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CAECAL MICROFLORA COMPOSITION IN BROILERS FED SORGHUM BASED DIETS CONTAINING FEED ENZYMES

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ABSTRACT. This study was conducted to investigate whether dietary enzymes alter the caecal microbial profile of broilers fed sorghum-based diets. Four sorghum-based diets (918 g sorghum/kg diet) were prepared. One was the control diet and three had enzymes (xylanase, phytase and protease) added. Broilers, 35-day-old, were reared (8 birds/cage) in an environmentally controlled shed and randomly allocated to replicated (n=4) assay diets and free access to feed and water all time. On day-42, birds were euthanized and caecal contents collected, pooled on a per/pen basis and frozen (-20 °C). The DNA was extracted from caecal samples using a bead-beating protocol and the V2V3 region of the bacterial 16S rRNA gene amplified by PCR. Amplicons were separated on sequence difference using Denaturing Gradient Gel Electrophoresis (DGGE) and microbial profiles generated and compared.

The DGGE profiles, when analysed, indicated that there was approximately 80% similarity between caecal microflora in all types of the diet treatments. This suggests that there was no overall difference between any of the profiles and therefore the addition of different types of feed enzymes in a sorghum-based diet had no impact on the overall composition of the broiler caecal microflora.

Keywords: sorghum, feed enzymes, caecal microbial, denaturing gradient gel electrophoresis (DGGE)

INTRODUCTION

Sorghum is one of the most important cereal grains in the world. It is used in human and animal nutrition and has the advantage that it is suitable to be grown in drought stressed climates (Ali *et al.,* 2009; Mohammed *et al.,* 2010). It is a grain of potentially high nutritive value (Salinas *et al.,* 2006) and can have a highly variable crude protein (CP) 5.44 to 12.9% content. In addition, it contains the anti-nutritive factors, polyphenols, phytate and kafirins (Duodu *et al.,* 2003) which may interfere with protein and carbohydrate availability. As a result, broilers fed sorghum based diets

may have reduced growth rates, depressed feed conversion ratios and variable breast meat yields (Selle *et al.,* 2010).

Feed enzymes either in combination or alone can help to increase sorghum digestibility in meat type chicken by reducing the impact of the anti-nutritive factors. As suggested by Cadogan *et al.* (2005), the use of feed enzymes including a combination of protease, amylase and xylanase showed significant increments in body weight gain and feed intake: 3.7% and 4.9% respectively of broilers fed a sorghum-based diet.

The beneficial effects of feed enzymes may in part be due to modification of the caecal microflora. The caecal microflora, among other attributes, contributes to feed digestion, normal caecal function and prevention of caecal colonisation by pathogenic bacteria. The microbes also have their own specific function depending on the species distribution and total population of the microbes (Bedford and Apajalahti, 2001). Any changes in diet may severely affect the total population and or metabolic activity of resident microbes in the caecal.

As the feed enzyme is an additive to animal feed, it has been improved for animal growth. The utilisation of feed enzymes in animals' diet has been proven profitable, especially in chickens. The poultry producers in the world are very concerned about their poultry growth and production thus have chosen enzymes as feed additives instead of using a drug growth promoter. The roles of the enzymes are well known as aiding the digestibility and absorption of nutrients, reducing the pathogenic bacteria and promoting the growth of gut microflora (Walsh *et al.,* 1994). Besides, it is safe and also can increase the environmental quality by the reduction of pollutants such as ammonia, phosphate and nitrogen (Marquardt and Bedford, 1997).

The aim of this study is to examine the effect of feed enzymes on the composition of the caecal microflora of broiler chickens fed a sorghum-based diet. It is hoped the information gathered from this study will alleviate the nutrition based solutions for the poultry industry which is a fast growing entity dependent largely on innovative nutritional sources.

MATERIALS AND METHODS

Animal and management

All the bioassay measures undertaken in these studies involving birds were pre-approved by the University of Queensland Animal Care and Ethics Committee and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

The MR-Buster (red) sorghums were provided by Feedworks, Australia. Sorghum was hammer milled in the Feed Mill, Gatton campus and used for mixing diets. A total of four basal assay diets were prepared (4-cage/diet) which comprised of Diet 1: red sorghum (control); Diet 2: red sorghum + xylanase; Diet 3: red sorghum

+ phytase and Diet 4: red sorghum + protease. Feed ingredients for an assay diet were mixed in a feed mixer available at the Poultry Unit, Gatton. The ingredients of assay diets were mixed thoroughly and stored in plastic feed bins labelled with diet number for the experiment.

