Improved performance of broilers by structural manipulation of feed: Evidence from gizzard development, nutrient digestibility, gut microflora and gene expression

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Declaration

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree qualification.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

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Preface

This thesis has been written and edited in a journal article format. I have made every effort to minimise the repetition of materials between chapters. However, some overlap remains, particularly in the methodology sections.

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List of Abbreviations

GDM	Geometric mean diameter
GIT	Gastrointestinal tract
OH	Oat hulls
SB	Sugarcane bagasse
SBP	Sugar beet pulp
SFH	Sunflower hulls
RH	Rice hulls
NE	Necrotic enteritis
CC	Coarsely ground corn
FC	Finely ground corn
CGC	Coarsely ground corn
FGC	Finely ground corn
N	Nitrogen
AME	Apparent metabolizable energy
AMEn	Apparent metabolizable energy corrected to nitrogen equilibrium
NSP	Non-starch polysaccharides
CCK	Cholecystokinin
VIP	Vasoactive intestinal peptide
GIP	Gastrin releasing peptide
Cp	Clostridium perfringens
FPD	Footpad dermatitis
HB	Huck burn
BB	Breast blister
GLM	General linear method
AEC	Animal Ethics Committee
ANOVA	Analysis of variance
SCFAs	Short chain fatty acids
FCR	Feed conversion ratio
SID	Standard ileal digestible
dM+C	Digestible methionine + cysteine
dArg	Digestible arginine
dLys	Digestible lysine

dThr	Digestible threonine
D	Day
g	Gram
h	Hour, hours
kg	Kilogram, kilograms
m	Metre, metres
min	Minute, minutes
mL	Millilitre, mililitres
spp.	Species
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
F	Forward
R	Reverse
Ta	Annealing temperature
MC	moisture content
%	Percent
Na	Sodium
ATP	Adenosine triphosphate
bp	Base pair
LSD	Least Significant Difference
ATP1A1	ATPase Na+/K+ transporting subunit alpha 1
AMY2A	Pancreatic alpha 2A amylase
APN	Aminopeptidase N
ASCT1	Alanine, serine, cysteine, and threonine transporter
B^0AT	Solute carrier family 6, member 19
bo,+AT	Solute carrier family 7, member 9
CAT1	Cationic amino acid transporter-1
CAT2	Cationic amino acid transporter-2
CCK	Cholecystokinin
CCK1R	Cholecystokinin type 1 receptor
CELA1	Chymotrypsin-like elastase family, member 1
CELA2A	Chymotrypsin like elastase family member 2A
EAAT3	Excitatory amino acid transporter 3

GLUT1	Glucose transporter-1
GLUT2	Glucose transporter-2
LAT1	L type amino acid transporter-1
PGA5	Pepsinogen A
PGC	Pepsinogen C
PepT1	Peptide transporter-1
PepT2	Peptide transporter-2
PNLIP	Pancreatic lipase
rBAT	Solute carrier family 3, member1
SI	Sucrase isomaltase
y+LAT1	y^+ L amino acid transporter-1
y+LAT2	y^+ L amino acid transporter-2
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
TBP	TATA-Box binding protein

List of publications

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Summary

The legislation to ban the use of in-feed antibiotics in the poultry industry in European Union and voluntarily removal of antibiotics in animal feed have led to the emergence of wet litter problems, imbalanced gut microflora and reduced nutrient digestibility due to enteric disorders such as necrotic enteritis. Thus, the performance, health and welfare of broilers are affected and the profitability in the industry compromised. There has been a concerted effort to find alternative strategies to modulate gut microflora, augment the immune response and reduce pathogens through management and nutritional intervention, such as the inclusion of fibre and large particle size in diet, in an attempt to improve health and digestive efficiency of broilers. The current thesis examined a variety of strategies to minimise and tackle the issues that raised after the ban of in-feed antibiotics in the poultry industry.

Chapter 1 presents the summary of literature related to fiber and ingredient particle size and their potential roles in poultry nutrition and management.

Chapter 2 examined the effect of pelleted wheat straw as a bedding material on broiler performance, gut microflora and welfare in contrast to other litter sources commonly used in the broiler industry. The feed conversion ratio (FCR) of birds reared on pelleted straw was improved compared ($P<0.05$) to that of birds raised on rice hulls. However, it was observed that the birds reared on wood shavings had higher relative gizzard weight at d 24 compared to those reared on pelleted straw ($P<0.05$). Further, birds reared on pelleted wheat straw had a lower incidence of foot pad lesions than those on chopped straw and shredded paper on d 24 ($P<0.001$) and 29 ($P<0.01$). The study demonstrated the potential benefits to using pelleted wheat straw as a bedding material.

Chapter 3 investigated the effect of free choice oat hulls (OH) on performance, gut microflora and gizzard development in broilers during a mild (subclinical) necrotic enteritis (NE) challenge. On d 16, birds given OH had lower feed intake ($P<0.05$) and tended to have lower ($P=0.062$) FCR compared to those without access to OH. Birds accessed OH had heavier gizzards ($P<0.05$) compared to those without OH at d 35. The OH fed birds had an elevated caecal succinic acid concentration only in the unchallenged group. This study indicated a positive role of OH through improved

gizzard function and increased succinic acid in the gut but its role in controlling NE was not observed.

Chapter 4 evaluated the effect of a lignocellulose-rich fiber source and corn particle sizes and their interactions on growth performance, caecal microflora, nutrient digestibility, gizzard development and litter quality in broilers. The combination of coarsely ground corn (CC) and 2% of lignocellulose improved FCR at d 10 ($P<0.05$). Birds fed CC had lower FCR than those fed FC ($P<0.05$) at d 24 and 35. Ileal gross energy and protein digestibility increased in birds fed CC compared with those fed FC at d 24 ($P<0.05$). Relative gizzard weight was higher ($P<0.05$) in birds fed CC as compared to those fed FC. Birds consumed 2% dietary lignocellulose had decreased counts of cecal *Clostridium* spp. compared to those with 1% lignocellulose ($P<0.05$) at d 24. On d 35, lignocellulose had significantly decreased ($P<0.05$) litter moisture content compared to the control. These findings suggest that CC plays a significant role in improving broiler performance and gizzard development whereas dietary lignocellulose is beneficial to litter quality.

Chapter 5 examined the effect of sugarcane bagasse (SB) and corn particle size on growth performance, litter quality, gizzard development, gut microflora, and welfare of birds fed normal and high salt feed. Interactions on performance were observed between SB supplementation and corn particle size on d 24. FCR was improved by 2% SB supplementation in birds fed CC but increased in birds fed FC ($P<0.001$) on d 24. Weight gain of birds fed 2% SB was higher in birds fed CC but not in those fed FC ($P<0.05$). On d 35, birds fed 2% SB had a higher weight gain ($P<0.001$) compared to those without SB. SB reduced gizzard pH and increased the relative gizzard weight in birds fed FC diet but not CC diet ($P<0.05$). In addition, ileal *Bacillus* spp. were increased in birds fed SB ($P<0.05$) compared to those fed control diet on d 24. These findings suggest that SB independently or in combination with CC improves performance in older birds regardless of Na level in diets possibly through improved gizzard development and gut microflora of birds.

Chapter 6 investigated the effect of dietary SB and corn particle size on digestibility of nutrients in birds fed normal and high salt feed. The inclusion of CC in the diet resulted in improved ileal protein digestibility ($P<0.05$), while the addition of 2% dietary SB increased starch digestibility in the duodenum ($P<0.05$), distal jejunum

(P<0.001) and distal ileum (P<0.001) and increased protein digestibility in distal ileum (P<0.01). SB only increased ileal energy digestibility in birds fed the diet with FC and 0.16% Na. These findings demonstrate that SB supplementation and CC inclusion in diet are able to improve nutrient digestibility. It can be recommended that appropriate fibre sources such as SB and coarsely ground corn be used in broiler diets to improve feed efficiency and growth performance.

Chapter 7 evaluated how fibre supplementation and corn particle size affect expression of genes encoding digestive enzymes and nutrient transporters in digestive system. The addition of 2% SB upregulated pepsinogen A and C in pancreas of only CC fed birds. The mRNA expression of both pepsinogen A ($P < 0.01$ and $R = -0.534$) and C ($P < 0.01$ and $R = -0.588$) were negatively correlated with FCR on d24. The addition of 2% SB also upregulated pancreatic amylase (AMY2A) and intestinal cationic amino acid transporter-1 (CAT1). The inclusion of CC in broiler diet upregulated duodenal amino peptidase N (AMN), jejunal alanine, serine, cysteine and threonine transporter-1 (ASCT1), and ileal peptide transporter-2 (PepT2). These findings provide evidences that the dietary fibre and particle size improve performance of birds through the modulation of at least some of the digestive enzymes and nutrient transporters.

Overall, the application of different fibre sources in diets, as free choice or in litter has been proved to benefit broiler chickens with regard to performance, health and/or welfare. Similarly, the inclusion of coarsely ground corn showed same effect independently or in combination with fibre. These benefits appeared to be the results of improved gizzard development, more optimised gut microflora, increased digestibility of nutrients and energy, and modulated expression of genes encoding digestive enzymes and nutrient transporters in the birds. The outcomes of the thesis provide evidences that the broiler industry can improve bird performance through the manipulation of physical structural components of feed as a measure of management to tackle the issues caused by the removal of antibiotics from animal feed.

Chapter 1

General introduction

Poultry production has undergone a substantial increase compared to other agriculture and animal food-producing sectors during the past half century throughout the world. There are many challenges that affected the evolution of the poultry industry in the past decade. Pressures from increasing feed cost, pursuing better feed efficiency and live performance, animal welfare, and environmental impact relative to poultry production have been recognized as main challenges to the poultry industry. In addition, recent consumer shift from interest in productive efficiency to public security resulted in worldwide concern about the use of in-feed antimicrobials, resulting in a complete ban in the use of in-feed antibiotics in the European Union in 2006. A number of problems have arisen as a result of the ban, namely the emergence of enteric and systemic diseases and issues with wet litter and bird welfare and thus lessen the production efficiency (Timbermont, et al., 2011; Collett, 2012; M'Sadeq, et al., 2015). As a consequence, there has been a continuous endeavour for nutritionists worldwide to find alternative strategies to modulate gastrointestinal tract and gut microflora, augment the immune response and reduce pathogens through management, nutritional intervention in an attempt to improve health and digestive efficiency of broilers. A range of nutritional interventions including increasing grain particle size, using whole grain, and including different sources or levels of dietary fiber and other feed additives, such as probiotics, bacteriophages, enzymes and phytobiotics are currently under investigation (Caly, et al., 2015; Jiménez-Moreno, et al., 2016; Zaefarian, et al., 2016). A well-developed gizzard that achieved by feeding coarse ingredient particles and different sources of fiber has been reported to improve gut health and bird performance (Choct, 2009). The major focus of this thesis is to investigate the efficacy of different sources of fiber and corn particle size on gizzard development, gut health and productivity in broilers. The specific objectives of this study were to:

1. Evaluate the impact of pelleted wheat straw as a new source of bedding material on broiler performance, gut microflora, gizzard function and welfare in contrast to other litter sources commonly used in the broiler industry.
2. Assess the response of broilers under subclinical NE challenge to free choice oat hulls on performance, gizzard development and caecal microflora.

3. Investigate the effect of different sources of fiber and corn particle sizes and their interactions on growth performance, nutrient digestibility, microflora, gizzard development and litter quality in broiler chickens.
4. Evaluate the effects of increased corn particle size and inclusion of sugarcane bagasse on expression of genes encoding digestive enzymes and nutrient transporters in broilers.

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Chapter 4

The response of broilers to oat hulls as free choice feeding under a mild necrotic enteritis challenge

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4.1 Abstract

Structural fibres have been reported to enhance performance, intestinal function and modify the composition and quantity of the microbial population in the chicken gastrointestinal tract. It is hypothesised that insoluble fibre in oat hulls may be able to improve gut health and thus reduce intestinal Clostridium perfringens number. This study assessed the impact of free choice oat hulls (OH) on performance and gut microbiota in broilers during a mild (subclinical) necrotic enteritis (NE) challenge. A total of 240 day-old male Ross 308 chicks were assigned to 24 cages in a 2 × 2 factorial arrangement of treatments. Factors were: challenge - or +; and OH - or +. On d 16, challenged birds had lower weight gain and feed intake ($P<0.05$) compared to unchallenged birds. On d 16, birds given OH had lower feed intake ($P<0.05$) and tended to have lower ($P = 0.062$) feed conversion ratio (FCR) compared to those without access to OH. Bird performance, however, was not affected by OH nor by challenge on d 24 and 35. The birds given OH had heavier gizzards ($P<0.05$) compared to those without OH at d 35 but not at d 13 or 16. Increased numbers of C. perfringens ($P<0.001$) and reduced numbers ($P<0.05$) of Lactobacillus and Salmonellae were observed in the caecal contents of challenged birds on d 16. Challenged birds had a lower concentration of caecal acetic acid ($P<0.01$) compared to unchallenged birds at d 16. The birds given OH had lower concentrations of caecal acetic ($P<0.05$), propionic ($P<0.05$), and valeric ($P<0.01$) acids compared to those without access to OH. An OH by challenge interaction on succinic acid concentration was observed on d 16 ($P<0.05$). The OH fed birds had an elevated caecal succinic acid concentration only in the unchallenged group. This study indicated a positive role of OH through improved gizzard function and increased succinic acid in the gut but its role in controlling NE was not conclusive.

