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Role of voluntary litter consumption by broiler chickens on gut function and gut health

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Executive Summary

Poultry housed in floor pen systems are able and likely to consume litter materials from the floor. How consumption of different types of litter materials affects growth performance, nutrient digestibility, gut function and gut health in poultry is a largely unexplored research area. Recent data from an experiment with egg-laying hens performed at NULS in Norway (CRC project 03-27 "Use of different dust bathing materials for layers – Effect on nutrient digestion, gut physiology and welfare") has indicated that the type of litter material in the litter bath affects feed intake and feed utilisation. Hens with access to paper had a higher feed intake compared with control birds with no access to litter material and with birds with access to wood shavings, thus resulting in a poorer feed utilisation.

A total of six experiments with broilers were conducted in this project. Three experiments were done in Norway at the Norwegian University of Life Sciences (NULS) and a further three experiments were conducted in Australia at the University of New England (UNE) and Inghams Enterprises Pty Ltd. Experimentation in both countries was necessary because NULS has unique expertise and facilities not available in Australia, and because of quarantine restrictions, it is not possible to bring gut and feed samples from Norway to Australia to be examined at UNE and SARDI. Furthermore, independent experimentation in Australia based on results obtained in Norway was necessary to validate results under Australian conditions.

The experiments in both countries were designed to test the following hypotheses:

i) Consumption of coarse/hard litter stimulates gizzard activity and decrease the risk of gizzard lesions in the koilin layer and inflammation of the mucosa.

ii) Birds housed on a coarse/hard litter type ingest less litter than birds housed on a soft and fine litter and the reduced quantity of litter consumed results in increased feed intake and improved growth performance.

iii) Consumption of coarse/hard litter, even in a small quantity, stimulates gizzard development and function and consequently improves breakdown of the feed as well as nutrient digestibility. The more functional gizzard also plays a positive role in the control of undesirable bacteria, such as *Clostridium perfringens* and *Salmonella* spp., and protozoan sporocysts such as *Eimeria* spp. This is expected to have beneficial impact on bird health and productivity.

The experiments conducted at the Norwegian University of Life Sciences (NULS) indicate a significant consumption among broilers of litter from the floor. However, consumption of litter was low when material was provided separately in a raised feeder trough. Similar to what was demonstrated in layers (CRC project 03-27), broilers showed a 50% increase in gizzard weight when given access to litter due to the requirement for grinding of hard coarse particles. Also young broilers showed a phenomenal grinding activity to smaller median and mean particle sizes when given coarse particles in feed or litter materials. Stimulation of the gizzard activity increased the digestibility of starch. This may be caused partly by the increased surface area arising from finer grinding of feed particles due to increased gizzard size. In addition, increased digestibility and feed utilisation may be related partly to increased enzyme activity, and in particular maltase activity, in the intestine.

The severity of gizzard lesions gradually reduced with age of the chickens, whereas the *C. perfringens* counts were lowest on day 19 and increased until day 32. Inclusion of oat hulls

was the most important predictor of gizzard scores. This was particularly clear on day 19. On day 32 there was a significant reduction in gizzard lesions only when birds were fed oat hulls and had access to litter. Access to litter was the most important predictor of *C. perfringens* counts in this trial. This was also particularly clear on day 19. On day 32 a specific combination of the two factors was necessary to exert a significant effect on *C. perfringens* counts. This combination (lack of added oat hulls and denied access to litter) was associated with increased *C. perfringens* counts. These results indicate that availability of non-soluble fibres can influence significantly both the severity of gizzard inflammation and the number of *C. perfringens* in caeca. Fibres in the feed and as litter appear to be interacting in their effects.

The first of three experiments in Australia was conducted at UNE and involved graded levels of hardwood saw dust litter incorporated in a commercial diet at 0, 0.75. 1.5, 3, 6 and 12% levels and fed to broiler chickens for 35 days. Inclusion of 12% hardwood sawdust in the feed significantly increased the relative weight of gizzard and proventriculus and improved apparent ileal digestibility of starch, but had no effects on feed intake, weight gain, feed conversion, or mucosal morphometry. These results are consistent with previous reports from UNE as well as in NULS experiments, that high fibre consumption from diet and litter can significantly stimulate the development of gizzard and improve apparent ileal digestibility of starch. Reduced numbers of enterobacteria in the gizzard and small intestine are indicative of the potential benefits from ingestion of hardwood litter.

The second Australian experiment was conducted in the Inghams Enterprise research facility in Leppington, New South Wales. The aim was to investigate the effects of two types of litter (paper and hardwood sawdust) in combination with a low and high fibre diets in a larger scale broiler growth study conducted under near commercial conditions. Overall, bird weight was not affected by the diet and litter treatment, however, diet and litter interactively affected feed conversion during the first 3 weeks of treatments. It appeared that the high fibre diet was beneficial to feed conversion of birds only when birds were unable to obtain hard particles from litter material, whereas it was detrimental if birds were able to consume hardwood sawdust litter.

High fibre diet feeding and apparent consumption of hardwood litter stimulated gizzard development in the present experiment. The combination of a high fibre diet and hardwood litter had an additive effect on gizzard growth. This may suggest that the quantity or structure of fibre contained in the high fibre feed used in the present experiment improved the gizzard growth, but was insufficient, which led to birds seeking an additional source in the form of hardwood litter.

High fibre diet feeding reduced enterobacteria in the ileum, and hardwood litter consumption elevated the number of lactic acid bacteria in the caecum, which was confirmed by T-RFLP analysis. In addition, the high fibre diet significantly reduced total anaerobes only in chickens housed on paper litter, and apparent consumption of hardwood litter consumption slowed the growth of duodenum villi. Conversely, no change of *C. perfringens* counts was observed among the treatments.

The hypothesis tested in third Australian experiment conducted at UNE was that enhanced gizzard development through increased dietary fibre and/or ingestion of hardwood litter would provide birds with a degree of protection when exposed to a strain of *C. perfringens* strain known to induce severe necrotic enteritis NE. The NE challenge procedure was highly successful in that birds exposed to Cp via oral gavage showed severe symptoms of necrotic

enteritis, which resulted in depressed live weight gain and raised feed conversion and mortality. Anticipated protection from enhance gizzard development was not evident, possibly because dietary fibre and litter type had no effects on relative gizzard weight of birds measured at day 14. On the other hand, there were indications at day 17 of an interaction between diet and litter type on gizzard weight. In birds raised on paper, those given a low fibre diet had smaller gizzards than those given a high fibre diet, whereas birds raised on hardwood were unaffected by dietary fibre. On the other hand, there was no difference due to dietary fibre level on gizzard size of birds subsequently challenged.

In conclusion, there can be little doubt that increased dietary fibre and/or ingestion of hardwood litter stimulates the development and functional capacity of the gizzard. Gizzard enhancement, through increased fibre ingestion, led to improvements in apparent ileal starch digestibility, by a mechanism not involving pancreatic amylase activity or mucosal morphology. However, these changes in gut function did not result in consistent improvements in growth or feed efficiency, and did not provide birds with a degree of protection from necrotic enteritis induced by *C. perfringens*.

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Introduction

Poultry housed in floor pen systems are able and likely to consume litter materials from the floor. How consumption of different types of litter materials affects growth performance, nutrient digestibility, gut function and gut health in poultry is a largely unexplored research area. Recent data from an experiment with egg-laying hens performed at NULS in Norway (CRC project 03-27 "Use of different dust bathing materials for layers – Effect on nutrient digestion, gut physiology and welfare") have indicated that the type of litter material in the litter bath affects feed intake and feed utilisation. Hens with access to paper had a higher feed intake than control birds with no access to litter material and birds with access to wood shavings, resulting in a poorer feed utilisation. The consumption of litter from the litter baths was 4 g/hen/day for wood shavings and 11 g/hen/day for paper. The weights of the empty gizzard and gizzard contents were considerably higher ($P<0.05$) for hens with access to wood shavings as compared with the other treatments. This indicates that the type of litter material available for consumption by the bird plays a role for gizzard development and functionality. The latter is supported by the findings that access to wood shaving decreased the amount of large particles in duodenum whereas the opposite was observed for hens with access to paper as the litter material. Thus, stimulation of the gizzard by consumption of small amounts of hard litter materials, such as wood shavings, seems to increase the grinding capacity of the gizzard and induce more efficiently breakdown of the feed, which may improve digestion in the small intestine of the bird. However, these results were obtained with hens and it is not known whether similar responses will be found in young birds, such as broiler chickens.

Ali *et al*. (2009) reared broilers on different types of litter including rice hulls, softwood sawdust, pine shavings, reused single batch litter (originally based on pine shavings), hardwood sawdust, shredded paper and chopped straw. They noted that although some litter materials stimulated gizzard development, this did not lead to differences in feed intake and growth to 42 days of age. Nevertheless, birds housed on pine shavings or rice hulls had significantly higher relative gizzard weights compared with birds housed on shredded paper or chopped straw.

Increased gizzard weight and improved gizzard function have been observed in broilers given a coarse feed or feed containing coarse components, such as whole grains (Engberg *et al*., 2004, Svihus *et al*., 2004). Inclusion of whole grains in the feed was found to reduce the duodenal mean particle size and improve starch digestibility as compared with ground grains (Hetland *et al*., 2002). Whole wheat has been reported to increase ileal digestibility and apparent metabolisable energy AME (Svihus *et al*., 2004).

Another important aspect of stimulating gizzard development is the potential positive role of a functional gizzard in control of bacterial populations. Whole wheat feeding has been reported to reduce the intestinal number of lactose-negative enterobacteria (i.e. *Salmonella* spp) as well as the number of *Clostridium perfringens* (Engberg *et al*., 2004). Similar results were observed in broiler chickens experimentally infected with *Salmonella typhimurium*. Following infection, lower numbers of *S. typhimurium* were found in the gizzard and ileum of birds receiving whole wheat as compared to pellet-fed birds. Beside this, whole wheat feeding also significantly reduced the numbers of *C. perfringens* in the intestinal tract of the birds (Bjerrum *et al*., 2005). These results indicate that a functional gizzard may act as a barrier organ preventing potential pathogenic bacteria from entering the distal digestive tract. Thus, if access to gizzard stimulating litter materials has a significant impact in broiler

chickens, choosing the right litter material may have important health implications in relation to reduce the prevalence of *Salmonella spp.* in chickens and consequently in chicken meat and also in relation to reduce occurrence of necrotic enteritis which is strongly associated with *C. perfringens*.

Objectives

The aim of the present project is to investigate the consumption of different types of litter by broiler chickens on feed intake and growth performance, gizzard function and gut development, nutrient digestibility and gut microflora. The project will follow up of the previous CRC project 03-27 *Use of different dust bathing materials for layers – Effect on nutrient digestion, gut physiology and welfare*.

The experiments were designed to test the following hypotheses:

i) Consumption of coarse/hard litter will stimulate gizzard activity and decrease the risk of gizzard lesions in the koilin layer and inflammation of the mucosa.

ii) Birds housed on a coarse/hard litter type will ingest less litter than birds housed on a soft and fine litter and the reduced quantity of litter consumed will result in increased feed intake and improved growth performance.

iii) Consumption of coarse/hard litter, even in a small quantity, will stimulate gizzard development and function and consequently improve breakdown of the feed as well as nutrient digestibility. The more functional gizzard will also play a positive role in the control of undesirable bacteria, such as *Clostridium perfringens* and *Salmonella spp.*, and protozoan sporocysts such as *Eimeria spp*. This will have beneficial impact on bird health and productivity and improve safety of the meat product.

Outline of experimental work

A total of six experiments with broilers were conducted in this project. Three experiments were done in Norway at the Norwegian University of Life Sciences (NULS) and a further three experiments were done in Australia at the University of New England (UNE) and Inghams Enterprises Pty Ltd.in collaboration with the South Australian Research & Development Institute (SARDI). The justification for conducting experiments in two widely-separated countries was mainly to exploit research facilities and expertise in both Norway and Australia that were not readily available in one or other country. In addition, it was considered essential that some of the research work done in Norway should be validated under Australian conditions, in recognition of differences in litter materials and feed ingredients available in the two countries.

Experiment 1 (Norway) - Interaction between litter type and dietary fibre level

The effect of litter type and dietary fibre level on litter consumption, performance, nutrient digestion and gizzard function in broilers was investigated. Use of a hard litter material (gizzard stimulating when consumed) and a soft/fine material (not stimulating the gizzard) were compared in control groups housed with and without litter.

Experiment 2 (Norway) - Effect of litter on secretion of digestive enzymes

As shown in CRC project 03-27, improvement in nutrient digestibility in laying hens is associated with gizzard stimulation by litter consumption. This was studied in broiler chickens housed in single-bird cages in a climate-controlled room, and fed a low-fibre diet with or without inclusion of coarse/hard fibre.

Experiment 3 (Norway) - Effect of litter on gizzard lesions

The causal factors behind gizzard inflammation are unknown. Since the feed structure and litter consumption is thought to play an important role of gizzard stimulation, knowledge about how these factors affect the koilin layer in the gizzard is important. This experiment examined the effects of feed structure and litter access.

Experiment 4 (Australia) - Effect of forced litter consumption on gut development, nutrient digestibility and gut microflora

This experiment investigated the effects of forced litter consumption by inclusion of graded levels (0 - 12%) of hard-wood sawdust in a commercial diet.

Experiment 5 (Australia) - Interaction between litter type and dietary fibre level

An experiment based on outcomes of the experiment with broilers was conducted by Inghams Enterprises to investigate the effect of litter in combination with different fibre content in the feed on performance, organ development, mucosal morphometry, and gut microbial community profiles.

Experiment 6 (Australia) - Effect of litter type on necrotic enteritis

The effect of consumption of litter on disease resistance and growth performance of broiler chickens was investigated in a challenge model involving experimental induction of necrotic enteritis.

Experiments 1-3 conducted in Norway are described in Chapter 1, and the three experiments conducted in Australia are described in Chapters 2-4.

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Chapter 1. Interaction between litter type and dietary fibre level on gizzard size, grinding ability

Introduction

At NULS in Norway, we have been increasingly aware of frequent gizzard lesions in birds without clinical symptoms; this was observed in birds from our experiments as well as in birds from commercial flocks. There are several documented and proposed causes of gizzard inflammation. The most obvious cause is toxins; termed gizzerosine, associated with fishmeal. This toxin acts on hydrogen receptors and stimulates gastric acid secretion at low concentrations. Since gizzerosine is formed from lysine and histidine, it could be speculated whether free synthetic amino acids under influence of overheating could combine with other feed components to form toxic compounds. Furthermore, the gastro-duodenal cycle is of importance to digestive functions, but may also be of importance to the integrity of the gizzard lining and mucosa beneath. It has been speculated whether a well-regulated cycle is necessary to maintain a functional koilin layer and so impaired cycle may increase the risk for defects of the koilin layer and in turn mucosal inflammation. Since feed structure and level of insoluble dietary fibre structures stimulate gizzard activity largely, it can be hypothesised that coarse feed structure or consumption of litter can stimulate the gastroduodenal cycle and so decrease the risk for defects of the koilin layer and inflammation of the mucosa.

Three experiments were conducted to test the following hypotheses:

i) Consumption of coarse/hard litter will stimulate gizzard activity and decrease the risk of gizzard lesions in the koilin layer and inflammation of the mucosa.

ii) Birds housed on a coarse/hard litter type will ingest less litter than birds housed on a soft and fine litter and the reduced quantity of litter consumed will result in increased feed intake and improved growth performance.

iii) Consumption of coarse/hard litter, even in a small quantity, will stimulate gizzard development and function and consequently improve breakdown of the feed as well as nutrient digestibility. The more functional gizzard will also play a positive role in the control of undesirable bacteria, such as *Clostridium perfringens* and *Salmonella spp.*, and protozoan sporocysts such as *Eimeria spp*. This will have beneficial impact on bird health and productivity and improve safety of the meat product.

Materials and Methods

Experiment 1

Birds, management and housing

Over 100 day-old male Ross 308 were placed on pens until seven days of age with *ad libitum* access to commercial starter feed. At day 7, 100 chicks were moved to single bird cages with wire mesh floor, and half of them fed on high fibre feed and the other half on low fibre feed. Both feeds were optimised to contain similar amount of energy and protein on weight basis despite the considerable difference in dietary fibre level. One half of the bids on each diet had no access to litter, while the other half of the bids on each diet had *ad libitum* access to litter weighed out into a separate cup (trough) linked to the cage wall. Thus, a total of 25 animals per treatment were allocated to a 2 x 2 factorial design with the factors being dietary fibre and litter consumption. Composition of the diet is shown in Table 1.1.

Table 1.1 Composition of experimental diets (in g/kg).

Ingredients	Low fibre	High fibre
Wheat	300	327
Oats	-	300
Dehulled oats	389	-
Fish meal	40	40
Soybean meal	175	182
Maize gluten meal	10	40
Soy oil	20	20
Vegetable fat	30	55
Ground limestone	7	7
Monocalcium phosphate	9	9
Vitamin/mineral/amino acids	20	20

Feed, litter and chicks were weighed weekly until five weeks of age.

Collection of tissue and intestinal contents

At 14 days of age, 10 chickens per treatment were dissected, and contents from gizzard, duodenum, and ileum were quantitatively collected and frozen in small boxes. Prior to dissection, feed and litter consumption behaviour were synchronised by including a darkness period followed by lights on for the last two hours prior to dissection. The remaining 15 birds/treatment were dissected in a similar way on day 35.

To determine the gizzard's grinding ability at different age, particle size distribution was measured on the duodenal content of all animals using laser diffraction technology (Olaisen *et al*., 2001). Feed and feces were collected quantitatively between 27 and 30 days of age for determination of apparent metabolisable energy. Gross energy content of diets and faeces was determined on a Parr 1281 adiabatic calorimeter (Moline, Illinois, USA).

Calculations and statistical analyses

Apparent metabolisable energy (AME_n) content for the each bird was corrected for nitrogen retention by assuming that weight gain consisted of 160 g/kg protein and that the energy equivalent was 34.36 kJ/g N gained (Bourdillon *et al*., 1990). Digestibility coefficients for protein (N*6.25) and starch were calculated from the ratios between each of these compounds and titanium dioxide in the feed and ileal contents.

One-way and two-way analyses of variance were performed using the GLM procedure of SAS software (SAS Institute Inc., Cary, NC, USA). Significant differences between treatments were determined by using the Ryan-Einot-Gabriel-Welsh F-test. Square root of MSE was used as a measure of random variation.

