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Control over life-long productivity by dietary manipulation immediately post hatch

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Executive Summary

This project sought to identify alternatives to antibiotics, using two separate strategies; one based on novel extracts obtained from native Australian and New Zealand plants, and the other based on manipulations to feed processing. In the first strategy, extracts were obtained from *Arthropodium cirratum* (Rengarenga lily), *Cordyline australis* (Cabbage tree); a seaweed, *Undaria pinnatifida* (Undaria), Rengarenga lily and *Acacia pycnantha* (Acacia extract). The Rengarenga lily and Cabbage tree contained predominantly fructose and glucose (82-95 %), the Acacia extract contained mainly galactose (78 %) and arabinose (22 %) while Undaria extract contained fucose (55 %) and galactose (44 %). These extracts were supplemented in diets and fed to broiler chickens, in comparison with existing commercial products and the antibiotic, Zn bacitracin. The dietary inclusion of fructans from Rengarenga and a commercial product, Frutafit® affected broiler performance differently and in a dose-dependent manner. In the second feeding trial, supplementation with extracts from Cabbage tree, Acacia, and Undaria seaweed had no effect on performance, gut morphology, microbial populations, and organic acid concentrations in the digestive tract of broilers. The high levels of Acacia extract and Undaria extract significantly ($P<0.05$) reduced the population of coliform bacteria in the ileum compared to the negative control group. The population of *Clostridium perfringens* (Cp) in the caeca was reduced ($P<0.05$) by plant extracts, but not in the ileum. The antibiotic supplement reduced the population of Cp in both the ileum and caeca compared with the negative control group.

The Lactobacilli isolated from the ileum and caeca of birds on diets supplemented with extracts of Cabbage tree, seaweed, Undaris, and exudates from *Acacia pycnantha* belonged to four distinctive groups: *L. salivarius* group (group I), *L. crispatus* group (group II), unidentified Lactobacillus species (group III), and *L. johnsonii* (group IV). All four groups fermented the monosaccharide, galactose at varying levels and disaccharides; sucrose, trehalose, and palatinose. Sucrose was the substrate which was the most fermented by isolates from all four groups. The Rengarenga lily and Acacia extracts were tested in a necrotic enteritis model, in comparison with the commercial products, Fibregum® and Raftifeed®-IPE. The birds on the antibiotic-supplemented diet showed better performance throughout the experimental period. The Fibregum-supplemented group had a lower ($P<0.05$) NE related mortality compared to the Acacia extract-supplemented group, but this mortality was not different ($P<0.08$) when compared to the negative control group. Fibregum decreased ($P<0.05$) the number of *C. perfringens* in the caeca before Cp challenge but this effect was not significant after Cp challenge, although the values tended to be lower than those of the negative control group. Total serum IgY level did not differ between treatment groups of chickens before challenge but the Fibregum supplement resulted in an increase ($P<0.05$) in IgM concentration compared to those fed with Acacia and lily extracts. Such stimulation is useful in preparing birds to respond to a disease challenge.

The focus of the second strategy was to manipulate gastrointestinal function through feeding of diets based on grains processed to different degrees, using different processing techniques. The results of the feeding trials revealed that feeding an intermediate sized, roller-milled sorghum diet enabled the best feed conversion compared to whole, fine and intermediate hammer-milled sorghum. Nitrogen-corrected apparent metabolisable energy (AMEn) was improved by feeding whole sorghum. The weight of the proventriculus was inversely proportional to the particle size of the feed while the relative gizzard weight of birds fed the whole sorghum diet higher ($P<0.05$) than that of birds on ground grain. At three and five weeks of age, the relative weight of the duodenum was increased ($P<0.05$) by feeding wheat relative to sorghum. The pH of the gizzard content was inversely proportional and the pH of the duodenum digesta was proportional to the particle size of the feed across age differences ($P<0.05$). There were no significant differences between treatments in proximal jejunal histomorphology. Feed texture tended to have some effect on the villus height ($P<0.10$) and villus height to crypt depth ratio at 7 and 21 days of age ($P<0.10$ and $P<0.05$, respectively).

Under commercial rearing conditions, the 43d body weight was unaffected by type of diet or processing method but tended to be higher on a coarse hammer-milled wheat diet than on the other

diets. The FCR on whole-wheat diet was 3 % better than on the coarse, hammer-milled diet, which had the poorest FCR. The fine hammer-milled wheat-based treatment, without antibiotic supplements, yielded a lower ($P<0.05$) ileal starch digestibility than all other treatments. The weight of the gizzard from the whole wheat-fed birds was also 15 and 8 % higher than that of birds on the fine hammer-milled antibiotic-supplemented diet at 21 and 43d of age, respectively. The population of Lactobacilli in the gizzard was highest ($P<0.03$) in birds raised on the coarsely ground wheat diet and lowest on the whole wheat diet. Total anaerobe counts were also higher ($P<0.001$) in the ileum of chickens on the former diet than in other chickens. The morphometry of the jejunal mucosa was unaffected by dietary treatment.

The results demonstrate that the plant extracts alter the microbial composition of the GIT but did not significantly improve growth and feed efficiency. Feeding whole or coarsely milled grain in the diet appears to confer some advantage to intestinal development but there is no conclusive evidence that this practice alone would substitute for the use of antibiotics in poultry diets. Perhaps, it may be necessary to test mixed proportions of the different extracts, to identify any synergy between the different compounds. It is also not known if combining the two strategies will be of some benefit to feed utilization and growth of broiler chickens.

1. Introduction

Since the mid-1940s, many broad-spectrum antibiotics have been used intensively by the poultry industry as therapeutic and prophylactic agents to improve the health and well-being of birds; maximize efficiency of production and product quality, and to control diseases. However, in recent years, there has been growing concern that this use of antibiotics is leading to an increase in antibiotic-resistance in human and animal pathogens (Aarestrup, 1999; Ferket, 2004). In addition, the use of antibiotics may negate many beneficial properties conferred by gastrointestinal bacteria of chickens (Poole *et al*., 2004). Therefore, in the EU, the application of antibiotics as prophylactics has been banned, and countries such as Australia, Canada and the USA, are considering also banning the use of antibiotics in feed or setting up programmes to reduce their overall use. The restriction on the use of in-feed antibiotics has resulted in an increase in enteric disorders such as NE and the widespread occurrence of ill-defined intestinal dysbacteriosis in poultry (Bager *et al*., 2002; Grave *et al*., 2004).

Currently, many countries are experimenting with alternative feed additives that may be used to alleviate the problems associated with the withdrawal of antibiotics from poultry feed. In this context, there has been increased interest in the use of biological products, including naturally occurring additives such as microbial enzymes, probiotics, prebiotics, synbiotics, organic acids and plant extracts (phytobiotics) as alternatives to in-feed antibiotic additives (Bedford, 2000; Wenk, 2003; Ferket, 2004).

Although the modes of action of in-feed antibiotics (IFAs) are not fully understood, the main effects are thought to be mediated via the gut-associated bacteria (Gaskins *et al*., 2002). Therefore, modification of the chicken gut microflora has currently become an important objective of the poultry industry, when introducing natural alternatives to conventional chemotherapeutic agents. Many investigations have been made to explore the wealth of medicinal plants, which can be used for growth promotion as well as for therapeutic treatment in humans and animals (Guo *et al*., 2003; Wenk, 2003; Cornelison *et al*., 2006). Plant and fungal bioactive compounds such as oligosaccharides and polysaccharides have recently received increasing attention as potential IFA replacements in poultry feed (Iji & Tivey, 1998; Guo *et al*., 2004b; Lan *et al*., 2004). However, little research has been done to investigate the effects of prebiotics and bioactive compounds on nutrient digestibility and gut morphology of broiler chickens. Among the vast sources of plant materials from which prebiotic and bioactive compounds may be extracted are many herbs and plants found in Australia and New Zealand. These are rich in largely untapped compounds which may have potential commercial uses. Furthermore, the health benefits of Australian and New Zealand plant extracts have been empirically known for thousands of years by Aboriginal and Maori populations (Cambie & Ferguson, 2003).

However, these extracts have not been tested on poultry. The present study not only could open up new supply channels for basic raw materials for the poultry industry, but by doing so it could generate possible alternatives to IFAs that are being used in the poultry feed industry.

Apart from inclusion of products in diets, feed ingredients can be processed in such a way to regulate the structure and function of the gastrointestinal tract (GIT). The results of such intervention are not clearcut and may be dependent on grain type and quality (Parsons *et al*., 2006). Augmentation of suitable husbandry practices with appropriate nutrition will allow improved broiler production and health, by helping the bird's physiological systems to function at optimum. That is, nutritionists need to present a feed to the broiler that more accurately meets its needs, not only on a nutritional level, but also on a physiological level, such that the bird is more able to effectively digest and absorb the feed due to improved physiological responses to the feed itself.

Manipulation of the particle size of poultry feed has been shown to be able to address some or all of the issues raised above. It has thus become an issue of importance to the Australian broiler industry in recent times, where producers are striving for the best performance from their stock, whilst minimising costs, to increase profitability. Most of the work on the effects of feed particle size on broiler health and nutrition has been conducted in Europe and the USA in recent times, and published work has been restricted to cage trials.

It was hypothesized in the present study that dietary supplementation with plant extracts or commercially available prebiotic compounds with a similar chemical composition to the plant extracts would exert prebiotic effects and selectively stimulate growth and/or activity of favourable bacteria in the GIT, thereby improving the gut development, health and performance of broilers. The project was also conducted to provide clarification and insight into how feed particle size manipulation can influence broiler performance, gut development and health, both under experimental and Australian field conditions. For purposes of clarity, most parts of this report are divided into two sections (A and B), representing the two broad strategies of the research.

1.1 Objectives

The objectives of this research were:

- To isolate and characterise prebiotic compounds from selected Australian and New Zealand plants;
- To investigate the effects of these plant extracts and commercially available prebiotic compounds on performance, organ development, gut morphology, microbial populations and microbial activity in broilers;
- To characterise and identify the ileal and caecal microflora stimulated by the plant extracts: with emphasis on the populations of lactobacilli and bifidobacteria;
- To evaluate the effects of water-soluble prebiotic extracts on performance, gut morphology, gut microflora composition and humoral immune responses of broiler chickens subjected to a NE disease challenge model involving oral inoculation with *Clostridium perfringens*;
- To determine the effects of grain type, feed particle size and form on gut organ development and digestive processes and the relationship between these response variables and performance;
- To assess changes in morphology, digestive enzyme activity and nutrient digestibility of birds on diets varying in physical and chemical structure, and
- To determine the effects of grain type, feed particle size and form on bacterial populations and how this influences nutrient digestion, digesta physiochemical characteristics and bird performance.

2. Methodology

2.1 Strategy A

2.1.1 Isolation of water-soluble carbohydrates

The underground parts (rhizome) of *Arthropodium cirratum* (Rengarenga lily) and third order branches (stems) of *Cordyline australis* (Cabbage tree) were collected in mid-winter of 2003 from a nursery in Wellington, New Zealand. The exudate from *Acacia pycnantha* (Golden wattle) was obtained from trees grown in the Adelaide area of South Australia in December 2003. The *Undaria pinnatifida* seaweed samples were collected from Point Arthur, Wellington Harbour, New Zealand in June 2003 and the algae were washed thoroughly with seawater, followed by tap water, to remove soil particles and epiphytes. The raw plants were ground and extracted through various procedures developed at the IRL, New Zealand, to obtain the products that were included in the diets. These procedures are fully reported in the PhD thesis submitted to the University of New England by Janak Vidanarachchi (Vidanarachchi, 2006).

2.2 Analytical techniques and measurements

The DM content of extracts was determined gravimetrically according to the Association of the Official Analytical Chemists Official Method 934.01 (AOAC, 2002). The nitrogen content of the extracts was determined according to the DUMAS combustion technique following the method described by Sweeney (1989) using a LECO® FP-2000 automatic nitrogen analyser (Leco Corp., St Joseph, MI, USA). The amino acid composition of the plant extracts was determined using a gas chromatography-mass spectrometry (GC-MS) method described by Persson and Nasholm (2001). Total water-soluble carbohydrates (WSC) were determined by the phenol-sulphuric acid method using glucose as a standard (Dubois *et al*., 1956).

2.2.1 Constituent sugar analysis

The reductive hydrolysis and acetylation method of Stevenson and Furneaux (1991) was used to convert the constituent sugars in water-soluble carbohydrate fractions into alditol acetate derivatives. This method utilises *in situ* reduction with N-methylmorpholineborane (MMB) during hydrolysis to prevent degradation of 3,6-anhydrogalactose units.

2.2.2 High performance size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS)

Ten milligrams (10 mg) of each of the water-soluble extracts were dissolved in 2 mL of 0.1M lithium nitrate ($LiNO_3$) and heated at 95˚C for 15 min. Another 3 mL of 0.1M $LiNO_3$ was added and left overnight. The SEC-MALLS system consisted of a Waters® 2690 XE Alliance separations module, a Waters® 490 E programmable multi-wavelength detector set at 280 nm, a DAWN-EOS multi-angle laser light-scattering detector (Laser Photometer) with a laser at 690 nm (Wyatt Technology Corp., Santa Barbara, CA, USA), and a Waters® 2410 refractive index monitor. Samples (0.2 mg/mL) were filtered (0.45 μm) before injection (100 μL) and eluted with 0.1M LiNO3 containing 0.02 % NaN_3 (0.7 mL/min) from two columns (TSK-Gel G5000PWXL and G4000PWXL, 300 x 7.8 mm, Tosoh Co., Tokyo, Japan) connected in series. Data for molecular weight determination and conformation were analysed using ASTRA software (Wyatt Technology Corp., Santa Barbara, CA, USA) with a specific refractive index (dn/dc) of 0.145 mL/g (determined experimentally).

2.2.3 Determination of fructan contents and thin-layer chromatographic (TLC) analyses of fructans

Fructan content in Rengarenga lily extract and Cabbage-tree extract was determined using the full Megazyme fructan assay procedure (Megazyme, 2004). Structural composition of fructans was analysed by TLC using a method described by Sims (2003).

2.2.4 Extraction of genomic DNA for PCR amplification

Bacterial isolates (240) were thawed, streaked on Rogosa agar (Oxoid, CM0627) and incubated at 39˚C for 48 h in anaerobic jars (Oxoid Ltd, Hampshire, UK) in an anaerobic environment consisting of <1 % O_2 and 9-13 % CO_2, generated using anaerobic AnaeroGen® sachets (AN0025A, Oxoid Ltd, Hampshire, UK). A single colony was then picked, inoculated to 10 mL MRS broth in a sterile screw cap tube. The cells were grown overnight at 39˚C and 1.0 mL of bacterial suspension transferred into an Eppendorf tube and harvested by centrifugation (14,500 *x g*, 5 min) in an Eppendorf centrifuge (Eppendorf 5415 D, Eppendorf AG, Hamburg, Germany). Following other intermediate processes, the DNA was precipitated and purified using the DNeasy® Tissue kit (QIAGEN Pty Ltd., Doncaster, VIC, Australia) following the manufacturer's instructions. The 16-23S rDNA (16S rRNA gene and the entire 16S-23S rRNA intergenic region) were amplified from DNA extracted from isolates by PCR using the primers Lb16a and 23-1B, as reported by Guan *et al*. (2003).

