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The Bacterial Community of the Chicken's Intestinal Tract: Impact of Xylanase Supplement

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ABSTRACT

The effect of xylanase supplementation on the profile of the bacterial community of the gastrointestinal tract of layer chicken was investigated using PCR-DGGE analysis. Thirty four common bands, belonging to *Lactobacillus acidophilus* and *Lactobacillus salivarius*, were excised from the DGGE gel, amplified, cloned and sequenced. Sequence analysis of the clones revealed that 79.4% of sequences from the intestine were related to those of *Lactobacillus* spp., while the remaining 20.6% belonged to the four families, *Clostridiaceae* (8.8%), *Streptococcus* (5.8%), *Bacteroides* (3%) and *Enterococcus* (3%). Bacteria belonging to the genus *Lactobacillus* spp. were the predominant bacteria across the different treatments and segments of the gastrointestinal tract of chicken.

Keywords: Bacterial community, Denaturing Gradient Gel Electrophoresis (DGGE), intestinal tract, xylanase

INTRODUCTION

Dietary enzymes supplementation has been widely used in poultry diets in attempts to improve nutrient utilization, health, quality improvement of the product and reduce pollution, as well as to increase the choice of content of ingredients which are acceptable for inclusion in diets (Elmenaway *et al.,* 2010). Apart from that, it was added to the poultry diet to improve the nutritional value of feeds and gut microflora of birds (Anjum & Chaudhry, 2010). Exogenous enzymes have been available for many years, and their use in animal diet has increased exponentially, mainly due to increasing cost of feeding. It can be used to reduce the negative impacts of some feed constituents such as non-starch polysaccharides (NSP) on gut conditions and overall utilisation of dietary energy and proteins (Choct *et al.,*

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2006). The adverse effects of soluble NSP on nutrient digestion and absorption in poultry are due to the ability of the soluble NSP to increase the viscosity of the digesta. Apart from that, it can also modify the physiology and the ecosystems of the gastrointestinal tract (Choct, 2002). High gut viscosity can lead to poor bird performance. It is thought that soluble NSP acts together with the glycocalyx of the intestinal brush border and thickens the rate-limiting unstirred water layer of the mucosa, decreasing the efficiency of nutrient absorption through the intestinal wall (Johnson & Gee, 1981).

The addition of feed enzymes such as xylanase in poultry diets could cleave the large molecules of NSP into smaller polymers, thereby reducing digesta viscosity and increasing the nutritive value of the feed (Bedford *et al.,* 1991; Choct & Annison, 1992). The use of xylanase has shown not only to improve energy utilisation of wheat based chicken diet but also eliminate *Clostridium perfringens* in broiler chicken fed low apparent metabolizable energy (AME) diet (Choct *et al.,* 2006). Supplementation of xylanase in poultry diet seems to decrease the microflora fermentation in the small intestine but increase in the large intestine and ceaca (Steenfeldt *et al.,* 1998). Apart from that, the caecal content pH also decreases due to high production of short-chain fatty acids caused by an increase of microbial fermentation. The increase of the fermentation was indicated by degradation of cell wall arabinoxylans in the enzyme-supplemented diets that increases the amount of material available for microbial fermentation in the ceaca (Steenfeldt *et al.,* 1998). A few studies have been reported that xylanase supplement could change the development of gut microflora, including those attached to the mucosa using microbial culturing techniques (Bjerrum *et al.,* 2006; Hubener *et al.,* 2002; Vahjen *et al.,* 1998). However, the effects of xylanase alone on the bacterial population in the intestinal tract of poultry using molecular culture-independent techniques are less likely to have been reported since most of the published works are actually involved a combination of a few exogenous enzymes (Bjerrum *et al.,* 2006; Torok *et al.,* 2008).

The present work was designed to particularly determine the influence of xylanase on the development of selected groups of intestinal bacterial community of chickens. An understanding of the bacterial population in the intestinal tract of the chickens will allow us to detect changes in the flora and to analyze the effects of food animal management changes. The information presented in this paper may permit us to manipulate intestinal flora with the intention of enhancing intestinal health and feed conversion. Independent molecular techniques were used to examine the bacterial profile in the different segments of the intestines, while the identification of the common bacterial groups was based on the 16S rRNA gene sequence.