Key words: Necrotic enteritis, Caecal microflora, Performance, Gizzard, Oat hulls

4.2 Introduction

Necrotic enteritis (NE) is a widespread and economically important enteric disease in broiler flocks (Van der Sluis, 2000). The financial cost of NE has been estimated to be US $6 billion per year to the world's poultry industry (Wade and Keyburn, 2015). In broiler flocks, the mild (subclinical) form of NE, which is not manifested by clear signs or symptoms (Skinner et al., 2010), is more common than the clinical form (Kaldhusdal, 2000), which has visible signs of the disease and leads to high mortality. The subclinical NE mostly results in higher feed conversion ratio and lower gain, which cannot be regained via compensatory growth in the modern broiler, thus leading to massive economic losses (Kaldhusdal and Hofshagen, 1992; Kaldhusdal et al., 1999; Engström et al., 2003). In addition, the disease causes wet litter (Williams, 2005) and possible contamination of poultry products for human consumption (Timbermont et al., 2011). NE is known to change the composition and quantity of the gastrointestinal flora (Wu et al., 2014; Stanley et al., 2014). It was reported that counts of *lactobacillus* decreased following NE challenge (Dahiya et al., 2005; Feng et al., 2010). The causative agent of NE is *Clostridium perfringens,* a ubiquitous bacterium found in soil, dust, feed, animal feces, used poultry litter, and the intestines of normally healthy animals (Ewing and Cole, 1994; Wages and Opengart, 2003). Although *C. perfringens* is the primary causative agent of subclinical NE, contributory factors alter the intestinal microbial balance to favor the proliferation of *C. perfringens* allowing them to colonise the upper intestines. Predisposing factors include diet composition, management related stress, and presence of other intestinal diseases such as coccidiosis (*Eimeria maxima and E. acervulina*) (Shane et al., 1985; Kaldhusdal and Løvland, 2000; McDevitt et al., 2006; Wu et al., 2014). Traditionally, NE has been controlled by in-feed antibiotics (Williams, 2005). However, consumer pressure and legislation to ban the use of in-feed antibiotics in the poultry industry in the EU has increased the incidence of enteric disorders including NE in broilers (Caly et al., 2015). The strategies to control NE without the reliance on antibiotics have focused on improving gut health using a range of feed additives, such as probiotics, prebiotics, bacteriophages, enzymes and phytobiotics (Caly et al., 2015; M'Sadeq et al., 2015). It is speculated that structural fibres play an important role in enhancing performance and intestinal health by selectively stimulating the growth and/or activity of beneficial bacteria in

gut. Insoluble fibre, a key component of structural fibres, certainly improves intestinal barrier function and host immunity by reducing pathogen load (e.g., Clostridia), and enhancing short chain fatty acid production (Glenn and Roberfroid, 1995; Amerah et al., 2009; Choct, 2009; Slavin, 2013; Jiménez-Moreno et al., 2016; Kheravii et al., 2016b). It has also been reported that feeding birds insoluble fibre improves gizzard function and stimulates mucosal layer, and increases gut motility, thereby reducing *C. perfringens* adhering to the mucosal surface in the distal part of the gastrointestinal tract (Kalmendal et al., 2011). Wu et al. (2011) and Kheravii et al. (2017) reported that ingestion of litter by broilers enhances gizzard development in birds. Furthermore, a well-developed gizzard increases digesta retention time in the proximal part of the gastrointestinal tract and enhances secretion of HCl in the proventriculus, thereby resulting in reduced gizzard pH (Kimiaeitalab et al., 2016). This, in turn, has an antimicrobial impact on pathogenic bacteria entering the distal part of digestive tract (Engberg et al., 2002). Jiménez Moreno et al. (2011) reported that the inclusion of oat hulls, which contain 847 g/kg lignified insoluble fibre (Hetland and Svihus, 2001), in broiler diets improved gizzard function and reduced the counts of caecal *C. perfringens*. The present study assessed the response of broilers under subclinical NE challenge to free choice oat hulls (OH) on performance, gizzard development and caecal microflora.

4.3 Material and methods

4.3.1 Design and Husbandry

A total of 240 1-d-old male Ross 308 chicks were obtained on the day of hatch from the Baiada Hatchery in Tamworth, NSW, Australia. On arrival, chicks were randomly allocated to 24 multi-tiered brooder cages measuring (600 × 420 × 23 cm) with six replicates per treatment and 10 birds per replicate. The cages were physically partitioned according to the challenge treatments (12 cage per partition). A 2 × 2 factorial arrangement of treatments was employed with the factors being: challenge – without (-) or with (+); and free choice OH – without (-) or with (+). Each cage was equipped with 2 nipple drinkers and a trough feeder, and water and feed were available *ad libitum*. The feeders were divided into two portions: first portion had 4 access holes to the feed and the second portion either had two holes to access the free choice OH (OH+) or blocked holes (OH-). The lighting, relative

humidity and temperature followed the Ross 308 strain (Aviagen, 2014) management guidelines.

4.3.2 Diets and Oat hulls

All birds were fed the same diets in three phases: starter (placement to d 10), grower (d 10–24), and finisher (d 24–35). The diets were formulated with wheat, sorghum, soybean meal, meat meal, and canola meal according to the Ross 308 nutrient specifications (Table 4.1). The diets were thoroughly mixed and pelleted at 65^{0}C. The diets were also assayed for crude protein, fat, and fibre, ash, NDF, ADF, moisture content, insoluble non-starch polysaccharides (NSP), soluble NSP and free sugars (Table 1). The OH was obtained locally from a commercial supplier (Grazag Company, Armidale, NSW, Australia).

4.3.3 Animal ethics

This experiment was approved by the Animal Ethic Committee of the University of New England (Approval No: AEC14-068). All bird management procedures including health care, husbandry and use of laboratory animals fulfilled the requirements of the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

4.3.4 Bird performance

On d 16, 24 and 35, birds and leftover feed were weighed and mortality recorded daily. Feed conversion ratio was adjusted for mortality. Live bird feed intake was calculated as FCR × weight gain during the measured periods.

4.3.5 Lesion scoring

On d16, two birds were randomly selected from each pen and euthanised by cervical dislocation. Duodenum, jejunum and ileum were excised and scored following the lesion scoring system reported by Prescott et al. (1978).

Table 4. 1 Ingredient composition, and calculated and analysed nutrients of the experimental diets (g/kg)

Ingredient Name	Starter	Grower	Finisher
Sorghum	250	285	306
Wheat	319	333	330
Soycomil K SPC	-	20.0	-
[1]SBM	247	107	85.2
Canola meal solvent	70.3	127	141
Meat meal	40.0	60.0	65.0
Canola oil	40.0	45.0	55.8
Limestone	11.03	4.87	3.95
Dicalcium Phosphate 18P/21Ca	8.42	4.37	1.92
Xylanase powder (500g/mt)	0.500	0.500	0.500
NaCl	1.076	0.665	0.584
Na bicarb	2.00	2.00	2.00
[2]Vit (0.5 kg/mt inclusion)	0.500	0.500	0.500
[3]TM (0.75 kg/mt inclusion)	0.750	0.750	0.750
Choline Cl 60%	0.824	0.902	0.838
L-lysine HCl 78.4	3.84	3.69	2.59
DL-methionine	3.52	2.62	2.08
L-threonine	1.96	1.50	1.19
Calculated nutrients, g/kg			
ME, kcal/kg	3,025	3,130	3,178
Crude protein	230	216	202
Crude fat	63.6	73.6	82.9
Crude fibre	27.0	28.8	28.9
Digestible arginine	13.1	11.4	10.2
Digestible lysine	12.9	11.5	9.70
Digestible methionine	6.50	5.53	4.85
Digestible Met + Cys	9.40	8.40	7.60
Digestible tryptophan	2.39	1.93	1.73
Digestible isoleucine	8.60	7.52	6.79
Digestible threonine	8.30	7.30	6.50
Digestible valine	9.83	8.86	8.16
NDF	110	122	127
ADF	45.28	51.29	53.74
Soluble NSP	8.09	8.60	8.52
Insoluble NSP	61.6	63.0	61.1
Calcium	10.5	9.00	8.50
Phosphorus availability	4.50	4.50	4.20
Sodium	1.60	1.60	1.60
Chloride	2.18	2.00	1.73
Choline, mg/kg	1,600	1,500	1,400
Linoleic (18:2)	19.2	20.5	21.5
Analysed nutrients composition (g/kg) *"as is"* basis			
Crude protein	237	224	215
Crude fat	62.0	74.2	82.5
Crude fibre	29.9	34.6	34.9

Ash	60.7	54.6	47.9
NDF	105	109	142
ADF	44.9	56.0	57.0
Total insoluble NSP	70.4	70.4	73.6
Total soluble NSP	7.44	8.10	8.35
Free sugar	33.0	29.3	25.8

[1]Soybean meal.
[2] Vitamin concentrate supplied per kilogram of diet: retinol, 12000 IU; cholecalciferol, 5000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 µg; biotin, 200 µg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.
[3] Trace mineral premix supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

4.3.6 Analysis of short chain fatty acids (SCFAs)

The caecal SCFAs was analysed according to method described by Jensen et al. (1995) with minor modifications. Frozen caecal samples were defrosted and homogenised. Approximately 1 g of homogenised caecal sample from two birds at d 16 was suspended in 1 mL of internal standard (0.01 M 2-ethylbutyric acid), thoroughly mixed and centrifuged at 2050 × g for 15 min at 5 °C. Then, 2.5 mL of diethyl ether and 0.5 mL of concentrated HCl (36%) were added to 1 mL of the supernatant and thoroughly mixed by using a vortex mixer. Meanwhile, using the same method, a blank and an internal standard solution were prepared by replacing 1mL of the supernatant with the same amount of water and standard acid mixture respectively. The mixture was centrifuged at 2050 × g at 5°C for 15 min. An aliquot of 400 µL of the supernatant was transferred to a gas chromatograph vial (2 mL) and mixed with 40 µL of N-tert-butyldimethlsilyl-N-methyltrifuoroacetamide (MTBSTFA) and incubated at 80 °C for 20 min. The mixture was left at room temperature for at least 48 hours. The caecal SCFAs were measured using a Varian CP3400 CX gas Chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA).

4.3.7 Gizzard measurements

On d 13, 16, and 35, empty gizzards and gizzard contents were weighed and recorded along with bird weight using a digital scale. Gizzard contents of 2 birds were collected and homogenised to measure pH by using a calibrated pH meter (EcoScan pH 6). The relative gizzard weight was calculated as mass per unit of live body weight (g/100 g of live body weight).

4.3.8 Caecal content collection

On d 16, the caecal contents from two birds per pen were pooled and mixed in specimen containers and stored at -20°C until SCFA quantification. During collection of the samples, approximately 2 g of caecal digesta from the homogenised caecal content in specimen containers were transferred into a 2-mL Eppendorf cap lock tube, snap-frozen in liquid N_2, and stored at -20˚C until DNA extraction for bacterial quantification.

4.3.9 Extraction of caecal DNA

Caecal DNA was extracted following the protocol of ISOLATE II Plant DNA Kit (Bioline, NSW, Australia) with slightly modification. Approximately 200 mg of freshly defrosted caecal content were placed in a 2-mL Eppendorf tube contained 300 mg of glass beads. An aliquot of 450 µl Lysis Buffer PA1 was added to the samples and thoroughly mixed with a vortex mixer. The samples were transferred to a block bead mill (Retsch GmbH & Co, Haan, Germany) to disrupt at a frequency of 30/s for 5 min prior to being heated at 95°C for 5 min. The digesta was lysed and homogenised after adding 200 µL and then 100 µL of Extraction Buffer with vortex-mixing following each addition. An aliquot of 10 µL of RNase was added to 600 µL of the lysate in a 1.5-mL microcentrifuge tube in order to remove RNA. The solution was incubated at 65˚C for 10 min. The incubated mixture was centrifuged for 1 min at 11,000 × g to pellet potential impurities. An aliquot of 450 µL of Binding Buffer was used to capture DNA by vortexing thoroughly and then centrifuging for 1 min at 11,000 × g. Then 400 and 700 µL of Wash Buffer PAW1 and PAW2 respectively were added at independent steps to purify DNA, centrifuged for 1 min at 11,000 × g to remove the wash buffer and to dry the silica membrane completely. An aliquot of 50 µL of Elution Buffer was used to elute DNA into a 1.5-mL Eppendorf tube.