One hundred and twenty-eight 35-day-old birds were obtained and randomly chosen to 16 cages (8-bird/cage) and assigned to different dietary treatments. The experimental shed, metabolic cages and other facilities were washed and disinfected before the commencement of experiment.

On day-42, all the birds were euthanized. Samples from all birds within a cage were pooled, frozen immediately (-20 ^{o}C) after collection, and subsequently freeze-dried (Christ®, Quantum Scientific, Australia).

Laboratory analyses

The pooled caecum samples from eight birds were mixed thoroughly. Approximately 180-220 mg of sample was transferred to a 2 ml labelled microcentrifuge tube and was placed on ice prior to DNA extraction. DNA extraction procedure was based on instruction in the QIAGEN Stool Kit with minor modification. The DNA concentration and purity was measured using a spectrophotometer. The concentration was determined by measuring the wavelength absorbance at 260-280 nm (BioSpec-nano Shimadzu®).

The DNA concentration also was determined by running an agarose gel electrophoresis. Two sets of DNA concentration were prepared, of which one set is the original concentrated solution of purified template DNA and the other set is a duplicate 10-fold dilution of the DNA. The dilution of DNA concentration was made for observing the difference of the reaction between the concentrated and the 10-fold diluted DNA.

Each sample was prepared and set up in a 1.5 mL Eppendorf tube, containing 4 µL of Fermentas 6× Loading Dye and 6 µl of PCR product. The sample was loaded into wells using the appropriate DGGE loading tips and a 20 µL pipette. 1 ul reference ladder as described by (Klieve *et al.,* 2007), was loaded into three lanes as reference. Amplification was done according to (Milinovich *et al.,* 2008) by denaturing gradient gel electrophoresis (DGGE) (DCode System, Bio-Rad Hercules, CA, USA). The separation of the PCR products was performed on 8% acrylamide gels with 30-60% formamide/urea gradients. Two gels were run in 0.5× TAE buffer (Tris-acetate, 0.04 M; EDTA, 0.001 M) at 60 °C with 100 volt for 18 hours. The DNA bands which were shown in the gels were visualized by silver staining.

RESULTS

Throughout the experimental period the birds appeared clinically normal and there was no morbidity and mortality.

The concentration of nucleic acids obtained from the caecal contents are shown in Table 1. There is a difference of DNA concentration among the four replicates within each treatment. For Diet 1, the line graph (Figure 1), demonstrates the variation between replicates which was also very obvious between diets 2, 3 and 4.

PCR products on agarose gel

Band fragments at 200 bp were obtained from all samples of 16S rRNA V2-V3 region as shown in Figure 2. Band fragments from non-diluted DNA extraction (sample 1 to 16) were brighter than band fragments from diluted DNA extraction at dilution 1:10. It was observed that there was a slightly different reaction between the concentrated and the 10-fold diluted DNA.

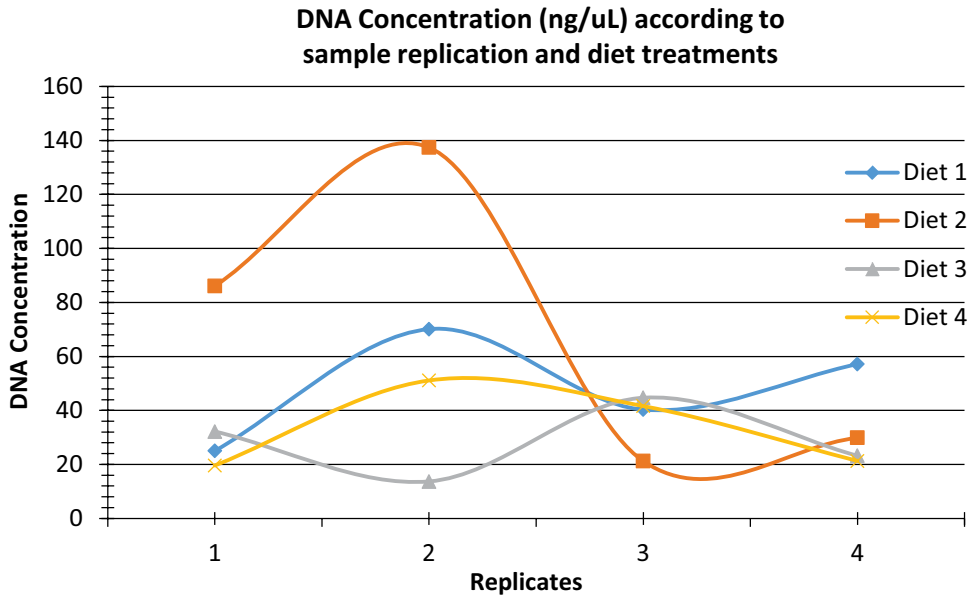

Figure 1. Concentration of DNA of caecal samples from all diet treatments.