MATERIALS AND METHODS

Birds and Their Management

A total of 36, twenty-seven-week old, ISA Brown layers were raised for a period of five weeks before being euthanized for digesta collection. This study was conducted according to the animal ethics guidelines set by the Animal Ethics Committee of the University of Queensland (AEC Approval Number: SAS/372/08/eirdc/fei) in 2010. At the beginning of the experiments, the birds were randomly divided into two equal treatment groups, with 6 replicates (cages) of 3 birds per replicate each. The birds were fed with a basal diet *ad libitum* that did not contain animal protein, growth-promoting antibiotics or coccidiostats. The birds were randomly assigned to either control group (basal diet with no enzyme added, T1), and basal diet + xylanase (T2) (2,500U/kg diet, Sigma-Aldrich, Selangor, Malaysia). The enzyme was added according to the recommendations of the manufacturers. Each basal diet contains wheat, soybean meal and ground corn as major ingredients to provide 11.28 MJ, ME/kg, 15.53% crude protein, 0.5% methionine plus cyctine, 0.7% available lysine and 3.5% calcium. The composition of the basal experimental wheat based diet is shown in Table 1.

Collection of the Samples

At the end of the five weeks feeding period, all the birds were euthanized, while the intestine and caeca contents were removed according to the procedure by Yang *et al.* (2008). Briefly, the small intestine was divided into three segments: duodenum (from the gizzard outlet to the end of the pancreatic loop), jejunum (from the pancreatic loop to Meckel's diverticulum), and ileum (from Meckel's diverticulum to the caecal junction). The contents of the small intestine and large intestine were aseptically flushed with ice-cold phosphate buffered saline (PBS) at pH 7.4 into sterile

TABLE 1
The basal commercial experimental wheat-based diet

Ingredients	g kg^{-1}
Wheat	670.0
Soybean meal	191.0
Vegetable Oil	28.0
Corn meal	83.0
Di-calcium Phosphate	18.0
Salt	3.0
Vitamin-trace mineral premix[a]	7.0

[a]Contents per kg premix: 4.6 mg trans-retinol; 122.5 µg cholecalciferol; 28 mg DL-α-tocopheryl acetate; 2.8 mg menadione; 2.1 mg thiamine; 11.2 mg riboflavin; 21 mg calcium pantothenate, 42 mg niacin; 7 mg pyridoxine; 2.8 mg folic acid; 21 µg cyanocobalamine; 140 µg biotin; 105 mg Mn; 70 mg Zn; 7 mg Cu; 2.2 mg Mo; 420 µg Co; 1.4 mg I; 28 mg Fe; 140 µg Se; 420 mg choline chloride; 175 mg ethoxyquin.

containers, placed over ice and transported immediately to the laboratory for bacterial analysis.

Culture-independent Techniques

Genomic DNA was extracted from the contents of the different segments of the intestines using the non-ionic detergent cetyltrimethylammonium bromide (CTAB) with beads beating, as described by Wright *et al.* (1997). Briefly, approximately 1 gram of the digesta samples were centrifuged (12,000 ×g) for 5 min and the supernatant was removed. Then, 200 mg of silica/ zirconium beads were added and the pellet was resuspended in 800 µl CTAB isolation buffer (100mM Tris-HCl, pH8; 1.4M NaCl; 20mM EDTA (sodium salt); 2% hexadecetyltrimethylammonium bromide). The samples were homogenised in a bead beater for 2 min, cooled on ice and homogenised for another 2 min. The samples were then incubated at 70°C for 20 min, followed by centrifugation at 10,000 g for 10 min. The aqueous phase was transferred to a new microfuge tube and 500 µl phenol/ chloroform/ isoamyl alcohol (25:24:1) were added, vortexed, and centrifuged at 13,000 g for 10 min. Again, 500 µl of the upper aqueous layer was removed and dispensed into another micro-centrifuge tube. DNA was precipitated with 300 µl of isopropanol at room temperature for 5 min. DNA was collected by centrifugation at top speed (14,000 g) for 15 min and the nucleic acid pellet was washed with 1 ml 70% ice-cold ethanol. The pellet was incubated at 70°C for 10 min before centrifuging again at 14,000 g for 10 min. The ethanol was removed and the pellet was air-dried before resuspending it in 50 µl of DNA/RNA free water. DNA from the extracted samples was quantified using a nanodrop spectrophotometer (BioSpec-nano Shimadzu, Queensland, Australia) to check for the quantity and quality of DNA.