Experiment 2

Birds, management and housing

A total of 100 day old male Ross 308 broilers were housed in battery cages with *ad libitum* access to commercial starter diet. At seven days of age, 90 of the bids that varied by less than 20% in weight from the flock mean were moved to single bird cages with wire mesh floor. One half of the birds were

fed on the high-fibre diet, and the other half was fed on the low fibre diet, as used in Experiment 1 (Table1.1). Feed and weight gain were measured weekly.

Collection of tissue and intestinal contents

At 35 days of age, all chickens were fasted for six hours. 40 chickens on each feed were dissected as follows: 8 chickens/feed after fasting prior to access to feed, 8 chickens/feed after 30 minutes feeding, 8 chickens/feed after 90 minutes feeding, 8 chickens/feed after 180 minutes feeding and 8 chickens/feed after 300 minutes feeding. Gizzard and gizzard were weighed. A standardised five centimetre segment of upper jejunum was collected and frozen in liquid nitrogen. Pancreas was also weighed and frozen in liquid nitrogen. Jejunum and pancreas was analysed for maltase and amylase activity, respectively, at the National Veterinary College, Oslo.

Statistical analyses

One-way and two-way analyses of variance were performed using the GLM procedure of SAS software (SAS Institute Inc., Cary, NC, USA). Significant differences between treatments were determined by using the Ryan-Einot-Gabriel-Welsh F-test. Square root of MSE was used as a measure of random variation.

Experiment 3

Birds, management and housing

A total of 360 broiler chickens (Ross 308) were placed on 24 pens with 15 birds per pen at 4 days of age. One half of the pens had rubber matting on the floor, and the other half had litter on the floor. One half of the pens on each floor type were fed on the diet without oat hulls and the other half on a diet with 5% coarse oat hulls produced by diluting the basis diet. This represents a 2 x 2 factorial design with the factors being dietary fibe (low and high) and litter (absent and present). Birds and feed were weighed at the start, day 19, and day 32 (at finish).

Low level of feed dietary fibre (no oat hulls) was designated Dietary fibre = 0. High level of feed dietary fibre (added oat hulls) was designated Dietary fibre = 1. All chickens were housed in cages with a floor ensuring that the birds had access to their excreta. Rubber mat as floor type was designated Litter = 1. Floor dressed with wood-derived litter was designated Litter = 2.

Gizzard scores were based on gross lesions found in the koilin layer and on the mucosal surface, according to a predetermined scoring system (National Veterinary Institute, Oslo, Norway). The minimum possible score in this system was 0, and the maximum possible score was 24. Scores in this experiment ranged from 2 to 13. A total of 48 gizzards (12 per treatment) per sampling day were examined.

Table 1.2 Composition of diet.

Ingredients	g/kg
Wheat	708.54
Fish meal	50.00
Soybean meal	170.00
Soy oil	30.00
Vegetable fat	30.00
Ground limestone	15.00
Monocalcium phosphate	11.00
Vitamin/mineral/amino acids	15.46

Collection of tissue and intestinal contents

At day 19 and 32, two birds per pen were dissected and digesta samples from the duodenum and ileum were taken for particle size distribution and starch content determination, respectively.

Gizzard scores were based on gross lesions found in the koilin layer and on the mucosal surface, according to a predetermined scoring system (Unpublished data, National Veterinary Institute, Oslo, Norway). The minimum possible score in this system was 0, and the maximum possible score was 24. Scores in this experiment ranged from 2 to 13. A total of 48 gizzards (12 per treatment) per sampling day were examined.

Caecal contents were collected from 24 (12 per study factor level and 6 per treatment group) chickens per sampling day. Quantification of *C. perfringens* was based on cultivation from diluted samples on blood agar dishes. Specimens of 1 to 3 grams caecal contents from each bird were diluted to give a 10^{-1}solution. Two series of 10^{-2} to 10^{-7} dilutions were subsequently made, and from each of these dilutions 0.1 ml was plated on blood agar plates. The plates were incubated anaerobically at 37 °C for 18-24 hours. Counts were based on numbers of colonies surrounded by the typical double-haemolysis on blood agar. Selected colonies were examined more closely (Gram staining and microscopy, and biochemical tests) to ensure the identity of *C. perfringens.* Recorded counts were transformed to $\log_{10}$ of the original counts (LogCP). The lower detection limit was 1,000 *C. perfringens* per gram caecal contents. The values ranged from below the detection limit to log 9.0. The median $\log_{10}$ counts per sampling days were 4.2, below lower detection limit (3.0) and 3.2 on days 6, 19 and 32, respectively.

Statistical analyses

Gizzard lesion and *Clostridium perfringens* count data were analysed using the software package Stata version 10.1. Gizzard lesion data were analysed using graphic plots, analysis of variance (data from days 6 and 19) and Kruskal Wallis test (data from day 32). *Clostridium perfringens* count data were $\log_{10}$-transformed, and analysed using graphic plots, median regression analysis and Kruskal Wallis analysis.

Results and discussion

Both experiment 1 and 3 clearly illustrated at least as good performance with access to dietary fibre and litter fibre as the concentrated control diets (Table 1.3 and Table 1.6). The measured consumption level of wood shavings was quite low, less than 1 gram/day (Table 1.4). The data for gizzard weights also clearly show that voluntary litter consumption among young broilers is very low. However, the individual variation was high.

In contrast, the dietary fibre level caused considerably difference for the gizzard size and -activity. Assuming that hulled oats consist of 20% hulls, the high fibre diet in experiment 1 consist of 6% oat hull fibre. This resulted in approximately 60% increase in gizzard weight, and a huge increase in weight of gizzard contents. The less response of gizzard weight of litter confirms the low numerical appetite for litter. However, the individual variation seems to be high. This phenomenon is in strong contrast to observations for layers which show high appetite for litter from litter bath when access in general (CRC Project 3.27). However, experiment 3 with birds on littered floor showed a more profound effect of litter on gizzard function due to higher appetite. Hetland *et al*. (2005) observed that the amount of bile acids and NDF in gizzard content increased significantly when birds consumed wood shavings. It is believed that a functioning gizzard should be large and muscular, and able to retain feed components. This, in turn, results in better regulation of digestive processes, leading to improved digestibility of nutrients.

Similar feed utilisation among control birds and birds with access to wood shavings indicate that the grinding cost of wood shavings in the gizzard and handling cost through the gut is completely compensated by the utilisation of nutrients from the digestive processes. In correspondence to in layers, broilers with access to wood shavings and oat hulls resulted in improved starch digestibility. However, the effect seems to be partly dependant on inclusion in the feed since the appetite mechanism does not seem to be developed as well as in older birds. However, in both experiments on littered floor, the effect is significant.

In agreement with results from CRC Project 3.27, improved nutrient utilisation seem to be related to gizzard activity, which again can be related to the interaction between gizzard and intestine. This may be caused by the fact that the structure of the feed is often too fine to meet the need for gizzard stimulation. In layers and broilers (Hetland *et al*., 2003) access to wood shavings and oat hulls resulted in improved starch digestibility. In several experiments wood shavings have been shown to increase gizzard weight by 50%. Improved nutrient utilisation may be related to this phenomenon because of the role of the gizzard in the gastroduodenal refluxes, which regulate the passage through the anterior tract prior to digestion.

Hetland *et al*. (2002; 2003; 2005) illustrated that broilers have a remarkable ability to grind all feed components in the gizzard down to a relatively narrow range of particle sizes. The particle size distribution data of the current experiment illustrates that the gizzard of layers can grind feed components even more extensively than that of broilers (Table 1.5 and 1.6). However, CRC Project 3.17 illustrated that the grinding capacity or grinding functionality may be dependant on litter source. Hard fibre structures such as wood shavings need to be ground before entering the small intestine, and the gizzard activity, as indicated by the gizzard size, is strongly stimulated by such components in the feed or environment. In contrast, the measurement of gizzard size suggests that paper does not stimulate gizzard activity, even though the consumption of paper was twice the amount of wood shavings. The particle size data show that particle size of intestinal digesta is positively related to gizzard size.

The project also indicates that the more rapid starch digestibility due to structural components may be related to increased secretion of maltase from the intestine (Table 1.5). In that experiment, no clear effect of structural components on secretion of amylase and pancreas weights was revealed in the experiments. However, Svihus *et al*. (2004) observed increased pancreas weight in broilers due to whole cereals, although random variation was high for such measurements. Anyway, this observation supports the hypothesis that gizzard activity stimulates other functions of the gut. However, we could expect a combination of the better preparation of nutrient substrate during gizzard grinding and increased secretion of degrading components improve the digestion.

In experiment 3, a highly significant effect of dietary fibre on gizzard score was detected. High levels of dietary fibres reduced the severity of gizzard lesions, as suggested by Figures 1.1 and 1.2. A tendency (P=0.07) for interaction between dietary fibre and litter was caused by the fact that the reducing effect of dietary fibre on gizzard lesions was most pronounced when the chickens had access to litter, and only on the borderline of significance (p=0.06) when there was no litter available.

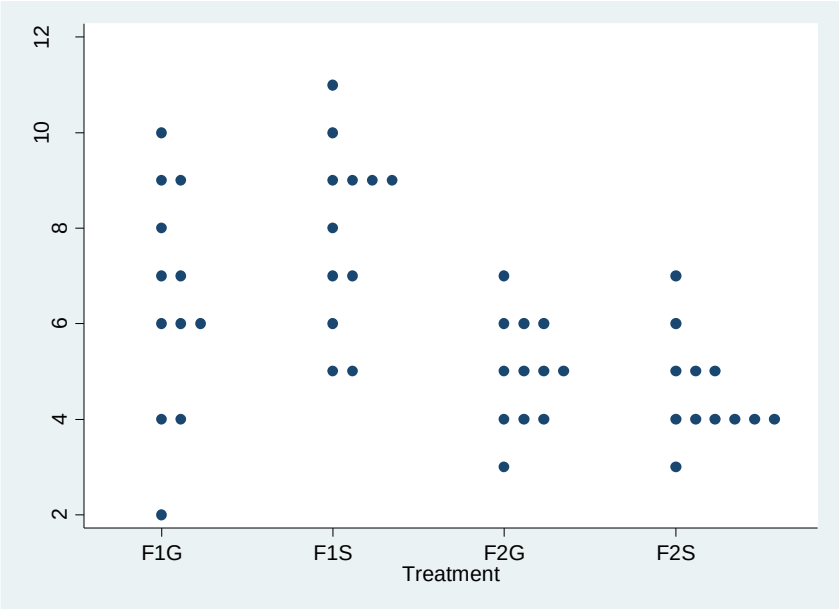

Figure 1.1 Gizzard score (Y axis) versus combined treatments of Dietary fibre (F1=no oat hulls added, F2=oat hulls added) and Litter (G=no access to litter, S=access to litter) on day 19.

The effect of dietary fibre level and access to litter depended on the duration of exposure to these factors and/or the age of the chickens. The severity of gizzard lesions was gradually reduced with age of the chickens, from a median score of eight on day 6 to a median score of four on day 32. The study factors were introduced on day 4; only two days before our first sampling day. We therefore cannot fully evaluate the effect of the study variables at this age. The effect was most clearly demonstrated on day 19. At this age inclusion of oat hulls significantly reduced the severity of gizzard lesions. The data also showed that the effect of oat hulls was strongest among chickens with access to litter. On day 32 the interaction between the two study variables was even more important. At this age there was a significant effect of the study variables only among chickens offered feed with oat hulls that also had

access to litter. These findings strongly suggest that the level of insoluble dietary fibres is an important predictor of the severity of gizzard lesions, and that this effect can be modified by access to litter.

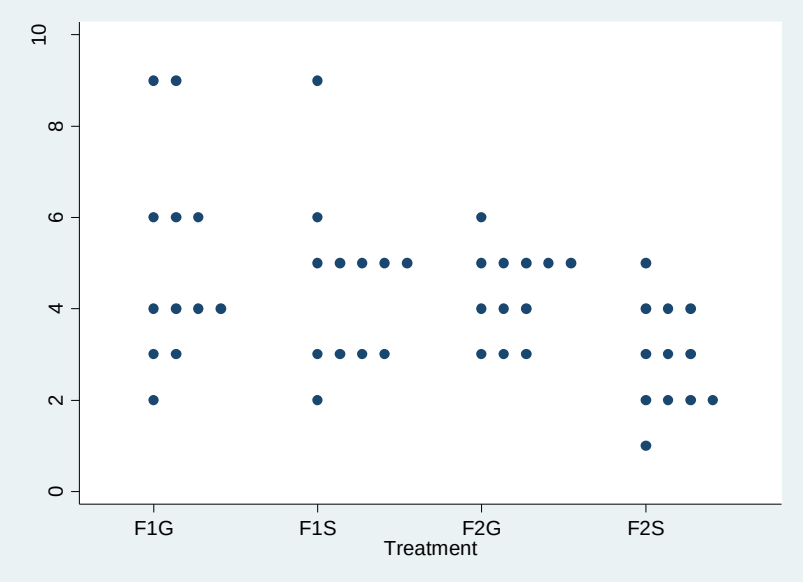

Figure 1.2 Gizzard score (Y axis) versus combined treatments of Dietary fibre (F1=no oat hulls added, F2=oat hulls added) and Litter (G=no access to litter, S=access to litter) on day 32.

At day 19, 15 out of 24 samples were recorded with counts below the detection limit for *C. perfringens*, and 11 of these low-level samples originated from chickens with access to litter (Figure 1.3). At this age availability of litter seemed to be associated with reduced bacterial counts. There was no apparent association between dietary fibre and counts at day 19. At 32 days of age 6 out of 24 samples were recorded with caecal counts below the detection limit, and five of these originated from chickens with access to litter (Figure 1.4).

Litter was found to show significant ($p<0.03$) effect on caecal counts while dietary fibre showed no statistically significant effect. Figure 4 also suggest that the effect of litter on gizzard scores was more pronounced on day 19 than on day 32. A Kruskal Wallis analysis of treatment groups on day 32 indicated significant ($p=0.02$) differences, and a comparison of the two treatments with highest counts (treatments 1 and 4) confirmed that there was a significant ($p=0.045$) difference between treatment 1 (no oats added to the feed, and no access to litter) and the three other treatment groups. At this age the 75 percentile of *C. perfringens* counts in treatment group 1 was log 6.4.

The *C. perfringens* counts of caecal contents in this trial varied considerably with age, from a median count of log 4.2 on day six to a median count below the lower detection limit on day 19 and a median count of log 3.2 on day 32. No significant effect of dietary fibre or litter could be found on day 6, only two days after the first exposure of the chickens to the study variables. However, on days 19 and 32 analysed together, and on day 19 analysed separately, there was a significantly reducing effect of access to litter on *C. perfringens* counts. This effect was not statistically significant ($p = 0.12$) when data collected on day 32 were analysed separately. However, on day 32 the *C. perfringens* counts of treatment group 1 were

significantly higher than the counts of all other groups, suggesting a detrimental combined effect of a lack of access to non-soluble fibres either through the diet or from litter. A high level of Dietary fibres did not significantly influence caecal counts of *C. perfringens* on its own, but appeared to contribute to a reduction of bacterial counts on day 32.

Conclusions

The experiments indicate a significant consumption of litter from the floor when housed on littered floor. However, the appetite for litter was low when given separately. In correspondence to layers, broilers also showed up to 50 % increase in gizzard weight when access to litter due to the requirement for grinding of hard coarse particles. Also young broilers showed a phenomenal grinding activity with smaller median and mean particle sizes behind the gizzard when given coarse particles in feed or litter materials. Stimulation of the gizzard activity increased the digestibility of starch. This may be caused by the more finely ground feed particles due to the biological gizzard grinding. Furthermore the increased digestibility and feed utilisation may be caused by increased enzyme activity, and in particular maltase activity in the intestine. Occurrence of gizzard lesions and caecal counts of *C. perfringens* were affected by dietary fibre and access to litter affected in this trial. These findings suggest that dietary fibre and floor bedding may be of importance to gastrointestinal health and microflora also under commercial conditions.

