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Molecular Characterisation of Lactobacilli Isolated from Ileal and Caecal Digesta of Broilers Fed with Prebiotic Plant Extracts

J.K. Vidanarachchi, L.L. Mikkelsen[1], P.A. Iji[1] and M. Choct[2]

Department of Animal Science
Faculty of Agriculture
University of Peradeniya, Sri Lanka

ABSTRACT. *Molecular characterisation was carried out for lactobacilli isolated from ileal and caecal digesta of broiler chickens fed on water-soluble prebiotic carbohydrate extracts (10 g/kg) obtained from Cabbage tree (Cordyline australis), seaweed (Undaria pinnatifida) and exudates from Acacia pycnantha. Genomic DNA was extracted from lactobacilli isolated from Rogosa agar the 16-23S rDNA intergenic spacer regions were amplified and subjected to Amplified Ribosomal DNA Restriction Analysis (ARDRA) using HaeIII enzyme. Partial 16S rRNA gene sequences of major genotypic groups were determined and compared sequences in the GeneBank using the Basic Local Alignment Search Tool (BLAST) algorithm. The ARDRA and partial 16S rRNA gene sequencing revealed four distinctive groups of lactobacilli: Lactobacillus salivarius (group I), L. crispatus (group II), unidentified Lactobacillus species (group III), and L. johnsonii (group IV). These Lactobacillus species are dominant in the ileal and caecal digesta of broilers. The L. johnsonii was mainly detected in the ileal and caecal digesta of chicken supplemented with the Acacia extract.*

INTRODUCTION

There is a considerable interest in understanding how the gut microflora can be modified, particularly through diet. Some dietary carbohydrates, that are selectively fermented by beneficial microorganisms in the lower part of the gastrointestinal tract (GIT), are generally known as prebiotics (Gibson and Roberfroid, 1995). These beneficial bacteria are thought to create conditions unfavourable to the growth of pathogens and as a result, the use of prebiotic compounds have recently gained further interest in the poultry industry as an alternative to the use of in-feed antibiotic growth promoters. microflora, and accurate identification of unknown isolates is now achieved by sequence analysis of 16S rRNA (Vaughan *et al*., 2000).

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[1] Gut Microbiology Laboratory, Faculty of Science, University of New England, Armidale, NSW 2351, Australia.

[2] Gut Microbiology Laboratory, Faculty of Science, University of New England, Armidale, NSW 2351, Australia.

[1] [2] Australian Poultry CRC, University of New England, PO Box U242, Armidale, NSW 2351, Australia.

In the previous feeding experiment on caged broilers the inclusion of prebiotic plant extracts in the diets resulted in increased *lactobacillus* counts in the ileal and caecal digesta (Vidanarachchi *et al.*, 2006). The aim of the present study was to perform a molecular characterization of lactobacilli isolated from ileal and caecal digesta of broiler chickens fed with three different prebiotic plant extracts using Amplified Ribosomal DNA Restriction Analysis (ARDRA) and gene sequence analysis of 16S rRNA.

MATERIALS AND METHODS

A total of 192 Cobb day-old male broilers were allocated to 4 treatments of 6 replicates (8 birds per replicate). Three different prebiotic extracts were prepared from Cabbage tree (*Cordyline australis*), Acacia (*Acacia pycnantha*) and Undaria seaweed (*Undaria pinnatifida*), and were included at 10 g/kg level in the diets. A non-supplemented diet was used as a negative control. The Cabbage tree extract contained mainly fructans and the water-soluble carbohydrates extracted from Undaria seaweed were composed mainly of sulphated oligosaccharides. The Acacia extract was composed of naturally-occurring arabinogalactans. Chickens had *ad libitum* access to feed and water. On day 35, three chickens were selected at random from each replicate and euthanized by cervical dislocation. Subsequently, the abdominal cavity was opened and the small intestine was ligated and removed. The contents of the ileum were collected into sterilized plastic containers.

