immune stimulus are indicated in order to comprehensively characterise their immunomodulatory and potential health-promoting capacity.

Intestinal mucin

Intestinal mucin is important in the protection of the epithelium and the regulation of microbial binding and colonisation (Lievin-Le Moal and Servin 2006). Dietary additives appeared to have the capacity to influence mucin sub-type production. As is the case with the microbiota and immune system, more research is needed to understand which mucin compositions facilitate optimal bird health and performance. Different mucin compositions may promote the colonisation of distinct microbial profiles, which will also influence the immune status of the bird. This project and other recent studies suggest that these components of the intestinal environment can be manipulated by dietary means. The identification of the optimal intestinal environment, and an understanding of which dietary additives can facilitate the development of this environment in a reliable manner, is crucial to the maintenance and potential improvement of bird performance in the absence of in-feed antibiotics.

Intestinal gene expression

Our preliminary microarray experiments in high and low performing birds indicate that there is a link between AME and gene expression. Our analyses indicate that genes involved in immunity and nutrient transport were among the subsets of genes differentially expressed in high and low performing birds. Future studies are required to further investigate the relationship between AME and intestinal gene expression, and to identify key genes and pathways underlying any performance differences. Additionally, a nutrigenomics approach can be adopted to determine if particular feed additives can improve bird performance and/or flock uniformity.

The integrated picture

This project has highlighted the variable effects that nutrition can have on the intestinal environment. This entire data set can now be further examined in order to study relationships amongst the different components of the intestinal environment and bird performance within individual birds, in order to identify other key factors underlying variable bird performance in broilers fed the same diet; a

common phenomenon in the poultry industry (Hughes and Choct 1997). The identification of changes in these components, linked to improved performance of individual birds, may indicate areas for future strategic research.

This study highlighted the fact that changes made to the diet have the capacity to significantly influence the overall intestinal environment, and, whilst not observed in the current project, these changes can potentially be beneficial or damaging to bird health and performance. It is important that we continue to improve our understanding of these individual aspects of intestinal health, including the microbiota and immune status, and further develop our knowledge of the interactions amongst these components and any subsequent effects on bird health. In light of pressure for withdrawal of antibiotics from broiler feed, the assessment of the impact of candidate natural alternatives on healthy and diseased birds is crucial for the sustained viability of the poultry industry.

Importantly, the current project has successfully used a multi-disciplinary approach to investigate the influence of nutrition on the intestinal environment of broiler chickens. This project has brought together experts in the fields of immunology, microbiology, molecular biology, genetics, nutrition and intestinal physiology in order to simultaneously assess the impact of antibiotics and natural alternatives on intestinal health and broiler performance. This project highlights the capacity of the Australian Poultry CRC to fund ambitious, multi-institutional, multi-disciplinary projects which tackle key industry problems. The collaborative research team brought together within this project have the capacity to use and expand upon the techniques and database established within this project to further extend our knowledge of how the chicken intestine is influenced by nutrition, how different components of the intestinal environment interact with one another, and how this may influence bird performance and disease resistance. Research into this area will also no doubt incorporate an increased focus on emerging scientific fields including nutrigenomics, metabolomics and microbiomics.

Implications

- Our findings indicate that in-feed antibiotics will not improve bird performance in all conditions and that sub-optimal conditions are necessary to produce a detectable performance improvement. This supports previous the finding by Hooge (2004) that when birds are not exposed to stressors, performance between diets with and without antibiotics often does not differ. Future work in this arena should attempt to experimentally induce these slightly sub-optimal conditions, which are more likely to simulate the conditions apparent in the broiler industry compared to the more sanitary conditions of a small-scale research facility.

- Factors including bird type, level of cleanliness, climate and housing conditions are likely to influence whether a feed additive (antibiotic or otherwise) may have a beneficial effect.

- Within the optimal conditions created in the current series of studies, none of the dietary additives assessed had a negative effect on bird performance, indicating no damaging effects associated with their dietary inclusion.

- Dietary additives have the capacity to alter components of the intestinal environment, including the microbiota, the immune system, and microbial activity. Our results, and other studies, also indicate likely diet-induced effects on intestinal mucin dynamics.