The variable V3 region of 16S rDNA was enzymatically amplified in the PCR with primers to conserve regions of the 16S rRNA genes. The nucleotide sequences of the primers are as follows: 341f-GC (5'-CCTACGGGAGGCAGCAG-3'); and 534r (5'-ATTACCGCG GCTGCTGG-3'). Primer 341f has an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5' end. PCR amplification was performed with a MJ Research PTC-100 thermal cycle machine (MJ Research, Inc., Watertown, Mass.). A 50 µl volume PCR reaction contained with 5 µl 10x PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 3 µl 50 mM $MgCl_2$, 1 µl 10 mM dNTPs (Qiagen, Victoria, Australia), 1 µl 10 µM of each primer, 0.3 µl of 5 U/µl of Platinum *Tag* DNA polymerase (Invitrogen, Carlsbad, NM, USA), 250 ng of genomic DNA and DNA/RNA free water to 50 µl. The samples were first incubated for 5 min at 94°C to denature the template DNA. This hot start technique was performed to minimize non-specific annealing primers to non target DNA. The initial hot-start was then followed by 15 cycles of the following parameters: 94°C for 30 s, 65°C for 30 s, and 68°C for 1 min. At the temperature of 65°C, the temperature was decreased by 1°C every second cycle until a touchdown at 55°C was accomplished. Another set of annealing

parameters were as follows: 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min, and it was run for 20 cycles. This procedure reduces the formation of spurious by-products during the amplification process (Muyzer *et al.,* 1993). Primer extension was carried out at 68°C for 5 min. The PCR products were analyzed first by electrophoresis in 1% (wt/vol) agarose gel under ultraviolet (UV) illumination after ethidium bromide staining of the gel. Then, the amplified samples were separated by using DGGE (DCode System; Bio-Rad, Hercules, CA, USA) on 8% acrylamide gels with 30-60% formamide/urea gradients. Electrophoresis was performed at 60°C and 100 V over 18 h in 0.5 x TAE buffer (Tris-acetate, 0.04 M; EDTA, 0.001M). After that, the gels were rinsed in ddH2O, fixed in a solution of 10% ethanol, 0.5% acetic acid and silver stained. The DNA bands on the gel were visualized following electrophoresis by using a computer scanner (HP Scanjet G2410, UK).

A total of 34 different bands were gently excised from the gel. The gel and the bands were examined by manually observing some common bands to all dietary treatments, while those restricted to individual treatment were chosen for further analysis and sequencing. DNA of the gel plugs was amplified as described previously for pre–DGGE PCR and then subject to electrophoresis using a 2% agarose gel. Purified PCR products were then ligated into pGEM–T Easy vector (Promega, Madison, USA) and transformed into *E. coli* Top10 cells. Sequencing was performed on 34 transformed colonies representing the bands using the T7 primer found within the pGEM–T vector. A more definitive analysis of the population diversity was undertaken by comparing the 16S rRNA gene sequences of the isolates from this study with those found in public databases of NCBI.

Sequences were edited manually using CHROMAS Lite (ver. 2.0) before being assembled using the programme called SEQMAN (ver. 3.34, DNASTAR Inc.). The sequence data were aligned using Greengenes (DeSantis *et al.* 2006) and their nearest-neighbour for each sequence was identified. Both the chickens intestinal clones and their nearest neighbour sequences were manually imported into the ARB software package release 07.07.11 (Ludwig *et al.,* 2004) for fine alignment with similar sequences and to remove any alignment errors. A neighbour-joining tree was constructed (Saitou & Nei, 1987) for each enrichment media using the Kimura-2 parameter (Kimura, 1980) model in PHYLIP (Felsenstein, 1993) with 1000 bootstrap re-samplings.