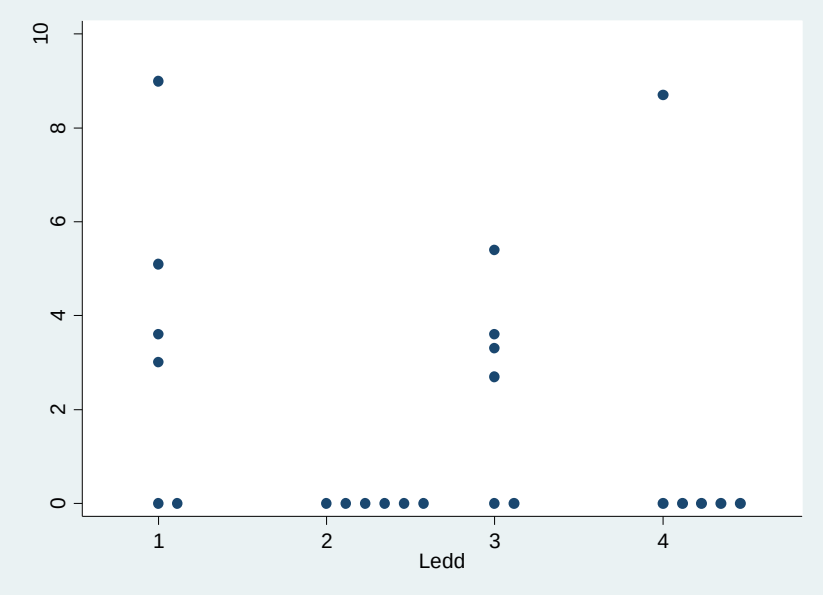

Figure 1.3 Caecal *C. perfringens* level (LogCP) versus combined treatments of dietary fibre and litter (Treatment 1= no oat hulls added, no access to litter, Treatment 2 = no oat hulls added, access to litter, Treatment 3 = oat hulls added, no access to litter, Treatment 4 = oat hulls added, and access to litter) on day 19

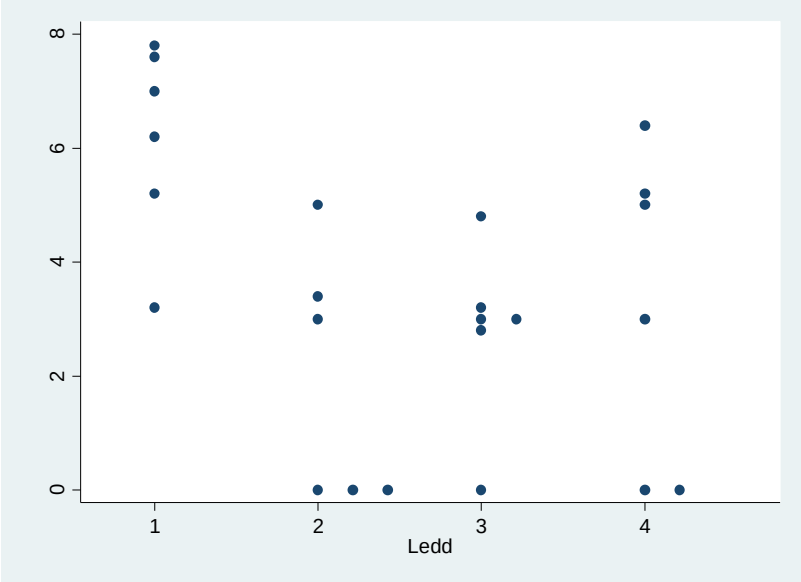

Figure 1.4 Caecal *C. perfringens* level (LogCP) versus combined treatments of dietary fibre and litter (Treatment 1= no oat hulls added, no access to litter, Treatment 2 = no oat hulls added, access to litter, Treatment 3 = oat hulls added, no access to litter, Treatment 4 = oat hulls added, and access to litter) on day 32

Table 1.3 Performance and gizzard characteristics for birds in Experiment 1

	Low fibre		High fibre		P-values			RSD
	No litter	Litter	No litter	Litter	Dietary fibre	Litter	Interaction	
Weight gain, 7-35 days	2015	2052	2117	2137	0.0174	0.4527	0.8269	144
Feed consumption, 7-35 days	3256	3380	3258	3240	0.3185	0.4406	0.3011	256
Feed/gain, 7-35 days	1.62	1.64	1.54	1.51	<0.0001	0.8945	0.1664	0.078
Empty gizzard, g/kg live wt, 35 days of age	9.0	8.5	14.4	15.2	<0.0001	0.7212	0.1307	1.71
Gizzard content, g/kg live wt, 35 days of age	0.016	0.16	6.3	7.2	<0.0001	0.31188	0.4692	2.01
Empty gizzard, g/kg live wt, 17 days of age	13.4	15.1	22.6	22.3	<0.0001	0.2902	0.1538	1.79
Gizzard content, g/kg live wt, 17 days of age	0.5	1.16	9.2	6.8	<0.0001	0.2577	0.0461	1.95
Weighted mean particle size, µm, 35 days	48		26		<0.0001			14.7
Limit for smallest 10 percentages, µm , 35 days	5		5		0.0010			0.40
Median particle size, µm, 35 days	23		13		<0.0001			3.8
Limit for largest 10 percentages, µm, 35 days	131		53		0.0007			50.1
AMEn, 17-19 days of age	13.3		13.6		0.2019			0.618

Table 1.4 Mean and individual variation of litter consumption from separate trough in Experiment 1 for birds fed on diets with different fibre level.

Feed	Mean	St dev	Minimum	Maximum
High fibre	0.27	0.28	0.0	0.96
Low fibre	0.92	0.91	0.0	2.74

Table 1.5 Enzyme secretion in different organs of bird in Experiment 2

	Diet with Oat hulls				Diet without oat hulls				P-values			RSD
Time, minutes	0	30	90	180	0	30	90	180	Oat hulls	Time	Litter*time	
Maltase mg/g tissue	47.4	49.9	49.8	53.0	45.2	43.3	41.8	42.0	0.0001	0.8575	0.1933	4.564
Amylase, U/ g pancreas	22.6	15.2	16.4	12.7	21.0	14.2	12.8	14.3	0.3808	0.0006	0.5301	4.144
Amylase U total pancreas	99.2	65.8	71.0	58.5	82.3	53.7	47.3	68.2	0.1643	0.0286	0.4104	24.06
Empty gizzard, g/kg	13.5	15.5	15.8	15.6	8.9	8.5	8.9	10.2	<0.0001	0.2147	0.3477	1.86
Gizzard content, g/kg	5.5	6.2	7.8	7.0	0.0	0.1	0.4	0.4	<0.0001	0.3845	0.7116	1.92
Pancreas, g/kg	2.17	1.95	1.96	2.02	1.94	1.77	1.59	2.07	0.1697	0.3505	0.7051	0.44
Weight gain, g		1850				1828			0.6640			221
Feed consumption, g		2961				3082			0.1056			371
Feed/gain, g/g		1.61				1.69			<0.0001			0.089

Table 1.6 Performance, gizzard characteristics, particle size distribution for Experiement 3

	No oat hulls		Oat hulls included		P-values			RSD
	No litter	Litter	No litter	Litter	Dietary fibre	Litter	Interaction	
Weight gain, g, 6-19 days	702	669	676	660	0.1693	0.0566	0.4800	30.06
Weight gain, g, 19-32 days	1255	1251	1273	1300	0.2352	0.6745	0.5816	67.04
Weight gain, g, 6-32 days	1957	1919	1949	1960	0.6205	0.6802	0.4486	77.11
Feed consumption, g, 6-19 days	1056	933	941	879	0.0002	<0.0001	0.1175	45.42
Feed consumption, g, 19-32 days	2125	2013	2079	2053	0.9531	0.1620	0.3800	116.41
Feed consumption, g, 6-32 days	3181	2945	3021	2931	0.1623	0.0136	0.2376	146.86
Gain/feed 6-19 days	0.67	0.71	0.72	0.75	<0.0001	0.0001	0.3585	0.022
Gain/feed 19-32 days	0.59	0.62	0.61	0.64	0.0599	0.0141	0.6693	0.024
Gain/feed 6-32 days	0.62	0.65	0.65	0.67	0.0044	0.0006	0.4469	0.018
Empty gizzard, g, 19 days of age	15.5	21.9	25.6	26.3	<0.0001	0.0037	0.0169	4.01
Empty gizzard, g, 32 days of age	27.6	31.0	44.9	43.4	<0.0001	0.5303	0.0939	4.88
Weighted mean particle size, µm, 19 days	160.2	120.3	88.6	109.9	0.0748	0.6821	0.1809	77.9
Limit for smallest 10 percentages, µm , 19 days	6.7	6.5	5.7	5.4	0.1923	0.7538	0.9598	2.72
Median particle size, µm, 19 days	80.8	53.8	42.2	50.7	0.1804	0.5496	0.2539	53.14
Limit for largest 10 percentages, µm, 19 days	445.9	346.4	247.6	321.7	0.0692	0.8332	0.1544	207.4
Weighted mean particle size, µm, 32 days	236.5	263.8	136.1	171.9	<0.0001	0.0908	0.8169	63.21
Limit for smallest 10 percentages, µm , 32 days	6.4	7.9	8.8	10.1	0.0092	0.1024	0.9205	2.91
Median particle size, µm, 32 days	87.3	134.1	80.4	97.1	0.1363	0.0336	0.3047	50.09
Limit for largest 10 percentages, µm, 32 days	705.8	729.8	355.8	457.0	<0.0001	0.1755	0.4005	157.5
Starch content, % of ileal content, 19 days of age	14.4	3.5	1.7	2.3	<0.0001	0.0001	<0.0001	4.16
Starch content, % of ileal content, 32 days of age	12.2	9.6	1.1	1.25	<0.0001	0.1042	0.0622	2.61

Chapter 2. Effect of forced litter consumption on gut development, nutrient digestibility and gut microflora

Introduction

Poultry housed in floor pen systems are able and likely to consume litter materials from the floor that can significantly affect their production traits (Malone and Chaloupka, 1983). How consumption of litter materials affects performance and gut function in poultry is largely an unexplored research area. Previous experimental work supported by the Poultry CRC (Ali, 2009) demonstrated several points; (1) 2-week and 4-week old broilers housed in floor pens ate appreciable amounts of litter detected by examination of gizzard contents, (2) there were noticeable bird to bird differences in gizzard size and litter consumption, and (3) litter consumption varied according to type of litter used, which implied that particle size and/or hardness of litter may be important.

The aim of the present study was to investigate the effect of forced litter feeding on growth performance, gut development and starch digestibility in broiler chicken. The approach taken here was to incorporate specified amounts of hardwood sawdust litter material in experimental diets to ensure that individual birds ingested litter. Alternative approaches of attempting to measure actual litter intake, or by using naturally-occurring markers in litter to estimate litter consumption, were considered impractical within the current project.

Materials and Methods

Birds and diets

384 day-old male Cobb broiler chickens (Baiada hatchery, Kootingal, NSW, Australia) were raised in 48 brooder cages (8 birds per cage) in a temperature-controlled room. Each cage was randomly assigned to one of six treatments.

From day of arrival to day 14, birds in all groups were given *ad lib* access to a standard commercial starter diet, and adulterated with different levels of hardwood sawdust litter at the proportions of 0, 0.75, 1.5, 3.0, 6.0 and 12.0%, and fresh water. From day 15 to day 35, birds were fed commercial grower ration with same proportions of sawdust incorporated.

Growth performance and feed conversion ratio

Feed consumption and live weight of the birds were measured on days 7, 14, 21, 28 and 35 of the experiment. Body weight gain and feed conversion ratios were calculated on those days.

Sample collection

Three and two birds were randomly chosen in each cage and sacrificed at days 21 and 35, respectively, for sample collections. All birds were euthanised by cervical dislocation, total body weight of each bird was recorded, and gizzard together with proventriculus, small intestines, pancreas, liver, spleen and bursa were removed and weighed individually. The duodenum from one bird of each treatment replicate was collected and fixed in 10% buffered formalin for subsequent histological examination. The pancreas was collected from different bird of each treatment replicate and snap-frozen in liquid nitrogen until required for analysis of enzyme activity.

The contents of the gizzard, ileum and caeca were pooled separately for the birds sacrificed in each replicate, pH of the contents was measured, and approximately 1 g of the digesta was collected for microbial culture, and the remaining digesta was stored for determination of volatile fatty acid analysis. Approximately 3 cm section of ilea (including digesta) mid-point between Meckel's diverticulum and caecal tonsils, and one caecal lobe (including contents) per bird were sampled for T-RFLP analysis.

Volatile fatty acid

For measurement of volatile fatty acid (SCFA, lactic and succinic acid) concentrations, about 2.0 g of thawed gizzard, ileal and caecal sample was suspended in 1.0 mL of 0.01M 2-ethylbutyric acid as internal standard and thoroughly mixed, followed by centrifugation at 25,000 x g at 5°C for 20 min. To a sample of 1 mL supernatant, 0.5 mL of concentrated HC1 (36%) and 2 mL of diethyl ether were added and thoroughly mixed, followed by centrifugation at 2000 x g at 5°C for 15 min. Four hundred μL of supernatant (the ether phase) were mixed with 40 μL N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA), and incubated at 80^{o}C for 20 min. The mixture was then left at room temperature for at least 48 hrs before concentration quantification of the organic acids. The concentration of organic acids was quantified using a Varian **CP**3800 gas chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA).

The chromatographic apparatus included a Varian CP3800 CX gas chromatograph fitted with a flame ionization detector (FID) and a Varian *C. perfringens* 8200 autosampler (Varian Analytical Instruments, Palo Alto, CA, USA) equipped with a capillary column (0.32 mm internal diameter, 30 m length and 0.25 μm film thickness) (Alltech ECONO-CAP^{TM}, Alltech Associations Inc., Deerfield, IL, USA). The initial and final oven temperatures were 70°C and 240°C, respectively, and the injector and FID temperatures were 240°C and 280°C, respectively. Ultra high purity helium was used as the carrier gas (40 cm/sec). Varian Star 5.52 chromatography workstation (integration system) software (Varian Analytical Instruments, Palo Alto, CA, USA) was used for data processing. Total organic acid concentration is the sum of the all organic acids observed in a sample, expressed as μmol/g digesta. Molar percentages of individual organic acids are expressed as mol/100 moles.

Microbial profiling

Enumeration of intestinal bacteria

Intestinal digesta samples in pre-reduced salt medium were homogenised for 2 min in CO2-flushed plastic bags using a MiniMix® bag mixer (Interscience, St. Nora, France) and serial diluted in 10-fold increments according to the technique of Miller and Wolin (1974). An aliquot (100 □L) was plated on the following media. Total anaerobic bacteria were enumerated on Wilkins-Chalgren anaerobic agar (Oxoid, CM0619) after incubation at 39°C for 7 days in an anaerobic cabinet (Model SJ-3, Kaltec Pty. Ltd., Edwardstown, SA, Australia). Coliform bacteria and lactose-negative enterobacteria were counted on MacConkey agar (Oxoid, CM0115) after aerobic incubation at 39^{o}C for 24 h. The population of *C. perfringens* (Cp) were counted on Tryptose-Sulfite-Cycloserine and Shahidi-Ferguson Perfringens agar base (TSC & SFP) (Oxoid, CM0587) mixed with egg yolk emulsion (Oxoid, SR0047) and Perfringens (TSC) selective supplement (Oxoid, SR0088E) according to the pour-plate technique, where plates were overlaid with the same agar after spreading the inoculums. Bacterial numbers were expressed as log10 CFU/g digesta.

Terminal-restriction fragment length polymorphism

Total nucleic acid, including that of the representative bacterial population, was extracted from freeze-dried ileal and caecal samples by a modified South Australian Research & Development Institute (SARDI) proprietary method. The bacterial ribosomal DNA from the extracted material was amplified with universal 16S bacterial primers, one of which was labelled with the fluorescent dye (Torok *et al*., 2008). The resulting amplicons were restricted with a specific recognition sequence restriction enzymes and electrophoretically separated on a capillary DNA sequencer (ABI 3730, Applied Biosystems). Data were analysed using GeneMapper (Applied Biosystems) to determine positions of terminal restriction fragments (TRF). Data points from GeneMapper analysis were validated and outputs generated for statistical analysis using queries within a custom built database. Queries in the database were used to compare duplicate T-RFLP profiles and identify synonymous fragment sizes (±2 bp). DNA quantity, as measured by total relative fluorescence between duplicates, was standardized and peaks that fell below the background threshold of 75 relative fluorescent units were excluded using an iterative method. For each sample a derivative profile was then created from the average position and height of reproducible T-RFs. T-RFs ≥ 1% of the total relative peak height per sample were used in subsequent calculations. The resulting fragments were treated as operational taxonomic units (OTUs), representing particular bacterial species or taxonomically related groups. OTUs obtained from the ileum and caeca of the 54 broiler chickens were analysed using multivariate statistical techniques (PRIMER 6 and PERMANOVA+β1, PRIMER-E Ltd., Plymouth, UK). These analyses were used to examine similarities in chicken gut microbial communities and identify OTUs accounting for differences observed in microbial communities (Torok *et al*., 2008).

Ileal digestibility of starch

Apparent ileal digestibility of starch 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 digestibility. Diets and ileal digesta were analysed for starch as described below. The apparent ileal digestibility of starch was calculated using the following formula.

$$\text{Apparent nutrient digestibility} = \frac{(\text{Starch}/\text{AIA})_{\text{diet}} - (\text{Starch}/\text{AIA})_{\text{ileum}}}{(\text{Starch}/\text{AIA})_{\text{diet}}}$$

where, $(\text{Starch}/\text{AIA})_{\text{diet}}$ = ratio of starch and acid insoluble ash in diet, and $(\text{Starch}/\text{AIA})_{\text{ileum}}$ = ratio of starch and acid insoluble ash in ileal digesta.

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 HC1, following the method described by Vogtmann *et al*. (1975) and Choct and Annison (1990). In brief, samples were weighed accurately (diet, 3 g; ileal digesta, 1 g) into Pyrex® brand Gooch-type crucibles (porosity 4 μm) and dried overnight at 105°C in a forced-air convection oven. After cooling and weighing, the samples were ashed overnight at 480°C in a Carbolite CWF 1200 chamber furnace (Carbolite, Sheffield, UK). The crucibles were gently boiled twice in 4M HC1 for 15 min and the acid was removed through suction. The residues (AIA) were washed with distilled water, and the crucibles were dried overnight at 105°C and weighed. The acid insoluble ash content was calculated using the following equation:

$$\text{AIA (g/kg dry matter)} = \frac{(\text{Crucible} + \text{Ash weight}) - (\text{Crucible weight})}{(\text{Crucible} + \text{Dry sample weight}) - (\text{Crucible weight})} \text{x1000}$$

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). 20 mg of feed and 100 mg freeze-dried ileal digesta in duplicates were used for the analysis. To aid dispersion, 0.2 mL of 80 % (v/v) ethanol was added into the samples with mixing by vortex followed by addition of 2.0 mL dimethyl sulphoxide, and the mixture was incubated at 100°C for 10 min. Three millilitres of thermostable α-amylase (*EC* 3.2.1.1, Megazyme Australia Pty. Ltd., Warriewood, NSW, Australia) in 50 mM MOPS buffer (3-[N-morpholino]propanesulfonic acid, pH 7.0, Sigma-Aldrich Co., St. Louis, MO, USA) were added and the mixture was incubated at 100°C for 30 min. Four millilitres of 200 mM sodium acetate buffer (pH 4.5) and 0.1 mL of amyloglucosidase (EC 3.2.1.3, Megazyme Australia Pty. Ltd., Warriewood, NSW, Australia) were added and the solution was incubated at 50°C for 1 h. The reaction mixture was then centrifuged at 3000 x g for 10 min. An aliquot of 0.1 mL supernatant was mixed with 3 mL of Glucose Determination Reagent (GOPOD), and incubated at 50°C for 20 min. The absorbance was measured at 510 nm and 1 cm light path length using a Hitachi 150-20 spectrophotometer (Hitachi Science Systems Ltd., Ibaraki, Japan). It was assumed that the free glucose in ileal digesta derived from starch.

Pancreatic amylase activity

Pancreatic samples were analysed for amylase activity. Briefly, 2g of pancreas was cut and defrosted in 20mL buffer containing 100mM mannitol and 2mM HEPES at pH of 7.1. The samples were homogenised by vortexing for 1 min and filtered through a Buchner funnel. Homogenate was blended at for 30 sec, and used for subsequent analysis. 100 µL of the homogenate was combined with 500µL 50mM phosphate buffer (pH 7.2) and 500µL pre-warmed solution of soluble potato starch (2mg/mL). The mixture was incubated at 39° C for 60 min. One mL of the solution containing 0.2% Triton X-100 and 0.5 M This-HCl buffer at pH of 7.02 (at 37^{o}C) was added to stop the reaction by incubation at 39C^{o} for20 min. After the samples cooled, the absorbance was read at 246 nm and compared against the standard GOPOD (Roche Diagnostics).