- Significant alterations to the intestinal microbiota and the immune status may not deter from performance. Indeed, a high level of performance was observed in birds with a range of different microbial compositions. This phenomenon was also observed for changes in immune parameters and microbial activity.

- Throughout this experiment we observed that similar microbial compositions can have a different activity in terms of VFA production, whilst different microbiota can have a similar level of activity. Therefore, it is important to concurrently investigate microbial composition and activity. Future studies of this nature may also focus on microbial gene expression.

- The MCFA mixture and *L. johnsonii* strain assessed did not impart the same benefits as ZnB in protection from NE. However, this does not imply that other MCFA mixtures or other bacterial strains will produce the same results. Further studies investigating different compounds/organisms and strategically-selected combinations of different compounds are needed in order to identify candidate replacements for in-feed antibiotics.

- This project has produced a large amount of information on the impact of diet-induced shifts on the intestinal environment of individual birds. This can now be used in a data mining exercise to identify if particular shifts are associated with improved or decreased performance of that particular bird. This may identify targets for future research into improving bird health and performance by optimising gut health and gut function.

- A multi-disciplinary collaborative research team has been established which can tackle key industry issues associated with poultry health and nutrition. The techniques and database established during this project and by members of this collaborative team can be expanded upon in the future to further improve our understanding of how nutrition influences gut health, how different components of the intestinal environment interact, and how changes in the intestinal environment influence bird health and performance.

Recommendations

- Antibiotics, and alternatives, will not necessarily have a performance-enhancing effect when there is no "challenge" on the birds, or if birds are housed in optimal conditions. Therefore future research in this area should attempt to simulate housing conditions that are more indicative of industry hygiene levels.

- Further investigation into the role of the microbiota on bird performance is needed. This should encompass studies into the microbial species linked with high and low performance. Identification of these species may lead to novel probiotic products, or dietary additives that can facilitate the manipulation of the microbiota to increase levels of these beneficial species, and decrease levels of bacteria associated with low performance.

- More investigation into the repeatability of diet-induced effects on the microbiota is needed, in order to determine if a desirable microbiota can consistently be reproduced in broilers by the inclusion of dietary additives (antibiotic-based or otherwise).

- Studies investigating links between bird performance and the microbiota should also consider microbial activity, as a comparable activity can be facilitated by a different microbiota and vice versa.

- It is unlikely that one compound/organism will have a similar effect to that of antibiotics (in terms of growth performance and disease prevention). Therefore, a targeted approach consisting of strategically-selected combinations of compounds may be more successful. Potential synergistic and/or antagonistic activities of these combinations must be considered.

- Further evaluation of the effects that antibiotic alternatives have on different components within the intestinal environment may provide a "database" to facilitate strategic selection of these compounds based on desired outcomes.

- The selection of the optimal feed additives (or combinations) must be investigated in multiple scenarios (for example, optimal conditions, sub-optimal conditions, and infectious challenge), as bird responses may differ significantly.

- As seen for other components of the intestinal environment, the current project and other recent studies suggest that dietary additives may influence the dynamics and composition of intestinal mucin. This will be important in the protection of the epithelia and may influence other important factors including the intestinal microbiota and mucosal immune system. An

improved understanding of intestinal mucin dynamics and knowledge of a dietary means to support the development of an optimal mucin composition for the establishment and maintenance of a healthy intestinal environment will be important.

- Further work is needed in the field of nutrigenomics. Firstly, the level of natural variation in intestinal gene expression amongst birds must be known, and from there one can investigate the effects of dietary manipulation on the expression of key groups of genes. Assessment of the impact of gender and disease on gene expression is also needed.

- Preliminary studies have indicated that there are important changes in gene expression between high and low performing birds. This phenomenon should be investigated further and may lead to future nutrigenomics-based research to improve the performance of low-performing birds, and promote flock uniformity.