The amplified 16-23S rDNA intergenic spacer regions of *Lactobacillus* isolates were digested with the restriction endonuclease HaeIII enzyme (restriction enzyme isolated from *Haemophilus aegptius*) according to the manufacturer's instructions (New England BioLabs, Brisbane, QLD, Australia). Restriction digestion products were electrophoretically resolved in a 2 % agarose gel containing 5 µL of GelStar® nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA) for 4h at 90 V and band patterns were viewed by UV transillumination and digitised on an Infinity CN-3000 Gel Documentation System (Vilber Lourmat, Cedex, France).

2.3.5 Isolation and characterization of bacteria

All colonies from MTPY agar plates and twenty randomly selected colonies from Rogosa agar (lactobacilli) from the highest dilution of each sample were isolated and grown in TPY broth and MRS broth (Oxoid, CM0359), respectively. Isolates were grown at 39˚C in the anaerobic cabinet for three days for bifidobacteria and two days for lactobacilli and stored at -20˚C in 30% (v/v) sterilized glycerol. Subcultures of isolates from MTPY agar were grown in 10 mL of TPY broth at 39˚C overnight. Cells from isolates that did not produce gas during growth (99/402) were then harvested by centrifugation at 5,500 *x g* for 10 min and tested for the presence of F6PPK activity as described by Orban and Patterson (2000). The sequences of the 16S rRNA genes determined in this study were deposited with the GeneBank nucleotide database under the accession number DQ676992.

2.2.6 Sequencing of 16S rRNA gene

Amplified PCR products generated with the primer pair TH008-PH1522 were purified and concentrated using the QIAquick® PCR purification kit (QIAGEN Pty Ltd., Doncaster, VIC, Australia) as described by the manufacturer. DNA concentrations of the purified products were determined with an ND-1000 NanoDrop Spectrophotometer (Biolab Ltd, Mulgrave, VIC, Australia) in order to adjust the DNA concentrations in sequencing mixtures. The sequences of the 16S rRNA genes determined in this study were deposited with the GeneBank nucleotide database under the accession numbers DQ676989 for isolate group I, DQ676990 for isolate group II, DQ832760 for isolate group III, and DQ676991 for isolate group IV.

2.2.7 Fermentation characteristics of lactobacilli

Fermentation characteristics of each group of Lactobacillus species were performed with the Ph-48 generalized PhenePlate system (BactusAB, Huddinge, Sweden). This is based on interval measurements of colour changes, visualized by the pH indicator (bromothymol blue), resulting from bacterial metabolism of two sets of 46 freeze-dried substrates including low–molecular-weight carbohydrates (mono-, di-, and trisaccharides), carbohydrate derivatives (sugar alcohols, sugar acids, and glucosides), organic acids, urea, and ornithine.

2.2.8 Enzyme-linked immunosorbent assay (ELISA)

Total antibody titre concentrations of IgY, IgM, and IgA in serum were determined before Cp challenge (14 d), and at 7 d after first challenge (21 d) using a sandwich ELISA. Blood samples were collected from the jugular vein into 7-mL serum tubes and clotted at room temperature (RT) (25˚C) for 2 h, and serum was separated from the cells by centrifugation (Beckman Instruments Inc., Palo Alto, CA, USA) at 2,300 *x g* for 5 min and stored at -20˚C. The specific IgY antibodies against the **α**-toxin of Cp in blood serum were determined as described by Heier *et al*. (2001), with some modifications

2.2.9 Bird husbandry

All birds used under Strategy A were Cobb strain, obtained from a local hatchery (Baiada hatchery, Kootingal, NSW, Australia). They were vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease prior to collection. At 1 d of age, chicks were randomly placed in brooder cages (42 cm x 75 cm x 25 cm dimension) with wire floor and with a floor space of 0.32 m^2/cage. The cages were randomly assigned to one of six dietary treatments with the appropriate number of replicates per treatment. The temperature was set at 33-34˚C during the first week and gradually decreased by 3˚C per week until 24-25˚C was reached by the third week. Relative humidity was between 65 and 70 %. A photoperiod of 24 h from 1 to 21 d of age, and 18 h from 22 to 42 d of age was maintained. Each pen was equipped with a feeding and water trough placed outside and also an excreta collection tray. Water and feed were provided ad libitum. Weekly BWG and FI per cage were measured and FCR, adjusted for mortality, calculated on a cage basis. Birds were observed twice daily for general health.

2.3 Strategy B

Three experiments were conduct to address the second strategy of this project. Some of the methods were common to those used for strategy A, and are described in section 2.6.0. Specific methods used for this strategy are described in the following sections.

The first of the three experiments was conducted at the Agricultural University of Norway, Ås, approximately 20km south-east of Oslo. The feeding trial work was conducted inside a temperature and light controlled environment on campus. The experiment commenced in May 2004 and was completed in June 2004.

Feed was processed at The Agricultural University Center for Feed Technology Ltd. (FôrTek), situated on the University campus. All sample analyses were conducted on campus. Dry feed and digesta particle size analysis was carried out using a Mastersizer 2000 LASER diffraction particle size analyser, with Sirocco 2000 and Hydro 2000G accessories (Malvern Instruments Ltd., UK) used for dry and wet samples, respectively.

2.3.1 Treatments

A semi-purified diet consisting of 75 % sorghum and 12 % isolated soy protein was formulated. Prior to processing, the sorghum was sieved to remove excess debris and dust. Four treatments, differing in mean particle size and processing method used to modify the sorghum grain were compared. Treatments were based on sorghum processing type before pelleting and are described by method of milling, if any, and were as follows: Whole sorghum (WS); Hammer-mill – 3.0 mm screen (HM3); Roller-mill – 0.15 mm roller spacing (RM0.15), and Hammer-mill – 1.0 mm screen (HM1).

Diet 3 (RM0.15) was ground to have a mean equivalent particle size of Diet 2 (HM3). Diets 'WS' and 'HM1' were used to represent extreme variations from the intermediate particle sizes used in diets HM3 and RM0.15. The HM3 diet was used as a control, based on current processing methods used in feed milling. All dietary treatments were manufactured using the same formulation, which was semi-purified to reduce the confounding effects of other factors, and excluded the use of antibiotics/growth promotants and coccidiostats.

2.3.2 Feed preparation

After sieving, the sorghum needed for the experiment was divided into four portions corresponding to the four treatments. After a 1mm screen was fitted to a commercially used 18.5kW hammermill (Münch-Edelstahl, Wuppertal, Germany, licensed by Bliss, USA), one portion of sorghum was fed through the machine to yield the sorghum used for diet treatment 4 (HM1). The same hammer-mill was then fitted with a 3 mm screen, and a second portion of sorghum was fed through the mill to yield the sorghum component for diet treatment 2 (HM3). A representative sample of the HM3 was taken and dry sieved using a Retsch AS 200 (F. Kurt Retsch GmbH & Co., Haan, Germany) and the mean particle size calculated.

Using trial and error, the two sets of a commercial 13kW roller-mill (Model DP 900-12, Roskamp, Indiana, USA) were adjusted to yield an equivalent mean sorghum particle size to the HM3 sorghum. The roller spacings were 0.15 mm at the top rollers and 0.15 mm at the bottom rollers. The resulting milled sorghum was used in the third dietary treatment (RM0.15). The sorghum for the first treatment was left unprocessed prior to mixing. All dietary components, except for soy oil, were added to the sorghum and mixed thoroughly using a Dinnisen mixer (Pegasus Menger 400 1, Sevenum, Holland).

All diets were steam-conditioned in a double conditioner (Münch-Edelstahl, Wuppertal, Germany) for 60 seconds at an average conditioning temperature of 75˚C, and soy oil was added at this stage. The diets were then steam-pelleted (Münch-Edelstahl, Wuppertal, Germany, RMP 350,100, 5000kg.h^{-1} capacity) using a 3 mm die (42mm thick). The pellet press had two closed-end corrugated rolls set at approximately 0.25 mm to the inner surface of the pellet die. For each diet, a pellet sample was taken immediately as the pellets fell from the press and stored in a polystyrene box fitted with an electronic thermometer to measure post-pelleting temperature. All diets were cooled after pelleting in a Miltenz Counter Flow Cooler (Auckland, New Zealand, 2000kg.h^{-1} capacity). Average post-pelleting temperatures, energy consumption and production rates of the pellet press of the four diets are presented in Table 2.1.

Table 2.1: Production characteristics of the four sorghum diets through a commercial steam conditioner and pellet press (Experiment one).

Treatment	WS	HM3	RM0.15	HM1
Pellet temperature (^{o}C)	83.9	82.3	81.8	84.0
Power consumption (A)	83.9	82.3	81.8	84.0
Production rate (kg/hr)	750-800	800	800	800
Feeder rate (%)	40-45	45-50	45-50	45-50

2.3.3 Measurements

Body weights were recorded at 11 days of age, 14 days of age and weekly thereafter. Feed refusals were weighed at the time of bird weighing, and additions to each feeder were allocated on a needs basis and weighed each time.

At 21 days of age, one bird per cage was euthanazed using CO_2 gas for dissection. The proventriculus, gizzard, duodenum, jejunum, and ileum were weighed with and without digesta contents. Gizzard and duodenum digesta pH was recorded. Digesta was collected from the duodenum and immediately refrigerated for particle size analysis. All organs were cleaned of external fat and mesentery before weighing. At 35 days of age, the dissection procedure was repeated.

At 25d all birds were fasted for six hours, feeds and birds were weighed, and excreta trays placed under birds for excreta collection. Excreta were collected for three consecutive days at the same time of day that the trays were placed under the birds on the first day of collections. Daily collections of excreta were pooled and frozen at -20˚C. The birds and feeds were weighed at the commencement and completion of excreta collection. Excreta were used for nitrogen corrected apparent metabolisable energy (AME_n) determination.

2.3.4 Marker preparation and administration

Chromium mordanted hay (used as a solid phase marker) was prepared according to the method outlined by Udén *et al.* (1980). The prepared hay was ground using a 1 mm screen on a Christy-Hunt hammermill, desiccated, and pressed into 1000±5 mg tablets using a manual screw-down tablet press. Cobalt-ethylenediamine tetraacetic acid (Co-EDTA) powder, used as a liquid phase marker, contained in a commercially available 1.0cm^3 gelatine capsule was also used to measure digesta transit time. Cobalt-EDTA was prepared according to the method outlined in Udèn *et al.* (1980). Birds received two 1000 mg Cr-mordanted hay tablets each, and one Co-EDTA capsule in a one-off (force-fed) administration. All birds were allowed *ad libitum* access to feed and water immediately before and after marker administration. Markers were administered to ten 29d-old birds per treatment over clean excreta collection trays, with excreta collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24h post marker administration for marker retention time and recovery analyses. Samples were frozen after each addition of fresh excreta, and until the time of analysis.

2.4 Experiment 2

Based on the findings of the first experiment, it was concluded that there was an effect of the particle size of the grain used in the diet. It was postulated that the type of grain used in the diet would also have an effect on the performance of the broiler, and thus a second grain of greatest significance to the Australian broiler industry (wheat) as well as sorghum was used in the second experiment. The hypotheses for the second experiment were that; the particle size of the grain in the pelleted diets would affect performance, gut development, and enteric microbial status, irrespective of grain type, and, the aforementioned parameters will be affected by grain type.

The second experiment was conducted at the University of New England Animal House facility, located in Armidale, New South Wales, Australia, during September and October of 2005. Armidale is located at 30˚30'E, 151˚40'S and is approximately 1000 m above sea level. Due to its altitude, and distance from the coast, Armidale is in a cool-temperate climate zone, with mean daily temperatures ranging between 3.7˚C and 17.6˚C in September, and 7.0˚C to 21.2˚C in October (Australian Bureau of Meteorology, 2006). Climate-controlled rooms were thus needed for the experiment.

2.4.1 Experimental facilities

The birds were housed in two climate-controlled rooms for the brooder phase, and two temperature controlled rooms for the grower phase. Birds were housed in their respective treatments groups from 1 day of age until the end of the experiment. All analyses were conducted at the Department of Animal Science laboratories, located on the university campus.

2.4.2 Treatments

Four treatments were used in the experiment to test the stated hypotheses. The treatments consisted of a 2 x 2 factorial, with grain type and particle size comprising the two factors. Within the grain type factor, the two levels were wheat and sorghum, and within the particle size factor, the two levels were coarse and fine particle size. The four treatments used were: Fine wheat (WF); Coarse wheat (WC); Fine sorghum (SF), and Coarse sorghum (SC).

The diet formulations used were semi-purified, in an attempt to eliminate confounding factors in the diet, such as fibre. The diets were also free of antibiotic/growth promotant and coccidiostat, as these additives would most certainly affect the microbial ecology of the digestive tract.

2.4.3 Feed preparation

Approximately 50% of the wheat and sorghum used for the treatments was ground using a small roller-mill (Wolf Engineering & Millwrights, Victoria, Australia) powered by a 0.75kW Teco Electrical and Machinery Company Ltd. (Taiwan) motor. Both rollers used, had approximately 2.75 flutes.cm^{-1}, with flutes protruding from the roller by approximately 4 mm, and a 0.5 mm flattened top. The remaining grain was milled using a hammer-mill (JAS. Smith Pty. Ltd., Ballarat, Victoria, Australia), used for small scale grinding, operated at 2500 RPM (approximately 2000 kg.hr^{-1} capacity) and was powered by a 15kW (25.8A) (Australian Electrical Industries Pty. Ltd., Australia) motor. After milling, and prior to mixing with the remaining ingredients in a small rotary four-blade mixer operated (at 23 RPM), each of the grains was sieved using a soil sieving machine (custom made for the University of New England's School of Agronomy and Soil Science), vibrating at an approximate amplitude of 3 cm at a frequency of approximately 20 Hz, fitted with a 2.0 mm sieving screen. Screenings collected from under the sieve were used for the 'fine' treatments, and residue on the top of the screen was used for the 'coarse' treatments.

Celite® was added as a source of acid insoluble ash, as an indigestible marker for nutrient digestibility analyses. Cellulose was added to the sorghum diet to serve as an energy diluent, whilst semi-pure wheat starch (Fielder's™ Wheaten Corn Flour) was added to the wheat diet to increase the metabolisable energy content.

All diets were "cold" pelleted using a 7.5 kW Templewood Hawkesley (Slough, Bucks, England) cold pelleter fitted with a 4.0 mm die, moving at 126 RPM past two pellet cutter blades. The mean pellet length for all diets was approximately 6mm. The actual feeding trial was conducted as described in section 2.4.1.

2.5 Experiment Three

The third experiment was held under commercial conditions on rice husk litter to give a 'real world' perspective to the body of research, as Experiments One and Two were conducted under experimental conditions in cages.