Lactobacilli were enumerated on Rogosa agar (Oxoid, CM0627) after anaerobic incubation at 39^{o}C for 48 hrs in anaerobic jars (Oxoid Ltd, Hampshire, UK) with an anaerobic environment (<1% O_2 and 9 - 13% CO_2), generated using anaerobic AnaeroGen™ sachets (AN0025A, Oxoid Ltd, Hampshire, UK). Twenty randomly selected colonies from Rogosa agar from the highest dilution of samples from all four treatments were carefully isolated and sub-cultured in MRS broth (De Man, Rogosa, Sharpe; Oxoid, CM0359). Five isolates from each replicate (30 isolates per treatment group) were randomly collected for molecular characterization of lactobacilli from the ileum (n = 120) and the caeca (n = 120). The cells were grown overnight at 39^{o}C, and 1.0 mL of bacterial suspension was transferred into an Eppendorf tube and harvested by centrifugation (14,500xg, 5 min) in an Eppendorf centrifuge. Extraction and purification of genomic DNA were carried out using the DNeasy® Tissue kit (QIAGEN Ptv Ltd., Doncaster, VIC, Australia) following the manufacturer's instructions and the 16 - 23S rDNA (16S rRNA gene and the entire 16S-23S rRNA intergenic region) were amplified by Polymerase Chain Reaction (PCR) using the primers, Lb16a and 23-1B (Table 1) as described by Vidanarachchi (2006). The amplified 16-23S rDNA intergenic spacer regions of *Lactobacillus* isolates were digested with the restriction endonuclease *Hae*III (1.0 U/μL, 2 hrs at 37^{o}C) according to the manufacturer's instructions (New England BioLabs, Brisbane, QLD, Australia). Then the digested products were electrophoretically resolved on a 2% agarose gel containing 5 μL of GelStar® nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA) for 4 hrs at 90 V and band patterns were visualized by UV transillumination and

digitised on an Infinity CN-3000 Gel Documentation System (Vilber Lourmat, Cedex, France).

Table 1. Primers used for amplification and sequencing of 16 - 23S rDNA.

Primer	Direction	Nucleotide sequence (5'→ 3')	Use
Lb16a	Forward	GTG CCT AAT ACA TGC AAG TCG	ARDRA
23-1B	Reverse	GGG TTC CCC CAT TCG GA	ARDRA
THOO8	Forward	AGR GTT YGA TTM TGG CTC AG	Sequencing
PH1522	Reverse	AAG GAG GTG ATC CAG CCG CA	Sequencing

Amplified PCR products generated with the primer pair TH008-PH1522 were purified and concentrated using the QIAquick® PCR purification kit (QIAGEN Pty Ltd., Doncaster, VIC, Australia) as described by the manufacturer. Partial sequences of 16S rRNA gene of lactobacilli isolates were determined with the primer TH008 (Table 1) and the sequencing reactions were performed with a GenomeLab™ Dye Terminator cycle sequencing quick start kit (Beckman Coulter, Inc., Fullerton, CA, USA) as described by the manufacturer. The reaction mixtures were amplified in an Eppendorf PCR Thermal Cycler (MasterCycler®, Eppendorf AG, Hamburg, Germany). Sequencing was carried out using the Beckman Coulter CEQ 8000 Genetic Analysis System and sequences were analysed using the CEQ 8000 software package (Beckman Coulter, Inc., Fullerton, CA, USA). Resulting 16S rRNA gene sequences were subjected to sequence comparisons in the GeneBank (National Center for Biotechnology Information, Bethesda, MD, USA) using the Basic Local Alignment Search Tool (BLAST) algorithm. The sequences of the 16S rRNA genes determined in this study were deposited with the GeneBank nucleotide database under the accession numbers DQ676989 for isolate group I, DQ676990 for isolate group II, DQ832760 for isolate group III and DQ676991 for isolate group IV.

RESULTS AND DISCUSSION

Two hundred and forty *Lactobacillus* isolates from ileal and caecal digesta were identified using the *Hae*III-ARDRA method, and the results are summarised in Table 2. In the present study, distinct results of ARDRA analysis of the 16 - 23S rRNA genes divided the *Lactobacillus* isolates (240) into four genotypic groups (Plate 1): group I (161 isolates), group II (40 isolates), Group III (4 isolates), and group IV (33 isolates) (Table 2). The genotypic group I was the most abundant group of *Lactobacillus* species in both ileum and caeca. Genotypic group II was observed in ileal isolates of 4 (13%) from the negative control treatment, 11 (37%) from 10 g/kg cabbage tree extract-supplemented group, and 3 (10%) each from the 10 g/kg seaweed extract- and Acacia extract-supplemented groups. The genotypic groups III and IV were not detected in ileal and caecal digesta of birds fed with

the negative control and 10 g/kg cabbage tree extract-supplemented diets. Of the 240 16-23S rDNA ARDRA profiles analysed, group IV was the most abundant in the 10 g/kg Acacia extract-supplemented group at 43% in the ileum and 63% in the caecum. The genotypic group III was found in one isolate in ileal and two isolates in caecal contents of 10 g/kg Undaria seaweed extract-supplemented group. From the caecal isolates (30) of 10 g/kg Acacia extract-fed group, only one isolate had ARDRA band patterns similar to genotypic group III. Two isolates from ileal content (Table 2) of 10 g/kg Acacia extract-fed group generated *HaeIII*-ARDRA banding patterns that did not match any of the four

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

genotypic groups reported in this study.