Table 1. Determination of nucleic acid concentration of sample from each diet treatment.

Composition	Nucleic acid concentration (ng/uL)			
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**
	Red sorghum (control)	Red sorghum + xylanase	Red sorghum + phytase	Red sorghum + protease
Replicate 1	25.00	86.09	32.2	19.6
Replicate 2	70.12	137.38	13.7	51.1
Replicate 3	40.29	21.22	44.7	41.6
Replicate 4	57.17	29.85	23.2	21.3

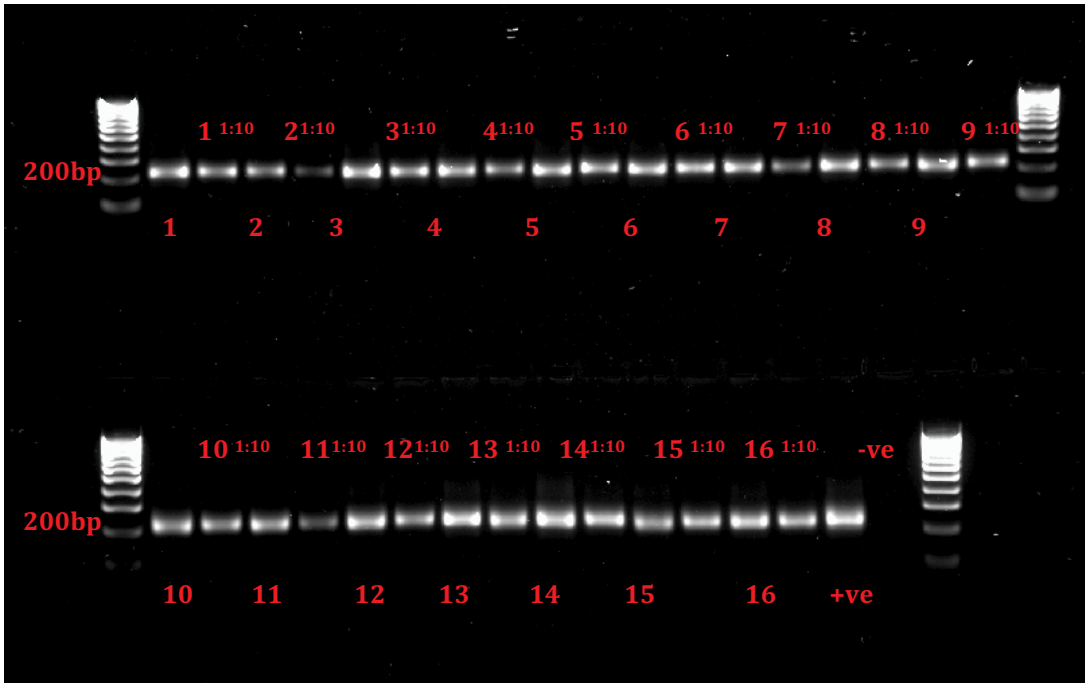

Figure 2. 16SrRNA amplified by PCR separated on 1% agaros gel.