4.3.10 Quantification of caecal bacteria

The quantification of caecal bacterial groups, *Bifidobacterium* spp., *Lactobacillus* spp., *Bacillus* spp., *Ruminococcus* spp., *Bacteroides* spp., *Salmonella* spp., *C. perfringens and* Enterobacteriaceae were achieved by using the methods of Wise and Siragusa (2006). The extracted caecal DNA was diluted twenty times in autoclaved Milli-Q water. The Rotorgene 6000 real-time PCR machine (Corbett, Sydney,

Australia) was employed for qPCR assay of the desired bacteria from the extracted cecal DNA. The PCR was performed in duplicate for each sample in 10 µL of reaction. For PCR reactions, a SYBR Green containing Mix (SensiMix SYBR No-Rox, Bioline, Sydney, Australia) was applied for all groups of bacteria except *C. perfringens* for which the SensiFAST Probe SYBR No-ROX (Bioline, Sysdney, Australia) was used. The reaction in a volume of 10 µL contained 5 µL of 2× SensiMix, 300 mM of each primer and 2 µL of DNA template. However, for *C. perfringens*, the reaction in a volume of 10 µL contained 5 µL of 2× SensiFAST Probe, 400 mM of each primer, 500 mM of probe and 2 µL of DNA template. The below specific 16S rRNA primers and/or probe were used for quantification of different groups of bacteria: GCG TCC GCT GTG GGC and CTT CTC CGG CAT GGT GTT G for *Bifidobacterium spp*. (Requena, et al, 2002); CAC CGC TAC ACA TGG AG and AGC AGT AGG GAA TCT TCC A for *Lactobacillus spp*. (Wise and Siragusa, 2007); GCA ACG AGC GCA ACC CTT GA and TCA TCC CCA CCT TCC TCC GGT for *Bacillus* spp. (Zhang, et al., 2015); GGC GGC YTR CTG GGC TTT and CCA GGT GGA TWA CTT ATT GTG TTA A for *Ruminococcus* spp. (Ramirez-Farias, et al., 2009); GAG AGG AAG GTC CCC CAC and CGC TAC TTG GCT GGT TCA G for *Bacteroides* spp. (Layton, et al., 2006); CGT TTC CTG CGG TAC TGT TAA TT and AGA CGG CTG GTA CTG ATC GAT AA for *Salmonella spp*.; CAT TGA CGT TAC CCG CAG AAG AAG C and CTC TAC GAG ACT CAA GCT TGC for the *Enterobacteriaceae*; CGG YCC AGA CTC CTA CGG G and TTA CCG CGG CTG CTG GCA C for the total bacteria; and CGC ATA ACG TTG AAA GAT GG and CCT TGG TAG GCC GTT ACC C; TaqMan probe: 5'-FAM-TCA TCA TTC AAC CAA AGG AGC AAT CC-TAMRA-3' for the *C. perfringens*:

The PCR was performed according to the following cycles: DNA templates were denatured at 95 ˚C for 10 min followed by 40 cycles of denaturation at 95 ˚C for 30 sec and annealing 63 ˚C for 45 and extension at 78 ˚C for 45 sec for all the primers except *Salmonella* spp and *C. perfringens* primers which the annealing/extension was conducted at 56/72 and 55/70 ˚C respectively. A threshold cycle averaged from the duplicate samples was used for data analysis. Serial dilutions of linearised plasmid DNA (pCR®4-TOPO Vector, Life Technologies, Carlsbad, USA) inserted with respective bacterial amplicons were used to construct a standard curve. The

concentrations of the plasmid DNA were measured using NanoDrop ND-8000 (Thermo Fisher Scientific, Waltham, USA) prior to the serial dilutions. The number of target DNA copies was calculated from the mass of DNA taking into account the size of the amplicon insert in the plasmid. Bacteria numbers were expressed as $\log_{10}$ (genomic DNA copy number)/g digesta.

4.3.11 NE challenge

The NE challenge was performed according to Wu et al. (2014) with modifications. *Eimeria acervulina* (batch E1-4/14-042), *E. brunetti* (batch E9-7/14-012A) and *E. maxima* (batch E2-6/14-043) were obtained from Bioproperties Pty. Ltd. (Glenorie, NSW, Australia). *C. perfringens* type A strain EHE-NE18 (CSIRO Livestock Industries, Geelong, Australia) was incubated overnight at 39˚C in 100 mL of sterile thioglycollate broth (USP alternative; Oxoid) followed by subsequent overnight incubation of 1 mL of the previous culture in 100 mL of cooked meat medium (Oxoid), and then in 700 mL of thioglycollate broth (USP alternative; Oxoid) containing starch (10 g/L) and pancreatic digest of casein (5 g/L) to obtain the challenge inoculum. On d 9, birds in the challenge room were inoculated with 5000 sporulated oocysts each of *E. maxima* and *E. acervulina* and 2500 sporulated oocysts of *E. brunetti* in 1 mL of 1% (w/v) sterile saline. Unchallenged birds received 1 mL of 1% (w/v) sterile saline as a sham treatment. On d 14 and 15, the challenge group were inoculated *per os* with 2 mL of *C. perfringens* suspension (10×10^7 CFU/mL).

4.3.12 Statistical analysis

All data were analysed using the General Linear Models (GLM) procedure of SPSS statistics version 22 (IBM, Armonk, New York, US) for the main effect of challenge and free choice OH and interactions. SCFA data were transformed as ($\log_{10}$+1) to achieve normal distribution of the data. Differences between mean values were determined using the Tukey HSD test at the level of $P < 0.05$.

4.4 Results

4.4.1 Broiler performance

Performance results are presented in Table 4.2. On d 16, challenged birds had lower weight gain and feed intake ($P<0.05$) compared to unchallenged birds. The FCR in

challenged birds tended to be higher (P = 0.052) compared to unchallenged birds. On d 16, birds given OH had lower feed intake (P<0.05) compared to those without access to OH. Birds with access to OH tended (P = 0.062) to have lower FCR compared to those without access to OH. However, on d 24 and 35, the challenge and free choice OH had no effect on weight gain, feed intake, FCR and livability. No challenge × free choice OH interaction was observed for any performance parameters during all the trial period (P>0.05).

4.4.2 Relative gizzard weight, gizzard contents and pH

As shown in Table 4.3, at 13 d, i.e., 4 days following *Eimeria* inoculation, the relative gizzard weight, gizzard contents and pH were not affected by *Eimeria* inoculation. However, on d 16, birds challenged with NE exhibited decreased gizzard contents compared to unchallenged birds, whereas the relative gizzard weight and pH were not affected by challenge. On d 13 and 16, the free choice OH had no effect on the relative gizzard weight, gizzard contents and pH. However, the birds given OH had heavier (P<0.05) gizzards compared to those without access to OH at d 35. No significant differences were observed on gizzard contents and pH between birds with and without access to OH at d 35. On the other hand, challenged birds had lower gizzard content at d 16, and higher gizzard pH (P<0.05) at d 35 compared to those unchallenged birds. However, no differences were observed in relative gizzard weight and gizzard content between challenged and unchallenged birds at d 35 (P>0.05). No challenge × free choice OH interaction was observed for relative gizzard weight, gizzard contents or pH throughout study (P>0.05).

4.4.3 Bacterial quantification

On d 16, the challenge led to significant changes in the counts of caecal microflora (Table 4.4). Increased numbers of *C. perfringens* (P<0.001) and Enterobacteriaceae (P<0.05) and reduced numbers of *Lactobacillus* (P<0.05) and *Salmonellae* (P<0.05) were observed in the caecal contents of challenged birds. However, no changes were observed in caecal *Bifidobacteria*, *Bacillus*, *Ruminococcus*, *Bacteroides* or total bacteria as a result of challenge (P>0.05). The OH had no effect on caecal bacterial counts on d 16. An OH by challenge interaction was observed for Enterobacteriaceae

group counts ($P<0.001$). In the unchallenged birds, those without OH had lower counts of Enterobacteriaceae compared with those accessed to OH but no effect of OH was observed in challenged birds. Also, the challenged birds had higher counts of Enterobacteriaceae than unchallenged birds without accessed to OH.

Table 4. 2 Impact of free choice OH on the performance in broilers challenged with NE at d16, 24, and 35

Treatments		FCR			Weight gain (g/bird)			Feed intake (g/bird)			Livability		
Challenge	OH	D0-16	D0- 24	D0-35	D0-16	D0- 24	D0-35	D0-16	D0- 24	D0-35	D0-16	D0- 24	D0-35
No	No	1.179	1.276	1.444	623	1321	2241	736	1686	3236	97	97	93
No	Yes	1.154	1.258	1.417	602	1276	2273	695	1605	3218	100	100	100
Yes	No	1.194	1.258	1.414	577	1300	2323	688	1633	3284	98	98	97
Yes	Yes	1.180	1.264	1.401	562	1312	2307	663	1650	3231	100	100	98
Main effect													
Challenge													
No		1.167	1.267	1.430	612	1299	2257	715[a]	1646	3227	98	98	97
Yes		1.187	1.261	1.408	569	1306	2315	675[b]	1642	3257	99	99	98
OH													
No		1.187	1.267	1.429	600	1311	2282	712[a]	1659	3260	98	98	95
Yes		1.167	1.261	1.409	582	1294	2290	679[b]	1628	3224	100	100	99
P value													
Challenge		0.052	0.610	0.082	0.008	0.776	0.258	0.013	0.887	0.614	0.542	0.542	0.701
OH		0.062	0.661	0.114	0.241	0.519	0.875	0.037	0.287	0.555	0.078	0.078	0.066
Challenge × OH		0.607	0.353	0.579	0.844	0.275	0.636	0.599	0.103	0.763	0.542	0.542	0.257

[a, b] Means sharing the same superscripts are not significantly different from each other at P <0.05

Table 4. 3 Impact of free choice OH on the relative gizzard weight, gizzard pH and contents in broilers challenged with NE at d13, 16, and 35

Treatments		Relative gizzard weight			Gizzard content			Gizzard pH		
Challenge	OH	D13	D16	D35	D13	D16	D35	D13	D16	D35
No	No	2.45	2.11	1.03	12.91	17.38	14.78	2.89	2.97	3.17
No	Yes	2.44	2.02	1.16	12.01	15.65	20.32	2.89	2.88	2.89
Yes	No	2.41	2.11	1.08	11.67	13.60	18.19	2.71	2.98	3.33
Yes	Yes	2.48	2.10	1.21	11.62	14.57	19.37	2.77	3.06	3.24
Main effect										
Challenge										
No		2.45	2.07	1.09	12.46	16.51[b]	17.55	2.89	2.92	3.03[b]
Yes		2.44	2.11	1.14	11.64	14.09[a]	18.78	2.74	3.02	3.28[a]
OH										
No		2.43	2.11	1.05[b]	12.29	15.49	16.49	2.80	2.97	3.25
Yes		2.46	2.06	1.18[a]	11.81	15.11	19.85	2.83	2.97	3.06
P value										
Challenge		0.963	0.615	0.343	0.316	0.028	0.737	0.077	0.468	0.048
OH		0.751	0.498	0.017	0.551	0.714	0.362	0.695	0.984	0.140
Challenge × OH		0.656	0.586	0.931	0.599	0.202	0.553	0.725	0.517	0.463

[a, b] Means sharing the same superscripts are not significantly different from each other at P <0.05

Table 4. 4 Impact of free choice OH on caecal microflora (log10 CFU) in broilers challenged with NE at d16

Treatments		Lactob-acillus	Bifidob-acteria	Bacillus	Rumino-coccus	Bacteroides	Enteroba-cteriaceae	C. perfringens	Salmonella	Total bacteria
Challenge	OH									
No	No	8.40	8.46	8.67	9.39	4.52	6.54[b]	0.00	6.97	10.00
No	Yes	8.54	8.71	8.96	9.30	4.62	7.19[a]	0.00	6.92	9.99
Yes	No	8.26	8.70	8.74	9.43	4.56	7.39[a]	9.58	6.77	10.06
Yes	Yes	8.15	8.58	9.03	9.29	4.98	6.98[ab]	9.49	6.75	10.03
Main effect										
Challenge										
No		8.47[a]	8.59	8.81	9.34	4.57	6.86[b]	0.00[b]	6.94[a]	9.99
Yes		8.21[b]	8.64	8.88	9.36	4.77	7.18[a]	9.54[a]	6.76[b]	10.05
OH										
No		8.33	8.58	8.71	9.41	4.54	6.96	4.79	6.87	10.03
Yes		8.34	8.64	8.99	9.30	4.80	7.09	4.75	6.84	10.01
P value										
Challenge		0.035	0.666	0.764	0.765	0.185	0.023	< 0.001	0.026	0.282
OH		0.913	0.601	0.214	0.132	0.087	0.327	0.853	0.658	0.649
Challenge × OH		0.304	0.133	0.988	0.723	0.290	0.001	0.853	0.861	0.859

[a, b] Means sharing the same superscripts are not significantly different from each other at P <0.05

4.4.4 NE lesions

On d 13, which was between *Eimeria* and *C. perfringens* challenges, there were no significant differences (P>0.05) of lesion scores between treatments for the duodenal, jejunal and ileal samples. On d 16, no significant differences (P>0.05) of lesion scores were detected between treatments for all parts of small intestine (data not shown).