Mucosal morphometric analysis

Formalin-fixed duodenum samples were processed in an automatic tissue processor (TOSCO, Thomas Optical & Scientific Co., Melbourne, Australia) in consecutive steps of dehydration by serial ethanol solutions (30% to 100%), clearing by xylene, and infiltration by paraffin. The tissue was embedded in paraffin using a Histo Embedding Centre (Leica EG 1160, Leica Microsystems, Bensheim, Germany). Embedded samples were subsequently sectioned at a thickness of 5 µ with a rotary microtome Leitz 1516 (Leica Microsystems, Bensheim, Germany). The tissue sections on the slides were stained using Harris's hematoxylin (George Gurr Ltd., London, UK), and eosin (Gur Certistain, VWR International Ltd., Poole, UK), and mounted with DPX (distrene polystyrene xylene) mountant. The sections were viewed under a Leica DM LB microscope (Leica Microscope GmbH, Wetzlar, Germany) and the images captured with a Sony Exwave HAD SSC-DC83p colour video camera (Sony Corporation, Tokyo, Japan). Morphometric indices were determined using computer-aided light microscope image processing analysis software VideoPro 32 package (Leading. Edge Pty Ltd,

Adelaide, Australia). Villus height and crypt depth were measured in 15 vertically, well-oriented, intact villi and crypts. All measurements were calibrated with a micrometer.

Statistical analyses

All data except T-RFLP results were analysed using the statistical package Minitab® for Windows 12.1 (Minitab Inc., State College, USA). An analysis of variance was performed using the one way ANOVA procedure with significant differences among the diets determined by Tukey Multiple Comparison test of means. T-RFLP data were analysed as described in the related section above.

Results

Growth performance of birds

Growth performance (feed intake, weight gain and feed conversion) was not affected by forced consumption of litter in feed at any stage in this experiment (Tables 2.1 and 2.2).

Relative organ weights

The relative weight of gizzard plus proventriculus at 21 and 35 days of age appeared to increase with increasing level of litter in the feed, but was significantly greater only for birds given the highest level (12%) compared with any other treatment (Tables 2.3 and 2.4). The gizzard and proventriculus were not measured separately. Other organs were unaffected by litter consumption.

Ileal digestibility and pancreatic amylase activity

The apparent ileal digestibility of starch was significantly higher in birds given the two highest levels of litter in feed (6 and 12%) compared with other treatments (Table 2.5). Amylase activity in the pancreas was unaffected by dietary treatment (Table 2.5).

Volatile fatty acid and pH analysis in intestinal organs

Gizzard pH was significantly lower in birds receiving 12% litter in feed compared with other treatments (Table 2.6), whereas litter consumption had no effects on pH in the ileum and caeca. Similarly, there was no effect of dietary treatment on SCFA concentrations in the gizzard, ileum and caeca (Table 2.6). Consumption of litter (3, 6 and 12%) significantly lowered the molar proportions of lactic acid in the caeca, but not in the gizzard or ileum (Table 2.6).

Mucosal morphometry

There was no significant difference of the villa lengths and crypt depths in the duodenum of birds fed diets containing different proportions of hardwood sawdust at either 21 or 35 days of age (Table 2.7).

Table 2.1 Growth performance of birds fed the experimental diets[1]

	Litter in feed (g/100g)						SE	One way[2] ANOVA
	0	0.75	1.5	3	6	12		
Body weight (g)								
Day 1 (overall)	36						0.4	NS
Day 7	134	132	127	130	130	129	3.1	NS
Day 14	350	352	331	354	351	339	10.2	NS
Day 21	767	791	764	808	754	754	23.7	NS
Day 28	1169	1253	1238	1262	1207	1229	30.7	NS
Day 35	1695	1844	1849	1736	1803	1802	81.2	NS
Body weight gain (g)								
Day 1-21	731	754	728	771	718	718	23.6	NS
Day 21-35	928	1053	1084	1059	1049	1047	60.8	NS
Day 1-35	1597	1749	1758	1642	1709	1709	80.8	NS

[1] Values are means and pooled standard errors (n = 7). Values within a row not having the same letter are significantly different.

[2] NS, P>0.05; *, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, *P* < 0.001.

Table 2.2 Feed intake and feed conversion ratio (FCR) of birds fed the experimental diets[1]

	Litter in feed (g/100g)						SE	One way[2] ANOVA
	0	0.75	1.5	3	6	12		
Feed intake (g)								
Day 1-7	121	116	118	123	120	125	3.3	NS
Day 1-21	1201	1207	1271	1233	1222	1244	37.4	NS
Day 21-35	1847	1906	2064	1960	1954	2081	80.2	NS
Day 1-35	2810	2862	3007	2946	2951	3018	88.7	NS
FCR								
Day 1-7	1.23	1.24	1.39	1.30	1.30	1.37	0.04	NS
Day 1-21	1.67	1.67	1.77	1.67	1.70	1.77	0.06	NS
Day 21-35	1.81	1.76	1.83	1.89	1.84	1.77	0.07	NS
Day 1-35	1.63	1.57	1.61	1.64	1.67	1.61	0.05	NS

[1] Values are means and pooled standard errors (n = 7). Values within a row not having the same letter are significantly different.

[2] NS, P>0.05; *, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, *P* < 0.001.

Table 2.3 Relative organ weights (g/ 100 g body weight) birds 21 days of age fed the experimental diets[1]

	Litter in feed (g/100g)						SE	One way [2] ANOVA
	0	0.75	1.5	3	6	12		
Proventriculus + gizzard	3.1ab	2.8a	3.3ab	3.1ab	3.4b	4.6c	0.20	***
Small intestine	7.1	7.1	7.4	6.4	6.2	6.4	0.41	NS
Pancreas	0.3	0.3	0.3	0.3	0.3	0.3	0.02	NS
Liver	3.2	3.1	3.1	3.3	3.3	3.3	0.11	NS
Spleen	0.1	0.1	0.1	0.1	0.1	0.1	0.01	NS
Bursa	0.2	0.2	0.2	0.2	0.1	0.2	0.02	NS

[1] Values are means and pooled standard errors (n = 7). Values within a row not having the same letter are significantly different.

[2] NS, P>0.05; *, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, *P* < 0.001.

Table 2.4 Relative organ weights (g/ 100 g body weight) of birds 35 days of age fed the experimental diets[1]

	Litter in feed (g/100g)							One way[2]
	0	0.75	1.5	3	6	12	SE	ANOVA
Proventriculus + gizzard	2.0ab	2.3b	1.8a	2.1ab	2.4b	3.3c	0.16	***
Small intestine	4.9	4.5	5.1	4.8	4.3	4.4	0.27	NS
Pancreas	0.2	0.2	0.2	0.2	0.2	0.2	0.01	NS
Liver	2.3	2.5	2.5	2.7	2.4	2.5	0.11	NS
Spleen	0.1	0.1	0.1	0.1	0.1	0.1	0.01	NS
Bursa	0.2	0.2	0.2	0.2	0.2	0.2	0.02	NS

[1] Values are means and pooled standard errors (n = 7). Values within a row not having the same letter are significantly different.

[2] NS, P>0.05; *, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, *P* < 0.001.

Table 2.5 Apparent ileal digestibility of starch (%),and pancreas amylase activity (µmol/mg protein/min) on 35d of experiment[1]

	Litter in feed (g/100g)						
	0	0.75	1.5	3	6	12	ANOVA[2]
Starch	63.0a	63.5a	63.5a	69.8a	86.0b	91.4b	***
Amylase	0.241	0.238	0.243	0.212	0.249	0.232	NS

[1] Values are means and pooled standard errors (n = 7). Values within a row not having the same letter are significantly different.

[2] NS, P>0.05; *, 0.05 > P > 0.01; **, 0.01 > P > 0.001; ***, P < 0.001.

Table 2.6 pH, total concentration (mM) and molar proportions of volatile fatty acids (VFA) in intestinal content on 35d of experiment[1].

		Litter in feed (g/100g)				SE	One way[2] ANOVA
		0	3	6	12		
Gizzard	pH	3.52a	3.44a	3.23a	2.59b	0.21	*
	Total VFA	2.68	3.29	0.05	3.87	1.10	NS
	% Acetic acid	28.7	24.7	34.3	21.2	6.85	NS
	% Lactic acid	70.7	69.9	63.6	77.3	6.890	NS
Ileum	pH	7.77	7.42	7.50	7.75	0.18	NS
	Total VFA	6.95	3.75	3.82	4.53	2.47	NS
	% Formic acid	3.7	4.8	9.7	5.2	2.35	NS
	% Acetic acid	14.5	18.6	19.7	19.0	3.53	NS
	% Lactic acid	81.6	76.1	70.3	75.7	4.88	NS
Caeca	pH	5.51	5.91	5.59	5.49	0.15	NS
	Total VFA	94.3	127.1	103.7	110.4	16.08	NS
	% Acetic acid	63.2	72.1	68.9	63.2	2.63	NS
	% Propionic acid	2.0	5.0	3.1	3.1	0.81	NS
	% Butyric acid	15.0	12.7	17.2	14.4	1.57	NS
	% Valeric acid	0.5	0.7	0.6	0.6	0.17	NS
	% Lactic acid	3.9a	0.9b	0.9b	1.3b	0.58	**
	% Iso-branched acids	0.3	0.6	0.6	0.1	0.20	NS

[1] Values are means and pooled standard errors (n = 7). Values within a row not having the same letter are significantly different.

[2] NS, P>0.05; *, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, *P* < 0.001.

Table 2.7 Mucosal morphometry (μ) of duodenum of birds on 21d and 35d of experiment[1].

	Litter in feed (g/100g)						SE	One way[2] ANOVA
	0	0.75	1.5	3	6	12		
Day 21								
Villus length	1629.1	1629.4	1457.8	1670.6	1430.9	1658.4	101.4	NS
Crypt depth	229.69	229.45	208.19	208.35	222.69	213.22	14.60	NS
Day 35								
Villus length	1903	1847.7	1997.5	1737.5	1730.2	1996.2	292.5	NS
Crypt depth	138.08	130.02	129.56	134.57	131.41	129.96	25.52	NS

[1] Values are means and pooled standard errors (n = 7). Values within a row not having the same letter are significantly different.

[2] NS, P>0.05; *, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, *P* < 0.001.

Table 2.8 Microbiological counts (log10 CFU/g digesta) in intestinal content on d21 of experiment[1].

		Litter in feed (g/100g)				SE	One way ANOVA[2]
		0	3	6	12		
Gizzard	Total anaerobic bacteria	6.55	6.73	6.28	6.47	0.34	NS
	Lactic acid bacteria	6.53	6.65	5.98	6.36	0.37	NS
	Enterobacteria	4.97a	3.80b	3.38b	3.40b	0.33	**
	C. perfringens	3.11	2.91	3.28	3.13	0.12	NS
Ileum	Total anaerobic bacteria	8.01	7.64	7.46	8.03	0.25	NS
	Lactic acid bacteria	7.80	7.78	7.43	7.99	0.27	NS
	Enterobacteria	5.97	5.27	5.08	4.95	0.33	NS
	C. perfringens	3.76	3.46	3.38	3.62	0.24	NS
Caeca	Total anaerobic bacteria	9.68	9.55	9.13	9.68	0.11	NS
	Lactic acid bacteria	9.58	9.54	9.23	9.67	0.19	NS
	Enterobacteria	8.14	8.04	7.70	7.79	0.21	NS
	C. perfringens	6.58	6.74	5.64	6.72	0.51	NS

[1] Values are means and pooled standard errors (n = 7). Values within a row not having the same letter are significantly different.

[2] NS P>0.1, * P < 0.05, ** P < 0.01, *** P < 0.001.

Microbial profiling

Intestinal microflora by enumeration

In 21 day old chickens, the number of enterobacteria (assessed by the plate-counting method) was significantly lower in birds fed 12, 6 and 3% litter compared with the control group ($P < 0.01$), however, this effect in gizzard was not observed in 35 day old chickens, instead a significantly lower number of enterobacteria was observed in the ileum of birds fed 12% litter compared with the other treatment groups ($P < 0.05$). The populations of total anaerobic bacteria, lactic acid bacteria and *C. perfringens* were not affected by the dietary treatments in the present experiment (Tables 2.8 and 2.9).

Table 2.9 Microbiological counts (log10 CFU/g digesta) in intestinal content on d35 of experiment[1].

		Litter in feed (g/100g)					
		0	3	6	12	SE	One way ANOVA[2]
Gizzard	Total anaerobic bacteria	6.99	7.07	7.02	6.79	0.34	NS
	Lactic acid bacteria	6.32	5.85	6.48	6.13	0.47	NS
	Enterobacteria	3.79	3.72	4.32	3.16	0.41	NS
	C. perfringens	2.81	2.84	3.3	2.86	0.17	NS
Ileum	Total anaerobic bacteria	7.51	7.71	7.49	7.76	0.29	NS
	Lactic acid bacteria	7.19	7.01	6.9	7.23	0.37	NS
	Enterobacteria	5.33a	5.56a	5.16a	3.94b	0.37	*
	C. perfringens	2.99	3.03	3.05	3.05	0.06	NS
Caeca	Total anaerobic bacteria	9.36	9.47	9.76	9.31	0.22	NS
	Lactic acid bacteria	8.8	8.64	9	8.87	0.16	NS
	Enterobacteria	7.18	7.49	6.95	6.87	0.25	NS
	C. perfringens	5.62	6.58	5.73	5.85	0.51	NS

[1] Values are means and pooled standard errors (n = 7). Values within a row not having the same letter are significantly different.

[2] NS P>0.1, (*) P < 0.1, * P < 0.05, ** P < 0.01, *** P < 0.001.

Terminal-restriction fragment length polymorphism

One-way ANOSIM comparing diets containing hardwood sawdust with the commercial control diet confirmed that only the commercial diet containing 12% hardwood sawdust resulted in differing gut microbial communities in both the ilea and caeca (Table 2.10). The difference between gut microbial communities of birds raised on the commercial diet versus commercial diet containing 12% hardwood sawdust is graphically shown for the ilea (Figure 2.1) and the caeca (Figure 2.2) in the nMDS ordinations.

Table 2.10 One-way ANOSIM of ileal and caecal microbial communities associated with dietary treatments. The Global *R* statistic (**bold**) and significance level (*italics*) are shown for comparisons with the control diet containing no hardwood sawdust.

		Litter in feed (g/100g)		
		3	6	12
Ilea	No sawdust	**0.022**, *0.214*	**0.031**, *0.227*	**0.095**, *0.036*
Caeca	No sawdust	**-0.011**, *0.539*	**0.068**, *0.085*	**0.114**, *0.022*

Similarities in gut bacterial communities among birds on the same diet were calculated with SIMPER for birds on the commercial diet and birds on the commercial diet containing 12% hardwood sawdust. Within the ileum, gut bacterial communities were more similar for birds on the commercial diet containing 12% hardwood sawdust than birds on the commercial control diet and were 63% and 43% similar, respectively. Within the caeca, similarity in gut bacterial community composition was the same (45%) for birds on both the commercial diet and commercial diet containing 12% hardwood sawdust. Dissimilarity in bacterial communities between the two diets was 49% in the ileum and 56% in the caeca. OTUs contributing to the top 50% of dissimilarity in bacterial community composition between diets were identified within the ileum and caeca. Three OTUs within the ileum (Table 2.11), and eleven OTUs within the caeca (Table 2.12), were identified as good discriminators between diets. Two of these diet-associated OTUs (178 and 566) were common to both the ileum and caeca.

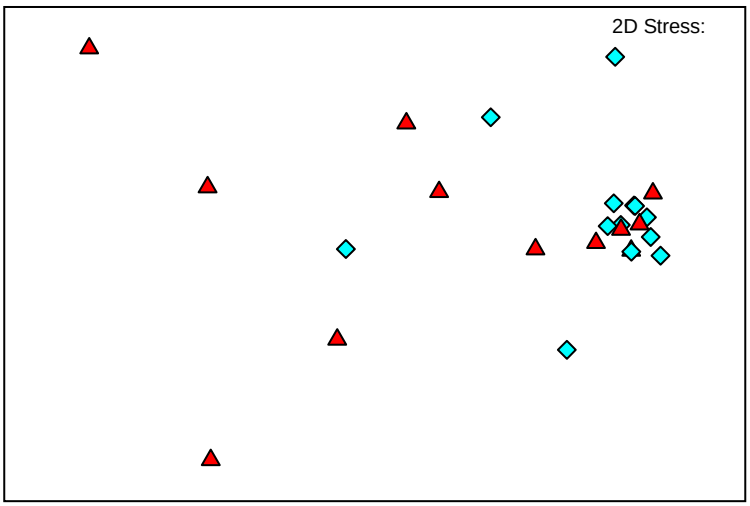

Figure 2.1 nMDS ordination of ileal microbial communities identified by either the commercial diet (▲) or the commercial diet containing 12% hardwood sawdust (◆). The ordination is based on Bray-Curtis similarities calculated from standardised and 4th-root transformed OTU abundances.

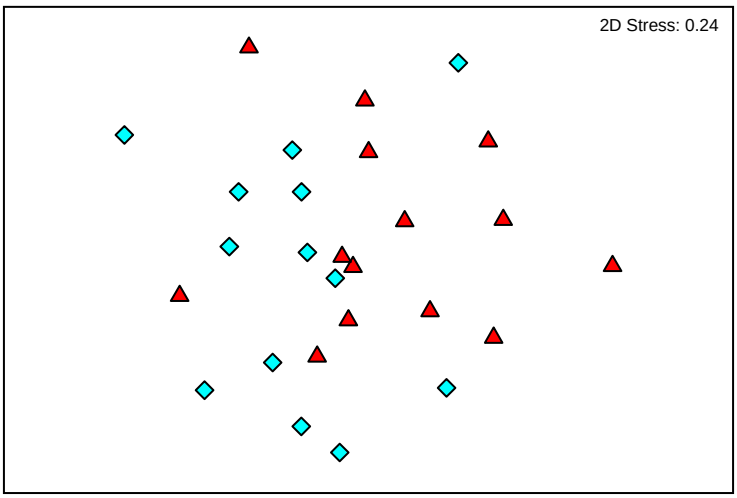

Figure 2.2 nMDS ordination of caecal microbial communities identified by either the commercial diet (▲) or the commercial diet containing 12% hardwood sawdust (◆). The ordination is based on Bray-Curtis similarities calculated from standardised and 4^{th}-root transformed OTU abundances.