The experiment was conducted at the Bartter Enterprises Pty. Ltd. grow-out testing facility, located near Hanwood in southern central New South Wales (34.33˚S, 146.03˚E) at an elevation of

approximately 126m. The experiment was conducted in autumn from March to April, 2006, with average daily temperatures ranging from 13.6˚C to 28.1˚C in March, and from 9.4˚C to 22.9˚C in April (Australian Bureau of Meteorology, 2006).

The testing facility was a modified commercial grow-out shed, originally capable of holding approximately 30,000 broilers under contemporary stocking density legislation. Within the shed, 96 pens (5.28m^2) capable of holding 90 birds at 2.1 kg per bird (maximum of 36 $kg.m^{-2}$ stocking density), were available for use for the experiment, but only 84 pens were used. The shed was tunnel ventilated, with the temperature controlled from the front annex of the shed via an electronic control panel, set to decrease the shed temperature from 32˚C at one day of age, down to 21˚C at 21 days of age, and this temperature was maintained thereafter. The shed was illuminated using 8 W fluorescent light globes, providing between 12 and 16 Lux at shed floor level throughout the experimental period.

2.5.1 Hypotheses and aims

Based on the findings of the previous experiments, it was hypothesised that the level of grain processing and the means by which the grain was processed would influence the performance and gut physiology of the birds. Furthermore, it was postulated that the birds receiving a coarse wheat particle would perform better than birds receiving a fine particle, and that birds fed rolled grain would have a lower feed conversion ratio, than birds fed hammer-milled grain of an equivalent particle size. The expected differences in performance between particle size and milling type groups of treatments, was hypothesised to be due to differences in enteric microbial status, digesta pH, and starch digestibility, brought about by a more developed and more efficient digestive tract.

The experimental aims were to determine if, under commercial conditions with birds placed on litter, wheat particle size and milling type would affect performance, gut development, and enteric microbial status of male and female broilers.

2.5.2 Bird husbandry

Twelve single pen replicates of seven dietary treatments were randomly allocated to 84 pens, with 90, one day old Ross 308 broilers placed per pen replicate (7560 birds total). Treatments were allocated to pens in a randomised block design. The seven treatments were as follows: HM3.2A - Commercial grind (hammer-mill 3.2mm screen) with IFA; HM3.2 - Commercial grind (hammer-mill 3.2mm screen); HM35 - Hammer-mill intermediate grind (no screen, 35% feed rate); HM55 - Hammer-mill coarse (no screen, 55% feed rate); RM35 - Roller-mill intermediate (0.3 mm roller spacing, maximum pressure, minimum feed rate); RM55 - Roller-mill coarse (0.3 mm roller spacing, maximum pressure, maximum feed rate), and WW - Whole wheat, passed through roller mill with minimum pressure and maximum flow rate (most grains with stress fissures).

Birds were placed in groups of 90 into each pen and fed a commercially formulated, crumbled, pre-starter diet, without an IFA or coccidiostat, until 6 days of age. The birds were then started on their respective experimental diets in a coarse crumble formulated as per a commercial starter diet (from 7 to 17 days of age). The method and degree of wheat processing for the starter, grower and finisher diets was maintained for each treatment and was conducted using commercially available feed processing equipment. Birds were fed a grower and finisher diet, from approximately 18-35 days of age and 36-43 days of age respectively, depending on individual pen feed consumption. All feeds were fed to the birds on an *ad libitum* basis. Water was supplied to each pen via common drinking lines from day one to day 43.

Samples for analyses were taken at 21 and 41 days of age. Birds used for sampling - one per pen – were humanely euthanazed using cervical dislocation. Birds not used for dissections were processed at 43 days of age at Bartter's commercial processing plant, whereupon final bird weights were taken for performance calculations.

2.5.3 Feed preparation

All treatments were based on the same formulation within a diet, with differing levels and methods of wheat processing being the only difference between dietary treatments. The formulations of the pre-starter, starter, grower and finisher rations were contemporary commercial feeds, the composition of which is not able to be published due to their commercial nature. The diets were wheat/soy based, with animal protein, vitamin and mineral premix, xylanase and an anticoccidial compound added to meet the nutritional and health requirements of the birds. Four formulations were used (pre-starter, starter, grower and finisher) and fed depending on the growth stage and requirements of the birds at that stage.

All diets were processed at the Bartter Enterprises Pty. Ltd. feed processing site located near Hanwood; used exclusively for Bartter's breeder and broiler feed manufacture in the Riverina district of NSW. The same batch of wheat was used for all diet treatments within each diet (pre-starter, starter, grower or finisher).

Hammer-milled grain was processed through a computer-controlled, variable speed and flow rate hammer-mill, with a 8-10 tonne.hr^{-1} capacity using a 3.2mm screen. Roller-milled grain was processed through a commercial roller-mill, with flow rate and roller pressure adjusted manually by hopper chute aperture and roller spacing adjustments. Milled grain particle sizes were maintained throughout the experiment – from starter through to finisher – for each treatment.

Wheat for treatments 1 and 2 was milled through a 3.2mm screen fitted to the hammer-mill, as is commercial practice. Treatment 3 utilised wheat from the hammer-mill, with the screen removed, and the flow rate adjusted until the desired texture was achieved (35% flow rate). This treatment's grain was an intermediate particle size between the coarse roller-milled (treatment 6), and the 3.2 mm hammer-milled wheat. The coarse hammer-milled wheat for treatment 4, was achieved by passing grain through the hammer-mill with no screen, at a flow rate of 55 %.

The wheat for the remaining three treatments (5, 6 and 7) was processed to some degree using the roller-mill. Treatment 5 grain was rolled to an intermediate particle size by adjusting the rollers to be very close to each other; increasing the pressure on the rollers when grain was passed through the mill, and restricting the flow of grain to maximise the crushing of grain in the mill. This was done to achieve an equivalent particle size to the intermediate hammer-milled grain. The same process was repeated, with less pressure exerted on the rollers by widening the between roller space fractionally, but again minimising the flow of grains through the mill. This created an equivalent texture to that of the coarse hammer-milled grain. Grain for the final treatment was again passed through the roller-mill, with the minimum of pressure and maximum flow rate exerted on the grain. This process allowed the grain to be stressed enough to cause one or more small fissures in the grain, but not enough to crush the grain into pieces.

Portioning and mixing of all feed ingredients was automated using a computer system, directed and monitored by the feed plant staff. After mixing to the prescribed formulation, the diets were conditioned, then pelleted through a 3 mm pellet die fitted to a 38 t.hr^{-1} capacity steam pelleter, with a minimum draw of 200A, and a maximum draw of 575A. The energy output from the pelleter, and conditioner temperature were measured and recorded for each diet.

2.5.4 Analytical procedures

Particle sizes of feed, milled grain and duodenal digesta

Feed and grain particle size measurements for feeds/grain used in Experiment One, were conducted using LASER diffraction (Mastersizer 2000, with Sirocco 2000A attachment for dispersion of dry particulate samples, Malvern Instruments Ltd., U.K.). The Mastersizer equipment was computer

controlled, using Mastersizer v.5.13 software© (Malvern Instruments, Ltd., U.K.) Use of the Mastersizer 2000 allowed a detailed description of the particle size distribution of the milled grain and pre-pellet mixed feed, also supplying the mean surface area, mean geometric diameter based on the volume and the mean geometric diameter based on the surface area of the particles within a sample.

For Experiments Two and Three, grain particle size was determined by sieving a weighed sub-sample of the ground grain through a series of analytical sieves with apertures of 2630, 2000, 1700, 1180, 1000, 850, 600, 500, 250 and 125 µm. Sieving for each sample was standardised using an oscillating sieving machine (Fritsch GmbH Analysette 3E, Idar-Oberstein, Germany), for 120 s with an amplitude of 5.0mm. The weight of each fraction, remaining on the surface of each sieve, was then weighed and recorded. Based on the assumption that the distribution of particle sizes was normal within each screening (the fraction that passes through a sieve), the average particle size of that screening would be the mean of the aperture diameters of the sieve the fraction passed through, and that of the sieve whose surface the sample was collected from. The sum of the proportions of each fraction multiplied by the mean particle size of those fractions, respectively, equals the mean particle size of the original sample.

2.5.5 Intestinal membrane-bound and pancreatic enzyme activity

Enzyme activity was analysed on samples obtained from Experiments Two (jejunum and pancreas tissue) and Three (jejunum tissue only). Jejunal tissue homogenate was prepared for protein, sucrase, maltase and leucine-aminopeptidase analyses following the method described by Shirazi-Beechey *et al.*(1991). Pancreatic homogenate was prepared for chymotrypsin activity according to modified methods of Gertler and Nitsan (1970), with modifications made by Nitsan *et al.* (1974).

Protein and enzyme assays

Protein concentration of the jejunal and pancreatic tissue samples was measured, as the activity of enzymes is expressed as activity per unit of protein. Protein assays were conducted according to the methods of Bradford (1976). Sucrase and maltase assays were done according to the method described by Dahlqvist (1964). Leucine aminopeptidase activity (LAP) was conducted according to the method described by Miura *et al.* (1983).

The determination of pancreatic amylase activity was based on the method of Miller *et al.* (1960) with modifications made by Iji *et al.* (2003). Chymotrypsin activity was determined based on the method outlined by Serviere-Zaragoza *et al.* (1997).

2.5.7 Digesta flow marker analyses

The chromium content of the feed, and faecal samples was determined according to the method outlined by Udèn *et al.* (1980). Cobalt content in the feed and excreta were measured according to the method outlined by Williams *et al.* (1962). The calculations used to determine the mean retention time of the markers for each diet were outlined by Ferrando *et al.* (1987).

2.6 General methods applied to both strategies

The following methods were used for experiments reported under both strategies. Occasionally, there were minor modifications to fit with specific requirements of each experiment.

2.6.1 Experimental diets

All diets were formulated to meet the requirements set by the National Research Council (1994) for broilers using the "PRO-4 Standard Edition" software package (Version 1, Agri-Data Systems, Inc., Annapolis, Maryland, USA). When necessary, Celite™(Food Chemicals Cod*ex g*rade, Celite Corp.,

Lompar, CA, USA), a source of acid-insoluble ash, was added (5 g/kg) to finisher diets as an indigestible marker. The grains in the experimental diets were hammer-milled using a 5-mm screen, and all diets were mixed and cold-pelleted at the University of New England.

2.6.2 Collection and processing of samples

Birds to be killed for samples were selected at random from each replicate and euthanazed by cervical dislocation or CO_2 asphyxiation. To synchronise the feeding pattern of the birds, light was switched off for 2 h, followed by at least 1 h light before the chickens were sacrificed. Subsequently, the abdominal cavity was opened and the small intestine was ligated and removed. The contents of the ileum were collected into plastic containers. The ileal digesta samples were frozen immediately after collection, subsequently lyophilized (Martin Christ Gerfriertrocknungsanlagen, GmbH, Osterode am Harz, Germany), ground to pass through a 0.5 mm sieve (Cyclotec 1093 sample mill, Tecator, Höganes, Sweden), and stored at -20˚C in airtight containers until chemical analyses were conducted. In experiments in which histology was conducted, approximately 2.5 cm of the middle portion of the ileum was excised from one bird per replicate, flushed with PBS buffer (pH 7.6) and fixed in 10 % (v/v) neutral buffered formalin for histomorphological analysis.

2.6.3 Organ weights

The weights of the proventriculus, gizzard, and small intestine without content, pancreas, bursa of Fabricius, caeca, spleen and liver without gall bladder were recorded at sampling. The duodenum is the region from the outlet of the gizzard to the distal attachment of the pancreas, the jejunum, distally from the end of the pancreatic loop to Meckel's diverticulum, the ileum distally from Meckel's diverticulum to 1 cm above the ileo-caecal junction. In some of the experiments, the length and weight of each segment were recorded, as was the body weight of the bird from which they were excised. The 'gut mass index' was subsequently calculated as an indication of mass per unit weight of body or length of the GIT region.

2.6.4 Acid-insoluble ash

The concentration of acid-insoluble ash in the feed, freeze-dried ileal digesta and excreta was determined after ashing the samples and treating the ash with boiling 4M HCl, following the method described by Vogtmann *et al*. (1975) and Choct and Annison (1990).

2.6.5 Apparent metabolisable energy (AME) bioassay

Apparent metabolisable energy (AME) evaluation was conducted over a period of 4 days at the chosen age. Clean excreta trays were placed under each AME cage, droppings were collected daily, dried at 80˚C to a constant weight in a forced-draught oven and collections from each pen were pooled for analysis. Care was taken to avoid contamination with feed, feathers, scales and debris. The moisture content of the excreta voided was measured. Diet and excreta were ground to pass through a 0.5 mm screen using a Cyclotec sample mill. Gross energy contents of diets and excreta were determined using an IKA bomb calorimeter system, C7000 with Cooler C7002 (IKA®-Werke GmbH & Co, Staufen, Germany) standardized with benzoic acid. Apparent metaboli*S*able energy of diets was calculated using the equation below and values were corrected for zero nitrogen retention using a value of 34.4 MJ per g nitrogen retained (Hill & Anderson, 1958).

$$\text{AME} = \frac{\text{FI} \times \text{GE}_{\text{diet}} - \text{Excreta output} \times \text{GE}_{\text{excreta}}}{\text{FI}}$$

where, FI si feed intake, GE_{diet} is gross energy content in diets and $GE_{excreta}$ is gross energy content in excreta.

2.6.6 Digestibility of nutrients

Apparent ileal digestibilities of protein, fat, starch and DM and the AME as a proportion of the gross energy of feed were estimated from the analyses of feeds, freeze-dried ileal digesta and excreta; an indigestible acid-insoluble marker was used to calculate digestibilities. Diets and ileal digesta were analysed for DM, protein, fat and starch as described below. The apparent ileal digestibility of protein, fat, starch and DM were calculated using the following formula. All values are expressed on a DM basis.

$$Digestibil\,ity = (1 - \frac{digesta\ \ nutrient\ \ (g/kg)/digesta\ \ AIA\,(g/kg)}{diet\ \ nutrient\ \ (g/kg)/diet\ \ AIA\,(g/kg)}) \times 100$$

where AIA is acid insoluble ash in diet or digesta.

2.6.7 Proximate analysis

The dry matter content was determined gravimetrically according to the Association of Official Analytical Chemists Official Method 934.01 (AOAC, 2002) as described in Section 3.2.2.1.

The total starch content of the diets and ileal digesta was determined using the Megazyme Total Starch Assay Kit (Megazyme Australia Pty. Ltd., Warriewood, NSW, Australia) based on the method developed by McCleary *et al*. (1994). Diets and ileal digesta were analysed for protein by the method of Sweeney (1989) using a LECO® FP-2000 automatic nitrogen analyser (Leco Corp., St. Joseph, MI, USA). The crude fat content of the ileal digesta samples was determined gravimetrically by the Soxhlet extraction procedure using Association of the Official Analytical Chemists Official Method 920.39 (AOAC, 2002).