4.4.5 Caecal SCFAs

On d 16, the caecal concentrations of SCFAs were significantly affected by challenge and OH (Table 4.5). The challenged birds had lower concentrations of caecal acetic acid (P<0.01) and succinic acid (P<0.05) compared to unchallenged birds, while challenge had no effect on the concentrations of propionic, isobutyric, butyric, isovaleric, valeric and lactic acids (P>0.05). The OH had no effect on the concentrations of isobutyric, butyric, and isovaleric acids. However, the birds given OH had lower concentrations of acetic (P<0.05), propionic (P<0.05), and valeric (P<0.01) acids and a higher concentration of succinic acid compared to those without access to OH. Furthermore, the birds with access to OH tended to have a higher concentration of lactic acid (P = 0.075) compared to those without access to OH. Interestingly, there was no significant challenge × free choice OH interaction observed for all SCFAs except for succinic acid concentration (P<0.05). Succinic acid concentration in the caeca was reduced by challenge of birds only when the birds had access to OH. On the other hand, OH access elevated succinic acid concentration only in the unchallenged birds.

Table 4. 5 Impact of free choice OH on caecal SCFAs (µmol/g digesta,) in broilers challenged with NE at d 16

Treatments Challenge	OH	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Lactic acid	Succinic acid
No	No	111.6	4.13	0.449	15.65	0.094	1.64	0.331	8.07[b]
No	Yes	78.80	2.66	0.257	10.92	0.024	1.03	5.530	18.84[a]
Yes	No	68.72	3.83	0.300	11.92	0.072	1.28	0.823	7.42[b]
Yes	Yes	60.51	2.66	0.283	10.99	0.073	0.937	2.110	6.74[b]
Main effect									
Challenge									
No		95.22a	3.39	0.353	13.29	0.059	1.34	2.93	13.45[a]
Yes		64.61b	3.24	0.292	11.46	0.072	1.11	1.46	7.08[b]
OH									
No		90.18[a]	3.98[a]	0.374	13.79	0.083	1.46[a]	0.58	7.74[b]
Yes		69.66[b]	2.66[b]	0.270	10.96	0.048	0.983[b]	3.82	12.79[a]
P value									
Challenge		0.003	0.808	0.316	0.381	0.579	0.174	0.404	0.015
OH		0.031	0.043	0.095	0.183	0.152	0.008	0.075	0.047
Challenge × OH		0.178	0.811	0.156	0.364	0.138	0.404	0.269	0.026

[a, b] Means sharing the same superscripts are not significantly different from each other at P <0.05.

4.5 Discussion

Necrotic enteritis imposes a significant economic burden on the global broiler industry as it affects bird welfare and increases the risk of contamination of chicken for human consumption (Timbermont et al., 2011). The birds in this study were inoculated with *Eimeria* spp. and *C. perfringens* to produce a subclinical form of NE experimentally in order to investigate the possible role of free choice OH on performance, gut development and health in challenged birds.

Subclinical NE was successfully induced after inoculation with *Eimeria spp*. and *C. perfringens* as shown by the depression of weight gain, feed intake, numerically higher FCR of birds on d 16. A number of studies have shown impaired broiler performance when birds are challenged with subclinical NE (Cooper, 2007; Timbermont et al., 2011; Saleem, 2013). Most of studies also have shown improved broiler performance when birds are fed fibre supplementation in their diet at different ages (Hetland et al., 2003; González-Alvarado et al., 2010; Jiménez-Moreno et al., 2009, 2010, 2013a, 2016; Mateos et al., 2012; Kheravii et al., 2016b). The improvement in growth performance due to the addition of moderate amounts of structural fibre in the diet was more pronounced in young broilers (Jiménez-Moreno et al., 2009, 2010, 2013a, 2016). Hetland and Svihus (2001) reported increased feed intake in birds fed OH. In the current study, birds with free choice access to OH consumed less feed and tended to have lower FCR compared to those without access to OH.

It has been speculated that insoluble fibre might increase the release of cholecystokinin (Svihus et al., 2004), which acts through the vagus nerve to stimulate pancreatic enzyme secretion and gastro-duodenal reflux (Duke, 1992; Li and Owyang, 1993). This, in turn, may assist digestion and absorption of nutrients in the small intestine and hence result in performance improvement.

Several studies have shown that feeding birds with insoluble fibre, such as OH, increases gizzard size at different ages (Jiménez-Moreno et al., 2013b; Kimiaeitalab et al., 2016). Indeed, feeds need to be ground to certain particle size before leaving the gizzard. Jiménez-Moreno et al. (2013b) stated that the relative weight of the gizzard and its dry matter content were increased, and gizzard pH was reduced with OH inclusion (2.5 or 5 %) at all ages (d 6, 12, and 18). This is different from the findings of this study where younger birds (up to d 16) might not have eaten enough OH to increase the relative gizzard weight and reduce the pH, as it is possible that young birds prefer to consume less OH which is provided as free choice. However, the relative gizzard weight of the birds at d 35 increased with access to OH. A

large and well-developed gizzard is able to grind feed particles more thoroughly (Amerah et al., 2007), to elevate pancreatic enzyme secretion through increased release of cholecystokinin (Svihus, 2011), to increase proteolysis by pepsin, trypsin and other endogenous proteases in the small intestine, and to improve gastrointestinal tract motility (Ferket, 2000; Gonzalez-Alvarado et al., 2008) and to improve nutrient digestibility (Amerah, 2008). Thus, gut development and health can be enhanced (Choct, 2009). This study has shown that infection with a pathogenic bacterium *C. perfringens* strain led to the development of necrotic enteritis, which altered the populations of some specific organisms harboured in the caeca, such as *lactobacillus* spp. Feng et al. (2010) and Dahiya et al. (2005) noted that counts of *lactobacillus* decreased following *C. perfringens* challenge. Stanley et al (2012) suggested the artificial boosting of *C. perfringens* caused an imbalance in the numbers of Lactobacillales orders. The disruption of the gut flora resulted from *C. perfringens* infection could also disrupt the ability of *lactobacillus spp* to colonise. In the poultry industry, *lactobacillus* species are often regarded as probiotics due to their health promoting properties (Klaenhammer et al., 2008) that are characterised by reduced pathogens in the gut, improved immune function, and increased bird performance (Salim et al., 2013; Zhang et al., 2012). It is possible that a notable reduction in these beneficial probiotic bacteria may predispose chickens to the onset of the other bacterial diseases.

The structural components of feed that impart beneficial effects on the gizzard consist largely of fibre, which is the sum of NSP and lignin. In addition to the direct effects that fibre has on the gizzard, certain components of fibre bring about gut health benefits via the production of prebiotics *in situ*. The effect of OH was not notable on the number of the selected bacteria examined in this study. However, SCFAs as the products from the fermentation of dietary fibre by the anaerobic bacteria were clearly influenced by free choice OH. This may indicate the OH as the substrate significantly influenced the number of some groups of bacteria. For example, the birds with access to free choice OH had a higher amount of caecal succinic acid compared to those without access to free choice OH. It has been reported that succinic acid improves performance in broilers possibly due to its bactericidal properties (Broz et al., 2009).

Succinic acid has been shown along with other SCFAs to have immunomodulatory effects (Lawhon et al., 2002; Cavaglieri et al., 2003; Maslowski et al., 2009). It has been reported that succinate or succinic acid increased pro-inflammatory cytokines such as interleukin-8 and interleukin-1β (Graham et al., 2013; Tannahill et al., 2013), which play an important role in cell signaling as inflammatory reaction develops (Brat et al., 2005; Tannahill et al., 2013). In this study, the amount of caecal succinic acid in challenged birds with access to OH was lower than those unchallenged with access to OH. It is possible that challenged birds with

access to OH might have used the succinic acid for immunological purposes. Hence, offering birds with dietary fibre may not only affect overall bird performance, but may also contribute to immune function and health.

In conclusion, free choice OH was beneficial in improving bird performance at least at younger ages. The benefit of OH may be due to altered SCFA types and concentrations as a result of mediated changes in the gut microbiota. In addition, offering broilers with OH resulted in a well-developed gizzard that has been considered as the pacemaker organ for digestion and health in poultry.

4.6 Acknowledgements

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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Chapter 8

Upregulation of genes encoding digestive enzymes and nutrient transporters in the digestive system of broiler chickens by dietary supplementation of fiber and inclusion of coarse particle size corn

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8.1 Abstract

Background: Measures to improve bird performance have been sought due to the imminent phase out of in-feed antibiotics in poultry and continued demand for higher poultry feeding efficiency. Increasing grain particle size and dietary fibre may improve gizzard function, digestive efficiency and nutrient absorption. This study was conducted to evaluate the effect increased particle size of corn and inclusion of sugarcane bagasse (SB) on mRNA expression of genes encoding digestive enzymes and nutrient transporters in broilers.

Methods: A total of 336 day-old Ross 308 males were assigned in a 2 × 2 factorial arrangement of treatments with corn particle size - coarse 3576 µm or fine 1113 µm geometric mean diameter, and SB - 0 or 2% inclusion. Feed conversion ratio (FCR), weight gain and feed intake were measured from d 0-10 and d 10-24. The relative gizzard weight and mRNA expression of genes encoding digestive enzymes and intestinal nutrient transporters were measured on d 24.

Results: During d 10-24, a particle size × SB interaction was observed for FCR ($P < 0.01$), where birds fed coarsely ground corn (CC) with 2% SB had lower FCR than those fed CC without SB. A particle size × SB interaction was observed for both expression of pepsinogen A and C ($P < 0.01$) which were negatively correlated with FCR on d 24. Addition of 2% SB upregulated pepsinogen A and C only in CC fed birds. Further, 2% SB also upregulated pancreatic amylase (AMY2A) and intestinal cationic amino acid transporter-1 (CAT1). Inclusion of dietary CC upregulated duodenal amino peptidase N (APN), jejunal alanine, serine, cysteine and threonine transporter-1 (ASCT1), and ileal peptide transporter-2 (PepT2).

Conclusion: These results suggest that both SB and coarse particle size modulate expression of genes encoding important digestive enzymes and nutrient transporters and thus are directly related to bird performance. These findings provide insights into the combination effects of dietary fiber and particle size in the future management of broiler feeding.

Key words: Gene expression; sugarcane bagasse, fiber; particle size; amylase; pepsinogen; nutrient transporter; broilers

8.2 Introduction

Consumer pressure and legislation to ban the use of in-feed antibiotics in the EU and voluntarily removal of antibiotics from animal feed in other countries have supressed performance and profitability in broiler chickens due to enteric disorders (1, 2). The development of antibiotic alternatives to improve digestive efficiency is becoming a topic of broad interest for the poultry industry worldwide. Strategies to improve broiler digestive efficiency and performance without reliance on antibiotics have been the focus for improving gut health and manipulating development of the gastrointestinal tract (GIT). A range of nutritional interventions including; increasing grain particle size, using whole grain, and including different sources or levels of dietary fiber and other feed additives, such as probiotics, bacteriophages, enzymes and phytobiotics are currently under investigation (2-4).

The physical structure of feed ingredients and inclusion of dietary fiber may improve nutrient digestion and absorption as a result of increased gizzard size (3, 5) and enhanced secretion of HCl in the proventriculus (6). Low pH in the upper GIT is known to improve solubility and absorption of minerals (7) and increase pepsin activity (8). Well-developed gizzard musculature has been hypothesized to elevate pancreatic enzyme secretion including amylase and chymotrypsin through increased release of cholecystokinin (9). The inclusion of fiber or coarse particle size in broiler diets not only enhances gizzard development but also increases digesta retention time and gut reflux (9, 10). Slower digesta retention time improves nutrient digestion and absorption by increasing time of contact with absorptive cells (11). In the chickens, the small intestine is the main site of nutrient absorption. It is known that the glucose transporters: glucose transporter-1 (GLUT1) and glucose transporter 2 (GLUT2); amino acid transporters: Na+-dependent neutral amino acid transporters, such as B^oAT and ASCT1, cationic amino acid transporters, such as CAT1 and CAT2, and Na+-dependent neutral/cationic amino acid exchanger, such as y+ L amino acid transporter-1 and y+ L amino acid transporter-2, and peptide transporters, such as PepT1 and PepT2, in the small intestinal epithelium are closely associated with nutrient absorption capacity (12, 13). Although, structural components of the diet have been reported to improve nutrient digestibility and performance in broilers (3, 14-17), there are no investigations on nutrigenomic mechanisms underlying such improvements.