Table 2.11 OTU contribution to the dissimilarity in ileal microbial communities associated with diet. Average abundance of important OTUs in ileal microbial communities of birds fed either a commercial diet (C) or commercial diet supplemented with 12% hardwood sawdust (C + 12% HWS) are shown. OTUs are listed in order of their contribution ($\bar{\delta}_i$) to the average dissimilarity ($\bar{\delta}_t$ =49.47%) between diets. Percent contribution of individual OTUs and cumulative percent contribution to the top 50% of average dissimilarities are shown. OTUs contributing significantly to the dissimilarity between dietary treatments were calculated ($\bar{\delta}_i/SD(\delta_i)>1$) and are marked with an asterix *.

OTU	Average abundance		$\bar{\delta}_i$	$\bar{\delta}_i/SD(\delta_i)$	Individual contribution %	Cumulative contribution %
	Control	12% hardwood saw dust				
220*	1.06	0.45	4.16	1.25	8.41	8.41
178*	1.58	2.07	3.85	1.03	7.78	16.19
286	0.84	0.34	3.43	0.94	6.94	23.14
180	2.07	2.63	2.92	0.88	5.91	29.05
936	0.39	0.51	2.81	0.89	5.69	34.73
566*	1.52	1.35	2.59	1.01	5.23	39.96
296	0.69	0.14	2.45	0.85	4.96	44.92
222	0.61	0.13	2.26	0.83	4.57	49.49
284	0.55	0.00	2.16	0.56	4.36	53.85

Table 2.12 OTU contribution to the dissimilarity in caecal microbial communities associated with diet. Average abundance of important OTUs in caecal microbial communities of birds fed either a commercial diet (C) or commercial diet supplemented with 12% hardwood sawdust (C + 12% HWS) are shown. OTUs are listed in order of their contribution ($\overline{\delta}_i$) to the average dissimilarity $\overline{\delta}_t$(=56.34%) between diets. Percent contribution of individual OTUs and cumulative percent contribution to the top 50% of average dissimilarities are shown. OTUs identified as being good discriminators between diet are marked with an asterix*

OTU	Average abundance		$\overline{\delta}_i$	$\overline{\delta}_i$ /SD(δ_i)	Individual contribution %	Cumulative contribution %
	Control	12% hardwood saw dust				
288*	1.03	0.47	1.91	1.20	3.40	3.40
282*	0.30	0.99	1.79	1.17	3.18	6.58
90	0.35	0.82	1.74	0.89	3.09	9.67
296*	1.48	1.31	1.69	1.03	3.01	12.67
280*	0.94	0.26	1.68	1.25	2.99	15.66
296	0.41	0.71	1.68	0.94	2.98	18.64
178*	0.46	0.84	1.68	1.03	2.98	21.62
142*	1.06	0.83	1.54	1.10	2.74	24.36
566*	0.44	0.77	1.54	1.06	2.74	27.09
180	0.24	0.72	1.48	0.93	2.62	29.71
492	0.41	0.59	1.47	0.91	2.62	32.33
294	0.65	0.26	1.41	0.81	2.51	34.83
300*	0.71	1.14	1.35	1.08	2.39	37.22
536*	0.54	0.58	1.33	1.03	2.36	39.58
482*	0.80	0.73	1.29	1.06	2.29	41.88
140	0.57	0.47	1.28	0.99	2.27	44.15
396*	0.08	0.65	1.23	1.04	2.18	46.33
474	0.55	0.38	1.23	0.95	2.18	48.51
144	0.52	0.32	1.22	0.91	2.16	50.67

Discussion

Few studies have been reported on the effect of forced consumption of litter materials at different levels on growth performance, organ development, digestibility, microflora and mucosal morphometry in broiler chicken. In this experiment, we have clearly shown that birds receiving 12% hardwood sawdust in the feed significantly increased the relative weight of gizzard plus proventriculus. This result is consistent with previous reports as well in our NULS experiments that high fibre consumption from diet and litter can significantly stimulate the development of gizzard (Kubena et al., 1974, Preston et al., 2000, Hetland et al., 2005). As bird growth performance including feed intake, weight gain, and FCR was not affected by enforced hardwood sawdust consumption, it can be concluded that excess hardwood litter intake does not have detrimental effect on the birds under treatment. Moreover, apparent ileal digestibility of starch increased significantly with high levels of litter intake (6% and 12%) in comparison with intake of lower or no litter addition in feed, it is likely hardwood consumption can improve the function of gizzard, and possibly the small intestine. The lower pH of the birds receiving 12% than the birds in other groups may be related to the improvement starch digestibility, by a mechanism other than increased pancreatic amylase activity, which did not differ among dietary treatments.

In general, enterobacteria were observed to be reduced in the gut of the broilers receiving hardwood sawdust in the feed. Significant reduction, for instance, occurred on day 21, with enterobacteria counts being less in gizzard of birds fed 3%, 6% and 12% hardwood sawdust in feed than in the control group; on day 35, enterobacteria counts were less in the ileum of the birds fed 12% hardwood sawdust in feed than in other groups. Enterobacteria, mainly including groups of pathogenic bacteria such as *Salmonella* and *Escherichia coli*, have detrimental effects to bird health. Thus, the reduction of the enterobacteria by hardwood sawdust intake suggests a beneficial role of the consumption of hardwood sawdust litter to the bird health, and the proportion of the litter in total feed intake can be up to 12%.

Ileal and caecal microbial communities were significantly different for birds on the commercial diet containing 12% hardwood sawdust as compared with the commercial control diet. Within the ilea addition of 12% hardwood sawdust resulted in birds having a more similar ileal microbial community than birds on the commercial control diet. OTUs 566 and 178 were identified as contributing to differences in microbial community composition between birds on the commercial control diet and commercial diet containing 12% hardwood sawdust within both the ilea and caeca.

In spite of the possible benefits of enhanced gizzard development, the overall bird performance was not improved by feeding hardwood sawdust. This lack of effect is consistent with several other studies (Anisuzzaman and Chowdhury, 1996, Brake et al., 1993, Swain and Sundaram, 2000).

In conclusion, forced consumption of hardwood litter had no effect on growth performance despite enlargement of the gizzard and proventriculus in bird given a diet with 12% hardwood litter material, and increased digestibility of starch in the small intestine of birds given 6 and 12% levels, compared with birds given the commercial diet only.

Chapter 3. The effects of dietary fibre and litter type on gut development, nutrient digestibility and gut microflora

Introduction

Previous experimental work conducted in this project in Norway and Australia was done on a relatively small scale not conducive to detecting small differences growth performance in response to dietary treatments for other than short experimental periods. This experiment was conducted in a larger experimental grow-out facility over a 42-day period, and involved commercially-prepared starter, grower, finisher and withdrawal diets fed to a much larger number of birds, in order to increase the power of the experiment.

The aim of this experiment was to investigate the effects of two types of litter (paper and hardwood sawdust) in combination with a low and high fibre diets on growth performance, feed efficiency, organ development, mucosal morphometry, gut microbial communities and microbial fermentation products.

Materials and Methods

Birds and diets

720 day-old Cobb broiler chickens were raised for 6 weeks in 24 brooder cages in a temperature-controlled shed at Inghams Enterprise research facility in Leppington, New South Wales. Each cage was randomly assigned to one of four treatment groups in a 2 x 2 factorial design arrangement with six replicates per treatment (30 birds per cage). Three replicate cages within a treatment were male birds and the other three were female birds. Nutrient and dietary composition of the two experiment diets (high and low fibre) are shown in Table 3.1.

Growth performance and feed conversion ratio

Feed consumption and live weight of the birds were measured on days 7, 21, 35 and 42 of the experiment. Body weight gain and feed conversion ratios were calculated on those days.

Sample collection

Four birds were randomly chosen in each cage and sacrificed at days 35 for sample collections. All birds were euthanised by cervical dislocation, total body weight of each bird was recorded, and proventriculus, gizzard, small intestine, pancreas, liver, spleen and bursa were removed and weighed individually. The duodenum from one bird of each treatment replicate was collected and fixed in 10% buffered formalin for subsequent histological examination.

The contents of the gizzard, ileum and caeca were pooled separately for the birds sacrificed in each replicate. pH of the contents was measured, approximately 1 g of the digesta was collected for microbial culture, and the remaining digesta was stored for determination of volatile fatty acid analysis. Approximately 3 cm section of ilea (including digesta) mid-point between Meckel's diverticulum and caecal tonsils, and one caecal lobe (including contents) per bird were sampled for T-RFLP analysis.

Table 3.1 Diet composition.

Nutrients	Energy (MJ/KG)	Protein (%)	Fat (%)	Fibre(%)
Starter				
Control	12.8	23.5	6.2	3.0
High fibre	12.1	22.2	6.2	5.0
Grower				
Control	12.9	21.0	6.2	3.0
High fibre	12.2	20.0	6.2	5.0
Finisher				
Control	13.1	20.0	6.2	3.0
High fibre	12.4	19.0	6.2	5.0
Withdrawal				
Control	13.1	19.4	6.2	3.0
High fibre	12.4	18.4	6.3	5.0

Ingredients	Control (%)	High fibre (%)
Wheat	64.6	59.4
Soymeal	18.1	16.4
Meat meal	6.6	6.6
Expellor canola meal	5.0	5.0
Oat hulls	-	7.0
Poultry tallow	3.8	3.8
Vitamins & minerals	1.9	1.8

Volatile fatty acid

Measurement of volatile fatty acid was performed following the technique described in Chapter 2

Microbial profiling

Enumeration of intestinal bacteria and T-RFLP analysis were performed following the methods described in Chapter 2.

Mucosal morphometric analysis

Musocal morphometre analysis followed the method described in Chapter 2.

Statistical analyses

All data except T-RFLP results were analysed using the statistical package Minitab® for Windows 12.1 (Minitab Inc., State College, USA). An analysis of variance was performed using the two way ANOVA procedure and significant differences among the treatment groups were determined by Tukey Multiple Comparison test of means. T-RFLP data were analysed as described previously.

Results

Growth performance of birds

The growth performance, feed conversion and mortality results are summarised in Table 3.2. Bird body weight was not affected at any stage of the experiment except for day 7 (Table 3.2), when birds fed the high fibre diet were significantly heavier ($P < 0.05$) than birds fed the low fibre diet (179.9 versus 175.5 g/bird). No differences were detected in time taken to reach 2.45 kg live weight amongst the birds housed on different litter and dietary treatments. The interaction between diet and litter played a significant role on FCR in the first three weeks of the experiment ($P < 0.01$). In the period to day 7, the high fibre diet reduced FCR significantly only for birds housed on paper litter, but not for the birds housed on hardwood litter. To day 21, the high fibre diet significantly increased FCR only for the birds housed on hard wood litter, but not for the birds housed on paper litter. No significant mortality differences were observed amongst the groups.

Relative organ weights

Significant differences were noted in the empty gizzard weight and small intestinal weight relative to body weight amongst the animal groups housed on different litters or fed different diets (Table 3.3). Both litter and diet had significant effects on the empty gizzard weight relative to body weight. Relative gizzard weight was significantly higher ($P < 0.001$) for birds housed on hardwood (1.73 g/100g) compared with birds housed on paper (1.44 g/100g). Similarly, relative gizzard weight was significantly higher ($P < 0.001$) for birds fed the high fibre diet (3.01 g/100g) compared with birds fed the low fibre diet (2.72 g/100g). No interactive effects between diet and litter were found on the relative weights of any of the organs. Respective relative weights of proventriculus (0.38 ± 0.01), pancreas (0.20 ± 0.01), liver (2.61 ± 0.04), spleen (0.13 ± 0.01) and bursa (0.14 ± 0.01) were not significantly affected by diet or litter treatments.

Microbial profiling

Intestinal microflora by enumeration

The counts of the total anaerobic bacteria, lactobacilli, *C. perfringens* and enterobacteria in the gizzard were not significantly different ($P > 0.05$) in birds subjected to different dietary and litter treatments (Table 3.4). However, the interaction between diet and litter had a significant effect on the counts of lactic acid bacteria in the gizzard ($P < 0.05$). High fibre diet tended to reduce the numbers of lactic acid bacteria for birds housed on paper litter, but to increase the numbers of lactic acid bacteria for birds housed on hardwood litter. In the ileum, no significant differences were observed for the total anaerobic bacteria, lactic acid bacteria, lactobacilli and *C. perfringens*. However, the numbers of total enterobacteria were significantly affected ($P < 0.001$). The high fibre diet significantly reduced coliform bacteria ($P < 0.01$) and lactose-negative bacteria ($P < 0.01$). Similarly, the high fibre diet significantly reduced the counts of the total anaerobic bacteria ($P < 0.05$) in the caecum. The number of lactic acid bacteria in the caecum of the birds housed on hardwood litter was significantly higher ($P < 0.01$) than those housed on paper litter.

Table 3.2 Growth performance of birds housed on paper or hardwood litter and fed high and low fibre diets[1].

		Paper litter		Hardwood litter		Two-way ANOVA[2]		
		High fibre diet	Low fibre diet	High fibre diet	Low fibre diet	Litter	Diet	Diet x Litter
Body weight (g)	d7	183.0±1.6b	174.7±1.6a	176.8±2.2ab	176.3±2.5ab	NS	*	NS
	d21	926.7±26	889.2±14.2	918.0±16.9	916.7±15.9	NS	NS	NS
	d35	2155.8±76.1	2094.0±73.6	2153.0±59.7	2153.2±58.1	NS	NS	NS
	d42	2803.8±98.3	2676.7±100.6	2716.0±84.1	2719.3±75.7	NS	NS	NS
Age (d)	at 2.45kg	37.4±1.3	39.0±1.3	38.5±1.1	38.5±1.0	NS	NS	NS
FCR	D0-7	0.78±0.03a	0.86±0.02b	0.82±0.01ab	0.79±0.00a	NS	NS	**
	D0-21	1.21±0.01ab	1.22±0.01b	1.23±0.01b	1.17±0.01a	NS	*	**
	D0-35	1.52±0.01	1.54±0.02	1.52±0.02	1.48±0.02	NS	NS	NS
	D0-42	1.66±0.02	1.70±0.04	1.70±0.02	1.66±0.02	NS	NS	NS
Mortility (%)	d21	1.45±0.65	2.43±1.4	2.45±1.18	0.48±0.48	NS	NS	NS
	d35	1.93±0.61	4.40±1.26	4.40±1.66	2.93±1.87	NS	NS	NS

[1] Values are means and standard errors. Values within a row not having the same letter are significantly different.

[2] NS P>0.05, * 0.05 > *P* > 0.01, ** 0.01 > *P* > 0.001, *** *P* < 0.001.

Table 3.3 Relative organ weight of birds 35d of age housed on paper hardwood litter and fed high and low fibre diets[1].

		Paper litter		Hardwood litter		Two-way ANOVA[2]		
		High fibre diet	Low fibre diet	High fibre diet	Low fibre diet	Litter	Diet	Diet x Litter
Relative to body weight (g/100g)	Proventriculus	0.40±0.02	0.40±0.03	0.36±0.01	0.35±0.02	NS	NS	NS
	Empty Gizzard	1.68±0.05bc	1.20±0.05a	1.91±0.06c	1.55±0.06b	***	***	NS
	Small Intestines	2.79±0.12ab	3.04±0.08b	2.65±0.08a	2.98±0.10ab	NS	**	NS
	Pancreas	0.20±0.01	0.19±0.01	0.20±0.01	0.20±0.01	NS	NS	NS
	Liver	2.64±0.07	2.60±0.11	2.65±0.10	2.55±0.08	NS	NS	NS
	Spleen	0.12±0.01	0.11±0	0.16±0.03	0.12±0.01	NS	NS	NS
	Bursa	0.14±0.01	0.13±0.01	0.15±0.02	0.14±0.02	NS	NS	NS

[1] Values are means and standard errors. Values within a row not having the same letter are significantly different.

[2] NS P>0.05, * 0.05 > *P* > 0.01, ** 0.01 > *P* > 0.001, *** *P* < 0.001.

Table 3.4 Microbiological counts (log10 CFU/g digesta) in intestinal content of birds 35d of age housed on paper or hardwood litter and fed high and low fibre diets[1].

		Paper litter		Hardwood litter		Two way ANOVA[2]		
		High fibre diet	Low fibre diet	High fibre diet	Low fibre diet	Litter	Diet	Diet x Litter
Gizzard	Total anaerobes	6.66±0.44	7.52±0.33	7.46±0.16	7.32±0.22	NS	NS	NS
	Lactic acid bacteria	6.62±0.42	7.53±0.29	7.50±0.17	7.08±0.17	NS	NS	*
	Lactobacilli	6.25±0.40	6.92±0.29	6.63±0.12	6.60±0.13	NS	NS	NS
	Clostridium perfringens	2.99±0.06	3.55±0.32	3.42±0.26	3.35±0.14	NS	NS	NS
	Enterobacteria	4.52±0.12	5.02±0.50	3.88±0.79	4.89±0.20	NS	NS	NS
	Coliform bacteria	4.40±0.11	4.96±0.49	3.87±0.79	4.84±0.19	NS	NS	NS
	Lactose-negative bacteria	4.10±0.26	4.69±0.60	3.17±0.13	3.93±0.28	NS	NS	NS
Ileum	Total anaerobes	8.47±0.10	8.68±0.17	8.62±0.15	8.48±0.26	NS	NS	NS
	Lactic acid bacteria	8.51±0.17	8.59±0.13	8.78±0.15	8.46±0.24	NS	NS	NS
	Lactobacilli	8.00±0.14	8.30±0.16	8.15±0.23	7.98±0.28	NS	NS	NS
	Clostridium perfringens	4.04±0.39	3.62±0.18	3.52±0.17	3.63±0.31	NS	NS	NS
	Enterobacteria	5.21±0.21a	6.65±0.24b	5.24±0.25a	6.03±0.27ab	NS	***	NS
	Coliform bacteria	5.14±0.21a	6.45±0.28b	5.21±0.26a	5.89±0.30ab	NS	**	NS
	Lactose-negative bacteria	4.06±0.27a	5.84±0.31b	4.15±0.29a	5.02±0.35ab	NS	**	NS
Caecum	Total anaerobes	8.95±0.20a	9.58±0.20b	9.10±0.04ab	9.30±0.11ab	NS	*	NS
	Lactic acid bacteria	8.80±0.13a	8.95±0.09ab	9.27±0.12b	9.22±0.08ab	**	NS	NS
	Lactobacilli	8.45±0.13	8.69±0.19	8.82±0.06	8.88±0.16	NS	NS	NS
	Clostridium perfringens	5.77±0.64	5.03±0.73	5.31±0.67	5.81±0.54	NS	NS	NS
	Enterobacteria	7.62±0.20	7.56±0.25	7.11±0.19	7.44±0.13	NS	NS	NS
	Coliform bacteria	7.49±0.16	7.45±0.25	7.05±0.18	7.32±0.14	NS	NS	NS
	Lactose-negative bacteria	6.90±0.34	6.81±0.29	5.50±0.91	6.70±0.20	NS	NS	NS

[1] Values are means and standard errors. Values within a row not having the same letter are significantly different.