- The microbiota and intestinal immune system can play key roles in the prevention of intestinal diseases and bird performance. Further investigation into the roles of these systems on intestinal health and broiler performance is needed. Future research must also focus on understanding how these factors can be manipulated by the diet, and whether the microbiota or immune system can be improved via nutrition on a consistent basis to promote performance that is equivalent to that of antibiotics.

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Appendices

Appendix A: Abbreviations

AB	Alcian blue
AME	Apparent metabolisable energy
ANOSIM	Analysis of similarity
ATCC	American Type Culture Collection
BU1	Chicken B cell marker chB6
CD	Cluster of differentiation
Con A	Concanavalin A
cpm	Counts per minute
CT	Caecal tonsil
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid
DM	Dry matter
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribose nucleic acid
DPA	Docosapentaenoic acid
Dsc	Dice's similarity coefficient
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FA	Fatty acid
FACS	Fluorescence activated cell sorting
FAME	Fatty acid methyl ester
FB	Flow cytometry buffer
FCR	Feed conversion ratio
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate
FOS	Fructooligosaccharide
FSC	Forward scatter
GALT	Gut-associated lymphoid tissue
GE	Gross Energy
GEDC	Gross energy digestibility coefficient
GLM	General linear model
HRP	Horseradish peroxidase

IDE	Ileal digestible energy
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IMO	Isomaltooligosaccharide
IS	Internal standard
Lac	*Lactobacillus*
Lac	*Lactobacillus acidophilus*
Lc	Lactobacillus crispatus
LCGA	Group A acidophilus
Lf	Lactoferrin
Lg	*Lactobacillus gasseri*
Lj	*Lactobacillus johnsonii*
Lr	*Lactobacillus reuteri*
Ls	*Lactobacillus salivarius*
MOS	Mannanoligosaccharide
Mono	Monounsaturated
MCFA	Medium chain fatty acid
MHC	Major histocompatibility complex
MUFA	Monounsaturated fatty acid
NE	Necrotic enteritis
NRC	National Research Council
OD	Optical density
OTU	Operational taxonomic unit
Pa	*Pediococcus acidilactici*
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pp	*Pediococcus pentosaceous*
ppm	Parts per million
PUFA	Polyunsaturated fatty acid
RT	Real time
Sats	Saturated fatty acids
SCFA	Short chain fatty acid

SEM	Standard error of the mean
SI	Stimulation index
SIMPER	Similarity percentage
SPRD	Spectral red
SSC	Side scatter
TCR	T cell receptor
TMB	Tetramethylbenzidine
TOS	Transgalactooligosaccaride
T-RFLP	Terminal-restriction fragment length polymorphism
UPGMA	Unweighted pair group methods using arithmetic averages
VFA	Volatile fatty acid
ZnB	Zinc bacitracin

Appendix B: Publications derived from this project

1. Geier MS, Torok VA, Allison GE, Gibson RA, Janardhana V, Ophel-Keller K, Hughes RJ. Prebiotics and omega-3 polyunsaturated fatty acids influence the intestinal mucosal immune system and microbial communities of broiler chickens but do not improve performance (2008) *World's Poultry Science Journal* 64(Supp 2); 337.

2. Janardhana V, Broadway MM, Bruce MP, Lowenthal JW, Geier MS, Hughes RJ, Bean AGD. Impact of feed additives on immune function in the chicken (2008) *World's Poultry Science Journal* 64(Supp 2); 366.

3. Geier MS, Torok VA, Allison GE, Ophel-Keller K, Hughes RJ. Indigestible carbohydrates alter the intestinal microbiota but do not influence the performance of broiler chickens (2009) *Journal of Applied Microbiology* 106(5):1540-1548.

4. Geier MS, Torok VA, Boulianne M, Allison GE, Janardhana V, Guo P, Bean AGD, Hughes RJ. The effects of lactoferrin on broiler performance, gut microbial communities and intestinal mucosal immune system (2009) *Proceedings of the Australian Poultry Science Symposium* 20:81-84.