This study investigated the influence of fiber supplementation and increased corn particle size on broiler performance at the gene expression level. It was hypothesized that fiber and coarse particle size would stimulate secretion of digestive enzymes and nutrient transporters that would then accelerate the digestive activity in the intestine and improve feed conversion efficiency.

8.3 Materials and methods

8.3.1 Experimental design, bird management and diet

A total of 336 d-old male Ross 308 chicks were obtained on the hatching day from a local hatchery (Baiada Hatchery in Tamworth, NSW, Australia). The chicks were assigned in a 2 × 2 factorial arrangement of treatments with 2 particle sizes (coarse 3576 μm or fine 1113 μm geometric mean diameter) and 2 levels of sugarcane bagasse (0%, 2%). The geometric mean diameter (GMD) of corn particle size was determined according to the American Society of Agricultural Engineers (2003). The birds were randomly allocated to 4 treatments with 6 replicate pens each stocked with 14 birds. The broiler chicks were reared in pens measuring 75 cm × 120 cm to 24 d. Hardwood shavings were used as bedding with an initial depth of 7 cm. Each pen was equipped with a single tube feeder and 2 nipple drinkers. Feed and water were provided *ad libitum*. The lighting, relative humidity and temperature followed Ross 308 strain guidelines (18).

Table 8.1 shows the ingredient and nutrient composition of experimental diets. The diets were formulated to Ross 308 specifications (18). The composition of diets was diluted when 2% SB added over the top of the complete feed. All diets were thoroughly mixed and cold-pelleted (65^0C). The feeding program consisted of a starter (d 0 to 10), and grower (d 11 to 24).

The SB was provided by FCR Consulting Group, Brisbane. The composition of SB was determined ("as is" basis) for total non-starch polysaccharides (NSP) and lignin following the method described by Englyst et al. (19) and Kirk and Obst (20), respectively. The SB contained 6.1 g/kg free sugar, 191 g/kg lignin, 534 g/kg insoluble NSP and 1.9 g/kg soluble NSP.

This experiment was approved by the Animal Ethics Committee of the University of New England (Approval No: AEC 15-053). All bird management procedures

including health, care and use of laboratory animals were fulfilled with the Australian Code for the Care and Use of Animals for Scientific Purposes (21).

Table 8. 1 Composition and nutrient content of corn base diet (%)

Ingredients	Starter	Grower
Corn	60.6	62.3
Soybean meal	32.6	29.3
Meat and bone meal	3.00	3.60
Canola oil	0.644	1.91
Limestone	0.970	0.814
Dical phosphate	0.607	0.269
Phytase[1]	0.01	0.010
Salt	0.154	0.161
Na bicarbonate	0.219	0.200
Vitamin premix[2]	0.200	0.200
Choline	0.111	0.103
L-lysine HCl 784	0.305	0.226
D, L-methionine	0.392	0.336
L-threonine	0.204	0.148
TiO_2	60.6	0.500
Nutrients		
ME (kcal/kg)	3000	3100
ME (MJ/kg)	12.55	12.97
Crude protein	22.2	21.0
Crude fat	2.85	4.14
Crude Fiber	2.07	2.01
SID arginine	1.37	1.27
SID lysine	1.28	1.15
SID methionine	0.684	0.616
SID methionine + Cysteine	0.950	0.870
SID tryptophan	0.244	0.226
SID isoleucine	0.860	0.807
SID threonine	0.860	0.770
SID valine	0.992	0.939
Starch	35.8	36.8
NSP soluble	0.426	0.404
NSP insoluble	5.64	5.45
Calcium	0.960	0.870
Available Phosphorus	0.480	0.435
Sodium	0.160	0.160
Chloride	0.250	0.242
Choline	0.170	0.160

[1]Phyzyme XP5000G (100 g/mt) Dupont

[2]Vitamin-Mineral concentrate supplied per kilogram of diet: retinol, 12000 IU; cholecalciferol, 5000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamine, 16 μg; biotin, 200 μg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg; Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg; SID = Standard ileal digestible.

8.3.2 Measurement of growth performance and gizzard weight

On d 10 and 24 weight gain, feed intake and FCR were measured. On d 24, empty gizzards without proventriculi from 3 birds per pen were weighed and recorded along with corresponding bird weights. The relative gizzard weight was calculated as mass per unit of live body weight (g/100 g of live body weight).

8.3.3 Sampling and RNA Isolation

On d 24, one bird was randomly selected from each pen and killed by cervical dislocation. Around 2 cm from each duodenum, jejunum, ileum, proventriculus and pancreas was excised and flushed with 4°C PBS and collected into a 2 mL Eppendorf cap lock tube, snap-frozen in liquid N_2, and kept at –80°C until required for RNA extraction. For each sample, total RNA was extracted from the tissue after homogenization in TRIsure^{TM} (Bioline, Sydney, Australia) following the manufacturer's instructions. Total RNA quantity and purity was determined using a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). An RNA 6000 Nano kit was used to measure RNA integrity (RNA Integrity Number, or RIN) using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). The RNA samples were considered of high integrity if the RIN was higher than 7.5 (22).

8.3.4 cDNA Synthesis

The isolated RNA of each sample was reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Briefly, one microgram of total RNA from each sample was incubated in 2µl of 7× gDNA Wipeout Buffer at 42°C for 2 min in order to avoid genomic DNA contamination. Then the gDNA elimination reaction was mixed with reverse-transcription reaction components contained 1 µl of Quantiscript Reverse Transcriptase, 4 µl of 7× Quantiscript RT Buffer, and 1 µl of RT Primer Mix. The Rotorgene 6000 real-time PCR machine (Corbett, Sydney, Australia) was employed to incubate the mixture at 42 ˚C for 15 min and at 95 ˚C for 3 min in order to convert the RNA into cDNA. The cDNA was diluted three times with Nuclease-free water and stored at -20 ˚C until required.

8.3.5 Primer sources and design

In the current study, the primers were either sourced from previously published studies in chickens or designed using NCBI primer tool (https://www.ncbi.nlm.nih.gov/). Table 2 show the primers that were used in this study. Prior to qPCR analysis, Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Germany) was employed to check the primer specificity for each pair using Agilent DNA 1000 Kit (Agilent Technologies, Inc., Germany). Only primer pairs with specific amplifications were used in this study (Figure 8.1).

8.3.6 Real-time quantitative PCR (qPCR)

Quantitative PCR was performed in triplicates using a SYBR Green kit SensiFAST™ SYBR® No-ROX (Bioline, Sydney, Australia) with Rotorgene 6000 real-time PCR machine (Corbett Research, Sydney, Australia). The PCR reaction was performed in a volume of 10 µL containing 5 µL of 2× SensiFAST, 400 mM of each primer and 2 µL of cDNA template. After thermal cycling, amplification cycle (Cq) values for all genes were collected and imported into qBase+ version 3.0 (Biogazelle, Zwijnbeke, Belgium) software and analyzed against two previously optimized reference genes, HPRT1 and TBP. The qBase+ applied an arithmetic mean method to transform logarithmic Cq value to linear relative quantity using exponential function for relative quantification of genes (23, 24) and the output data was exported to SPSS statistics version 22 (IBM SPSS, UK) for further analysis.

Table 8. 2 Sequences of primers used for quantitative real-time PCR

Gene	Gene full name	Primer sequence (5′-3′)	Ta	size (bp)	Reference	Accession No.
ATP1A1	ATPase Na+/K+ transporting subunit alpha 1	F-GTCAACCCGAGGGATGCTAA R-ACTGCTACAATGGCACCCTG	60	179	This study	NM_205521.1
AMY2A	Pancreatic alpha 2A amylase	F-CGGAGTGGATGTTAACGACTGG R-ATGTTCGCAGACCCAGTCATTG	60	112	This study	NM_001001473.2
APN	Aminopeptidase N	F-AATACGCGCTCGAGAAAACC R-AGCGGGTACGCCGTGTT	60	70	(41)	NM_204861.1
ASCT1	Alanine, serine, cysteine, and threonine transporter (SLC1A4)	F-TTGGCCGGGAAGGAGAAG R-AGACCATAGTTGCCTCATTGAATG	60	63	(42)	XM_001232899.4
B^0AT	Solute carrier family 6, member 19 (SLC6A19)	F-GTGTTTGGAACCCTAAATACGAGG R-TAGCATAGACCCAGCCAGGA	60	72	This study	XM_419056.5
bo,+AT	Solute carrier family 7, member 9 (SLC7A9)	F-CAGTAGTGAATTCTCTGAGTGTGAAGCT R- GCAATGATTGCCACAACTACCA	60	88	(41)	NM_001199133.1
CAT1	Cationic amino acid transporter-1 (SLC7A1)	F-CAAGAGGAAAACTCCAGTAATTGCA R- AAGTCGAAGAGGAAGGCCATAA	60	75	(41)	XM_015277945.1
CAT2	Cationic amino acid transporter-2 (SLC7A2)	F-TGCTCGCGTTCCCAAGA R- GGCCCACAGTTCACCAACAG	60	67	(41)	XM_015285435.1
CCK1R	Cholecystokinin type 1 receptor	F-CACTTACTTCATGGGTATCTCTGTG R-GATGGCAACAAGGTTGAATGTAGA	60	55	(43)	AB214534.1
CCK	Cholecystokinin	F-AGGTTCCACTGGGAGGTTCT R-CGCCTGCTGTTCTTTAGGAG	60	152	This study	XM_015281332.1
CELA1	Chymotrypsin-like elastase family, member 1	F-AGCGTAAGGAAATGGGGTGG R-GTGGAGACCCCATGCAAGTC	60	75	This study	XM_015300368.1
CELA2A	Chymotrypsin like elastase family member 2A	F-GAGGGGAAGATGCAAGACCAT R-CCTTGCTCCTCAGCTTCTAGG	60	196	This study	NM_001032390.2
EAAT3	Excitatory amino acid transporter 3 (SLC1A1)	F-TGCTGCTTTGGATTCCAGTGT R- AGCAATGACTGTAGTGCAGAAGTAATATATG	60	79	(44)	XM_424930.5

Table 8.2 Cont'd

Gene	Gene full name	Sequence	Ta	size (bp)	Reference	Accession No.
GLUT1	Glucose transporter-1 (SLC2A1)	F-TCCTCCTGATCAACCGCAAT R-TGTGCCCCGGAGCTTCT	60	65	(44)	NM_205209.1
GLUT2	Glucose transporter-2 (SLC2A2)	F-TGATCGTGGCACTGATGGTT R-CCACCAGGAAGACGGAGATA	60	171	This study	NM_207178.1
LAT1	L type amino acid transporter-1 (SLC7A5)	F-GATTGCAACGGGTGATGTGA R- CCCCACACCCACTTTTGTTT	60	70	(41)	KT876067.1
PGA5	Pepsinogen A	F-TCCGTCTACCTGAGCAAGGAT R- AAGCAGGCGACGTACTTGTT	60	167	This study	NM_204878.1
PGC	Pepsinogen C	F-ATCGGGATTGAGGACTTCGC R- TGAAGACCTGGTTGGGAACG	60	115	This study	NM_204877.2
PepT1	Peptide transporter-1 (SLC15A1)	F-TACGCATACTGTCACCATCA R-TCCTGAGAACGGACTGTAAT	60	205	(45)	AY029615.1
PepT2	Peptide transporter-2 (SLC15A2)	F-TGACTGGGCATCGGAACAA R-ACCCGTGTCACCATTTTAACCT	60	63	(42)	NM_001319028.1
PNLIP	Pancreatic lipase	F-GCATCTGGGAAGGAACTAGGG R- TGAACCACAAGCATAGCCCA	60	113	This study	NM_001277382.1
rBAT	Solute carrier family 3, member1 (SLC3A1)	F-CCCGCCGTTCAACAAGAG R- AATTAAATCCATCGACTCCTTTGC	60	70	(41)	XM_426125.4
SI	Sucrase isomaltase	F-GCTTTAAGATGGGCAAGAGGAAG R- CCACCACCAGGCAAAAGAGG	60	65	This study	XM_015291762.1
y^+LAT1	y^+ L amino acid transporter-1 (SLC7A7)	F-TACTGAGGCTGACTGGAGGAA R- ACGACGTACAGCACAATATCTGG	62	227	This study	XM_418326.5
y^+LAT2	y^+ L amino acid transporter-2 (SLC7A6)	F-GCCCTGTCAGTAAATCAGACAAGA R-TTCAGTTGCATTGTGTTTTGGTT	60	82	(41)	NM_001005832.1
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	F-ACTGGCTGCTTCTTGTG R-GGTTGGGTTGTGCTGTT	63	245	(46)	NM_204848.1
TBP	TATA-Box binding protein	F-TAGCCCGATGATGCCGTAT R- GTTCCCTGTGTCGCTTGC	62	147	(47)	NM_205103 D83127