[2] NS P>0.05, * 0.05 > *P* > 0.01, ** 0.01 > *P* > 0.001, *** *P* < 0.001.

Terminal-restriction fragment length polymorphism

Multivariate statistical analysis was used to investigate differences in gut microbial communities from either the ilea or caeca of birds. Factors investigated were litter/dietary fibre composition and sex of birds. No significant differences were detected in the ileal microbial community composition among litter/diet combinations (global R=0.025, P=0.110) across both sexes or between sexes of birds (global R=0.033, P=0.095) across all litter/diet treatments. However, significant differences were detected in the caecal microbial community composition among litter/diet combinations (global R=0.089, P=0.001) across both sexes and between sexes of birds (global R=0.046, P=0.034) across all litter/diet treatments. Therefore, caecal microbial communities from male (n=12/treatment) and female (n=12/treatment) were further analysed separately. Significant differences ($P < 0.05$) were detected between: birds raised on paper and fed a low fibre diet versus birds raised on wood and fed either a low or high fibre diet; and birds raised on paper and fed a high fibre diet versus birds raised on wood and fed a high fibre diet for both males (Table 3.5) and females (Table 3.6).

Table 3.5 One-way analysis of similarity (ANOSIM) of caecal microbial communities associated with litter/diet for male birds. The R statistic (bold) and significance level (italics) are shown between litter/diet treatments for the caeca. The R-statistic value describes the extent of similarity between each pair in the ANOSIM analysis, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups. Global R=0.084 and P=0.006

	Paper		Wood	
	Low fibre	High fibre	Low fibre	High fibre
Paper + low fibre		**0.009**	**0.199**	**0.223**
Paper + high fibre	*0.371*		**0.031**	**0.116**
Wood + low fibre	*0.001*	*0.236*		**-0.048**
Wood + high fibre	*0.002*	*0.023*	*0.844*	

Table 3.6 One-way ANOSIM of caecal microbial communities associated with litter/diet for female birds. The R statistic (bold) and significance level (italics) are shown between litter/diet treatments for the caeca. Global R=0.094 and P=0.001

	Paper		Wood	
	Low fibre	High fibre	Low fibre	High fibre
Paper + low fibre		**0.025**	**0.128**	**0.172**
Paper + high fibre	*0.261*		**0.064**	**0.152**
Wood + low fibre	*0.018*	*0.080*		**0.047**
Wood + high fibre	*0.002*	*0.005*	*0.167*	

Table 3.7 Two-way crossed ANOSIM of caecal microbial communities associated with sex for litter material and dietary fibre level. The global R statistic (bold) and significance level (italics) are shown for each of the factors for males and females separately.

	Litter	Diet
Female	**0.140**, *0.002*	**0.036**, *0.157*
Male	**0.157**, *0.001*	**-0.019**, *0.692*

Multivariate statistical analysis showed that the composition of the caecal microbial community for both sexes was significantly different between litter materials but not between dietary treatments (Table 3.7).

Differences in caecal microbial communities of birds raised on paper or wood litter materials, irrespective of dietary treatment, are shown for both sexes in the nMDS ordination (Fig 3.1).

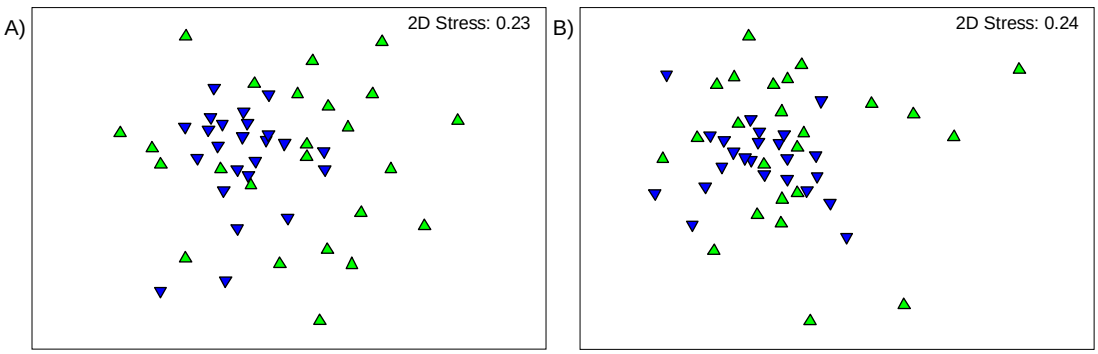

Figure 3.1 nMDS ordination of caecal microbial communities from male (A) and female (B) birds raised on either paper (△) or wood (▽) litter material irrespective of dietary treatment. The ordination is based on Bray-Curtis similarities calculated from standardised and 4th-root transformed OTU abundances. nMDS ordinations attempt to place all samples in an arbitrary two-dimensional space such that their relative distances apart match the corresponding pair-wise similarities. Hence, the closer two samples are in the ordination the more similar are their overall gut bacterial communities. "Stress" values (Kruskal's formula 1) reflect difficulty involved in compressing the sample relationship into the 2-D ordination.

Volatile fatty acid and pH analysis in intestinal organs

The volatile fatty acid levels in ileum and caeca and pH values in gizzard, ileum and caeca of 35 day old birds are summarised in Table 3.8. There were no significant differences in lactic acid and succinic acid in the ileum. In caeca, there was a significant effect of litter treatment on propionic acid ($P < 0.05$) and a significant interaction between diet and litter affected lactic acid ($P < 0.05$). Significant increase ($P < 0.05$) in propionic acid concentration was detected in the caeca of birds fed the high fibre diet (5.47 mM) compared with the birds fed the control diet (4.05 mM). The high fibre diet significantly decreased ($P < 0.05$) the concentration of lactic acid in the caeca of birds housed on paper litter, but increased the concentration of lactic acid in the caeca of birds housed on hardwood litter.

The pH value in the gizzard was significantly reduced ($P < 0.05$) by feeding the high fibre diet (3.64) compared with feeding the low fibre diet (4.18). In contrast, the pH values in the ileum and caeca were unaffected ($P > 0.05$) by diet or litter.

Table 3.8 SCFA concentration (mM) and pH in intestinal contents of birds 35d of age housed on paper or hardwood litter and fed high and low fibre diets[1].

		Paper litter		Hardwood litter		Two way ANOVA[2]		
		High fibre diet	Low fibre diet	High fibre diet	Low fibre diet	Litter	Diet	Diet x Litter
Ileum	Lactic acid	30.8±8.1	28.5±8.2	42.1±14.6	42.6±10.9	NS	NS	NS
	Succinic acid	0.25±0.11	0.22±0.08	0.35±0.16	0.33±0.1	NS	NS	NS
Caeca	Formic acid	0.66±0.03	0.70±0.02	0.70±0.03	0.47±0.19	NS	NS	NS
	Acetic acid	84.7±10.5	78.6±4.2	80.0±6.3	65.0±10.1	NS	NS	NS
	Propionic acid	4.24±1.01	3.47±0.57	6.70±0.95	4.62±0.82	*	NS	NS
	Isobutyric acid	0.32±0.10	0.35±0.09	0.44±0.08	0.40±0.12	NS	NS	NS
	Butyric acid	15.0±3.3	17.0±1.6	15.9±1.2	11.9±1.8	NS	NS	NS
	Isovaleric acid	0.13±0.05	0.17±0.05	0.19±0.03	0.62±0.39	NS	NS	NS
	Valeric acid	1.25±0.29	1.02±0.21	1.76±0.26	0.99±0.28	NS	NS	NS
	Lactic acid	0.36±0.14	0.96±0.26	0.72±0.29	0.11±0.11	NS	NS	*
	Succinic acid	14.8±1.5	18.8±4.6	18.3±4.7	8.0±4.1	NS	NS	NS
pH	Gizzard	3.42±0.15a	4.14±0.13ab	3.86±0.19ab	4.21±0.24b	NS	*	NS
	Ileum	6.80±0.25	6.86±0.31	6.61±0.30	6.27±0.28	NS	NS	NS
	Caeca	5.87±0.19	5.90±0.25	6.28±0.16	6.22±0.16	NS	NS	NS

[1] Values are means and standard errors. Values within a row not having the same letter are significantly different.

[2] NS P>0.05, * 0.05 > *P* > 0.01, ** 0.01 > *P* > 0.001, *** *P* < 0.001.

Table 3.9 Mucosal morphometry of the duodenum of birds 35d of age housed on paper or hardwood litter and fed high and low fibre diets[1].

	Paper litter		Hardwood litter		Two way ANOVA[2]		
	High fibre	Low fibre	High fibre	Low fibre	Litter	Diet	Diet x Litter
Crypt depth (μm)	277±21	285±8	316±21	362±49	NS	NS	NS
Villus length (μm)	1736±77	1679±111	1403±57	1654±83	*	NS	NS

[1] Values are means and standard errors.

[2] NS P>0.05, * 0.05 > *P* > 0.01, ** 0.01 > *P* > 0.001, *** *P* < 0.001.

Discussion

Although overall bird weight was not affected by the diet and litter treatments, diet and litter interactively affected FCR during the first 3 weeks of treatments. Based on this observation, it can be suggested that high fibre diet plays a beneficial role on FCR of birds only when birds cannot obtain hard particles from litter material, whereas it can be unfavourable if birds consume hardwood sawdust litter. As the high fibre diet in combination with hardwood litter may allow excessive fibre intake, the beneficial effect of high fibre was only produced on the birds housed on paper litter. Conversely, high dietary fibre can have negative impact on the birds housed on hardwood litter, as it was shown in this experiment that high fibre significantly increased FCR of birds raised on hardwood litter compared to those on paper litter.

Similar to the effect of forced hardwood sawdust consumption on gizzard development in our previous experiments (see Chapter 1 and 2), both high fibre diet feeding and hardwood litter consumption stimulated gizzard development in the present experiment. In contrast to (Hetland *et al*., 2005) where diet x litter interaction was found to have significant impact on gizzard weight, no interaction between diet and litter was evident here. Rather, the high fibre diet and hardwood litter had an additive effect on gizzard growth. This may suggest that the quantity or structure of fibre contained in the high fibre feed used in the present experiment improved the gizzard growth, but was insufficient, which led to birds seeking an additional source in the form of hardwood litter. In addition to the difference in gizzard weight between the treatments, high fibre diet also negatively affected pH in the gizzard. This is consistent with other studies that show stimulation of gizzard function can lead to a reduction in gizzard pH (Bjerrum *et al*., 2005, Engberg *et al.*, 2004, Hetland *et al*., 2005, Jimenez-Moreno *et al*., 2009) possibly through increased secretion HCl in the proventriculus (Duke, 1986). Surprisingly, in contrast to the effect of fibre, litter consumption did not show a significant effect on gizzard pH, although the litter effect on gizzard growth was significant. This result is also contradictory to the results described in Chapter 2 that forced consumption of hardwood sawdust litter led to a significant reduction in gizzard pH.

In other organs investigated in this study, effects of feed and litter consumption seemed insignificant on their development and pH values. Nevertheless, high fibre diet feeding reduced enterobacteria in the ileum, and hardwood litter consumption elevated the number of lactic acid bacteria in the caecum, which was confirmed by T-RFLP analysis. In addition, the high fibre diet significantly reduced total anaerobes only in chickens housed on paper litter,

and apparent consumption of hardwood litter consumption slowed the growth of duodenum villi. Conversely, no change of *C. perfringens* counts was observed among the treatments.

In conclusion, as with other experiments conducted in this project, ingestion of fibre either from litter or the diet stimulated gizzard growth, but did not result in consistent improvements in growth performance or feed conversion, and did not directly influence numbers of *C. perfringens* in gut contents.

Chapter 4: Effects of dietary fibre and litter type on necrotic enteritis

Introduction

Findings in this and the previous CRC project 03-27 clearly demonstrate that ingestion of even quite small quantities of litter with large and/or hard particles stimulates gizzard development. These findings are consistent with published studies showing increased gizzard weight and improved gizzard function in broilers given either a coarse feed or feed containing coarse components, such as whole grains (Engberg *et al*., 2004, Svihus *et al*., 2004).

Another important benefit arising from enhanced gizzard development is the potentially positive role of a functional gizzard in control of bacterial populations. Whole wheat feeding has been reported to reduce the intestinal number of lactose-negative enterobacteria (i.e., *Salmonella* spp) as well as the number of *C. perfringens* (Engberg *et al*., 2004). Similar results were observed in broiler chickens experimentally infected with *Salmonella typhimurium*. Following infection, lower numbers of *S. typhimurium* were found in the gizzard and ileum of birds receiving whole wheat as compared to pellet-fed birds. Beside this, whole wheat feeding also significantly reduced the numbers of *C. perfringens* in the intestinal tract of the birds (Bjerrum *et al*., 2005). These results indicate that a functional gizzard may act as a barrier organ preventing potential pathogenic bacteria from entering the distal digestive tract. Thus, if access to gizzard stimulating litter materials has a significant impact in broiler chickens, choosing the right litter material may have important health implications in relation to reduce the prevalence of *Salmonella spp.* in chickens and consequently in chicken meat and also in relation to reduce occurrence of necrotic enteritis which is strongly associated with *C. perfringens*. Alternatively, increased dietary fibre may have health benefits in terms of reduced enteric bacterial infections.

The aim of this experiment was to determine whether enhanced gizzard development achieved by either increased dietary fibre concentration or by ingestion of hardwood litter, had a beneficial impact on health and productivity of birds challenged with *C. perfringens.*

Materials and Methods

Birds and diets

1200 day-old Cobb male broiler chickens (Baiada hatchery, Kootingal, NSW, Australia) were raised for 4 weeks in 48 floor pens in a temperature-controlled room (33^{o}C-34^{o}C during week 1, decreased 3^{o}C each week to 24-25^{o}C by the third week) at Kirby research station at The University of New England. The birds were subjected to artificial fluorescence illumination of 16 h per day. Each cage was assigned to one of 8 treatment groups in a 2 x 2 x 2 factorial design arrangement (paper and hardwood litters, high fibre and control diets, challenged and unchallenged by Cp) with six replicates per treatment (25 birds/cage). Nutrient and dietary composition of the two experiment diets (high fibre and control diet) are shown as in Table 4.1.

Table 4.1 Diet composition of experiment 6

Ingredients	Starter		Finiser	
	Control (%)	High fibre (%)	Control (%)	High fibre (%)
Wheat	69.12	54.72	75.55	60.68
Soymeal	22.00	25.00	15.58	19.00
Cotton seed meal	5.00	5.00	5.00	5.00
Soy hulls	-	7.00	-	7.00
Oil	0.00	4.50	0.00	4.50
Lim stone/cal carb	1.10	1.10	1.10	1.10
DCP	1.70	1.70	1.70	1.70
Vitamins & minerals	0.20	0.20	0.20	0.20
Salt	0.40	0.40	0.40	0.40
Chioline	0.05	0.05	0.05	0.05
Lysine	0.30	0.25	0.30	0.25
Methionine	0.13	0.13	0.13	0.13

The birds were given the starter diet during days 1 to 7, 30% fish meal added to induce stress on GIT during days 8 to 14, 30% fish meal removed during days 15-21, and fed finisher during days 22 to 28. Birds had *ad lib* access to feed and water throughout the experiment.

Necrotic enteritis challenge

Birds were vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease. The research facility was thoroughly cleaned and disinfected prior to bird placement. To avoid cross contamination between challenged and unchallenged birds, a space of empty pen was kept on each side of the unchallenged pens.

On day 9, birds to be challenged were given per os a suspension of 2500 oocytes of *Eimeria acervulina* and *E. maxima* in 1 mL PBS (Bioproperties Pty Ltd., Glenorie, NSW). Birds in unchallenged group were given sterile PBS in place of *Eimeria*. On days 14, 15 and 16, birds to be challenged were inoculated per os with 1 mL of *C. perfringens* suspension at a concentration of 3.5 x 10^8 CFU/mL. A primary poultry isolate of *C. perfringens* type A (CSIRO Livestock Industries, Geelong) was incubated overnight at 39^{o}C in 1000 mL of thioglycollate broth containing starch (10 g/L) and casitone (5 g/L) to obtain the challenge inocula. Birds in unchallenged group received 1 mL of sterile thioglycollate broth.

Growth performance and feed conversion ratio

Feed consumption and live weight of the birds were measured on days 8, 15, 22 and 28 of the experiment. Live and adjusted body weight, body weight gain and feed conversion ratios were calculated.

Adjusted body weight gain within a particular period was defined as:

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

Adjusted FCR within a period was defined as:

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

Sample collection

On day 14 prior to *C. perfringens* inoculation, and on day 17, two birds were randomly chosen in each cage and sacrificed for sample collections. All birds were euthanised by cervical dislocation, total body weight of each bird was recorded, and proventriculus, gizzard, small intestine, pancreas, liver, spleen and bursa were removed and weighed individually.

The contents of the gizzard, ileum and caeca were pooled separately for the birds sacrificed in each replicate. pH of the contents was measured, and approximately 1 g of the digesta was collected for microbial culture, and the remaining digesta was stored for determination of volatile fatty acid analysis. Approximately 3 cm section of ilea (including digesta) mid-point between Meckel's diverticulum and caecal tonsils, and one caecal lobe (including contents) per bird were sampled for T-RFLP analysis.