2.6.8 Gut histomorphology

Formalin-fixed tissue slices from the ileum, each 5 to 6 mm thick, were enclosed in tissue cassettes (Bayer Diagnostics Australia Pty Ltd., Pymble, NSW, Australia). The tissues were processed over 19 h in an automatic tissue processor (TOSCO, Thomas Optical & Scientific Co., Melbourne, Australia), and embedded in paraffin using a Histo Embedding Centre (Leica EG 1160, Leica Microsystems, Bensheim, Germany). Processing consisted of serial dehydration with ethanol, clearing with xylol and impregnation with paraplast (wax). Embedded samples were subsequently sectioned at a thickness of 5 µm with a Rotary Microtome (Leitz 1516, Leica Microsystems, Bensheim, Germany). The tissue sections on the slides were stained using Harris's hematoxylin (Gerorge Gurr Ltd., London, UK), and eosin (Gur Certistain, VWR International Ltd., Poole, UK), and mounted with DPX mountant (Distrene Polystyrene Xylene), for histology (BDH Laboratory Supplies, Poole, UK). Slides were viewed on a Leica DM LB microscope (Leica Microscope GmbH, Wetzlar, Germany) and morphometric indices were determined using computer-aided light microscope image processing analysis system (SPOT 3.1, Diagnostic Instruments, Inc., Sterlin Heigths, MI, USA) as described by Iji *et al*. (2001).

2.6.9 Enumeration of intestinal bacteria

Samples in pre-reduced salt medium were homogenized for 2 min in CO_2-flushed plastic bags using a MiniMix® bag mixer (Interscience, St. Nom, France) and serially diluted in 10-fold increments in pre-reduced salt medium according to the technique of Miller and Wolin (1974). An aliquot (100 µL) was plated on the appropriate agar media, for enumeration of different species.

For the enumeration of bifidobacteria, the diluted samples were plated on modified tryptone-neutralized soy peptone-yeast extract agar (MTPY) (Petr & Rada, 2001), and incubated at 39˚C for 3 days in the anaerobic cabinet. The TPY agar was supplemented with mupirocin (100 mg/mL) and

glacial acetic acid (1 ml/L). Mupirocin was extracted from antimicrobial discs (200 µg; Oxoid, A MUP 200) as described by Rada *et al*. (1999).

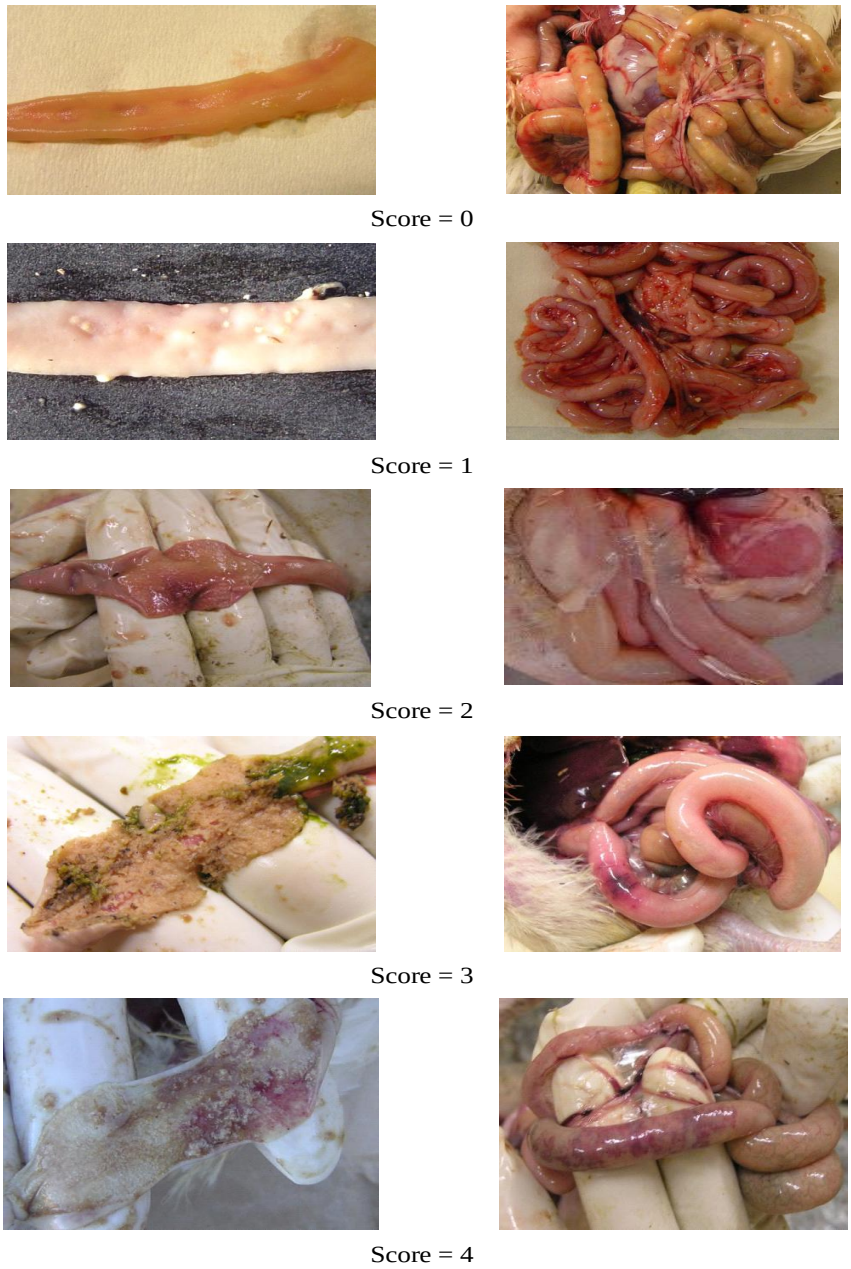

Plate 2.1: Gross appearance of the jejunal and ileum lesions, showing criteria for assigning necrotic enteritis scores

2.6.10 Measurement of organic acids

For measurement of organic acid (SCFA, lactic and succinic acid) concentrations, about 2.0 g of thawed ileal and caecal sample was suspended in 1.0 mL of 0.02 M-2-ethylbutyric acid and thoroughly mixed by using a vortex mixer, followed by centrifugation at 25,700 *x g* at 4˚C for 15 min in a Beckman model J2-21M Induction Drive centrifuge with a JA-21 rotor. To a sample of 1 mL supernatant fraction, 0.5 mL of concentrated HCl and 2 mL of diethyl ether were added and thoroughly mixed by using a vortex mixer, followed by centrifugation at 2060 *x g* at 4˚C for 15 min. Aliquots (360 µL) from the ether phase were recovered and mixed with N-methyl-N-tert-

butyldimethylsilyltrifluoroacetamide (MTBSTFA; 40 μL) for derivatisation of organic acids. The concentration of derivatised organic acids was quantified using a Varian CP 3400 C*X* *g*as chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA).

2.6.11 Necrotic enteritis challenge model

Necrotic enteritis tests involved challenge with *C. perfringens* and *Eimeria spp.* A gross pathologic diagnosis of NE in all dead birds and sampled birds was based on the presence of intestinal lesions typical of naturally occurring and experimentally induced NE, as described by Prescott *et al*. (1978) and Broussard *et al*. (1986): gas-filled small intestines, with confluent necrosis and sloughing of the mucosal surface of the intestinal tract which appeared as tan-orange pseudomembrane ("dirty turkish towel"-like appearance). 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 criteria of Prescott *et al*. (1978) with slight modifications as illustrated in Plate 2.1. All birds were examined twice daily and all dead chickens were immediately collected for postmortem analysis.

2.7.12 Statistical analysis

Data were analysed according to appropriate statistical design of each experiment. Percentage data were arcsine-transformed prior to analysis and data from all the response variables were analysed using the ANOVA procedures of SAS (SAS Institute Inc., 2000) or StatGraphics 5 Plus computer package (version 5.1 - Professional Edition, Manugistics Inc., Rockville, Maryland, USA). Variables having a significant F test were compared using Duncan's Multiple Range Test. Differences between mean values were considered significant at $P \leq 0.05$.

2.7.13 Animal ethics

All experimental procedures were approved by the University of New England Animal Care and Ethics Committee, and throughout the experiments, health and husbandry practices complied with the Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2004), for the Commonwealth of Australia and the Australian Model Code of Practice for the Welfare of Animals: Domestic Poultry (Primary Industries Standing Committee, 2002).

3 Results

3.1 Strategy A

3.1.1 Water-soluble prebiotic compounds from Australian and New Zealand plants: isolation and characterisation

The underground parts (rhizome) of *Arthropodium cirratum* (Rengarenga lily extract); third order branches of *Cordyline australis* (Cabbage tree extract); a seaweed, *Undaria pinnatifida* (Undaria extract), and exudates from *Acacia pycnantha* (Acacia extract) contained 576, 250, 275, and 794 g/kg DM water-soluble carbohydrates (WSCs), respectively. Constituent sugar analysis by gas-liquid chromatography (GLC) showed that extracts of Rengarenga lily and Cabbage tree contained predominantly fructose and glucose (82-95 %). The analysis also revealed that Acacia extract contained mainly galactose (78 %) and arabinose (22 %) while Undaria extract, contained fucose (55 %) and galactose (44 %). Thin-layer chromatography (TLC) showed that the fructan composition of Rengarenga lily extract and Cabbage tree extract was different. Cabbage tree extract contained 45% (w/w) fructans while Rengarenga lily extract contained 65 % (w/w) fructans (Table 3.1).

Table 3.1: Yield and chemical analyses of the plant extracts

	Plant extract			
	Lily extract	**Cabbage tree extract**	***Undaria* extract**	***Acacia* extract**
Yield of water-soluble carbohydrates (g/kg DM)	**576**	**250**	**275**	**794**
Dry matter (g/kg)	**938**	**880**	**885**	**930**
Total sugar content (g/kg DM)	**680**	**480**	**370**	**790**
Crude protein (g/kg DM)	**49**	**143**	**21**	**32**
Molecular weight (Da)	**~5,000[1]**	**N.D**	**511,000**	**30,000**
Polydispersity index[2] (M_w/M_n)	**1.10**	**-**	**1.46**	**1.07**
Fructan content (%, w/w)	**65**	**45**	**-**	**-**

N.D = Molecular weight is too low for determination. [1]approximate molecular weight only; close to limit of method. [2]Polydispersity index (M_w/M_n) = M_w (Weight average molecular weight) / M_n (Number-average molecular weight).

Table 3.2: The amino acid composition (weight percentage) of the plant extracts[1]

	Amino acid composition (g/100 g protein)				
Amino acid	**Lily Extract**	**Cabbage tree extract**	***Undaria* extract**	***Acacia* extract**	**Frutafit[2]**
Alanine	**11**	**10**	**10**	**5**	**6**
Glycine	**6**	**8**	**20**	**8**	**N.D**
Valine	**5**	**4**	**11**	**8**	**7**
Lecine	**6**	**6**	**11**	**11**	**7**
Isolecine	**3**	**3**	**4**	**4**	**7**
Proline	**5**	**4**	**3**	**7**	**6**
Methionine	**1**	**Tr**	**2**	**N.D**	**N.D**
Serine	**4**	**4**	**9**	**10**	**N.D**
Threonine	**3**	**3**	**6**	**2**	**1**
Phenylalanine	**2**	**3**	**4**	**3**	**N.D**
Aspartic acid	**8**	**9**	**6**	**12**	**30**
Hydroxyprolin	**2**	**3**	**6**	**23**	**N.D**
Glutamic acid	**16**	**26**	**6**	**6**	**36**
Lysine	**7**	**4**	**N.D**	**N.D**	**N.D**
Arginine	**12**	**4**	**N.D**	**N.D**	**N.D**
Histidine	**8**	**4**	**N.D**	**N.D**	**N.D**

[1]Average of duplicate determinations. [2]Commercially available fructooligosaccharide. N.D = Not detected. Tr = Trace amount (<1 wt%).

High performance size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS) showed that the extracts had varying weight average molecular weight due to differences in the average chain length of the major carbohydrates. Data for the amino acid compositions differed considerably depending on the type of extract (Table 3.2). Water-soluble carbohydrate extracts prepared from the four plant sources gave a wide range of WSC (250-794 g/kg DM) due to the different proportions of structural material in different species.

3.1.2 Fructans from Rengarenga lily (*Arthropodium cirratum*) extract and frutafit as prebiotics for broilers

The first animal study under this Strategy investigated the effect of dietary water-soluble carbohydrate extract from Rengarenga lily and a commercial product, Frutafit®, (both fructans) on the performance, organ weights, ileal digestibility and gut morphology in male Cobb broilers. There were six treatment groups: a negative control with no supplements, a positive control supplemented with 45 ppm Zn-bacitracin, and four test diets each supplemented with Rengarenga lily extract or Frutafit at 5 or 10 g/kg diet. Supplementation with low levels of Rengarenga lily extract and Frutafit in the diet did not affect productive parameters, whereas the inclusion of a high level of Frutafit had a negative effect on BWG and FI compared with birds fed the negative control diet (Table 3.3).

Table 3.3: Mean body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens fed diets supplemented with the Rengarenga lily extract and Frutafit[1]

	Period	Negative Control	Positive Control	Rengarenga lily extract		Frutafit		S.E.M.
				5 g/kg	10 g/kg	5 g/kg	10 g/kg	
BWG (g/bird)								
	1-21 d	**823^{b}**	**853^{a}**	**832^{b}**	**838^{ab}**	**828^{b}**	**781^{c}**	**17.6*****
	22-42 d	**1851^{b}**	**1926^{a}**	**1852^{b}**	**1858^{b}**	**1853^{b}**	**1831^{c}**	**15.7*****
	1-42 d	**2668^{b}**	**2779^{a}**	**2678^{b}**	**2681^{b}**	**2664^{b}**	**2613^{c}**	**23.5*****
FI (g/bird)								
	1-21 d	**1392^{a}**	**1407^{a}**	**1399^{a}**	**1407^{a}**	**1405^{a}**	**1336^{b}**	**15.2*****
	22-42 d	**3716**	**3722**	**3721**	**3716**	**3713**	**3680**	**39.8^{NS}**
	1-42 d	**5116^{a}**	**5129^{a}**	**5120^{a}**	**5121^{a}**	**5096^{a}**	**5048^{b}**	**45.5***
FCR								
	1-21 d	**1.69^{ab}**	**1.65^{b}**	**1.68^{ab}**	**1.68^{ab}**	**1.70^{a}**	**1.71^{a}**	**0.04***
	22-42 d	**2.01^{a}**	**1.93^{b}**	**2.01^{a}**	**2.00^{a}**	**2.00^{a}**	**2.01^{a}**	**0.03*****
	1-42 d	**1.92^{a}**	**1.85^{b}**	**1.91^{a}**	**1.91^{a}**	**1.91^{a}**	**1.93^{a}**	**0.02*****

[1]Least square means and pooled standard error of the mean (S.E.M.), $n = 6$.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$, results not sharing the same superscripts within a row are significantly different ($P<0.05$).