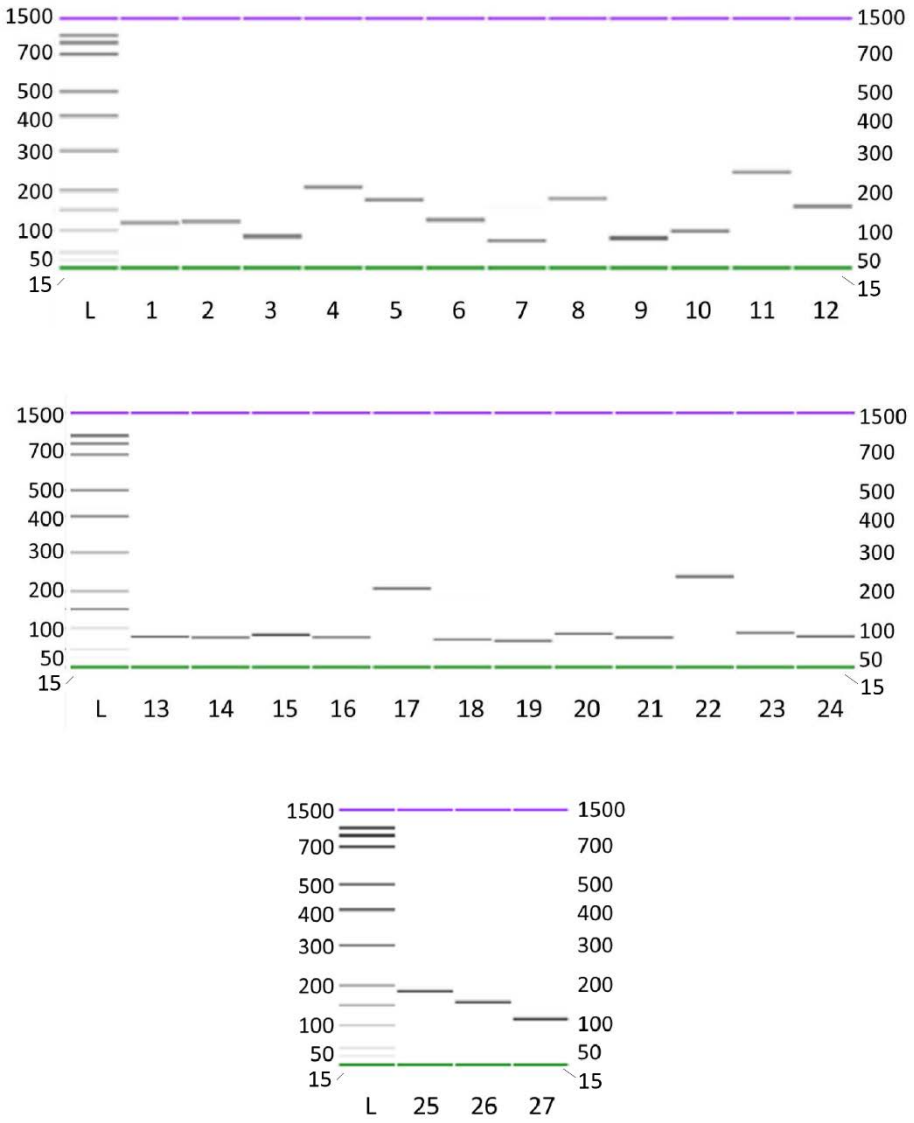

Figure 8. 1 Gel electrophoresis images of DNA fragments to show the specificity of the primers used in the current study.

8.3.7 Statistical Analyses

The data were analyzed using the General Linear Models (GLM) procedure of SPSS statistics version 22 (IBM SPSS, UK) for the main effect of particle sizes, and SB supplementation along with their interactions. Differences between mean values were determined using LSD test at the level of $P < 0.05$. Correlations between FCR and the expression levels of the genes were conducted using the procedures of SPSS statistics version 22 (IBM SPSS, UK).

8.4 Results

8.4.1 Performance and gizzard weight

The effect of corn particle size and SB on broiler performance is presented in Table 8.3. On d 10, weight gain and feed intake were significantly higher in birds fed FC than those fed CC ($P < 0.05$). FCR was impaired in birds fed the diet containing 2% SB compared to those fed the diet without SB treatment during d 0-10 ($P < 0.05$). No interaction was observed between particle size and SB in early age ($P > 0.05$). During d 10-24, significant particle size × SB interactions were observed for FCR ($P < 0.01$). The birds fed CC with 2% SB had lower FCR than those fed CC without SB. However, this was not the case when fine corn was fed. Similarly, CC reduced FCR compared to FC only when 2% SB was included in the diet. During d 10-24, birds fed SB were heavier than those fed without SB ($P < 0.05$).

The effect of corn particle size and SB on relative gizzard weight is presented in Table 8.4. On d 24, the broilers fed CC had heavier gizzards ($P < 0.05$) compared to those fed FC. A particle size × SB interaction was observed for relative gizzard weight ($P < 0.05$). Addition of 2% SB increased the relative gizzard weight only in the FC fed birds

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Table 8. 3 Effect of particle size and sugar cane bagasse on broilers growth performance

Treatments		D0-10			D10-24		
Particle size	SB	FCR	Weight gain (g/bird)	Feed intake (g/bird)	FCR	Weight gain (g/bird)	Feed intake (g/bird)
CC	0%	1.049	277	290	1.332[a]	1158	1542
FC	0%	1.053	279	294	1.333[a]	1181	1573
CC	2%	1.072	267	287	1.280[b]	1233	1579
FC	2%	1.074	281	302	1.351[a]	1189	1606
Main effect							
SB							
0%		1.050[b]	278	292	1.332	1169[b]	1557
2%		1.073[a]	274	294	1.316	1211[a]	1593
Particle size							
CC		1.061	272[b]	288[b]	1.306[b]	1196	1560
FC		1.063	280[a]	298[a]	1.342[a]	1185	1590
P value							
SB		0.005	0.279	0.557	0.143	0.037	0.171
Particle size		0.713	0.020	0.019	0.003	0.582	0.251
SB × particle size		0.877	0.097	0.134	0.004	0.090	0.940

[1] Within a column, values with different superscripts are significantly different from each other at $P < 0.05$.

Table 8. 4 Effect of particle size and sugar cane bagasse on relative gizzard weight at d 24

Treatments		Relative gizzard weight
Particle size	SB	
CC	0%	1.934[a]
FC	0%	1.713[b]
CC	2%	1.921[a]
FC	2%	1.922[a]
Main effect		
SB		
0%		1.823
2%		1.922
Particle size		
CC		1.927[a]
FC		1.817[b]
P value		
SB		0.060
Particle size		0.037
SB × particle size		0.036

[1]Within a column, values with different superscripts are significantly different from each other at $P < 0.05$.

8.4.2 Upregulation of pepsinogen A and C in proventriculus by sugarcane bagasse and coarsely ground corn

The mRNA expression of three genes was investigated in response to SB addition and corn particle size in the proventriculus as presented in Table 8.5. While a particle size × SB interaction was observed in the expression of genes PGA5 (A) and PGC (C; $P<0.01$), no effect of treatment was observed on the gene CKK from the proventriculus. It was shown that the combination of SB and CC significantly upregulated PGA5 and PGC compared to the expression of genes in the other three groups. Both pepsinogen A ($P < 0.01$ and $R = -0.53$) and C ($P < 0.01$ and $R = -0.59$) were negatively correlated to FCR on d24.

Table 8. 5 Effect of particle size and sugar cane bagasse on expression of proventricular genes at d 24

Treatments				
Particle size	SB	PGA5	PGC	CCK
CC	0%	0.984[b]	0.982 [b]	0.953
FC	0%	1.222[b]	1.097[b]	1.326
CC	2%	2.435[a]	2.638 [a]	1.016
FC	2%	0.898[b]	0.884[b]	1.160
Main effect				
SB				
0%		1.103	1.039	1.140
2%		1.666	1.761	1.088
Particle size				
CC		1.709	1.810	0.985
FC		1.060	0.990	1.243
P value				
SB		0.070	0.078	0.811
Particle size		0.039	0.047	0.239
SB × particle size		0.007	0.026	0.598

[1] The relative expression levels of the genes of respective treatment groups are expressed as means of normalized relative quantities (NRQ). Relative quantities for individual genes are scaled to the average across all unknown samples per target gene.
[2] Within a column, values with different superscripts are significantly different from each other at $P < 0.05$.

8.4.3 Upregulation of pancreatic AMY2A and CELA1 by sugarcane bagasse

The mRNA expression of seven pancreatic genes in response to feed SB addition and corn particle size was examined (Table 8.6). It was demonstrated that genes AMY2A and CELA1 were upregulated by 2% SB addition to the diets ($P<0.05$), while no response to SB was observed in other genes investigated, namely, ATP1A1, CCK1R, CCK, CELA2A, and PNLIP. Corn particle size did not affect the expression of pancreatic genes either as a main effect and no interactions were observed ($P > 0.05$).

Table 8. 6 Effect of particle size and sugar cane bagasse on expression of pancreatic genes at d 24

Treatments Particle size	SB	AMY2A	ATP1A1	CCK1R	CCK	CELA1	CELA2A	PNLIP
CC	0%	1.042	0.963	1.231	0.909	1.173	1.460	1.432
FC	0%	1.314	0.990	1.254	0.992	1.089	1.401	1.226
CC	2%	1.559	1.028	1.384	1.356	1.599	1.171	1.177
FC	2%	1.500	0.783	1.375	1.039	1.678	1.185	1.257
Main effect								
SB								
0%		1.178[b]	0.977	1.243	0.951	1.131[b]	1.431	1.329
2%		1.530[a]	0.906	1.380	1.198	1.639[a]	1.178	1.217
Particle size								
CC		1.301	0.996	1.307	1.133	1.386	1.316	1.304
FC		1.407	0.887	1.315	1.016	1.384	1.293	1.242
P value								
SB		0.024	0.543	0.398	0.162	0.040	0.115	0.428
Particle size		0.468	0.355	0.964	0.498	0.991	0.885	0.656
SB × particle size		0.263	0.252	0.922	0.253	0.724	0.813	0.317

[1] The relative expression levels of the genes of respective treatment groups are expressed as means of normalized relative quantities (NRQ). Relative quantities for individual genes are scaled to the average across all unknown samples per target gene.
[2] Within a column, values with different superscripts are significantly different from each other at $P < 0.05$

8.4.4 Upregulation of APN by coarsely ground corn and CAT1 by sugarcane bagasse in duodenum

The mRNA expression of nineteen genes was investigated in response to feed SB addition and corn particle size in the duodenum as presented in Table 8.7 and 8.8. It was shown that the CC diet significantly upregulated APN in the duodenum compared with expression of the gene in the FC diet group. No responses to CC diet were observed for other genes investigated, namely, ASCT1, ATP1A1, B^oAT, CAT1, CAT2, CCK1R, CCK, bo,+AT, GLUT1, GLUT2, LAT1, PepT1, PepT2, SI, y^+LAT2, and rBAT. Addition of 2% SB upregulated CAT1 in the duodenum compared to the expression in the birds without SB ($P < 0.05$). However, 2% SB did not affect the expression of the genes APN, ASCT1, ATP1A1, B^oAT, CAT2, CCK1R, CCK, bo,+AT, GLUT1, GLUT2, LAT1, PepT1, PepT2, SI, y+LAT2, and rBAT in the duodenum ($P > 0.05$). A particle size × SB interaction was observed for expression of gene y+LAT1 ($P < 0.05$), where 2% SB downregulated y+LAT1 only in the birds fed FC but not in those fed CC diet.