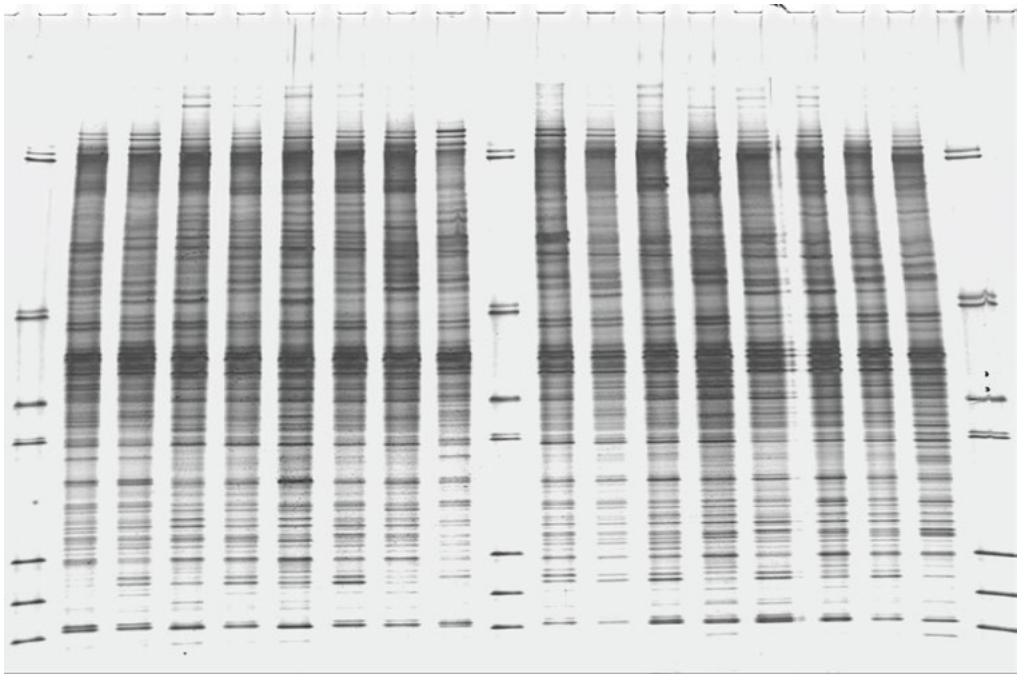

Figure 3. DGGE analysis of 16S amplicon band patterns.

Amplification by DGGE

Following DNA extraction, the V2V3 region of the bacterial 16SrRNA gene was amplified by PCR-based DGGE. Amplicons were separated on sequence difference using Denaturing Gradient Gel Electrophoresis and are displayed in Figure 3.

The similarity of the profiles among the 4 replicates of each dietary treatment was represented by Pearson correlation and Dice coefficient and is shown the dendrograms, Figures 4 and 5 respectively.

Based on Pearson correlation, the basis biodiversity of caecal microflora were divided into two main groups. Bands for s4 protease to s3 phytase were formed a group with 46% similarity of microflora composition with other group consisting s11 phytase to s5 control. Microbial contents between s4 protease to s2 xylanase were formed a cluster with s9 control to s3 phytase with 61% correlation. Meanwhile s11 phytase to s10 xylanase formed another cluster with s1 control to s5 control with correlation of 62%. A group of s4 protease to s2 xylanase formed a subclass of 70%, s9 control to s16 protease formed another subclass with s14 xylanase to s3 phytase for the percentage correlation of 72%. There was a great similarity between s11

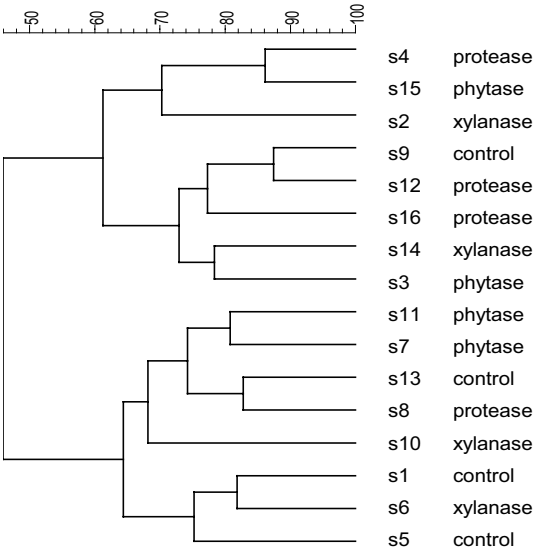

Figure 4. Denaturing gradient gel electrophoresis of caecal microflora of 16S amplicon band patterns. Pearson correlation for relative similarity of band patterns is indicated by their grouping on the dendrogram and the percentage coefficient (bar).

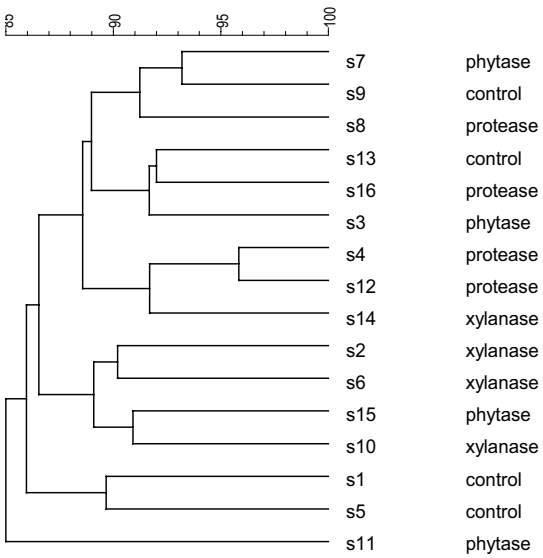

Figure 5. Denaturing gradient gel electrophoresis of caecal microflora of 16S amplicon band patterns. Dice coefficient for relative similarity of band patterns is indicated by their grouping on the dendrogram and the percentage coefficient (bar).

phytase to s8 protease with s10 xylanase to form a cluster with 66%. Sample for s1 control and s6 xylanase were formed a cluster of 74% with s5 control.