Volatile fatty acid

Measurement of volatile fatty acid was performed following the technique described in Chapter 2

Microbial profiling

Enumeration of intestinal bacteria and T-RFLP analysis were performed following the methods described in Chapter 2.

Statistical analyses

All data except T-RFLP were analysed using the statistical package SAS for Windows version 9.2 (SAS Institute Inc., Cary, NC, USA). Initially, 3-way analysis of variance was performed using the GLM procedure. A reduced linear model was fitted when interactions were not significant in the initial full model analysis. Significant differences between least squares means were detected by pair-wise t-tests. As mortality, volatile fatty acids, pH of digesta and bacterial enumeration were not normally distributed, these data were analysed by the non-parametric one-way analysis of variance procedure NPAR1WAY in SAS for Windows version 9.2 (SAS Institute Inc., Cary, NC, USA). T-RFLP data were analysed as described previously

Results

Growth performance and mortality

Growth performance demonstrated by adjusted live weight and adjusted feed conversion is summarised in Table 4.2. Only main effects of dietary fibre, litter type and challenge with Cp are shown as none of the interactions were significant ($P>0.05$). Mortalities are shown in Table 4.3

Table 4.2 Main effects of dietary fibre, litter type and challenge with *C. perfringens* (Cp) on adjusted live weight and adjusted feed conversion (mean ± SE).

	Adjusted live weight (g/bird)				Adjusted feed conversion (g feed/g live weight gain)			
Effect	Day 8	Day 15	Day 22	Day 28	Days 0-8	Days 0-15	Days 0-22	Days 0-28
High fibre	167 ± 13 ***	368 ± 44	841 ± 108	1329 ± 212	1.15 ± 0.12 **	1.35 ± 0.11 *	2.39 ± 1.25 *	2.46 ± 1.17
Low fibre	136 ± 11	361 ± 33	794 ± 100	1259 ± 122	1.35 ± 0.30 **	1.28 ± 0.14	1.90 ± 0.52	2.48 ± 2.31
Paper	149 ± 21	369 ± 43	806 ± 113	1253 ± 186 *	1.35 ± 0.30	1.30 ± 0.12	2.24 ± 1.15	2.59 ± 2.43
Wood	154 ± 18	359 ± 34	829 ± 99	1332 ± 156	1.15 ± 0.09	1.33 ± 0.14	2.05 ± 0.79	2.34 ± 0.97
Challenged	149 ± 18	343 ± 27 ***	802 ± 127	1191 ± 162 ***	1.30 ± 0.31	1.37 ± 0.11 *	2.76 ± 1.09 ***	3.32 ± 2.33 ***
Unchallenged	153 ± 21	385 ± 38	834 ± 79	1392 ± 122	1.20 ± 0.15	1.26 ± 0.13	1.53 ± 0.07	1.65 ± 0.07

Means within a main effect are significantly different; * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Table 4.3 Main effects of dietary fibre, litter type and challenge with *C. perfringens* (Cp) on mortality (means ± SE)

-	Days 0-8	Days 9-15	Days 16-22	Days 23-28
High fibre	1.2 ± 0.4	26.7 ± 3.6	12.7 ± 2.0	0.3 ± 0.2 *
Low fibre	1.2 ± 0.4	19.0 ± 2.7	11.8 ± 2.4	1.5 ± 0.5
Paper	0.8 ± 0.3	23.7 ± 3.2	10.7 ± 2.0	0.7 ± 0.3
Wood	1.5 ± 0.4	22.0 ± 3.3	13.8 ± 2.4	1.2 ± 0.5
Challenged	0.8 ± 0.3	35.3 ± 2.5 ***	19.3 ± 2.0 ***	0.8 ± 0.3
Unchallenged	1.5 ± 0.4	10.3 ± 1.4	5.2 ± 1.1	1.0 ± 0.4

Means within a main effect are significantly different if * $P<0.05$, *** $P<0.001$)

High dietary fibre provided by oat hulls had no effects on adjusted live weight except for birds that were significantly heavier at day 8. Similarly, there was a significant improvement in adjusted feed conversion in the period 0-8 days of age for birds given oat hulls. However, increased dietary fibre level was disadvantageous during the periods 0-15 and 0-22 days of age. Litter type had no effects except for increased live weight at 28 days of age (Table 4.2). Challenge with Cp had deleterious effects on live weight at 15 and 28 days of age. The numerical difference in live weight at day 22 was not significant ($P>0.05$). Challenge with Cp resulted in significantly poorer feed conversion in the periods 0-15, 0-22 and 0-28 days of age.

Challenge with Cp resulted in highly significant losses of birds in the periods 9-15 days and 16-22 days (Table 4.3). Litter type had no effect on mortality. Addition of oat hulls resulted in a small but significant improvement in mortality in the period 23-28 days of age.

Organ weights

The main effects of dietary fibre, litter type and challenge with Cp on relative organ weight at day 14 are shown in Table 4.4. None of the interactions were significant ($P>0.05$). Dietary fibre and litter type had no effects on any of the organ weights except for an increase in weight of the bursa which approached significance ($P=0.052$). Challenge with Cp resulted in a highly significant increase in weight of the small intestine and a significant decrease in liver weight.

Similarly, the main effects on relative weights of proventriculus, small intestine, liver, spleen and bursa at day 17 are shown in Table 4.5. Dietary fibre and litter type had no effects, and challenge with Cp resulted in significant increases in weights of the proventriculus, small intestine and bursa.

The relative weight of the gizzard was significantly affected by interactions between diet and litter type, diet and challenge, and litter type and challenge. These results are shown in Table 4.6. The 3-way interaction was not significant ($P>0.05$). Reduction in gizzard size was significant ($P<0.05$) in birds housed on paper litter and given a low fibre diet. Birds given the high fibre diet showed a greater decline in gizzard weight when challenged compared with birds on a low fibre diet.

Table 4.4 Main effects of dietary fibre, litter type and challenge with *C. perfringens* (Cp) on relative organ weights at day 14 (means ± SE)

	Relative organ weight (g tissue/kg live weight)						
Effect	Proventriculus	Gizzard	Small intestine	Pancreas	Liver	Spleen	Bursa
High fibre	8.41 ± 1.98	37.72 ± 5.11	104.33 ± 17.89	4.82 ± 0.72	38.61 ± 3.75	1.13 ± 0.32	1.64 ± 0.48 †
Low fibre	7.58 ± 1.19	35.20 ± 5.05	99.8 ± 17.55	5.07 ± 1.37	39.69 ± 6.86	1.18 ± 0.36	1.38 ± 0.42
Paper	8.39 ± 2.03	36.21 ± 5.73	102.80 ± 18.24	4.74 ± 1.14	40.25 ± 6.19	1.18 ± 0.30	1.48 ± 0.53
Wood	7.60 ± 1.11	36.71 ± 4.69	101.34 ± 17.47	5.15 ± 1.02	38.06 ± 4.58	1.13 ± 0.37	1.55 ± 0.40
Challenged	7.83 ± 1.35	37.11 ± 5.55	110.85 ± 18.31 ***	4.91 ± 1.28	37.49 ± 4.70 *	1.21 ± 0.31	1.42 ± 0.39
Unchallenged	8.16 ± 1.95	35.81 ± 4.83	93.30 ± 11.95	4.98 ± 0.86	40.82 ± 5.83	1.10 ± 0.35	1.61 ± 0.52

Means within a main effect are significantly different; * $P<0.05$, *** $P<0.001$

† Effect of diet approached significance $P=0.052$

Table 4.5 Main effects of dietary fibre, litter type and challenge with *C. perfringens* (Cp) on relative weights of proventriculus, small intestine, liver, spleen and bursa at day 17 (means ± SE)

	Relative organ weight (g tissue/kg live weight)				
Effect	Proventriculus	Small intestine	Liver	Spleen	Bursa
High fibre	6.99 ± 1.13	97.89 ± 14.88	42.00 ± 4.44	1.10 ± 0.21	1.52 ± 0.50
Low fibre	6.80 ± 0.99	97.53 ± 16.54	44.51 ± 6.03	1.16 ± 0.29	1.50 ± 0.36
Paper	7.20 ± 1.20 *	96.43 ± 17.46	41.95 ± 4.95	1.14 ± 0.29	1.54 ± 0.48
Wood	6.60 ± 0.81	99.00 ± 13.66	44.46 ± 5.60	1.12 ± 0.21	1.49 ± 0.38
Challenged	7.21 ± 1.20 *	108.88 ± 13.28 ***	44.42 ± 5.39	1.19 ± 0.30	1.75 ± 0.40 ***
Unchallenged	6.58 ± 0.79	86.54 ± 7.64	42.09 ± 5.24	1.07 ± 0.18	1.28 ± 0.31

Means within a main effect are significantly different; * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Table 4.6 Effects of interactions between diet and litter type, diet and challenge with *C. perfringens* (Cp), and litter type and challenge on the relative weight of gizzard at day 17 (mean ± SE).

Interaction		Relative gizzard weight (g tissue/kg live weight)
Diet	Litter type	
High fibre	Paper	36.45 ± 1.55 a
High fibre	Wood	35.04 ± 1.34 ab
Low fibre	Paper	29.64 ± 1.12 c
Low fibre	Wood	33.11 ± 0.89 bc
Diet	Cp	
High fibre	Unchallenged	39.11 ± 1.41 a
High fibre	Challenged	32.38 ± 0.50 b
Low fibre	Unchallenged	32.05 ± 1.01 b
Low fibre	Challenged	30.70 ± 1.23 b
Litter type	Cp	
Paper	Unchallenged	36.31 ± 1.78 a
Paper	Challenged	29.78 ± 0.82 c
Wood	Unchallenged	34.85 ± 1.42 ab
Wood	Challenged	35.30 ± 0.80 bc

Means within an interaction with a common letter are not significantly different (P>0.05)

Table 4.7 Effect of the interaction between litter type and challenge with *C. perfringens* (Cp) on the relative weight of the pancreas at day 17 (mean ± SE).

Interaction		Relative pancreas weight (g tissue/kg live weight)
Litter type	Cp	
Paper	Unchallenged	4.75 ± 0.15 a
Paper	Challenged	4.17 ± 0.12 b
Wood	Unchallenged	4.81 ± 0.18 a
Wood	Challenged	4.98 ± 0.12 a

Means with a common letter are not significantly different (P>0.05)

Birds housed on wood litter showed no decline in gizzard size when challenged, whereas birds on paper showed a significant decline in gizzard weight.

The relative weight of the pancreas was affected by interaction between litter type and challenge (Table 4.7). Birds housed on paper litter showed a significant decline in pancreas weight when challenged, whereas pancreas weight was unaffected by challenge when birds were housed on wood litter.

Volatile fatty acid and pH analysis in intestinal organs

The effects of dietary fibre, litter type and challenge with Cp on volatile fatty acids in ileal and caecal contents of birds at day 17 are summarised in Table 4.8. High dietary fibre significantly increased acetic, proprionic, isobutyric and butyric acids in caecal contents and depressed formic acid compared with low dietary fibre. There was a tendency for high fibre to increase lactic and succinic acids in ileal contents. Paper litter depressed concentration of succinic acid in caecal contents and tended to increase acetic acid in caecal contents. The challenge procedure significantly increased lactic and succinic acids in ileal contents, and depressed concentrations of formic and acetic acids in caecal contents. There were tendencies for challenge with Cp to raise concentrations of isobutyric acid and lactic acid in caecal contents.

The effects of dietary fibre, litter type and challenge with Cp on pH of contents of gizzard, ileum and Caeca at days 14 and 17 are summarised in Table 4.9. Dietary fibre and litter type had no effects on pH in gizzard, small intestine and caeca at day 14. The challenge procedure significantly raised pH in the small intestine and caeca at day 14. On day 17, high dietary fibre significantly increased pH of the gizzard whereas challenge with Cp lowered pH of the caeca.

Table 4.8 Main effects of dietary fibre, litter type and challenge with *C. perfringens* (Cp) on volatile fatty acids (mM) in ileal and caecal contents of birds on day 17 (mean ± SE).

.	Ileum		Caeca						
Effect	Lactic	Succinic	Formic	Acetic	Propionic	Isobutyric	Butyric	Lactic	Succinic
High fibre	33.8 ± 5.2 †	0.37 ± 0.10 ‡	0.31 ± 0.05 ***	52.1 ± 4.0 **	4.8 ± 0.6 **	0.42 ± 0.04 *	9.3 ± 0.9 *	3.5 ± 2.8	8.7 ± 2.3
Low fibre	22.1 ± 3.2	0.19 ± 0.03	0.75 ± 0.07	33.4 ± 3.8	2.3 ± 0.3	0.27 ± 0.04	6.1 ± 0.7	4.3 ± 1.7	13.55 ± 3.2
Paper	24.8 ± 4.9	0.20 ± 0.05	0.49 ± 0.07	49.9 ± 4.6 ‡	4.45 ± 0.7	0.38 ± 0.05	9.0 ± 1.0	1.4 ± 0.8	7.0 ± 1.2 *
Wood	31.1 ± 4.1	0.36 ± 0.09	0.47 ± 0.09	38.7 ± 4.3	3.05 ± 0.5	0.34 ± 0.04	6.9 ± 0.9	6.8 ± 3.8	14.9 ± 3.7
Challenged	38.1 ± 4.9 ***	0.44 ± 0.09 **	0.34 ± 0.07 *	37.3 ± 4.7 *	4.1 ± 0.8	0.42 ± 0.06 ‡	7.0 ± 1.0	7.6 ± 3.8 †	10.8 ± 3.2
Unchallenged	17.8 ± 2.9	0.13 ± 0.03	0.60 ± 0.07	51.0 ± 4.1	3.6 ± 0.5	0.31 ± 0.03	8.9 ± 1.0	0.7 ± 0.3	10.4 ± 2.3

Means within a main effect are significantly different; * $P<0.05$, ** $P<0.01$, *** $P<0.001$

† Difference approached significance $P<0.06$

‡ Difference approached significance $P<0.10$

Table 4.9 Main effects of dietary fibre, litter type and challenge with *C. perfringens* (Cp) on pH of contents of gizzard, ileum and caeca at days 14 and 17 (means ± SE).

	Day 14			Day 17		
Effect	Gizzard	Small intestine	Caeca	Gizzard	Small intestine	Caeca
High fibre	3.48 ± 0.32	7.03 ± 0.64	7.13 ± 0.46	3.46 ± 0.34 *	6.50 ± 0.51	6.81 ± 0.31
Low fibre	3.47 ± 0.25	6.91 ± 0.54	7.18 ± 0.60	3.27 ± 0.25	6.31 ± 0.52	6.96 ± 0.48
Paper	3.47 ± 0.24	6.88 ± 0.57	7.06 ± 0.47	3.31 ± 0.32	6.54 ± 0.52 †	6.83 ± 0.39
Wood	3.49 ± 0.32	7.06 ± 0.60	7.26 ± 0.56	3.42 ± 0.29	6.27 ± 0.48	6.95 ± 0.41
Challenged	3.52 ± 0.23	6.68 ± 0.58 ***	6.83 ± 0.50 ***	3.37 ± 0.24	6.29 ± 0.51	7.03 ± 0.36 ***
Unchallenged	3.43 ± 0.32	7.26 ± 0.44	7.48 ± 0.28	3.36 ± 0.37	6.52 ± 0.50	6.75 ± 0.41

Means within a main effect are significantly different; * $P<0.05$, ** $P<0.01$, *** $P<0.001$

† Effect of litter type approached significance $P=0.06$

Microbial profiling

Intestinal microflora by enumeration

As the digesta samples of the birds fed low fibre diet for bacterial culture were accidently lost, the analysis of the intestinal microflora data obtained through bacterial culture was performed only on samples from birds fed the high fibre diet.

On day 14 (one day after commencement of oral gavage with Cp), birds on hardwood litter had significantly higher counts of the total anaerobes, lactobacilli and lactic acid bacteria in the ileum, and a significantly lower number of anaerobes in the caecum (table 4.10). Challenge had no effects on numbers of different organisms in the ileum, but significantly raised numbers of enterobacteria and coliform bacteria in the caecum but depressed numbers of lactose-negative enterobacteria (Table 4.10).

In contrast to day 14, at day 17 litter type had no effects on bacterial counts in the ileum or caecum (Table 4.11), whereas challenge with Cp significantly increased numbers of lactic acid bacteria, *C. perfringens*, and enterobacteria in the ileum (Table 4.11), as well as *C. perfringens*, enterobacteria and lactose-negative enterobacteria in the caecum.

Terminal-restriction fragment length polymorphism

Multivariate statistical analysis was used to investigate differences in gut microbial communities from either the ilea or caeca of birds. Factors investigated were: age and treatment (challenge/diet/litter); influence of challenge status; and influence of dietary treatment in combination with litter material. Significant differences were detected in both the ileal and caecal microbial communities associated with age across all treatments; however, no significant differences were detected between treatment groups across both ages (Table 4.12). Therefore, ileal and caecal microbial communities for birds aged 14 and 17 days were further analysed separately.

Table 4.10 Main effects of litter type and challenge with *C. perfringens* (Cp) on CFU in ileum and caecum of birds on day 14 fed high fibre diet (mean ± SE).

		Litter type			Cp		
		Paper	Hardwood	Significance	Unchallenged	Challenged	Significance
Ileum	Total Anaerobes	6.97±0.31	8±0.18	**	7.35±0.35	7.65±0.18	NS
	Lactobacilli	6.81±0.33	7.84±0.27	*	7.31±0.39	7.34±0.26	NS
	Lactic acid bacteria	7.35±0.3	8.21±0.21	*	7.8±0.33	7.75±0.21	NS
	Clostridium perfringens	0.37±0.37	0.86±0.58	NS	0.41±0.41	0.87±0.58	NS
	Enterobacteria	2.05±0.71	2.97±0.72	NS	1.82±0.65	3.33±0.74	NS
	Coliform bacteria	2.05±0.71	2.94±0.72	NS	1.8±0.64	3.33±0.74	NS
	Lactose-negative enterobacteria	0±0	0.75±0.5	NS	0.35±0.35	0.41±0.41	NS
Caecum	Total Anaerobes	9.08±0.12	8.73±0.11	*	8.91±0.1	8.91±0.16	NS
	Lactobacilli	9.01±0.13	8.91±0.15	NS	8.98±0.11	8.94±0.17	NS
	Lactic acid bacteria	8.99±0.1	9.14±0.24	NS	9.1±0.13	9.03±0.22	NS
	Clostridium perfringens	3.44±1.04	4.5±1.09	NS	4.07±1.04	3.81±1.11	NS
	Enterobacteria	7.91±0.17	7.88±0.16	NS	7.64±0.15	8.17±0.14	*
	Coliform bacteria	7.82±0.18	7.77±0.21	NS	7.52±0.19	8.1±0.16	*
	Lactose-negative enterobacteria	5.09±0.89	4.35±1.05	NS	6.2±0.58	3.14±1.09	*

Table 4.11 Main effects of litter type and challenge with *C. perfringens* (Cp) on CFU in ileum and caecum of birds on day 17 fed high fibre diet (mean ± SE).