8.4.5 Upregulation of jejunal ASCT1 and y+LAT2 by coarsely ground corn and CAT1 by sugarcane bagasse

The mRNA expression of nineteen jejunal genes in response to feed SB addition and corn particle size was examined (Table 8.9 and 8.10). The expression of ASCT1 ($P < 0.01$) and Y+LAT2 ($P < 0.05$) in jejunum were upregulated in birds fed CC diet compared with those fed FC diet. Corn particle size did not affect other genes investigated: APN, ATP1A1, B^oAT, CAT1, CAT2, CCK1R, CCK, bo,+AT, GLUT1, GLUT2, LAT1, PepT1, PepT2, SI, y+LAT1, and rBAT, in jejunum ($P > 0.05$). Birds fed 2% SB had upregulated jejunal CAT1 compared to birds fed diet without SB ($P<0.05$). No responses to 2% SB were observed in other investigated genes: APN, ATP1A1, B^oAT, CAT2, CCK1R, CCK, bo,+AT, GLUT1, LAT1, PepT1, PepT2, SI, y+LAT1, and rBAT, in jejunum ($P > 0.05$). No interactions were observed between particle size and SB on expression of other investigated genes in the jejunum. However, a tendency for a particle size × SB interaction was observed for expression of CAT1 ($P = 0.077$) in the jejunum, where the birds fed CC diet with 2% SB inclusion tended to upregulate CAT1.

Table 8. 7 Effect of particle size and sugar cane bagasse on expression of duodenal genes at d 24

Treatments Particle size	SB	APN	ASCT1	ATP1A1	B^{o}AT	CAT1	CAT2	CCK1R	CCKD	EAAT3
CC	0%	1.458	1.128	0.958	1.079	0.920	1.071	1.231	1.049	0.979
FC	0%	0.897	0.848	0.967	1.114	0.813	1.196	1.232	1.304	1.187
CC	2%	1.367	1.056	1.176	0.900	1.3127	0.894	0.775	1.024	1.149
FC	2%	0.781	1.082	1.015	1.091	1.388	0.983	1.196	1.051	1.059
Main effect										
SB										
0%		1.177	0.988	0.962	1.097	0.867^{b}	1.133	1.231	1.177	1.083
2%		1.074	1.069	1.096	0.995	1.350^{a}	0.938	0.985	1.037	1.104
Particle size										
CC		1.413	1.092	1.067	0.990	1.117	0.9824	1.003	1.036	1.064
FC		0.839	0.965	0.991	1.102	1.100	1.0893	1.214	1.178	1.123
P value										
SB		0.689	0.419	0.203	0.444	0.037	0.088	0.202	0.508	0.924
Particle size		0.036	0.210	0.465	0.395	0.940	0.338	0.273	0.501	0.787
SB × particle size		0.961	0.135	0.411	0.556	0.675	0.870	0.273	0.587	0.499

[1] The relative expression levels of the genes of respective treatment groups are expressed as means of normalized relative quantities (NRQ). Relative quantities for individual genes are scaled to the average across all unknown samples per target gene.
[2] Within a column, values with different superscripts are significantly different from each other at $P < 0.05$.

Table 8. 8 Effect of particle size and sugar cane bagasse on expression of duodenal genes at d 24

Treatments Particle size	SB	bo,+AT	GLUT1	GLUT2	LAT1	PepT1	PepT2	SI	y+LAT1	y+LAT2	rBAT
CC	0%	0.947	1.133	1.371	1.240	0.953	1.981	0.941	0.887[b]	1.031	0.985
FC	0%	1.140	1.010	1.003	0.756	1.073	1.380	1.119	1.316[a]	1.015	1.154
CC	2%	1.001	1.115	1.106	1.100	1.228	1.351	1.170	1.101[ab]	1.144	1.066
FC	2%	1.132	0.883	0.904	1.101	0.992	1.233	1.052	0.926 [ab]	0.970	0.982
Main effect											
SB											
0%		1.043	1.071	1.187	0.998	1.013	1.681	1.030	1.101	1.023	1.069
2%		1.067	0.999	1.005	1.101	1.110	1.292	1.111	1.014	1.057	1.024
Particle size											
CC		0.974	1.124	1.239	1.170	1.090	1.666	1.055	0.994	1.087	1.026
FC		1.136	0.946	0.954	0.929	1.032	1.307	1.085	1.121	0.993	1.068
P value											
SB		0.875	0.541	0.389	0.424	0.579	0.323	0.618	0.545	0.822	0.746
Particle size		0.289	0.142	0.183	0.069	0.741	0.360	0.853	0.382	0.530	0.762
SB × particle size		0.837	0.643	0.691	0.068	0.314	0.536	0.370	0.047	0.602	0.367

[1] The relative expression levels of the genes of respective treatment groups are expressed as means of normalized relative quantities (NRQ). Relative quantities for individual genes are scaled to the average across all unknown samples per target gene.
[2] Within a column, values with different superscripts are significantly different from each other at $P < 0.05$

Table 8. 9 Effect of particle size and sugar cane bagasse on expression of jejunal genes at d 24

Treatments Particle size	SB	APN	ASCT1	ATP1A1	B^{o}AT	CAT1	CAT2	CCK1R	CCKD	EAAT3	bo,+AT
CC	0%	1.029	1.146	1.107	1.058	0.758	0.981	1.004	1.079	1.148	1.091
FC	0%	1.060	0.827	0.994	1.062	0.833	1.065	1.066	1.105	1.086	1.202
CC	2%	1.103	1.011	1.098	1.037	2.453	0.957	1.048	0.950	1.046	1.049
FC	2%	0.961	0.990	0.918	1.137	1.100	1.078	1.021	1.077	0.966	1.303
Main effect											
SB											
0%		1.044	0.986	1.051	1.060	0.796	1.023	1.035	1.092	1.117	1.147
2%		1.032	0.955	1.008	1.087	1.776	1.017	1.034	1.013	1.006	1.176
Particle size											
CC		1.066	1.078[a]	1.103	1.048	1.606	0.969	1.026	1.014	1.097	1.070
FC		1.010	0.863[b]	0.956	1.099	0.967	1.071	1.044	1.091	1.026	1.253
P value											
SB		0.917	0.628	0.705	0.873	0.019	0.943	0.993	0.586	0.428	0.902
Particle size		0.644	0.003	0.199	0.762	0.111	0.232	0.886	0.596	0.611	0.452
SB × particle size		0.472	0.117	0.765	0.778	0.077	0.827	0.718	0.726	0.948	0.769

[1] The relative expression levels of the genes of respective treatment groups are expressed as means of normalized relative quantities (NRQ). Relative quantities for individual genes are scaled to the average across all unknown samples per target gene.

[2] Within a column, values with different superscripts are significantly different from each other at $P < 0.05$

Table 8. 10 Effect of particle size and sugar cane bagasse on expression of jejunal genes at d 24

Treatments Particle size	SB	GLUT1	GLUT2	LAT1	PepT1	PepT2	SI	y+LAT1	y+LAT2	rBAT
CC	0%	1.074	1.794	1.120	1.131	2.087	1.288	1.017	1.143	1.154
FC	0%	1.001	1.276	0.844	1.108	1.659	1.044	1.048	0.984	1.105
CC	2%	1.132	0.973	1.004	1.026	1.633	1.001	1.011	1.029	0.997
FC	2%	0.886	0.682	0.947	0.952	1.611	0.871	1.012	0.896	1.003
Main effect										
SB										
0%		1.037	1.535[a]	0.982	1.119	1.873	1.166	1.032	1.063	1.129
2%		1.009	0.828[b]	0.975	0.989	1.622	0.936	1.011	0.963	1.000
Particle size										
CC		1.103	1.383	1.062	1.078	1.849	1.145	1.014	1.086[a]	1.075
FC		0.943	0.979	0.895	1.030	1.646	0.958	1.030	0.940[b]	1.054
P value										
SB		0.744	0.018	0.968	0.389	0.649	0.089	0.824	0.119	0.395
Particle size		0.075	0.157	0.309	0.747	0.713	0.163	0.865	0.028	0.886
SB × particle size		0.322	0.684	0.501	0.864	0.682	0.660	0.868	0.837	0.857

[1] The relative expression levels of the genes of respective treatment groups are expressed as means of normalized relative quantities (NRQ). Relative quantities for individual genes are scaled to the average across all unknown samples per target gene.
[2] Within a column, values with different superscripts are significantly different from each other at $P < 0.05$

Table 8. 11 Effect of particle size and sugar cane bagasse on expression of ileal genes at d 24

Treatments Particle size	SB	APN	ASCT1	ATP1A1	B^{o}AT	CAT1	CAT2	CCK1R	EAAT3	bo,+AT
CC	0%	1.046	1.099	0.906^{b}	0.923	0.986	1.0652	1.128	1.187	0.971
FC	0%	1.330	1.010	1.064^{ab}	0.926	0.936	1.0146	0.910	1.054	1.010
CC	2%	0.858	0.953	1.081^{a}	1.075	1.522	0.989	1.236	0.986	1.078
FC	2%	1.170	1.116	0.993^{ab}	1.155	2.074	1.005	0.918	1.353	1.090
Main effect										
SB										
0%		1.188	1.055	0.985	0.925^{b}	0.961^{b}	1.040	1.019	1.121	0.991
2%		1.014	1.034	1.037	1.115^{a}	1.798^{a}	0.997	1.077	1.170	1.084
Particle size										
CC		0.952	1.026	0.994	0.999	1.254	1.027	1.182	1.087	1.025
FC		1.250	1.063	1.029	1.041	1.505	1.010	0.914	1.204	1.050
P value										
SB		0.401	0.867	0.387	0.019	0.029	0.627	0.664	0.876	0.474
Particle size		0.156	0.765	0.554	0.583	0.489	0.846	0.056	0.709	0.844
SB × particle size		0.943	0.314	0.049	0.611	0.407	0.705	0.707	0.428	0.918

[1] The relative expression levels of the genes of respective treatment groups are expressed as means of normalized relative quantities (NRQ). Relative quantities for individual genes are scaled to the average across all unknown samples per target gene.
[2] Within a column, values with different superscripts are significantly different from each other at $P < 0.05$

Table 8. 12 Effect of particle size and sugar cane bagasse on expression of ileal genes at d 24

Treatments Particle size	SB	GLUT1	GLUT2	LAT1	PepT1	PepT2	SI	y+LAT1	y+LAT2	rBAT
CC	0%	1.202	0.900	1.101	0.963	3.520	1.142	1.171	1.055	0.980
FC	0%	1.080	0.877	1.064	0.937	1.426	1.281	0.886	1.008	1.113
CC	2%	1.081	1.091	0.914	1.144	3.224	0.920	1.124	0.939	0.993
FC	2%	0.947	1.105	1.167	1.122	1.984	1.164	0.976	1.125	0.995
Main effect										
SB										
0%		1.141	0.888	1.083	0.950	2.473	1.211	1.028	1.032	1.047
2%		1.014	1.098	1.041	1.133	2.604	1.042	1.050	1.033	0.994
Particle size										
CC		1.142	0.995	1.008	1.054	3.372[a]	1.031	1.147	0.998	0.987
FC		1.014	0.991	1.115	1.030	1.705[b]	1.223	0.931	1.067	1.054
P value										
SB		0.479	0.369	0.787	0.141	0.857	0.534	0.862	0.994	0.554
Particle size		0.477	0.986	0.491	0.842	0.031	0.481	0.097	0.525	0.452
SB × particle size		0.975	0.936	0.355	0.988	0.557	0.846	0.589	0.289	0.460

[1] The relative expression levels of the genes of respective treatment groups are expressed as means of normalized relative quantities (NRQ). Relative quantities for individual genes are scaled to the average across all unknown samples per target gene.
[2] Within a column, values with different superscripts are significantly different from each other at $P < 0.05$

8.4.6 Upregulation of ileal PepT2 by coarsely ground corn and CAT1 by sugarcane bagasse

The mRNA expression of eighteen ileal genes in response to feed SB addition and corn particle size was examined (Table 8.11 and 8.12). Birds fed the CC diet had upregulated ileal PepT2 compared to birds fed the FC diet ($P < 0.05$). The CC diet tended to upregulate CCK1R ($P = 0.056$) and y+LAT1 ($P = 0.097$) in the ileum. Corn particle size did not affect the expression of: APN, ASCT1, ATP1A1, B^oAT, CAT1, CAT2, CCK, bo,+AT, GLUT1, GLUT2, LAT1, PepT1, SI, y^+LAT2 and rBAT, in the ileum. Inclusion of 2% SB upregulated expression of B^oAT and CAT1 in the ileum compared to birds given no SB ($P < 0.05$). Inclusion of 2% SB had no effect ($P > 0.05$) on other genes: APN, CAT2, CCK1R, CCK, bo,+AT, GLUT1, LAT1, PepT1, PepT2, SI, y^+LAT1, and rBAT in ileum. A particle size × SB interaction was observed for expression of ATP1A1 in the ileum ($P < 0.05$), where 2% SB inclusion increased mRNA expression of ATP1A1 in the birds fed the CC diet but not in the birds fed the FC diet.