Table 2. Distribution of major genotypic groups of lactobacilli isolated from ileum and caeca of broilers at 35 days of age.

The partial sequence analysis of 16S rRNA gene in the four major types of ARDRA groups resulted in sequences with significant similarity (94.-.99%) to the 16S rDNA of known Lactobacillus species (Table 3). The group I isolates showed the closest relationship to *L. salivarius*, with high sequence similarities (>97%). The group II isolates had a high level of sequence similarities (>98%) to *L. crispatus*. Group III, which was observed only in four out of 240 isolates, showed a close relationship, with high levels of sequence similarities (98%) to an uncultured Lactobacillus species from chicken intestine (DQ057431) and comparatively low sequence similarities (94%) to a known species, *L. reuteri*. Additionally, the 16S rRNA gene sequences of this group are very similar to *L. vaginalis* (97%). The group IV isolates showed a close relationship, with high levels of sequence similarities, to *L. johnsonii* (99%).

Table 3. BLAST search results for 16S rDNA sequences obtained for major genotypic groups of *lactobacilli* isolated from ileum and caeca of broiler chickens.

Genotypic group	Closest relative in GeneBank (accession no.)	Percentage identity

Group I	*Lactobacillus salivarius* (DQ193532)	97%
Group II	*Lactobacillus crispatus* (AY335495)	98%
Group III	Uncultured bacterium from chicken intestine (DQ057431)	98%
Group IV	*Lactobacillus johnsonii* (AE017198)	99%

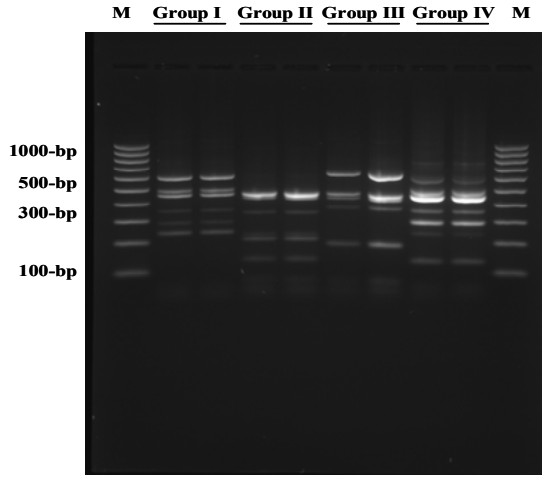

Plate 1. ARDRA patterns of 16-23S rRNA genes from lactobacilli isolated from ileum and caeca of broiler chickens.

Note: The amplified 16-23S rRNA gene fragments were digested with the restriction endonuclease *HaeIII* and resolved by electrophoresis on a 2% agarose gel. Lane M: molecular weight markers (100 - 1000 bp ladder).

Results from the present study are in agreement with the previous observations that *L. salivarius* is the predominant *Lactobacillus* spp. present in the intestinal tract of adult broilers (Engberg *et al.*, 2000). Out of the four major genotypic groups observed in this study, two groups (group II; *L. crispatus*, and group IV; *L. johnsonii*) belong to the *L. acidophilus* group (Klein *et al.*, 1998). According to the ARDRA patterns and gene sequence analysis results, the genotypic group IV (*L. johnsonii*) was predominantly detected in the ileal and the caecal digesta of chicken supplemented with Acacia extract. These results suggested that prebiotics such as arabinogalactans (Acacia extract) may have promoted the growth of *Lactobacillus* species *L. johnsonii*. It could be assumed that such specific groups would have fermented the water-soluble carbohydrates present in the Acacia extract.

CONCLUSIONS

In conclusion, *Lactobacilli* isolated on Rogosa agar from the ileum and caeca of broilers fed with prebiotic plant extracts could be identified using amplified ribosomal DNA restriction analysis and partial sequencing of the 16S rRNA gene. The predominant *Lactobacillus* species in ileal and caecal digesta of broiler chickens fed with prebiotic plants

extracts were *L. salivarius*, *L. crispatus*, unidentified *Lactobacillus* species and *L. johnsonii*. The current study suggests that *L. salivarius* is the predominant *Lactobacillus* species in ileal and caecal digesta of 35-day-old broiler chickens. The results also indicated that supplementation with Acacia extract supports the growth *of L. johnsonii* in the ileum and caeca of broilers.

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