The addition of an antibiotic to the diet significantly improved (P<0.05) the BWG and FCR of broilers relative to the negative control. Apparent ileal digestibility of dry matter, starch, protein and fat was not affected (P>0.05) by supplementation with both levels of lily extract and low level of Frutafit (Table 3.4). The apparent ileal digestibility of dry matter, protein and fat was decreased (P<0.05) by the high level of Frutafit. The apparent metabolisable energy (AME) of broilers fed the high level of Frutafit was approximately 0.2 MJ/kg DM lower than that of the negative control group. The addition

of Zn-bacitracin increased (P<0.05) the apparent ileal digestibility of fat. The relative weight of the liver was higher (P<0.05) in broilers supplemented with the high level of Frutafit than for negative control birds at 14 and 35 d of age. Feeding Rengarenga lily extract or Frutafit had no effect on the gut morphology of birds on d 14 and 35. It can be concluded that dietary inclusion of fructans from the two sources used in this study affected broiler performance differently and in a dose-dependent manner.

Table 3.4: Apparent ileal digestibility of major nutrients in broilers fed the experimental diets[1]

	Apparent Ileal Digestibility (%)			
	Dry matter	**Starch**	**Protein**	**Fat**
Negative control	**72.59[a]**	**93.57**	**77.21[a]**	**74.32[b]**
Positive control	**72.62[a]**	**93.81**	**77.19[a]**	**75.29[a]**
Lily extract (5 g/kg)	**72.45[a]**	**93.71**	**77.27[a]**	**74.34[b]**
Lily extract (10 g/kg)	**72.44[a]**	**93.73**	**77.26[a]**	**74.32[b]**
Frutafit (5 g/kg)	**72.56[a]**	**93.71**	**77.27[a]**	**74.50[b]**
Frutafit (10 g/kg)	**71.02[b]**	**93.48**	**76.04[b]**	**70.76[c]**
S.E.M.	**0.73**	**0.32**	**0.60**	**0.73**
Probability	*******	**NS**	******	*******

[1]Least square means and pooled standard error of the mean (S.E.M.), $n = 6$.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$, results not sharing the same superscripts within a column are significantly different ($P<0.05$).

Table 3.5: Mean body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens fed diets supplemented with plant extracts[1]

	Body weight gain (BWG; g/bird)		Feed Intake (FI; g/bird)		Feed conversion ratio (FCR)	
	1-21d	**1-35d**	**1-21d**	**1-35d**	**1-21d**	**1-35d**
Negative-control (NC)	**685**	**1822**	**1172**	**3340**	**1.71**	**1.83**
Positive-control (PC)	**716**	**1886**	**1163**	**3331**	**1.62**	**1.77**
Cabbage tree (5 g/kg)	**682**	**1845**	**1176**	**3351**	**1.72**	**1.82**
Cabbage tree (10 g/kg)	**671**	**1801**	**1178**	**3339**	**1.76**	**1.85**
***Undaria* (5 g/kg)**	**686**	**1831**	**1161**	**3345**	**1.69**	**1.83**
***Undaria* (10 g/kg)**	**667**	**1786**	**1162**	**3342**	**1.74**	**1.87**
***Acacia* (5 g/kg)**	**690**	**1847**	**1160**	**3356**	**1.68**	**1.82**
***Acacia* (10 g/kg)**	**693**	**1844**	**1159**	**3346**	**1.67**	**1.81**
S.E.M.[2]	**7.53**	**9.33**	**18.32**	**12.94**	**0.02**	**0.01**

[1]Results are given as least square means ($n = 6$). [2]S.E.M. = Pooled standard error of the least square means.

3.1.3 Prebiotic plant extracts for broilers: their effects on growth performance, intestinal morphology, microbial composition and activity

The second experiment was carried out to study the effects of WSC extracts from Cabbage tree, Acacia, and Undaria seaweed on performance, gut morphology, microbial populations, and organic acid concentrations in the digestive tract of broilers. Each plant extract was supplemented at two levels (5 g/kg or 10 g/kg) to the diets. The plant extracts had no effect on BWG, but both levels of Acacia extract improved (P<0.05) FCR during the first three weeks compared with the negative control group (Table 3.5). The high level of Undaria extract suppressed growth throughout the experimental period. The positive control group fed on antibiotic supplemented diet showed improved (P<0.05) BWG and FCR.

Ileal digesta viscosity was increased (P<0.05) and apparent ileal digestibility of fat was depressed (P<0.05) in birds fed the high level of Undaria extract compared to the negative control. The plant extracts increased (P<0.05) the numbers of lactobacilli in the ileum and caeca. The high levels of Acacia extract and Undaria extract significantly (P<0.05) reduced the population of coliform bacteria in the ileum compared to the negative control group. The population of *C. perfringens* (Cp) was reduced (P<0.05) in the caeca by plant extracts, but not in the ileum. The antibiotic reduced the population of Cp in both the ileum and caeca compared with the negative control group.

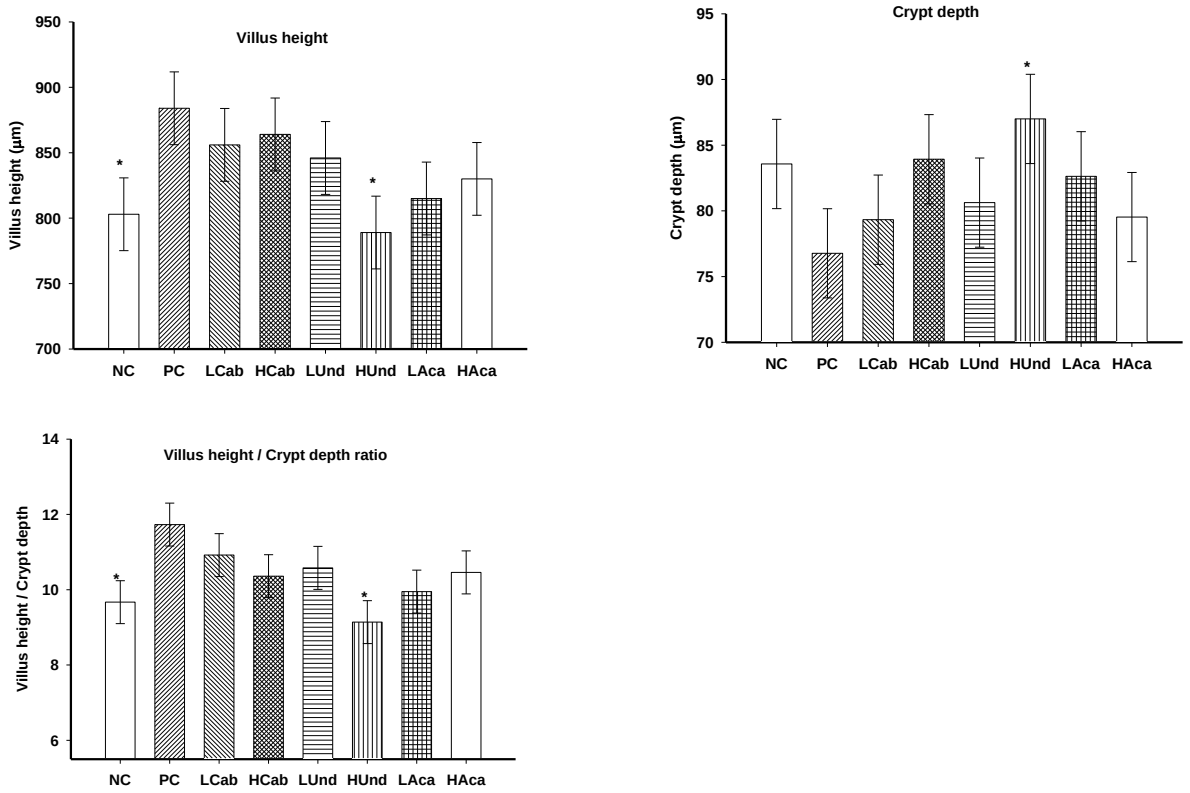

Figure 3.1: Effects of dietary plant extracts on the morphometric parameters of ileum at 35 days of age

Negative control (NC), positive control (PC), low level of Cabbage tree extract (LCab), high level of Cabbage tree extract (HCab), low level of *Undaria* extract (LUnd), High level of *Undaria* extract (HUnd), low level of *Acacia* extract (LAca) and high level of *Acacia* extract (HAca). (Results are given as least square means, *n*=6; error bars indicate pooled standard error of the least square means; bars with asterisks are significantly different from the positive control

The number of bifidobacteria was below the detection limit in most samples and not affected by dietary supplementation of plant extracts. The organic acid analysis indicated that plant extract affected microbial fermentation patterns in the ileum and caeca. The high level of Undaria extract reduced villus height in the ileum and the antibiotic diet resulted in higher ($P<0.05$) villus height and villus height/crypt depth ratio compared with the negative control group. The results from this study demonstrated that prebiotic plant extracts had no or negative effect on performance of broilers, but beneficially modulated the composition of the microflora in the ileum and caeca by increasing the number of lactobacilli and reducing harmful bacteria, such as potential pathogenic *E. coli* and Cp.

Table 3.6: Distribution of major genotypic groups of lactobacilli isolated from ileum and caeca of broilers at d 35

Treatment	Groups in ileum				Groups in caeca			
	I	II	III	IV	I	II	III	IV
Negative control	26	4	0	0	23	7	0	0
10 g/kg Cabbage tree extract	19	11	0	0	24	6	0	0
10 g/kg Seaweed extract	25	3	1	1	27	1	2	0
10 g/kg Acacia extract	12	3	0	13	5	5	1	19

3.1.4 Molecular and biochemical characterisation of lactobacilli isolated from ileal and caecal digesta of broilers fed prebiotic plant extracts

Molecular and biochemical characterisations were carried out for lactobacilli isolated from ileal and caecal digesta of broiler chickens fed with water-soluble prebiotic carbohydrate extracts (10 g/kg) obtained from Cabbage tree, seaweed, Undari, and exudates from *Acacia pycnantha*. Genomic DNA was extracted from lactobacilli isolated from Rogosa agar; the 16-23S rDNA intergenic spacer regions were amplified and subjected to Amplified Ribosomal DNA Restriction Analysis (ARDRA) using HaeIII enzyme. Partial 16S rRNA gene sequences of major genotypic groups were determined and compared to sequences in the GeneBank using the Basic Local Alignment Search Tool (BLAST) algorithm.

Table 3.7: Fermentation characteristics of four genotypic groups of lactobacilli isolated from broiler chickens[1]

	Lactobacillus isolates			
Substrate	Group I	Group II	Group III	Group IV
L-Arabinose	–	–	+/– (9.29)	+/– (9.98)
Galactose	+ (6.42)	+ (6.42)	+ (4.84)	+ (2.40)
Maltose	+ (3.15)	+ (3.30)	–	–
Cellobiose	–	–	–	+ (5.92)
Trehalose	+ (4.48)	+ (7.35)	+ (6.12)	+ (4.95)
Palatinose	+ (10.28)	+ (10.41)	+ (8.21)	+ (8.71)
Sucrose	+ (3.13)	+ (2.93)	+ (2.21)	+ (3.24)
Raffinose	+ (6.03)	+ (6.97)	–	+ (9.04)
D-fucose	–	+ (8.81)	–	+/– (10.19)

[1]Four representative isolates from each group were analysed. +, ability to ferment indicated substrate; – inability to ferment indicated substrate; +/–, variable results within a group. Values in brackets are absorbance values 620 nm x 10. A full list of test compounds is available on request.

The ARDRA and partial 16S rRNA gene sequencing revealed four distinctive groups of lactobacilli: *L. salivarius* group (group I), *L. crispatus* group (group II), unidentified Lactobacillus species (group III), and *L. johnsonii* (group IV) (Table 3.6). These Lactobacillus species are dominant in the ileal and caecal digesta of broilers. The *L. johnsonii* group was mainly detected in the ileal and caecal digesta of the Acacia extract-supplemented group. The Ph-48 generalized PhenePlate system was used to test the fermentation characteristics of the four groups of lactobacilli. All four groups fermented the monosaccharide; galactose at varying levels and disaccharides; sucrose, trehalose, and palatinose (Table 3.7). Sucrose was the substrate which was the most fermented by isolates from all four groups.

3.1.5 Natural plant extracts and prebiotic compounds as alternatives to antibiotics in broilers in a necrotic enteritis challenge model

The final experiment under Strategy A was conducted to determine the effects of two different water-soluble carbohydrate extracts (Rengarenga lily extract and Acacia extract), and two commercially available prebiotic compounds, Fibregum® and Raftifeed®-IPE, with similar chemical compositions to the plant extracts on performance, gut microflora composition, gut morphology and humoral immune responses of broiler chickens subjected to a necrotic enteritis (NE) challenge model involving oral inoculation with *C. perfringens* (Cp). The plant extracts and prebiotic compounds were added (10 g/kg) to a wheat-based diet.

Table 3.8: Mean body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens fed diets supplemented with plant extracts and prebiotic compounds[2]

	Body weight gain (BWG; g/bird)		Feed Intake (FI; g/bird)		Feed conversion ratio (FCR)	
	d 1 to 21	d 1 to 35	d 1 to 21	d 1 to 35	d 1 to 21	d 1 to 35
Unchallenged-control (UC)	**837**	**1993**	**1177**	**3551**	**1.41**	**1.78**
Negative-control (NC)	**690**	**1741**	**1055**	**3366**	**1.53**	**1.94**
Positive-control	**805**	**1998**	**1092**	**3296**	**1.36**	**1.65**
***Acacia* extract**	**725**	**1817**	**1099**	**3611**	**1.52**	**1.99**
Fibregum	**713**	**1841**	**1069**	**3534**	**1.50**	**1.92**
Lily extract	**692**	**1740**	**1087**	**3533**	**1.57**	**2.03**
Raftifeed	**684**	**1712**	**1036**	**3300**	**1.51**	**1.93**
S.E.M.[1]	**14.35**	**44.47**	**23.59**	**112.43**	**0.02**	**0.05**
Orthogonal contrasts						
UC vs. NC	**<0.001**	**<0.001**	**0.001**	**NS**	**0.001**	**0.02**
NC vs. PC	**<0.001**	**<0.001**	**NS**	**NS**	**<0.0001**	**<0.0001**
NC vs. *Acacia* extract	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
NC vs. Fibregum	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
NC vs. Lily extract	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
NC vs. Raftifeed	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

There were three control treatment groups: basal diet without Cp challenge (unchallenged control), basal diet with Cp challenge (negative control) and basal diet supplemented with 45 ppm active ingredients of Zn-bacitracin and 100 ppm monensin (positive control). Addition of plant extracts or prebiotic compounds neither improved performance nor reduced NE lesion scores in disease-challenged groups. The birds on positive control diet showed better performance throughout the experimental period (Table 3.8). An overall 8.8 % NE-related mortality was recorded, with mean jejunal and ileal lesion scores in dead birds ranging from 3.03 to 3.90 in all challenged groups except the positive control groups. Necrotic enteritis-specific deaths or clinical abnormalities were not observed with unchallenged control and positive control groups. Chi-square analysis revealed that the Fibregum-supplemented group had a lower ($P<0.05$) NE related mortality compared to the Acacia extract-supplemented group, but this mortality was not different ($P<0.08$) when compared to the negative control group. Fibregum decreased ($P<0.05$) Cp numbers in caecal contents before Cp challenge but this effect was not significant after Cp challenge, although the values tended to be lower than those of the negative control group (Table 3.9).