Phylogenetic Analysis

Clone sequences generated from the two segments of intestinal tract were assigned to phylotypes that were designated by "SI/LI" which stands for small and large intestine followed by treatments given (T1/T2) and the numbers which represent the clone number. The 34 sequences from the current study were aligned against each other, while 14 additional sequences were identified as nearest-neighbours and downloaded from

the Greengenes website. The sulphate-reducing archaea, *Archaeoglobus fulgidus* strain VC-16 (Klenk *et al.,* 1997) was used as the out-group for the tree.

RESULTS AND DISCUSSION

In total, 34 clones were successfully sequenced, and of these clones, 11 clones originated from T1, and the remaining 23 clones originated from T2. The sequence analysis of the clones revealed 13 sequences (38.2%) with 99% identity to known bacteria, while 13 sequences (38.2%) had 95 to 98% identity to their nearest relatives. The remaining eight sequences (23.6%) had <95% identity to any recorded entries in Greengenes or GenBank. Of the 34 clones examined, the most abundant sequences were homologous to Lactobacillaceae (79.4%), while Clostridiaceae and Streptococcaceae each accounted for 8.8% and 5.8% of the total (see Fig.1). The remaining clones were represented by the families Bacteroidaceae and Enterococcaceae, with each accounted for 3.0%.

The diversity and composition of the microorganisms at different sites of intestinal tract are shown in Table 2. At the species level, *Lactobacillus acidophilus* was the dominant species present in the intestinal tract and this was represented by 38.2% of the 34 clones, followed by *Lactobacillus salivarius* with 23.5%, *Lactobacillus reuteri* (14.7%), and *Clostridium* sp. (8.8%). Apart from these, 5.8% of the clones were related to *Streptococcus bovis* and the remaining clones were related to *Bacteroides uniformis, Enterococcus cecorium* and *Lactobacillus johnsonii* (Fig.2). Salanitro *et al.* (1974) enumerated anaerobic bacteria from the small intestinal tract of 14-day-old chicks and showed that the predominant bacteria retrieved from the small intestine were largely dominated by *Lactobacillus* spp. (33.8 to 59%), followed by *Streptococcus* spp. (8.9 to 16.8%), *E. coli* (14.7 to 33%) and eubacteria (9 to 24.3%). Meanwhile, species-to-species comparison between the small and large intestinal tracts of the birds revealed that *Lactobacillus reuteri*

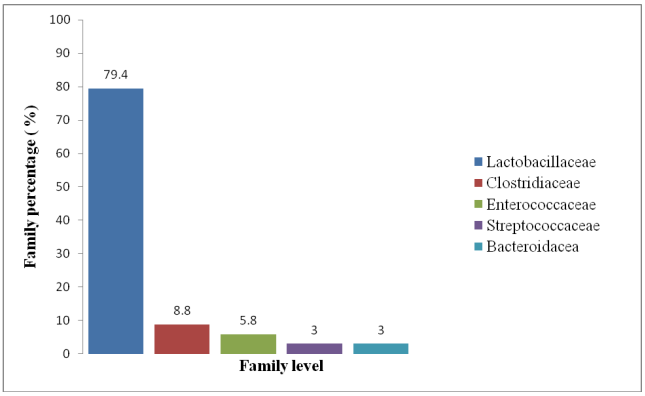

Fig.1: Distribution of bacteria derived from DGGE bands identified using the V3 region of 16S rDNA at family level

and *Lactobacillus salivarius* appeared to be present in both sections of the intestinal tract.