		Litter type			Cp		
		Paper	Hardwood	Significance	Unchallenged	Challenged	Significance
Ileum	Total Anaerobes	8.35±0.27	7.89±0.22	NS	7.79±0.18	8.44±0.28	0.06
	Lactobacilli	8.5±0.32	8.14±0.16	NS	8.01±0.14	8.63±0.31	0.08
	Lactic acid bacteria	8.4±0.24	8.29±0.18	NS	8.05±0.14	8.64±0.24	*
	Clostridium perfringens	2.66±0.83	1.6±0.7	NS	0.69±0.47	3.56±0.8	**
	Enterobacteria	4.08±0.83	4.37±0.66	NS	3.03±0.83	5.53±0.28	*
	Coliform bacteria	0±0	0±0	NS	0±0	0±0	NS
	Lactose-negative enterobacteria	4.08±0.83	4.37±0.66	NS	3.03±0.83	5.53±0.28	*
Caecum	Total Anaerobes	8.43±0.77	9.35±0.09	NS	9.17±0.11	8.61±0.79	NS
	Lactobacilli	9.14±0.21	9.23±0.12	NS	9.03±0.19	9.34±0.15	NS
	Lactic acid bacteria	9.33±0.2	9.38±0.09	NS	9.22±0.2	9.5±0.08	NS
	Clostridium perfringens	4.22±1.09	2.88±1.03	NS	0.52±0.52	6.57±0.63	***
	Enterobacteria	8.15±0.2	7.7±0.37	NS	7.37±0.34	8.47±0.14	**
	Coliform bacteria	1.74±0.91	2.79±1.01	NS	2.06±0.88	2.48±1.06	NS
	Lactose-negative enterobacteria	8.06±0.19	7.8±0.39	NS	7.37±0.34	8.49±0.15	**

Table 4.12 Two-way crossed analysis of similarity (ANOSIM) of ileal and caecal microbial communities associated with age and treatment. The global R statistic (bold) and significance level (italics) are shown for each of the factors within each gut section. The R-statistic value describes the extent of similarity between each pair in the ANOSIM analysis, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups.

	Age	Treatment
Ilea	**0.769**, *0.001*	**0.015**, *0.297*
Caeca	**0.734**, *0.001*	**-0.001**, *0.493*

Influence of necrotic enteritis challenge on ileal and caecal microbial communities from birds aged 14 and 17 days were investigated for each of the four dietary treatment and litter material combinations. There were no significant differences between necrotic enteritis challenged and unchallenged controls for any of the four dietary treatment/litter material combinations in either the ileal or caecal microbial communities of birds aged 14 or 17 days (Table 4.13). Although there was a trend towards a difference (P=0.061) between necrotic enteritis challenged versus unchallenged control group in the caecal microbial communities of birds aged 17 days which had been raised on the high fibre diet and paper litter (Table 4.13).

Effects of diet/litter on ileal and caecal microbial communities were investigated for necrotic enteritis challenged (Table 4.14) and unchallenged birds (Table 4.15) separately. No significant differences were detected in the ileal or caecal microbial communities among birds raised on the four different diet/litter combinations in either the necrotic enteritis challenged (Table 4.14) or unchallenged control (Table 4.15) groups. Therefore, multivariate statistical analysis showed that the composition of both the ileal and caecal microbial community were significantly different between birds aged 14 and 17 days but not among the eight treatments, between the challenge versus unchallenged groups nor among the diet/litter groups.

OTUs contributing to the top 50% of dissimilarity in bacterial community composition between age groups were identified within the ilea (Table 4.16) and caeca (Table 4.17) of birds separately. Ten OTUs within the ilea and nine OTUs within the caeca were identified as good discriminators for birds aged 14 and 17 days. None of these age specific OTUs were common to both gut sections.

Table 4.13 One-way analysis of similarity (ANOSIM) of necrotic enteritis challenge on gut microbial communities associated with gut section and age for each diet/litter combination.

		Paper litter		Hardwood litter	
Gut section	Age	Control diet	High fibre diet	Control diet	High fibre diet
Ilea	Day 14	**-0.100**, *0.812*	**0.113**, *0.134*	**0.000**, *0.420*	**0.044**, *0.357*
	Day 17	**0.050**, *0.262*	**-0.059**, *0.639*	**-0.085**, *0.792*	**0.046**, *0.266*
Caeca	Day 14	**-0.128**, *0.885*	**-0.207**, *0.991*	**-0.032**, *0.552*	**0.126**, *0.123*
	Day17	**-0.028**, *0.595*	**0.195**, *0.061*	**-0.075**, *0.643*	**-0.064**, *0.667*

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Table 4.14 One-way ANOSIM of gut microbial communities from

necrotic enteritis challenged birds associated with dietary treatment and litter material for each gut section and age category.

	Ilea		Caeca	
	Day14	Day 17	Day 14	Day 17
Diet/Litter	**-0.090**, *0.966*	**-0.050**, *0.727*	**-0.016**, *0.586*	**-0.102**, *0.927*

Table 4.15 One-way ANOSIM of gut microbial communities from unchallenged control birds associated with dietary treatment and litter material for each gut section and age category.

	Ilea		Caeca	
	Day14	Day 17	Day 14	Day 17
Diet/Litter	**0.112**, *0.077*	**0.067**, *0.134*	**0.002**, *0.439*	**0.021**, *0.343*

Table 4.16 OTU contribution to the dissimilarity in ileal microbial communities associated with age group. Average abundance of important age related OTUs in ileal microbial community composition are shown. OTUs are listed in order of their contribution ($\bar{\delta}_i$) to the average dissimilarity $\bar{\delta}$ (=76.27%) between age groups. Percent contribution of individual OTUs and cumulative percent contribution to the top 50% of average dissimilarities are shown. OTUs identified as being good discriminators between litter treatments are marked with an asterix *

OTU	Average abundance		$\bar{\delta}_i$	$\bar{\delta}_i$/SD(δ_i)	Individual contribution %	Cumulative contribution %
	Day 14	Day 17				
566 *	2.31	0.80	6.18	1.60	8.11	8.11
568 *	0.00	1.37	5.01	1.35	6.57	14.68
936 *	1.29	0.00	4.74	2.15	6.21	20.89
578 *	0.00	0.99	3.52	1.28	4.62	25.51
284	0.96	0.00	3.47	0.89	4.55	30.06
178 *	1.12	0.96	3.35	1.43	4.39	34.45
214 *	0.00	0.87	3.10	1.50	4.06	38.51
86 *	0.00	0.78	2.82	1.43	3.70	42.21
180 *	1.95	2.59	2.74	1.06	3.59	45.80
940 *	0.00	0.71	2.53	1.15	3.32	49.12
188 *	0.02	0.70	2.46	1.14	3.22	52.35

Table 4.17 OTU contribution to the dissimilarity in caecal microbial communities associated with age. Average abundance of important age related OTUs in caecal microbial communities are shown. OTUs are listed in order of their contribution ($\bar{\delta}_i$) to the average dissimilarity $\bar{\delta}$ (=61.52%) between ages. Percent contribution of individual OTUs and cumulative percent contribution to the top 50% of average dissimilarities are shown. OTUs contributing significantly to the dissimilarity between age groups were calculated ($\bar{\delta}_i/SD(\delta_i)>1$) and are marked with an asterix *.

OTU	Average abundance		$\bar{\delta}_i$	$\bar{\delta}_i/SD(\delta_i)$	Individual contribution %	Cumulative contribution %
	Day 14	Day 17				
96 *	0.04	2.00	5.89	3.59	9.57	9.57
156 *	0.00	1.27	3.82	3.17	6.20	15.78
92 *	0.09	1.11	3.22	2.44	5.24	21.01
76 *	0.09	0.86	2.67	1.55	4.34	25.36
296 *	0.44	0.92	2.33	1.21	3.79	29.14
544 *	0.88	1.18	2.29	1.31	3.72	32.86
94 *	2.85	2.26	2.12	1.32	3.45	36.31
72 *	0.00	0.68	2.10	1.17	3.41	39.72
180	0.34	0.56	2.02	0.83	3.28	43.00
294	0.57	0.30	1.89	0.95	3.07	46.07
220	0.53	0.30	1.70	0.93	2.77	48.84
210 *	1.36	1.84	1.66	2.17	2.70	51.54

Discussion

The hypothesis tested in this study was that enhanced gizzard development through increased dietary fibre and/or ingestion of hardwood litter would provide birds with a degree of protection when exposed to a strain of *C. perfringens* strain known to induce severe necrotic enteritis, with accompanying high mortality, and depression of live weight and feed conversion. Relatively high mortality (15.5% in the period 9-22 days of age) infers cross-infection from birds in challenged pens.

The NE challenge procedure was highly successful in that birds exposed to Cp via oral gavage showed severe symptoms of necrotic enteritis, which resulted in depressed live weight gain and raised feed conversion and mortality. Anticipated protection from enhanced gizzard development was not evident, possibly because dietary fibre and litter type had no effects on relative gizzard weight of birds measured at day 14. On the other hand, there were indications at day 17 of an interaction between diet and litter type on gizzard weight. In birds raised on paper, those given a low fibre diet had smaller gizzards than those given a high fibre diet, whereas birds raised on hardwood were unaffected by dietary fibre. On the other hand, there was no difference due to dietary fibre level on gizzard size of birds subsequently challenged.

In hindsight, it would have been instructive if measurements were taken on gizzards of birds that died from NE to determine whether these were indeed lesser in weight compared with those from birds which did not succumb. In this study it was noticed that an interaction between litter type and challenge tended to produce a lower gizzard weight in birds raised on paper litter and subsequently challenged, which is consistent with the idea that a bird with a poorly developed gizzard may be disadvantaged when faced with an enteric bacterial challenge, compared with flock mates with larger gizzards.

The general lack of effects of dietary fibre and litter type on relative organ weights at days 14 and 17, with the exception of an enlarged proventriculus in birds on paper litter, and reduced gizzard weight in birds given low dietary fibre and raised on paper, is not consistent with previous findings in this project and published studies (Ali, 2008; Hetland *et al*., 2003, 2005).

High dietary fibre resulted in increased concentrations of some short chain fatty acids (acetic, proprionic, isobutyric and butyric) which have been associated with protection of birds from enteric infections due to their bactericidal properties (Ricke, 2003). Nevertheless, these increases appeared not to protect birds from Cp; however, the Cp infection was very severe in this study. In less severe cases, perhaps elevation of SCFA would offset performance and mortality losses.

In conclusion, the hypothesis that enhanced gizzard development through increased dietary fibre and/or ingestion of hardwood litter provides birds with a degree of protection from necrotic enteritis induced by *C. perfringens* was not supported by results reported in this chapter.

General Discussion

The main objective of this project was to determine whether enhanced gizzard development through stimulation by dietary fibre and/or consumption of hard litter particles would lead to improved growth, more efficient feed conversion and protection from enteric bacterial infections such as *C. perfringens*.

The experiments conducted at the Norwegian University of Life Sciences (NULS) indicate a significant consumption among broilers of litter from the floor. However, consumption of litter was low when material was provided separately in a raised feeder trough. Similar to what was demonstrated in layers (CRC project 03-27), broilers showed a 50% increase in gizzard weight when given access to litter due to the requirement for grinding of hard coarse particles. Also young broilers showed a phenomenal grinding activity to smaller median and mean particle sizes when given coarse particles in feed or litter materials. Stimulation of the gizzard activity increased the digestibility of starch. This may be caused partly by the increased surface area arising from finer grinding of feed particles due to increased gizzard size. In addition, increased digestibility and feed utilisation may be related partly to increased enzyme activity, and in particular maltase activity, in the intestine.

The severity of gizzard lesions gradually reduced with age of the chickens, whereas the *C. perfringens* counts were lowest on day 19 and increased until day 32. Inclusion of oat hulls was the most important predictor of gizzard scores. This was particularly clear on day 19. On day 32 there was a significant reduction in gizzard lesions only when birds were fed oat hulls and had access to litter. Access to litter was the most important predictor of *C. perfringens* counts in this trial. This was also particularly clear on day 19. On day 32 a specific combination of the two factors was necessary to exert a significant effect on *C. perfringens* counts. This combination (lack of added oat hulls and denied access to litter) was associated with increased *C. perfringens* counts. These results indicate that availability of non-soluble fibres can influence significantly both the severity of gizzard inflammation and the number of *C. perfringens* in caeca. Fibres in the feed and as litter appear to be interacting in their effects.

The first of three experiments in Australia was conducted at UNE and involved graded levels of hardwood saw dust litter incorporated in a commercial diet at 0, 0.75. 1.5, 3, 6 and 12% levels and fed to broiler chickens for 35 days. Inclusion of 12% hardwood sawdust in the feed significantly increased the relative weight of gizzard and proventriculus and improved apparent ileal digestibility of starch, but had no effects on feed intake, weight gain, feed conversion, or mucosal morphometry. These results are consistent with previous reports from UNE as well as in NULS experiments, that high fibre consumption from diet and litter can significantly stimulate the development of gizzard and improve apparent ileal digestibility of starch. Reduced numbers of enterobacteria in the gizzard and small intestine are indicative of the potential benefits from ingestion of hardwood litter.

The second Australian experiment was done in the Inghams Enterprise research facility in Leppington, New South Wales. The aim was to investigate the effects of two types of litter (paper and hardwood sawdust) in combination with a low and high fibre diets in a larger scale broiler growth study conducted under near commercial conditions. Overall, bird weight was not affected by the diet and litter treatment, however, diet and litter interactively affected feed conversion during the first 3 weeks of treatments. It appeared that the high fibre diet was beneficial to feed conversion of birds only when birds were unable to obtain hard particles

from litter material, whereas it was detrimental if birds were able to consume hardwood sawdust litter.

High fibre diet feeding and apparent consumption of hardwood litter stimulated gizzard development in the present experiment. In contrast to published reports that a diet x litter interaction can have a significant impact on gizzard weight, no interaction between diet and litter was evident here. Rather, the high fibre diet and hardwood litter had an additive effect on gizzard growth. This may suggest that the quantity or structure of fibre contained in the high fibre feed used in the present experiment improved the gizzard growth, but was insufficient, which led to birds seeking an additional source in the form of hardwood litter. The high fibre diet also negatively affected pH in the gizzard. This is consistent with published studies that showed stimulation of gizzard function can lead to a reduction in gizzard pH, possibly through increased secretion of HCl by the proventriculus. Surprisingly, in contrast to the effect of fibre, litter consumption did not show significant effect on gizzard pH, although the litter effect on gizzard growth was significant. This result is also contradictory to the results described in Chapter 2 that forced consumption of hardwood sawdust litter led to a significant reduction in gizzard pH.

High fibre diet feeding reduced enterobacteria in the ileum, and hardwood litter consumption elevated the number of lactic acid bacteria in the caecum, which was confirmed by T-RFLP analysis. In addition, the high fibre diet significantly reduced total anaerobes only in chickens housed on paper litter, and apparent consumption of hardwood litter consumption slowed the growth of duodenum villi. Conversely, no change of *C. perfringens* counts was observed among the treatments.

The hypothesis tested in third Australian experiment conducted at UNE was that enhanced gizzard development through increased dietary fibre and/or ingestion of hardwood litter would provide birds with a degree of protection when exposed to a strain of *C. perfringens* strain known to induce severe necrotic enteritis NE. The NE challenge procedure was highly successful in that birds exposed to Cp via oral gavage showed severe symptoms of necrotic enteritis, which resulted in depressed live weight gain and raised feed conversion and mortality. Anticipated protection from enhanced gizzard development was not evident, possibly because dietary fibre and litter type had no effects on relative gizzard weight of birds measured at day 14. On the other hand, there were indications at day 17 of an interaction between diet and litter type on gizzard weight. In birds raised on paper, those given a low fibre diet had smaller gizzards than those given a high fibre diet, whereas birds raised on hardwood were unaffected by dietary fibre. On the other hand, there was no difference due to dietary fibre level on gizzard size of birds subsequently challenged.

In conclusion, there can be little doubt that increased dietary fibre and/or ingestion of hardwood litter stimulates the development and functional capacity of the gizzard. In this project, gizzard enhancement through increased fibre ingestion led to improvements in apparent ileal starch digestibility, by a mechanism not involving pancreatic amylase activity or mucosal morphology. However, these changes in gut function did not result in improved growth or feed efficiency, and did not provide birds with a degree of protection from necrotic enteritis induced by *C. perfringens*.

Implications

It would appear from the overall results obtained in this project that the alleged benefits arising from enhance gizzard development on growth performance and reduced enteric disease have yet to be proved, at least under Australian conditions. There can be little doubt that ingestion of hard particulate matter will enhance gizzard size, but there is doubt over whether increased gizzard size equates to increased gizzard function in terms of regulating the flow of fine digesta particles into the small intestine. Coarse and hard litter components may play a beneficial role in nutrient digestion and gut health, and thus nutrient utilisation, but it has yet to be shown that these phenomena lead to improvements into economically important factors such as growth, feed conversion efficiency, and overall health and welfare of broiler chickens.

Recommendations

- There is no doubt from this and many other studies that ingestion of hard particulate matter, either from the diet or by voluntary consumption of litter, will lead to increased gizzard size. There is, however, a lack of direct evidence that increased gizzard size *per se* is responsible for beneficial effects such as improved starch digestibility and reduction of undesirable gut microbiota such as enterobacteria, *C. perfringens* and Eimeria spp. This is an area that warrants further investigation.

- The lack of consistency of increased gizzard size on economically important factors such as growth rate, feed conversion, and mortality and morbidity is a concern, and worthy of further attention.

- Further work is needed to determine whether enhanced gizzard function (as opposed to increased gizzard size) is protective of gut health in birds subjected to less severe challenges from intestinal pathogens than was achieved in the project.

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