Table 3.9: Bacterial counts in ileal digesta of birds fed diets supplemented with plant extracts and prebiotic compounds at d 14 (before challenge)[2].

	Total anaerobic bacteria	Lactobacilli	Lactose-negative Enterobacteria	Coliforms	*Clostridium perfringens*
	——————Log_{10} CFU/g digesta——————				
Unchallenged-control	**9.76**	**7.19**	**7.55**	**8.76**	**9.00**
Negative-control	**9.72**	**7.64**	**7.38**	**8.72**	**9.03**
Positive-control	**9.31**	**6.66**	**6.95**	**8.75**	**3.53**
***Acacia* extract**	**9.64**	**8.24**	**7.00**	**8.21**	**9.29**
Fibregum	**9.30**	**7.29**	**6.78**	**8.18**	**8.35**
Lily extract	**9.53**	**8.00**	**6.82**	**8.72**	**9.03**
Raftifeed	**9.86**	**8.08**	**7.18**	**8.90**	**9.04**
S.E.M.[1]	**0.16**	**0.24**	**0.28**	**0.17**	**0.26**
Orthogonal contrasts	——————***Probability level of contrasts***——————				
UC vs. NC	**NS**	**NS**	**NS**	**NS**	**NS**
NC vs. PC	**NS**	**0.006**	**NS**	**NS**	**<0.0001**
NC vs. *Acacia* extract	**NS**	**NS**	**NS**	**0.04**	**NS**
NC vs. Fibregum	**NS**	**NS**	**NS**	**0.02**	**NS**
NC vs. Lily extract	**NS**	**NS**	**NS**	**NS**	**NS**
NC vs. Raftifeed	**NS**	**NS**	**NS**	**NS**	**NS**

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

Birds fed the plant extracts and Fibregum had higher ($P<0.05$) Lactobacillus counts in the ileal digesta than those of the negative control group on d 21. At seven days post-challenge, the concentration of specific IgY antibodies against the α-toxin of Cp in the serum was lower ($P<0.05$) in birds fed the positive control and Fibregum-supplemented diets in comparison with those from the negative control group (Table 3.9). The total serum IgY response did not differ between treatment groups of chickens before challenge. However, birds fed Fibregum had increased ($P<0.05$) IgM concentration compared to those fed with Acacia extract and lily extract. The Fibregum-fed group also had higher ($P<0.05$) IgA levels in serum than the positive control and lily extract-supplemented groups at 14 days but this effect did not persist to 21 days. The results from this study demonstrated that supplementation with

WSCs from the two plant sources was not effective in controlling NE. However, the prebiotic compound, Fibregum, was found to be effective in reducing NE-associated mortality and can be considered a novel prebiotic with some immunomodulatory effects. Addition of Zn-bacitracin, and monensin, was highly effective in counteracting the negative effects of the disease challenge.

3.2 Strategy B

3.2.1 Effects of particle size, milling method and grain type on broiler performance and apparent metabolisable energy

Two experiments were conducted to determine the effect of grain particle size and milling type on performance and nitrogen corrected apparent metabolisable energy (AMEn) of male broiler chicks to five weeks of age under experimental conditions.

Male broilers aged between10 and 35 d (Experiment One) were fed diets diets based on whole sorghum (WS), hammer-milled through a 3 mm screen (HM3) or roller-milled to a similar particle size distribution as HM3 (RM0.15), or hammer-milled through a 1 mm screen (HM1). In Experiment Two, male broilers were reared from one day of age to 35 days on their respective experimental diets containing either wheat (fine and coarse milled; treatments WF and WC respectively), or sorghum (fine and coarse; treatments SF and SC respectively).

Table 3.10: Mean performance values from 11 d - 35 d (Experiment One).

Treatment	WS	HM3	RM0.15	HM1	SEM	P value
21d						
BW gain[1] (g)	614.3^{b}	643.9^{a}	638.2^{ab}	615.2^{b}	8.8	0.032
Feed intake[2] (g)	868.7^{a}	875.7^{a}	837.5^{b}	823.8^{b}	10.1	<0.001
FCR[3] (g.g^{-1})	1.41^{a}	1.36^{b}	1.31^{c}	1.34^{b}	0.014	<0.001
35d						
BW gain[1] (g)	1823.3^{b}	1905.2^{a}	1916.4^{a}	1865.7^{ab}	26.3	0.047
Feed intake[2] (g)	2853.8	2902.2	2795.9	2804.5	37.4	NS
FCR[3] (g.g^{-1})	1.57	1.52^{b}	1.46^{c}	1.50^{b}	0.007	<0.001

^{a,b}Values in a row with unlike superscripts differ significantly (P<0.05). [1]Cumulative bodyweight gain from 11d of age. [2]Cumulative feed intake from 11d of age. [3]21d FCR calculated and averaged for the two birds from 11-21d of age, 35d FCR calculated from 11-35d of age and averaged for the two birds for the period 11-21d of age.

Table 3.11: Nitrogen corrected apparent metabolisable energy (AME_n) and AME_n:GE_{diet} ratio for the semi-purified sorghum diets (Experiment One).

AME/ Diet	WS	HM3	RM0.15	HM1	SEM	P value
AME_n[1]	13.85^{a}	13.47^{b}	13.51^{b}	13.60^{b}	0.08	0.005
AME_n:GE_{diet}[2]	0.807^{a}	0.791^{b}	0.786^{b}	0.783^{b}	0.005	0.003

^{a,b}Values within rows with unlike superscripts are significantly different. [1]AME_n calculated using excreta taken over three consecutive days (MJ.kg^{-1} DM). [2]AME_n:GE_{diet} ratio accounts for differences in GE of the feed (MJ.kg^{-1}).

Feeding an intermediate sized, roller-milled sorghum diet enabled the best feed conversion compared to whole, fine and intermediate hammer-milled sorghum (Table 3.10). Apparent MEn was improved by feeding whole sorghum (Table 3.11 and 3.12). Feeding sorghum resulted in improved feed conversion by and after three weeks of age, compared to feeding wheat, and an increase in grain

particle size was additive to the effect of grain type. Change in milled wheat particle size influenced feed conversion more than change in sorghum particle size. Apparent MEn increased with increasing particle size, but was not relative to performance. Differences in AMEn and performance between grains of similar particle sizes were attributed to perceived differences in soluble non-starch polysaccharide content.

3.2.2 Effect of grain particle size, milling method on digestive tract morphology and digesta pH of broilers raised in cages

The weight of the proventriculus was inversely proportional to the particle size of the feed (Table 3.13). At 21 days of age, birds fed the two intermediate particle sized diets had numerically lighter proventriculi than birds fed the WS diet. At 35 days of age, proventriculus size was numerically inversely proportional to the particle size of feed eaten, with proventriculi of birds in treatment HM1 being heavier (P<0.01) than those for all other treatments. The relative gizzard weight of birds fed the WS diet was heavier (P<0.05) when fed a larger particle sized feed at 21 days of age. Thus, the gizzards of birds fed the WS diet were heavier than the two intermediate particle sized diets, which were heavier than the gizzards of birds fed the HM1 diet (P<0.001). The difference in gizzard weight between the WS and intermediate particle sized treatments (HM3 and RM0.15) was not significant at 35 days of age, but gizzards from birds in treatment HM3 were lighter (P<0.001) than those from all other treatments. Wet gizzard content weight was proportional to gizzard relative weight, and was positively related (P<0.001) to particle size of feed at 35 days of age. The relative weight of all digestive tract organs decreased with age.

Table 3.12: Nitrogen corrected apparent metabolisable energy (AME_n) and AME_n:GE_{diet} ratio for the wheat and sorghum diets (Experiment Two).

AME/Diet	Diets				SEM	P value		
	WF	WC	SF	SC		Grain	Texture	Grain x Texture
AME_n[1]	11.22	11.21	12.54	13.42	0.30	<0.001	NS	NS
AME_n:GE_{diet}[2]	0.646	0.644	0.732	0.786	0.170	<0.001	NS	NS

[1]AME_n calculated using excreta taken over two consecutive days ($MJ.kg^{-1}$ DM).
[2]AME_n:GE_{diet} ratio accounts for differences in GE of the feed ($MJ.kg^{-1}$).

Table 3.13: Average relative organ weights (g/kg live-weight) of male broilers at 21 and 35 days of age (Experiment 1)[1].

Age/ Treatment	WS	HM3	RM0.15	HM1	SEM[2]	P value
21d						
Proventriculus	4.6[b]	4.3[b]	4.5[b]	5.1[a]	0.16	0.005
Gizzard	18.6[a]	16.8[b]	17.2[b]	14.4[c]	0.43	<0.001
Gizzard content	10.2[a]	9.2[a]	10.5[a]	4.4[b]	0.61	<0.001
Small intestine	49.3	47.8	47.4	46.9	0.81	NS
35d						
Proventriculus	3.3[b]	3.5[b]	3.6[b]	4.5[a]	0.18	<0.001
Gizzard	13.9[a]	13.4[a]	13.3[a]	11.4[b]	0.46	<0.001
Gizzard content	7.2[a]	5.9[ab]	5.5[b]	1.9[c]	0.55	<0.001
Small intestine	36.3	36.0	35.8	35.6	0.78	NS

[abc]Values within rows with unlike superscripts are statistically different. [1]Values for relative organ weight represent the weight of empty organs only. [2]Pooled standard error of the means. [3]Total tract weight is the sum of the weights of the empty digestive tract organs.

Results from Experiment Two demonstrate that the relative weight of the proventriculus was inversely proportional to the particle size of the feed eaten from 7 d through to 35 d (Table 14). The relative weight of the gizzard tended (P=0.108) to be proportional to feed particle size at 7 d, and was significantly so (P<0.001) at 21 d and 35 d. At three and five weeks of age, the relative weight of the duodenum was increased (P<0.05) by feeding wheat relative to sorghum.

Table 3.14: Relative organ weights (g/kg live-weight) from 7 d to 21 d (Experiment 2)[1].

Age/Organ	Diets				SEM[2]	P values		
	WF	WC	SF	SC		Grain	Texture	Grain × Texture
7 d								
Proventriculus	9.5	8.9	9.5	9.2	0.29	NS	<0.001	NS
Gizzard	35.1	37.6	36.3	37.5	1.14	NS	0.108	NS
Pancreas	4.1	4.4	4.5	4.6	0.15	0.055	NS	NS
Small intestine	143.8^{b}	154.2^{a}	149.1^{ab}	148.7^{ab}	2.56	NS	0.060	0.0416
21 d								
Proventriculus	5.5	5.3	6.0	4.9	0.29	NS	0.031	NS
Gizzard	16.6	20.2	17.8	19.5	0.58	NS	<0.001	NS
Pancreas	2.9	2.9	2.8	2.6	0.11	0.113	NS	NS
Small intestine	98.2	100.2	97.5	96.3	2.80	NS	NS	NS

^{abc}Values within rows with unlike superscripts are significantly different
[1]Values for relative organ weight represent the weight of empty organs only.
[2]Pooled standard error of the means.
[3]Total tract weight is the sum of the weights of the empty digestive tract organs, pancreas and liver.

The same pattern was observed for the ileum (P<0.05) from 7 d to 35 d. The relative weight of the jejunum was lower for sorghum fed birds than wheat fed birds at five weeks of age only. At 7 and 35 d, there was a significant treatment effect on ileum relative weight. Birds eating the SC diet had a lighter ileum than all other treatments, and those eating the SF diet had a lighter ileum than those being fed WC. Furthermore, ileum weight was inversely proportional to feed particle size for the sorghum diets (P<0.05), but proportional to particle size for the wheat diets. At 7 d, the total (relative) weight of the digestive tract was decreased (P<0.05) by feeding WF relative to feeding WC. The two sorghum treatments promoted total tract weights intermediate to the two wheat treatments. Sorghum-based diets decreased the relative digestive tract weight over wheat-based diets by five weeks of age.

Table 3.15: Digesta pH of male broiler chicks from seven to 35 days of age (Experiment 1).

Treatment	WS	HM3	RM0.15	HM1	SEM[1]	P value
21d						
Gizzard	3.4^{b}	3.4^{b}	3.4^{b}	3.9^{a}	0.08	<0.001
Duodenum	6.1^{a}	6.0^{ab}	6.0^{ab}	6.0^{b}	0.02	0.053
35 d						
Gizzard	3.1^{c}	3.5^{b}	3.5^{b}	4.2^{a}	0.09	<0.001
Duodenum	5.9^{a}	5.9^{ab}	5.8^{bc}	5.6^{c}	0.07	0.001

[1]Pooled standard error of the means.
^{abc}Values within rows with unlike superscripts are significantly different (P<0.05).

In Experiment One, the pH of the gizzard content was inversely proportional and the pH of the duodenum digesta was proportional to the particle size of the feed across age differences (P<0.05) (Table 3.15). Furthermore, birds fed the WS treatment had a lower gizzard pH and higher duodenal pH than HM1 fed birds, and the two medium particle size groups were intermediate. When comparing treatments, there were no significant differences between the mean pH values in the gizzard, ileum or

caeca for Experiment Two (Table 3.16). However, feeding a coarse particle, irrespective of grain type, reduced gizzard pH and increased duodenal pH relative to those of fine particle fed birds, and this effect became more marked with age. Feeding wheat reduced (P<0.001) caeca digesta pH relative to feeding sorghum at all age groups, but there was no apparent effect of particle size. There was a significant treatment effect on caecal pH at 21 d, where fine wheat increased caecal pH relative to coarse wheat and the inverse was true for sorghum. However, this pattern was not evident by 35 d.

Table 3.16: Digesta pH of male broiler chicks from seven to 35 days of age (Experiment 2)

Day/Origin of digesta	Diet				SEM[1]	P values		
	WF	WC	SF	SC		Grain	Texture	Grain x Texture
21 d								
Gizzard	3.7	3.3	3.6	3.4	0.07	NS	0.008	NS
Duodenum	6.0	6.0	5.8	6.2	0.08	NS	0.030	NS
Caeca	7.0[b]	6.6[b]	7.5[a]	7.8[a]	0.13	<0.001	NS	0.029
35 d								
Gizzard	4.1	3.6	4.1	3.5	0.07	NS	<0.001	NS
Duodenum	5.7	6.1	5.8	6.0	0.09	NS	0.028	NS
Caeca	6.3	6.4	7.3	7.6	0.12	<0.001	NS	NS

[a,b]Values within rows with unlike superscripts are significantly different (P<0.05).
[1]Pooled standard error of the means.

There were no significant differences between treatments in Experiment 2 for proximal jejunum histomorphology. Feed texture tended to have some effect on the villus height (P<0.10) and villus height to crypt depth ratio at 7 and 21 days of age (P<0.10 and P<0.05, respectively). However, the differences between the mean values for birds fed fine-milled vs.coarse-milled grains were not significant (0.05<P<0.10), but displayed a trend that was not evident at 35 days of age. At 21 days of age, birds fed coarser feeds had a higher (P<0.05) villus height to crypt depth ratio than fine-milled grain fed birds. At 35 days of age, it was observed that, birds fed wheat-based diets had a higher (P<0.05) villus height to crypt depth ratio than sorghum fed birds.