8.5 Discussion

This This study investigated responses of mRNA expression of genes encoding digestive enzymes and nutrient transporters in birds fed diets with SB and/or increased corn particle size. Gene expression in the proventriculus, pancreas and intestine of broiler chickens was increased in line with bird performance and gizzard development. It was demonstrated that 2% SB supplementation and CC particle inclusion in feed upregulated some genes encoding digestive enzymes, and nutrient transporters together with improved gizzard function and performance in birds up to d 24.

The combination of CC and SB improved broiler performance as demonstrated by lower FCR in birds fed CC together with SB. It is hypothesized that coarse particle size and structural fiber may have: 1) extended digesta retention time, leading to prolonged exposure of nutrients to endogenous enzymes; 2) promoted gut reflux, re-exposing undigested nutrients to digestive enzymes for better digestion; 3) created a better microenvironment for enzyme activity around coarse corn particles of SB that has a strong water-holding capacity (25); 4) enhanced gizzard activity, leading to

secretion of more digestive juices and better ability to grind feed particles. Indeed, both pepsinogen A and C were correlated with FCR on d24. The significantly greater mRNA expression of pepsinogen A and C in the birds fed CC diet supplemented with SB might increase the production of pepsin in proventriculus and improved FCR. The similarity in increase between mRNA of pepsinogen A and that of pepsinogen C suggests that the mechanism of stimulation of the two zymogens was similar and could have been caused by; physical effects of the combination of CC and SB on the oxyntic (or oxynticopeptic) cells of the proventriculus or, by one or both of the following possible mechanisms that may affect pepsinogen production: 1) the stimulating effect of coarse particles and fiber on gizzard function, in particular more frequent and powerful contractions which may potentially reflux the digesta back into the proventriculus repeatedly during each gizzard contraction and thereby allows for more proventricular secretions and thus re-expose the digesta to these two zymogens and pepsin; 2) Coarse particle and SB might cause more rapid duodenal-gizzard reflux and move the digesta back to the gizzard and then to proventriculus. Indeed, bile salts, associated with duodenum, have been reported to be found in the gizzard at low concentrations (26). It has been hypothesized that the structural components, such as whole or coarsely ground cereals, or fiber materials, in the diet induce an increase in gastrointestinal reflux via a well-developed gizzard (26, 27). However, in this study, the SB may have created an environment where gizzard contractions were enhanced and mucosal surface cleaned, leading to unhindered duodenal-gizzard reflux. This, in turn, could expose digesta to proventricular zymogens and enzymes repeatedly, resulting in better digestion of nutrients and a more dynamic foregut.

In recent years, the beneficial effects of fiber on gastrointestinal tract development and nutrient utilization in poultry have been discussed frequently. The source, physical structure and amount of fiber in the diet determine the effectiveness of fiber on birds (28, 29). For instance, previous studies have reported that insoluble fiber (ARBOCEL®) increased the activity of pancreatic enzymes such as chymotrypsin (30, 31). Furthermore, it has been suggested that oat hulls may stimulate pancreatic secretion of amylase and thus increase the activity of amylase in the jejunum (17). The significantly greater expression of pancreatic AMY2A and CELA1 in the birds fed diet supplemented with SB is at least partially responsible for better performance

in the present study. A number of studies have suggested that well developed gizzards in birds fed diets with large ingredient particles or fiber, improves gut motility, and thus digestibility and performance due to increased releases of cholecystokinin (CCK) which stimulates pancreatic enzyme secretion and gastro-duodenal reflux (3, 9, 17, 30, 32). Although a heavier gizzard weight accompanied by greater expression of proventricular pepsinogens and pancreatic AMY2A and CELA1 were observed in this study, the expression of both CCK and its receptor (CCK1R) in different tissues was not affected by particle size and SB addition. It has been previously reported that CCK at physiological concentrations has no influence on pancreatic secretion from isolated pancreatic acini in the chicken (33). It has also been stated that CCK plays a more important role as a pancreas-stimulating hormone in mammals than in birds (33). Furthermore, it was reported that the regulation of pancreatic secretion is controlled by numerous hormones such as melatonin and glucagon, regulatory peptides including C-natriuretic peptide, and neurotransmitters such as serotonin, vasoactive intestinal peptide (VIP) and gastrin releasing peptide (GIP) (34). Therefore, the mechanism underlying the heightened secretion of pancreatic enzymes by inclusion of fiber or large feed particles in chicken diets is not well understood and further investigation is warranted.

Several studies investigating structural components of diets, such as coarse particle size or fiber, have shown improved nutrient digestibility (3, 14-17, 35) via increased digesta retention time. Reports investigating the impact of corn particle size and fiber on digestive enzymes and nutrient transporters are scant or non-existent. In the present study, broiler performance was increased by inclusion of dietary coarse particle grain and SB and various nutrient transporters and digestive enzymes in the duodenum, jejunum and ileum were shown to be upregulated. For instance, SB upregulated B^0AT, the neutral amino acid transporter located at the brush border membrane, in ileum and CAT1, a transporter mediating the bidirectional transport of cationic amino acids, in duodenum, jejunum and ileum. Furthermore, CC upregulated duodenal APN, responsible for final digestion of peptides by N terminus cleavage, jejunal ASCT1, responsible for Na+-dependent neutral amino acid transporter, and y+LAT2, responsible for Na+-dependent neutral/cationic amino acid exchanger, and ileal PepT2, which transport di- and tripeptides. In fact, the upregulated nutrient transporters will not only improve nutrient absorption but also

play a vital role in the maintenance of intestinal barrier integrity and immune response. A deficiency of amino acids, such as alanine, cysteine, serine, threonine, arginine, and lysine, has long been known to impair immune function and increase the susceptibility of animals to infectious disease (36). Different mechanisms can be involved to elucidate the increase in expression of amino acid transporters in the gut. For example, CAT-1 mRNA expression level varies considerably in different tissues and cell types and can be modulated by a variety of stimuli, including cell proliferation, growth factors, cytokines, hormones, and nutrients (37). In the current study, a well-developed gizzard may generate stronger reverse peristalsis contractions that may stimulate the secretion of digestive enzymes and enzyme precursors in both the proventriculus and pancreas and consequently produce higher levels of substrates of nutrient transporters and thus upregulate those nutrient transporters in the gut. In general, three distinct sites of reverse peristalsis can be observed in the gastrointestinal tract of birds (38): 1) gastric reflux which transfer the digesta from gizzard to proventriculus via gastroduodenal contractions and this contraction cycle takes place 2-4 times per min.; 2) the small intestinal reflux which transfers digesta from the duodenum and jejunum into the gastric area and occurs about 4 times per 60 min.; 3) cloaca-cecal reflux, which transfers urinary nitrogen to the ceca via the colon (39). It has been well documented that structural components of the diet such as fiber and coarse or whole cereals enhance the gut motility and thereby increase the digesta retention time and better bird performance (27, 40). Therefore, SB and CC may have increased gut motility and digesta retention time, particularly in the upper part of digestive tract, and thereby enhanced the production of digestive enzymes, enzyme precursors and nutrient transporters. These active functional proteins promote digestion of nutrients and thus the growth and feed conversion efficiency of the birds.

8.6 Conclusions

In conclusion, the inclusion of either SB or CC in a pelleted diet is beneficial to the birds by improving performance likely through the upregulation of genes encoding digestive enzymes and nutrient transporters. The combination of CC and SB was more beneficial for the upregulation of some genes such as PGA5 and PGC. The results suggest enhanced gizzard development as a mode of action for higher production of digestive enzymes and nutrient transporters. These findings provide

insights on how dietary fiber and particle size independently or in combination can improve bird performance based on the analysis of gene expression. The knowledge obtained herein will be useful to understand the underlying mechanisms of how feed additives can improve nutrient digestibility and thus feed efficiency. Further, the outcomes lay a foundation for future research to elucidate the usefulness of fiber supplementation and coarse particle inclusion in feed in a nutrigenomic way.

Competing interests

The authors declare that they have no competing interests.

8.7 Authors' contributions

SKK performed the experiment and lab work, analyzed and interpreted the data, designed primers and drafted the manuscript. MC designed and supervised the experiment, and revised the manuscript critically. RAS supervised the experiment and critically revised the manuscript. SBW participated the design of the experiment, directed molecular laboratory work and data analysis, interpreted data, and critically revised the manuscript. All authors read and approved the final manuscript.

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8.9 Abbreviations

SB, sugarcane bagasse; FCR, feed conversion ratio; CC, coarsely ground corn; FC, finely ground corn; GIT, gastrointestinal tract; GMD, geometric mean diameter; SID, standard ileal digestible; NSP, non-starch polysaccharides; VIP, vasoactive intestinal peptide; GIP, gastrin releasing peptide; ATP1A1, ATPase Na+/K+ transporting subunit alpha 1; AMY2A, pancreatic alpha 2A amylase; APN, aminopeptidase N; ASCT1, alanine, serine, cysteine, and threonine transporter; B^0AT, solute carrier family 6, member 19; bo,+AT, solute carrier family 7, member 9; CAT1, cationic amino acid transporter-1; CAT2, cationic amino acid transporter-2; CCK, cholecystokinin; CCK1R, cholecystokinin type 1 receptor; CELA1, chymotrypsin-like elastase family, member 1; CELA2A, chymotrypsin like elastase family member

2A; EAAT3, excitatory amino acid transporter 3; GLUT1, glucose transporter-1; GLUT2, glucose transporter-2; LAT1, L type amino acid transporter-1; PGA5, pepsinogen A; PGC, pepsinogen C; PepT1, peptide transporter-1; PepT2, peptide transporter-2; PNLIP, pancreatic lipase; rBAT, solute carrier family 3, member1; SI, sucrase isomaltase; y+LAT1, y^+ L amino acid transporter-1; y+LAT2, y^+ L amino acid transporter-2; HPRT1, hypoxanthine Phosphoribosyltransferase 1; TBP, TATA-Box binding protein.

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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Chapter 9

9.1 General conclusion

The ban of in-feed antibiotics in the broiler industry have led to the emergence of wet litter, enteric disorders, imbalance of gut microflora and poor performance and nutrient digestibility thus reduced productivity and profitability. To minimise the effect of such issues, different alternative strategies have been used in this thesis to modulate the development and physiological status of upper gastrointestinal tract, gut microflora, and litter conditions so as to improve performance, health and welfare of the broiler chickens.

Wood shavings is characterised by high capacity of absorption moisture from the excreta of birds and thus to keep the environment suitable for the broilers to grow without developing lesions such as foot pad dermatitis and breast blisters. However, the wood shavings as bedding materials have become more scarce and costly since a rapid expanding of broiler production in the world and the ban of in-feed antibiotics which resulted in the emergence of wet litter due to enteric disorders. Experimental results suggest that a potentially novel bedding material, i.e., pelleted wheat straw, can be used for broilers. Pelleted straw litter was less caked than chopped straw and shredded paper whereas no significant differences were observed between pelleted straw, wood shavings and rice hulls. Pelleted wheat straw is beneficial to young birds' performance.

Consumption of fibre from feed or bedding materials by birds is an important aspect of nutritional management in the chicken meat production system. The results reported in this thesis demonstrate that birds consume fiber even when it's not included in the diet. For instance, birds accessed to free choice oat hulls (OH) had heavier gizzards than those without access to OH. Free choice OH is beneficial in improving bird performance at least at younger ages. Offering birds with OH alters concentrations and types of short chain fatty acid (SCFA) which may benefit bird performance and immunity.

It is well conceded that appropriate physical structure of feed ingredients and addition of certain sources of fiber enhance gut health, gizzard development, nutrient digestibility and performance in poultry. The inclusion of coarsely ground corn (CC)

in pelleted diet is beneficial to performance, nutrient digestibility, nutrient transporters and digestive enzymes, possibly through enhanced gizzard development in birds. The results reported in this thesis show that the supplementation of diets with 1 or 2% of lignocellulose-rich fiber source can improve litter quality without any adverse effect on broiler performance, nutrient digestibility, and gizzard development. On the other hand, the inclusion of 2% SB in pelleted diets showed improved performance, increased ileal beneficial microflora groups such as Bacillus spp., better gizzard development, higher nutrient digestibility and upregulated genes encoding digestive enzymes and nutrient transports. It, however, has no effect on litter quality.

The combination of lignocellulose and CC in pelleted diet improves FCR in young birds. However, the combination of CC and SB in pelleted diet enhances performance in older birds as well possibly through upregulated pepsinogens A and C and enhanced gizzard development. The results suggest stimulation of gizzard development and function may be a mode of action for SB.

These findings suggesting that it may be beneficial to include fiber and CC together in broiler diets as a tool for poultry producers to improve feed efficiency and growth performance. However, the choice of fiber sources may be critical so as to be used together with CC in broiler diet to improve bird performance. Therefore, further investigations on the types of fibres for such purpose is warranted.