The topology of the phylogenetic tree is shown in Fig.3. Of the 34 clones, 33 clones were clustered together with the *Lactobacillaceae, Streptococcaceae, Enterococcaceae* and *Clostridiaceae* families, while one clone derived from the family Bacteroidaceae was not included in the tree because its distance of similarity is less than 90 %. In the current study, 27 clones grouped within the family *Lactobacillaceae* were closely affiliated with member of the genera *Lactobacillus,* and an uncultured bacteria clone from the ileum and cecum of chicken (Bjerrum *et al.,* 2006). Two clones [SI(T3)_104 and SI(T3)_105] were

TABLE 2
The origin of the different clones, their closest related microorganisms and % identity

Origin	Treatment	No. of clones [a]	Nearest related microorganism	% sequence similarity
Small intestine	T1/T2	13	*Lactobacillus acidophilus* NCFM	98
	T1/T2	7	*Lactobacillus salivarius* UCC118	95
	T1/T2	2	*Lactobacillus reuteri* 100-23 ctg2179	94
	T1	1	*Enterococcus cecorium*	97
	T2	1	*Lactobacillus johnsonii* NCC 533	99
	T2	3	*Clostridium* sp. S6	90
	T2	2	*Streptococcus bovis* SB5	97
Large intestine	T1	3	*Lactobacillus reuteri* 100-23 ctg2179	100
	T2	1	*Bacteroides uniformis* ATCC 8492	88
	T2	1	*Lactobacillus salivarius* UCC118	95

[a] A total of 34 clones were examined

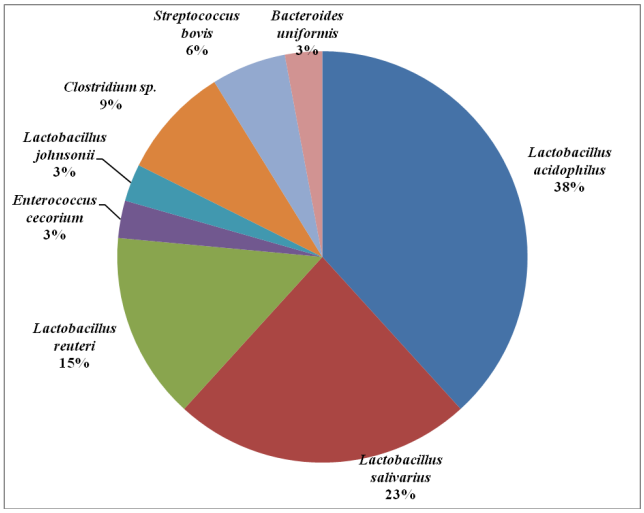

Fig.2: Distribution of bacteria derived from DGGE bands identified using the V3 region of 16S rDNA at species level

grouped within the Streptococcaceae and formed a cluster with *Streptococcus bovis*. Three cones were grouped together within the family *Enterococcaceae* and formed a cluster with *Clostridium* sp. Meanwhile, the remaining clone [SI(T1)_019] was grouped within *Enterococcus cecorum*, while clone LI (T2)_021 was grouped with an uncultured Turkey cecum clone (Scupham, 2007) (Fig.3).

The response of *Lactobacillus* spp. on xylanase supplementation in the diet appeared to have an effect, indicating that this group of bacteria is influenced by nutrient composition that was mediated by enzyme treatment (Teresa *et al.,* 2009). A study by Teresa and her co-workers (2009) on broilers fed wheat-soyabean-rape diets supplemented with xylanase preparation revealed that *Lactobacillus* spp. appeared