3.2.3 Effect of wheat milling type and particle size on productivity and intestinal development under commercial conditions

The 43d body weight was unaffected by type of diet or processing method but tended to be higher on the HM55 diet than on the other diets (Table 3.17). Feed conversion ratio on the WW diet was also 3 % better than on the RM55 diet, which had the poorest FCR. The HM3.2 treatment yielded a lower (P<0.05) ileal starch digestibility than all other treatments (data not shown).

Table 3.17: Performance of 43d broilers under commercial conditions.

Variable/diet	HM3.2A	HM3.2	HM35	HM55	RM35	RM55	WW	SEM[1]
Body weight (g)	2573.9	2546.8	2529.4	2588.0	2577.0	2509.0	2539.1	61.7
FCR	1.83	1.80	1.79	1.80	1.80	1.86	1.77	0.038
2.1 kg corr. FCR	1.64	1.62	1.62	1.61	1.61	1.69	1.59	0.046

[1]Pooled standard error of the means.

The weights of key visceral organs are shown in Table 3.18. At 21d of age, birds on the HM3.2A diet had the smallest (P<0.002) proventriculus. The weight of the proventriculus from birds on the other diets was similar to that of birds raised on the WW diet. The weight of the gizzard from the WW-fed

birds was also 15 % higher than that of birds on the HM3.2A diet. This pattern was repeated at 41d of age, with birds on WW having gizzards that were 8 % heavier than that obtained with the HM3.2A diet. There were no significant differences between the groups in the weight of the other organs measured at the two ages.

Table 3.18: Digestive organ weights (g/kg live-weight) of as-hatched broilers at 21 and 41d.

	HM3.2A	HM3.2	HM35	HM55	RM35	RM55	WW	SEM[2]
21 d								
Proventriculus	4.2^{c}	5.1^{ab}	4.6^{bc}	5.1^{ab}	5.3^{a}	5.0^{ab}	5.0^{ab}	0.18**
Gizzard	16.1^{b}	18.6^{a}	18.9^{a}	18.3^{ab}	20.0^{a}	18.1^{ab}	19.0^{a}	0.79*
Liver	30.6	31.6	32.6	35.6	33.4	33.7	33.3	1.32^{ns}
Total tract[3]	93.3	99.5	99.3	103.1	102.7	100.3	101.6	2.41^{ns}
41 d								
Proventriculus	3.1	3.2	3.1	3.1	3.0	3.3	3.2	0.17^{ns}
Gizzard	10.5	10.8	10.7	10.9	10.8	10.5	11.4	0.58^{ns}
Liver	23.8	24.6	24.6	25.7	22.7	25.5	24.5	1.16^{ns}
Total tract[3]	64.9	67.2	66.3	66.4	59.3	64.4	62.4	2.27^{ns}

[abc]Values within rows with unlike superscripts are significantly different (P<0.05), [1]Values for relative organ weight represent the weight of empty organs only, [2]Pooled standard error of the means, [3]Total tract weight is the sum of the weights of the empty digestive tract organs and liver.

Table 3.19: Bacterial counts ($log_{10}CFU.g^{-1}$ wet digesta) on selective agars from the gizzard, ileum and caeca of as-hatched broilers at 41d.

Diet	HM3.2A	HM3.2	HM35	HM55	RM35	RM55	WW	SEM[1]
Gizzard								
Coliforms, lact. -ve	2.4	2.9	2.8	2.9	3.4	3.5	2.8	0.27
Lactobacilli	5.5^{bc}	5.7^{bc}	6.2^{ab}	6.9^{a}	5.1^{c}	6.0^{abc}	5.2^{bc}	0.38*
C. perfringens	2.5	2.5	2.5	2.6	2.5	2.5	3.0	0.13
Total anaerobes	6.7	7.0	6.9	7.1	6.0	6.4	6.3	0.38
Ileum								
Coliforms, lact. -ve	4.6	4.8	4.3	5.2	4.2	4.6	5.2	0.44
Lactobacilli	7.8	7.5	7.5	8.0	6.7	6.9	7.1	0.39
C. perfringens	3.0	2.9	2.9	2.9	3.1	3.0	3.2	0.22
Total anaerobes	8.7^{ab}	8.3^{bcd}	8.6^{abc}	8.8^{a}	7.9^{d}	7.9^{d}	8.2^{cd}	0.15***
Caeca								
Coliforms, lact. -ve	7.0	6.8	6.7	6.5	6.8	6.9	6.7	0.24
Lactobacilli	8.2	8.0	8.3	8.3	8.3	8.0	8.3	0.17
C. perfringens	3.5	3.6	3.9	3.7	3.9	3.6	4.3	0.46
Total anaerobes	8.8	8.3	8.7	8.6	8.9	8.4	8.8	0.17

[1]Pooled standard errors of the means. [abcd]Means assigned different superscripts within a row differ significantly (P<0.05).

The population of Lactobacilli in the gizzard was highest (P<0.03) in birds raised on the HM55 diet, and lowest on the WW diet (Table 3.19). Total anaerobe counts were also higher (P<0.001) in the ileum of chickens on the RM55 diets than in birds on the other diets. There were no effects of diets or processing method in other microbial variables assessed.

The morphometry of the jejunal mucosa was also unaffected by dietary treatment (Table 3.20). There were no differences between treatments for digesta pH from 41 day old chicks (data not shown). However, when the two intermediate and coarse hammer- and roller-milled treatments were compared, feeding a coarser texture reduced ($P<0.05$) caecal pH.

Table 3.20: Morphometry of the jejunal mucosa of birds on different diets.

Variable/diet	HM3.2A	HM3.2	HM35	HM55	RM35	RM55	WW	SEM[1]
Villus height	1304.0	1180.0	1288.0	1260.0	1260.0	1400.0	1213.0	58.8
Crypt depth	464.0	434.0	437.0	466.0	396.0	409.0	404.0	23.2
Villus:crypt	2.2	2.9	3.0	2.7	2.3	3.5	3.0	0.21

[1]Pooled standard error of the means.

4 Discussion of Results

4.1 Strategy A

The findings of the current study add to the plethora of information available on alternatives to IFAs in animal feed. Typical of the nature of the research field, the plant extracts used in the current study yielded mixed results in terms of both bird performance and gut microflora. This highlights the challenges that researchers have to deal with when it comes to investigating multi-disciplinary areas of science, such as identification of alternatives to IFAs. Finding viable alternatives to IFAs will require a great knowledge and tools of nutrition, chemistry, immunology, microbiology, gut physiology, and disease. The current study certainly attempted to cover many of these disciplines and investigate their interactions.

4.1.1 Chemical properties of extracts

Water-soluble carbohydrates from the three plant sources and the seaweed differ in their physicochemical properties such as carbohydrate content, sugar composition, molecular weight, distribution of molecules (polydispersity) and degree of polymerisation. These are properties that would determine the biological effect of the extracts. For example, the Rengarenga lily and Cabbage tree extracts both contain fructans as their main carbohydrate but their degrees of polymerisation are different; Cabbage tree extract contains short chain fructans (oligofructans), while Rengarenga lily extract has long chain fructans. Acacia extract contains the highest amount of water-soluble carbohydrates of all the extracts, of which the main components are arabinogalactans. The WSCs extracted from Undaria seaweed are mainly galactose- and fucose- (galactofucans) containing carbohydrates. On the basis of current physicochemical analyses and literature findings the water-soluble carbohydrate compounds described above are prebiotic and bioactive in nature. Similar compounds have been shown to stimulate the growth of bifidobacteria and lactobacilli *In vitro* and *In vivo* in animals and humans (Kuda *et al*., 1998; Cherbut *et al*., 2003; Xu *et al*., 2003; Al-Tamimi *et al*., 2006). In the current study, the impact of the extracts on gut microflora was mixed. This could be due to genuine differences in chemical properties or failure to identify optimal levels of inclusion.

4.1.2 Biological response of birds to supplements

In the present study, none of the plant extracts or commercial prebiotic products at low dosage (5 g/kg) affected the FI of birds, whereas, at high dosage (10 g/kg), Undaria extract and Frutafit significantly reduced the FI of the birds compared with the negative control group.

The tested plant extracts and prebiotic products had a marginally positive effect, no effect or a negative effect on bird performance, depending on the type of plant extract or prebiotic product and its inclusion rate in the diet. This occurred despite the fact that the dietary inclusion levels (5 g and 10 g/kg) of plant extracts and prebiotic compounds were similar to the amounts used by some other researchers (Yusrizal & Chen, 2003a; Guo *et al*., 2004b). The BWG of birds given the diet supplemented with Undaria extract and Frutafit at the high dose was significantly lower than that of the negative control group, probably as a result of the effect of the former two supplements on FI. Although supplementation with Acacia extract (both low and high levels) produced an improvement in FCR of broilers reared in a clean environment (cages) during the first three weeks, the same extract failed to show any positive effect on performance or control of NE when the birds were challenged with Cp. The major hypothesis in many of the studies on similar products is their ability to improve health and growth of broiler chickens, and this has been realised in some of the studies (Kleessen *et al*., 2003a; Guo *et al*., 2004b; Cao *et al*., 2005). However, many studies have also shown that these products have either no effect or a reduction in performance of broilers, depending on the type of product and its inclusion rate, as was the case in the current study (Patterson *et al*., 1997; Wu *et al*., 1999; Gajewska *et al*., 2002).

When an antibiotic (Zn-bacitracin) was added to diets, the performance of birds was improved both under unchallenged conditions and challenged (with Cp) conditions. In the present study none of the supplements or the commercial products supported productivity or health to the same standard as the antibiotic supplement although the prebiotic product, Fibregum (arabinogalactans), tended to lower the levels of IgY antibody against Cp α–toxin, in agreement with lower Cp numbers and lower mortality. Experimental findings indicate that arabinogalactans can act as immunomodulators in animals because of the highly branched β–galactan fraction (Taguchi *et al*., 2004).

4.1.3 Intestinal development and function

In general, the plant extracts and commercial prebiotic products did not alter the ileal mucosal structure of birds reared under cage experiments or when subjected to Cp challenge. The high level of Frutafit (10 g/kg diet) impaired growth, reduced dietary AME and lowered the apparent ileal digestibility of fat and protein. Similar effects were observed with the higher level (10 g/kg) of Undaria seaweed extract used in the current thesis. These effects are consistent with the impaired biological responses observed in the same treatment groups. It appears that the microbial fermentation of large amounts of certain extracts or prebiotic products can impair nutrient utilisation in birds. This is further examined below (section 4.1.4).

4.1.4 Microbial dynamics and function

Although most of the exact mechanisms by which antibiotics improve poultry productivity are unknown, antibiotics generally tend to reduce the population of target microbes and may also alter the species composition of the gastrointestinal microflora (Visek, 1978; Thomke & Elwinger, 1998; Gaskins *et al*., 2002). It is therefore logical to hypothesise that any bioactive compound that achieves the same effects may be able to improve productivity and health of birds when included in the diet. Results from the present study demonstrate that plant extracts and the commercial prebiotic products modulated the microflora in the ileum and caeca of broilers reared under clean environmental conditions as well as under disease-challenge conditions. In the present study, the inclusion any of the plant extracts in feed increased the lactobacilli population in the ileal and caecal digesta of the birds and also reduced the number of Cp and coliforms in the caeca but had no effect on the growth of the birds. This is not unusual; the major role of IFA appears to be improvement in health, rather than growth. In-feed antibiotics rarely improve growth by more than 5 % (Rosen, 1995). These findings are in agreement with other studies showing that prebiotic and bioactive compounds from plants, herbs and mushrooms are fermented by lactobacilli *In vivo* and *In vitro*, leading to significant shifts in the bacterial community in the GIT of broilers and in fermentation medium, respectively (van Laere *et al*., 2000; Gajewska *et al*., 2002; Al-Tamimi *et al*., 2006). However, results from the NE challenge study in this thesis showed an increase in the lactobacilli numbers but no significant reduction in Cp

numbers in the caeca when birds were fed with a diet supplemented with the Acacia extract. This shows that although the prebiotic plant extracts stimulated the Lactobacillus populations under both unchallenged and challenge conditions, they were not effective in controlling Cp numbers in the face of Cp-associated NE challenge. The reason for these results is not clearly understood, and is an area that requires further research.

In the present study, one of the most interesting findings was that the inclusion of Acacia extract in the diets supported the growth of *L. johnsonii* in the ileum and caeca of the birds. As previously mentioned, Acacia extract improved the FCR during the first three weeks and reduced the population of intestinal Cp in the same cage experiment. This may be of practical importance because *L. johnsonii* is an indicator organism for "good microflora". In support of this, an *In vivo* study by La Ragione *et al*. (2004) revealed that a single oral dose of *L. johnsonii* is sufficient to suppress all aspects of colonization and persistence of Cp in chickens. The increased population of lactobacilli in birds fed the other plant extracts (Cabbage tree extract and Undaria extract) was dominated specially by *L. salivarius* and also *L. crispatus*. These birds had impaired growth performance and it was noted that when the birds were fed a diet supplemented with a high level (10 g/kg) of Undaria seaweed extract, beside reduced performance, the apparent ileal digestibility of fat was decreased. These findings suggest that an increase in Lactobacillus numbers and microbial fermentation activities in the GIT of birds resulting from supplementation with high levels of certain plant extracts or prebiotic products may not necessarily result in positive effects, instead they can have a negative impact on nutrient utilisation and performance. This may be due to the fact that certain *Lactobacillus spp*., such as *L. salivarius* are able to deconjugate bile acids and thereby reduce the fat digestion in animals (Gilliland & Speck, 1977; Knarreborg *et al*., 2002a). Out of the 240 Lactobacillus isolates tested, *L. salivarius* was the most abundant (67 %), followed by *L. crispatus* (17 %) and *L. johnsonii* (14 %).

It is claimed that prebiotic substances can stimulate the growth of bifidobacteria in the GIT of humans and animals and it is generally believed that they are helpful in maintaining a proper balance in gut microflora (Mitsuoka, 2002). Results obtained in the present study indicate that bifidobacteria numbers in ileum and caeca of birds fed with plant extracts were very low and in most of the replicates values were below detection limit.

4.2 Strategy B

4.2.1 Biological response to feed form and processing technique

The improvements seen in AMEn associated with milling type may have been a particle size effect, as roller-milling has been sown to produce larger particles than hammer-milling (Reece *et al*., 1985; Douglas *et al*., 1990; Nir *et al*., 1995). The current study found that the AMEn value of diets based on wheat is poorer than that of diets based on sorghum. It is generally accepted that different grains have different AMEn values due to many factors including – as in the case of wheat compared to sorghum – the presence of biologically significant proportions of soluble NSP in the grain (Choct *et al*., 1995; Steenfeldt *et al*., 1998; Choct *et al*., 1999; Svihus and Gullord, 2002).

In the first experiment under Strategy B, the results indicate that an intermediate particle size, irrespective of processing type, marginally improves bodyweight gain. However, FCR is significantly improved by using a roller mill rather than a hammer mill when preparing grain for a complete pelleted feed. This is in agreement with the work done by Nir *et al*. (1995), where rolled wheat and sorghum-based diets improved cumulative feed conversion by between five to 10 points depending on age and sex of the broilers. Interestingly, there was seemingly no particle size effect on FCR between both hammer-milled grain treatments. This result does not agree with previous observations, where feeding a fine particle sized diet to broilers has depressed feed efficiency (Nir *et al*., 1994a).