**Note: further publications are planned after completion of this final report.

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Project Title:	An integrated approach to understanding gut function and gut health of chickens
Poultry CRC Project No:	05-2
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Project Overview	This project addressed one of the Poultry CRC's primary objectives, *sustainable production of chicken meat without reliance on antibiotics*. We aimed to further understand the complex interactions amongst nutrition, intestinal bacteria, microbial activity, the mucosal immune system, gut structure and function and intestinal gene expression. We also studied how diet-induced changes to the intestinal environment subsequently altered bird growth and performance in healthy and challenged birds.
Background	One means by which the Australian chicken meat industry maintains a high standard of bird health and performance efficiency is by the in-feed addition of sub-therapeutic levels of antibiotics. Due to fears of antibiotic resistance, the European Union have recently banned in-feed antibiotics, with consumer pressure also driving other countries toward reduced usage. The absence of in-feed antibiotics has increased the susceptibility of birds to infectious diseases and has the potential to significantly reduce growth and performance. As such, the identification of natural alternatives to antibiotics is of importance to the industry in order to facilitate sustainable production of chicken meat without reliance on antibiotics. Current candidates for inclusion in poultry feed include prebiotics, probiotics, fatty acids, plant extracts and natural immunomodulatory compounds. These feed additives will all influence the intestinal environment including the microbiota, immunity, gut structure and function, and gene expression. The intestinal environment is complex and an improved understanding of how various facets interact with one another, and how they respond to dietary manipulation will facilitate the development of feeding regimens aimed at improving bird health and performance via the establishment and maintenance of an optimal intestinal environment, without reliance on antibiotics.
Research	This ambitious project utilised the multi-disciplinary skills set of CRC collaborators to simultaneously assess multiple intestinal parameters and bird performance. A series of bird metabolism experiments were performed in healthy birds to assess the effects of candidate antibiotic alternatives on bird performance and intestinal health compared to control diets with and without added antibiotics. We also performed an experiment in birds challenged with *Clostridium perfringens* in order to assess the capacity for selected antibiotic alternatives to prevent the onset of necrotic enteritis. Throughout the project we employed cutting edge techniques to study the intestinal microbial profiles (terminal-restriction fragment length polymorphism and denaturing gradient gel electrophoresis), microbial activity (volatile fatty acid measurement), immune status (enzyme-linked immunosorbance assay, flow cytometry, PCR, and live cell assays), mucin composition (histological staining) and intestinal gene expression (microarray).
Project Progress	Completed
Implications	• In-feed additives have the capacity to influence multiple aspects of the intestinal environment. These changes may or may not subsequently

	influence bird performance. • A high level of bird performance can be achieved under various microbial profiles and immune states. • The observation that a MCFA blend and a *Lactobacillus* strain could not prevent necrotic enteritis does not imply that other compounds/ organisms from the same classes will also fail. • It is more likely that strategically-selected combinations of compounds/ organisms will have the capacity to replace the actions of current in-feed antibiotics. • Preliminary analyses indicate that there may be a link between intestinal gene expression and AME values of individual birds.
Publications	• Geier MS, Torok VA, Allison GE, Gibson RA, Janardhana V, Ophel-Keller K, Hughes RJ. Prebiotics and omega-3 polyunsaturated fatty acids influence the intestinal mucosal immune system and microbial communities of broiler chickens but do not improve performance (2008) *World's Poultry Science Journal* 64(Supp 2); 337. • Janardhana V, Broadway MM, Bruce MP, Lowenthal JW, Geier MS, Hughes RJ, Bean AGD. Impact of feed additives on immune function in the chicken (2008) *World's Poultry Science Journal* 64(Supp 2); 366. • Geier MS, Torok VA, Allison GE, Ophel-Keller K, Hughes RJ. Indigestible carbohydrates alter the intestinal microbiota but do not influence the performance of broiler chickens (2009) *Journal of Applied Microbiology* 106(5):1540-1548. • Geier MS, Torok VA, Boulianne M, Allison GE, Janardhana V, Guo P, Bean AGD, Hughes RJ. The effects of lactoferrin on broiler performance, gut microbial communities and intestinal mucosal immune system (2009) *Proceedings of the Australian Poultry Science Symposium* 20:81-84. **Note: further publications are planned after completion of this final report.