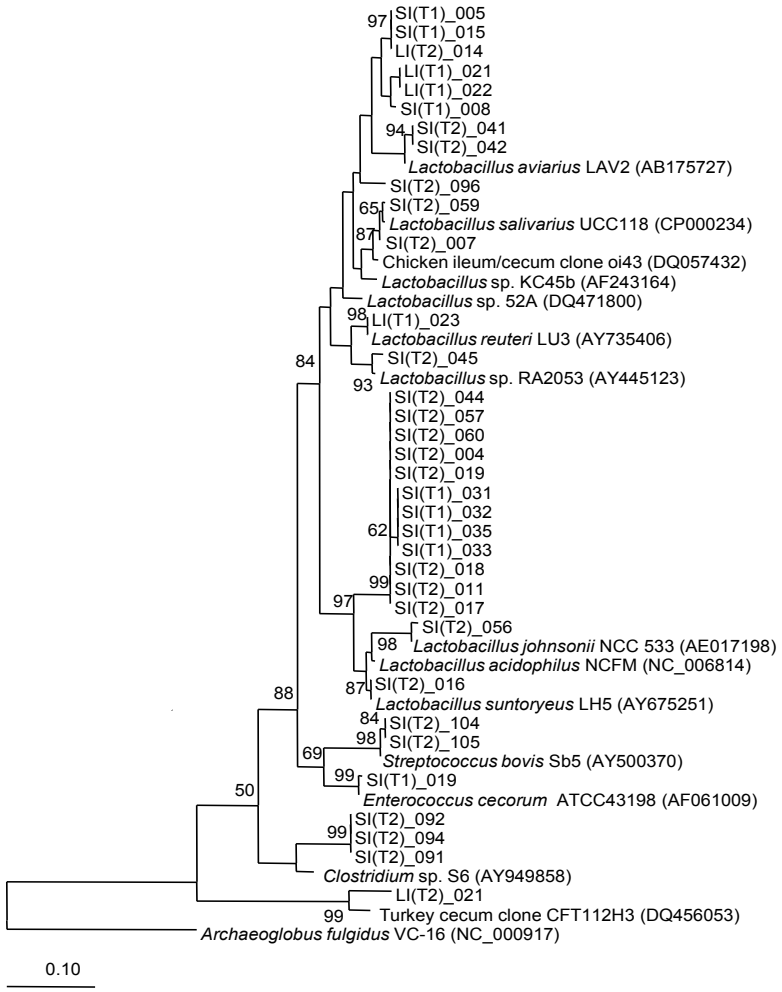

Fig.3: Distance-related phylogenetic tree of the lactic acid-producing bacteria from the different segments of the chicken intestine. Bootstrap values less than 50 are not indicated. The scale bar represents 10% sequence divergence. *Archaeoglobus fulgidus* VC-16 sequence was used as an out-group for rooting the tree.

to be present in high number. This finding is in agreement with the result of the present study, where high numbers of clone closely related with *Lactobacillus* spp. (79.4%) were detected from the intestine of the poultry fed with basal diet supplemented with xylanase. *Lactobacillus* spp. is considered as one of the most important bacterial groups that maintains the equilibrium of the microbial ecosystem (Pryde *et al.,* 1999). Our findings are similar, with *Lactobacillus* and *Streptococcus* accounting for 79.4% and 5.8% of the microflora, respectively. Various species of *Lactobacillus* have been extensively used as feed additives, either as mixed or pure cultures, and are considered beneficial to the monogastric animals (Fuller, 1999). The most dominating bands in this study were common for all the treatments and these belonged to *Lactobacillus acidophilus* and *Lactobacillus salivarius*.

High numbers of clone closely related with *L. acidophilus* were detected from the intestine. In broiler nutrition, this species, together with many other *Lactobacilli*, has been used as one of potential candidates for probiotics and has a beneficial effect on broiler performance (Kalavathy *et al.,* 2003). Supplementation of *L. acidophilus* cultures to chickens has significantly increased (P<0.05) the levels of amylase after 40 d of feeding trials (Jin *et al.,* 2000). It is believed that the lactobacilli colonizing the intestine may secrete the enzyme, therefore increasing the intestinal amylase activity (Sisson, 1989). The clones recognized as *L. salivarius* were detected in both the small and large intestinal tracts regardless of the treatment given. This is in agreement with that of a study by Al-Jassim *et al.* (2005) on the genetic diversity of lactic acid producing bacteria in the equine gastrointestinal tract, where *L. salivarius* were detected from the intestinal tract.

It is important to note that the PCR-DGGE approach allows determination of bacterial populations comprising more than 9% of the intestinal bacterial community (Zoetendal *et al.,* 1998). As it was not possible to obtain sequences from all bands in the gels and most of the retrieved sequences could not be aligned to any known bacterial species, it requires further investigation, particularly in relation to which intestinal microbiota could benefit from the xylanase supplementation and other methods such as the construction of 16S rRNA gene clone library that may be more suitable for this purpose.

CONCLUSION

Based on the findings of the current study, *Lactobacillus* spp. appeared to be the predominant bacteria across the different segments of the gastrointestinal tract of chicken. Further analysis is carried out to investigate the diversity using different molecular tools.

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