Dice coefficient based on band similarity has encountered 85% similarity where two groups were divided. Samples of s7 phytase to s5 control were formed a group and s11 phytase formed the second group.

Overall analysis of these two profiles (Pearson and Dice) indicates that there was an appropriate 80% similarity of gut microflora between the four dietary treatments.

DISCUSSION

The basal diet used in this study consisted of one major ingredient and it was so designed to remove any confounding factors by not including other major commercial feed ingredients. Therefore the only variation between diets was the enzyme added and the difference may have been insufficient to change the microflora. Before the experiment the birds had been reared together and were only fed the experimental diets for 7 days. This may have been insufficient time for microbial changes to occur as it has been suggested that 21 to 28 days are required (Al Jassim R., 2010, pers. comm). Choct *et al.* (2006) used a 21-day feeding trial to investigate the efficacy of xylanase supplementation in reducing between-bird variation for AME in broilers fed a wheat-based diet.

Perhaps the age of the birds contributed to the individual variation discussed below. A study by Mead and Adam (1975) (cited in Lan *et al.,* 2005) suggested that one of the factors affecting the composition of caecal microflora is the animal's age. According to Smith (1965) (cited in Lan *et al.,* 2005) and Coloe *et al.* (1984), the establishment of a bacterial community occurs within the first two weeks of life. However, after 42 days of age, lactobacillus has taken over and is dominant. Indeed, approximately 6 to 7 weeks is required for bacterial communities to colonize the caecum (Coloe *et al.,* 1984).

The processing of diets is one of the important factors that influence the feed intake, thus determine the transit time in the gastrointestinal tract of the chicken (Amerah *et al.,* 2007). Characteristics of materials from dietary composition which is in liquid form easily enter the caecum (Vergara *et al.,* 1989). This is because the anatomy of the caecum allows the liquid material to remain in this organ. Besides, any remaining coarse material will be fermented to produce several fatty acids. Only small amounts of produced nutrients are absorbed in this organ. Perhaps this is another factor that helps explain why the composition of the microflora did not change throughout the experiment.

Individual animals demonstrate considerable variation in their microflora population. There is wide variation in microflora populations from one animal to another (Klive A.V., 2010, pers. comm). This is shown by differences in DNA

concentrations between replicates in the present study. This indicates that in a group of broilers fed the same diet the population of caecal microflora differs from bird to bird in the group. Each replicate contained a population of caecal microflora of eight birds which may have to a huge variation. Zhou *et al.* (2007) investigated the appropriate sample size to be used to identify the composition of broiler intestinal microflora. A sample size of 5 birds per pool for caecal microflora was found appropriate to give significant results. In this experiment, 8 birds were pooled which was considered appropriate based on the previous study. Moreover pooling gives the advantage of reducing the degree of individual variation (Gong *et al.,* 2008). Therefore the effects of the experimental treatment could possibly be identified. On the other hand, Gong *et al.* (2005) (cited in Gong *et al.* 2008) and Richards *et al.* (2005) examined individual animal (pigs and poultry) to investigate the effect of antibiotics on intestinal microflora and found it difficult to determine the effect because the change of microflora composition might be confounded by experimental effects that are independent of variation due to antibiotic treatments.

CONCLUSION AND RECOMMENDATIONS

There was one major finding from the experimental study. Feed enzymes can improve the nutrient availability of grain sorghum. Secondly, as there was approximately 80% similarity between caecal microflora in all dietary treatments, suggesting that there was no overall difference between treatments in the composition of the broiler caecal microflora.

A number of limitations have been identified in the experimental design. If this work were to be repeated, birds should be fed experimental diets for at least 21 days and it would be useful to use a more commercial diet. Additional treatments could include enzyme combinations as this would overcome possible anti-nutritive factor interactions and reflect the usage of feed enzymes in the feed industry.

Perhaps future studies could establish a phylogenetic tree for the microflora at other intestinal locations such as the crop, duodenum, jejunum and ileum, in addition to the caecum, as microbial activity occurs throughout the digestive tract.

As feed resources become more expensive it is important to maximise nutrient availability using all potential strategies including feed enzymes. A greater understanding of how different approaches work, including through modification of the caecal microflora, will assist in refining the application of these strategies and securing a safe and affordable food supply. The results from this study further proves that sorghum can be used as an alternative feed source of poultry which is an expanding industry and new feed sources are needed to supplement the traditional corn-based feeds. As food safety and production are uppermost

concerns in most developing nations, this information will aid in improving poultry production for the nation in keeping with the National Agriculture Policy of Malaysia.

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