In the second experiment, the pre-pellet particle size of grain had the greatest influence on FCR; more so than on weight gain and feed intake, which is in agreement with studies conducted previously (Lott

et al., 1992; Nir *et al*., 1994a). However, other studies have shown that the pelleting process diminishes the effects of particle size and mill type, giving no differences between pre-pellet particle size treatments (Reece *et al*., 1985). Feeding sorghum allows better performance than feeding wheat irrespective of particle size, as seen by Nir *et al*. (1994a). However, it is evident that the particle size of the grain has a greater effect on FCR when wheat is fed as opposed to sorghum.

Within grain type, the effect of grain particle size on the performance of chicks fed wheat was greater than those fed sorghum. The results suggest that the effect of feed particle size on efficiency may be relative to the feeding value of the grain. This is a similar finding to the results of experiments conducted by Nir *et al*. (1994a) where the effect of particle size on feed efficiency was greatest in sorghum or wheat fed birds as no particle size effects were noted for corn in the same experiment, and similar mean particle sizes were attained for the three grind sizes across grains tested. Both experiments have demonstrated that a diet with coarse particle sized grain improves AMEn more than feed containing fine particles. Furthermore, this effect differs according to the grain used and is exacerbated when whole grain is used in the feed mix (Wu *et al*., 2004). The AMEn of the whole grain or coarse textured diets does not, however, relate to improved performance. This may be due to a higher maintenance energy requirement of birds with larger muscular gizzards, adapted to eating large particles. Also, as these birds were extracting more energy from their feed, the ratio of energy exerted (to liberate the energy from the feed) to the extra energy gained may have been higher for these birds than the birds eating processed grain.

It can be concluded that under experimental conditions, sorghum promotes better bird performance compared with wheat. Regardless of the time of introduction of the feed, e.g., from 1 or 10 d, the beneficial effects on performance of a coarse particle sized grain as part of the diet are apparent by 21 d and continue through to 35 d. The effect of feed particle size on FCR is greater for wheat fed birds than sorghum fed birds. Feed conversion can be improved by feeding intermediate-sized (approx. 600 µm) roller-milled sorghum compared to hammer-milled sorghum of similar mean particle size and particle size distribution. In the current study, the AMEn of the feed could be manipulated by the particle size of the diet, but was not correlated to broiler performance. The AME of wheat diets was not affected by particle size, but the AME of the sorghum diet was improved by offering a coarser particle.

4.2.2 Response under commercial production conditions

The significant differences in performance and gut development seen in broilers under experimental conditions were not well replicated in the current experiment. One key aspect of the current series of studies is the consumption of coarse particles by birds, leading to stimulation of gizzard development, which is, in turn, believed to modulate a cascade of physiological and endocrine changes in the gut. Thus, it should have been expected that under commercial conditions, broilers have access to the bedding material which they consume throughout the production cycle (Hetland, 2007). In the current study, rice husk was used as a litter material and it was observed during the experiment that the birds regularly consumed it as evidenced by the presence of rice husk in the gizzards of sample birds.

The pH of the digesta of the gizzard is affected by the activity of the gastric stomach and the fermentation of carbohydrates in the crop. Retention time in the crop is believed to be positively correlated to the degree of fermentation and production of SCFA (primarily by lactobacilli), and therefore inversely proportional to pH. Although the estimated concentration of lactobacilli in the gizzard was increased by feeding intermediate or coarse particle sized hammer-milled wheat or coarse roller-milled wheat, the pH was not significantly different between treatments. This would also suggest that the barrier function of the stomach to reduce the flow of pathogens to the intestine (Engberg *et al*., 2002; Engberg *et al*., 2004; Bjerrum *et al*., 2005) is reduced by the presence of litter. These effects have been observed recently in work presented by Bohorquez *et al*. (2006), where colonisation of the colifom bacteria, Salmonella, was enhanced in birds reared on litter. This may be due to the effects of litter on the digesta passage rate through the gizzard, as controlled by its

contractions, which may have been similar between treatments at 41 d (supported by gizzard weight), when the pH and microbiology readings were taken.

There appears to be a knock-on effect of a more developed gizzard on the overall physiology of the gut. As mentioned earlier, the gizzard may be regarded as a "pacemaker" organ, coordinating a number of gut physiological events such as immunity and secretory responses. Indeed, the gut is not only the organ where all nutrients are digested; it is also the largest immunological organ in the body. Thus, it is often implied that a more robust gut will make a healthier animal, which, in turn, digests and utilizes nutrients more efficiently. This link between enzyme activities, gut weight and growth performance has been elucidated by Hetland and his colleagues (Hetland and Svihus, 2001; Hetland *et al*., 2003) where the inclusion of oat hulls in a wheat-based broiler diet increased the gizzard weight, which coincided with a significant improvement (from 97 % to 99 %) in the digestibility of starch - the most important energy source in broiler diets - in the ileum. It was probably due largely to the massive increase in the amount of starch-degrading enzyme, amylase, secreted. In addition, the gizzard bile acid level increased in proportion to the amount of wood shavings retained in the gizzard. Since bile acids enter the intestine through the posterior duodenal loop, their levels in the gizzard contents give a good indication of gastroduodenal reflux, supporting the hypothesis that digesta reflux between the gizzard and the duodenum is increased by inclusion of insoluble fiber. Bile acids are strong emulsifiers and they facilitate nutrient solubilization in the gizzard by effective emulsification of liberated lipids. Lipids are released continuously from the diet by water dilution and protein degradation. An incomplete emulsification of dietary lipids could lead to formation of a protective lipid coating of nutrients in the lumen, resulting in impaired solubility, and hence eventual digestibility, of nutrients. The improvement in starch digestibility may, in part, be due to enhanced emulsification of lipids as a result of more bile acids being available.

The reduction in the total anaerobic count and a trend toward a reduction in total SCFA in the ileum of birds on the rolled diet compared to those on hammer-milled wheat may have been due to a reduction in the level and/or nature of fermentable nutrients in the ileum. However, the starch digestibility results do not support this hypothesis. The reduction in the viable lactobacilli from the gizzard and ileum of birds on intermediate sized rolled wheat diets compared to hammer milled wheat fed birds of equivalent particle size was unexpected. Lactobacilli have been suggested to be beneficial to poultry, where they are able to reduce coliform viability, enhance immune function and possibly performance (Chang and Chen, 2000; Engberg *et al*., 2002; Huang *et al*., 2004; Koenen *et al*., 2004). However, this study demonstrated that a reduction in the lactobacilli in the upper tract and intestine may not be of significance to the performance of birds on litter. The observed reduction in total anaerobes may be more important in this respect, where lower numbers of bacteria in the intestine and upper tract (the major and most energy efficient sites of digestion and absorption), may reduce the need for immune responses, and may also be indicative of enhanced digestive and absorptive processes in the absence of high amounts of SCFA and the resulting antimicrobial compounds (less substrates available for microbial proliferation). Similar total anaerobes have been previously observed when feeding whole wheat with a xylanase (Engberg *et al*., 2004).

The degree of litter consumption may have been relative to dietary treatment as a higher percentage of litter was perceived to be present in the gizzard and intestinal contents of birds on fine particle diets. This may have also been affected by age, where dietary particle size effects on the development of the stomach were evident in 21 d old birds but were not seen in 41 d old birds. Furthermore, feeding the HM3.2 diet increased gizzard weight by 21 d, which may have been associated with biologically significant litter consumption before this age. It was suspected that the incidence of infectious bursal disease in the flock in the latter stages of the trial increased the variability in bodyweight and feed conversion and may have masked any significant performance effects that may have otherwise been present by 41 d. As there are no known effects of feed particle size or milling type on the humoral immune status of broilers, it is unlikely that any level of protection would have been offered by the feeding treatments.

The results of the current study suggest that the relative size of the gizzard is proportional to the size of the grain particles fed, and that the inverse is true for the proventriculus. From the literature, the stimulation of the gizzard by a coarse grain fraction in the diet or ingested coarse fibre (such as litter material) seems to lead to its hypertrophy, reduced gizzard erosion and shrinkage of the proventriculus (Nir *et al*., 1994a; Taylor and Jones, 2004; Hetland, 2007). Indeed, birds that do not have access to litter or coarse insoluble fibre in the diet have been shown to have smaller gizzards than those that do (Deaton *et al*., 1973; Hetland and Svihus, 2003).

It is concluded that in commercial operations, the type of litter material used may be important in modulating gut development in broilers, which affects life-long production and health performance of the flock.

5 Recommendations

1. The plant extracts and commercial prebiotic products tested in the current study do not appear to be suitable as alternatives to IFAs in broiler diets. Although the products modulated the gut microflora composition of birds, this did not lead to an improvement in bird performance. The major contribution of this study to on-going research in the area is its breadth, with the evaluation of a few plant extracts and commercial products. This has laid a foundation for further studies, in which focus should be directed at one product at a time. This will facilitate the evaluation of a range of levels of supplementation, and the possibility of identifying an optimal level for use in diets.

2. There is a also a need for future research to elucidate the interaction between compounds that are present in different sources, their interactions with the gut microflora, and their effect on macronutrient utilisation in broilers. For instance, by conducting further studies to test the deconjugation ability of bile salts by the *Lactobacillus spp*. isolated and identified in the current study, one could determine their role in fat digestibility of broilers. It would also be interesting to look at how different *Lactobacillus spp.* that were isolated in this study affect the performance of broilers i*n vivo*. In the quest for alternatives to in-feed antibiotics, it should be considered whether combinations of different prebiotics and bioactive compounds can elicit diverse beneficial effects, exert synergistic effects, or perhaps have negative effects. In order to screen and test the prebiotic and bioactive compounds under clean environmental conditions, an experimental model should be developed which takes into account animal factors, dietary factors and management factors so that reproducible results can be translated to practice.

3. The effects of particle size on bird performance are different for different grains. For sorghum, processing grain to an intermediate particle size improves FCR over whole grain. Improvements in the AME of a diet by coarser grinding does not seem to be the reason for improved performance in wheat fed birds but may be one factor for improved performance of sorghum fed birds. Grain of an intermediate to coarse particle size improves the performance of broilers under experimental conditions, and roller-milling improves FCR over hammer milling to the same particle size. There is evidence to suggest that the effect of feed texture and milling type on broiler performance and AME may be related to the stimulation of the digestive organs and their function.

4. Feeding coarse particles in the form of the grain used in the feed can increase the relative weight of the gizzard, decrease proventriculus weight as well as the pH in the stomach and duodenum. Feeding wheat reduced caecal pH, and processing type (roller mill or hammer mill) did not affect organ morphology. The effect of particle size on digestive organ development and pH may influence the efficiency of the digestive processes in the broiler.

6 Implications

The project provides an insight into new areas of product development. Although none of the tested supplements was effective at replacing IFAs in poultry diets, we were able to identify the plant species

as those with active compounds that could be subjected to further processing and be useful in animal and human nutrition. Similar products are produced in other parts of the world but Australian producers may not have access to the raw material as much as they would to material that is present in the sub-continent (Australia and New Zealand). The identification of the active compounds in itself opens up avenue for further development through a range of chemical processes, including isomerisation, to obtain new products or possibly enhance the effectiveness of the native compounds.

In the area of feed processing, the project showed that saving can be made through less processing without detrimental effects on poultry productivity. Rather, there might be improvement in feed utilization and health of the birds that are maintained on minimally processed feeds.

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Yusrizal & Chen, T. C. (2003a). Effect of adding chicory fructans in feed on broiler growth performance, serum cholesterol and intestinal length. Int. J. Poultry Sci., 2: 214-219.

9.0 Plain English Compendium Summary

Project Title:	
Project No.:	UNE 03-4
Researcher:	Dr Paul A. Iji
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Objectives	
Background	The inclusion of antibiotics in poultry diet is under a cloud as disease agents have begun to develop resistance to these drugs. There is an intensive search for alternatives to the use of antibiotics around the world. This project examined two of the strategies that can be employed to the development of alternatives to antibiotic supplements. One of these strategies is to include extracts from local Australian and New Zealand plants in the diet, in order to kill or retard the growth of disease agents in poultry. Another strategy is to modify the feed in such a way as to improve intestinal growth and function. It was presumed that each of these strategies, separately, will be able to improve the health and productivity of broiler chickens.
Research	In the first strategy, extracts were obtained from four Australian and New Zealand plants – Rengarenga lily, Cabbage tree, Undaria (seaweed) and Acacia – and included in diets for broiler chickens. These were compared with an existing antibiotic supplement, Zinc bacitracin and commercial extracts, marketed by the feed industry. These extracts were predominantly sugar in composition but there were differences in chemical structures between the plant sources. In the second strategy, the effects of two major grains, sorghum and wheat, were compared, the grains having been milled to fine or coarse texture, or fed as whole grain. The grains were either hammer-milled or roller-milled. As in the first strategy, the diets were fed and compared to current commercial diets. One of the experiments examined the effects of feeding such diets under commercial rearing conditions – on the floor, in very large groups.
Outcomes	• The plant extracts changed the composition of intestinal microbes, and might improve the health of birds • The extracts did not grossly improve feed intake or body growth, when compared to Zinc Bactracin and commercial vegetable supplements • Feeding whole or coarsely milled grains resulted in enlarged gizzards and some changes in the microbial composition of the gut.
Implications	The plant extracts would be useful in the control of gut microbes, some of which cause disease but not improve body growth further. The feeding of whole or coarse grain would result into energy and time saving, and possibly improve the utilization of feed material.

Publications	1. Rodgers, N.J., Mikkelsen, L.L., Iji, P.A. and Choct, M. (2007). Effect of particle size and milling type on broiler performance under semi-commercial conditions. Recent Advances in Animal Nutrition in Australia 16, 273. 2. Vidanarachchi, J.V.A. (2006). Regulation of intestinal microflora and productivity of broiler chickens by prebiotics and bioactive plant extracts. PhD thesis, University of New England, Armidale, Australia. 3. Vidanarachchi, J.K., Mikkelsen, L.L., Sims, I., Iji, P.A. and Choct, M. (2006). Selected plant extracts modulate the gut microflora in broilers. Australia Poultry Science Symposium 18, 145. 4. Vidanarachchi, J.K., Mikkelsen, L.L., Sims, I., Iji, P.A. and Choct, M. (2005). Plant extracts from Australian native plants as alternatives to in-feed antibiotics in feed for broiler chickens. In: Avian gut function, health and disease. 28th Poultry Science Symposium, WPSA, UK, Bristol, UK, 15-17 September 2005, p. 51. 5. Vidanarachchi, J.K., Mikkelsen, L.L., Sims, I., Iji, P.A. and Choct, M. (2005). Phytobiotics: alternatives to in-feed antibiotics in monogastric animal diets. Recent Advances in Animal Nutrition in Australia 15, 131-144. There are plans to publish all the findings in peer-reviewed journals.