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Research article

Molecular tracking of cultivable lactic acid bacteria from digestive tract of Japanese quail (*Coturnix coturnix japonica*) fed with various grain sources

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Abstract

Lactic Acid Bacteria (LAB) possess many beneficial functions in the digestive tract of animals. The aim of the present study was to determine total LAB and Enterobacteriaceae counts and to track cultivable LAB isolates from proventriculus and small intestines of Japanese quails (*Coturnix coturnix japonica*) fed with various grain sources. Three treatment groups were fed with diets, designated as BAD, WAD or CAD, depending on replacement of 50% (w/w) of basal or control diet (CD) with barley, wheat and corn, respectively. The tracking of cultivable LAB isolates (n=66) was performed based on Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) and Amplified Ribosomal DNA Restriction Analysis (ARDRA) with combination of 16S rRNA gene sequencing. The average LAB counts within proventriculus samples were the highest for quails fed with WAD (5.47 cfu/g) and control diet (5.43 cfu/g). The highest LAB counts were detected in small intestine samples for quails fed with CAD (6.28 cfu/g). Although proventriculus and intestinal samples of BAD fed animals had the lowest LAB count, they had the most diverse LAB community in terms of number of species detected with molecular tracking. Moreover, ERIC-PCR analysis and sequencing revealed that *Lactobacillus salivarius* was the most commonly detected species in both parts of digestive tract of quails. Overall, feeding quails with different grain containing diets affected both total number and diversity of LAB species within proventriculus and small intestine samples. In addition, ERIC-PCR was more powerful method than ARDRA to monitor diet dependent changes in cultivable LAB composition within proventriculus and small intestine.

Keywords: ARDRA; digestive tract; ERIC-PCR; Japanase quail; Lactic Acid Bacteria

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Introduction

The microbiota of intestinal tract play important roles in health and growth of animals with its contribution to gut development, biochemistry, physiology, immune functions and nonspecific resistance to infectious host (Niba et al., 2009). The intestinal tract of poultry begins with esophagus and continuous down to crop, proventriculus, gizzard and small intestine (duodenum, jejunum, ileum, ceca) and ends with colon and cloaca (Pan and Yu, 2014). Each part of the intestinal tract harbors distinct microbial

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communities, therefore, numerous microbiota actually exist in each part of intestinal system. For example, the cecal contents exhibit greater levels of Clostridiaceae-related sequences as opposed to the ileum where more abundance of Lactobacillus-related sequences occurs (Lu et al., 2003). Microbiota of digestive tract have significant impacts on small intestinal functions, such as nutrient digestion and absorption, response to anti-nutritional factors (non-starch polysaccharides), pre- and probiotic feed additives and feed enzymes (Bedford and Apajalahti, 2000).

Starting with animal's birth, structure of microbiota inhabiting different parts of the digestive tract has undergone alterations until it has been reached to stable conditions where *Lactobacillus s*pecies become predominant in adult birds (Apajalahti and Kettunen, 2006). Modern poultry production practices can interfere with the development of indigenous intestinal flora (Feighner and Dashkevicz, 1987). It is also evident from many studies that different diets and age can influence the structure of intestinal bacterial community members in poultry (Torok et al., 2008).

Most extensive studies on avian digestive microbiota have been focused on commercially important species, such as broiler and laying chickens. Results of these studies demonstrated that members of four bacterial phyla (Firmicutes as the main dominating phylum; Actinobacteria, Bacteroidetes, and Proteobacteria) are found in intestinal tract (Torok et al., 2011). Compared to other avian species, less information is available on bacterial composition of intestinal community from Japanese quails. A recent extensive study on composition of quail's cultivable intestinal microbiota has been performed on Northern Bobwhite (*Colinus virginianus*) which is taxonomically related to *Coturnix coturnix japonica* (Su et al., 2014). The results of that study provided insights into the composition of cultivable bacterial microbiota from intestinal system of quails and remarked similarities in distribution of bacterial phyla with other avian species. Another report on bacterial diversity analysis of female quails indicated that the total numbers of facultative anaerobic and anaerobic bacteria in the upper intestine were smaller than in the intestine and Streptococcus (including Enterococcus), Lactobacillus, and Peptococcaceae were the main bacteria found in the intestine (Itoh et al., 1997). A most recent report was published on diet and host genotype dependant changes on ceca microbiota from *Coturnix coturnix japonica* (Liu et al., 2015). All previous reports also pointed out that *Lactobacillus crispatus*, *Lactobacillus salivarius*, *Lactobacillus reuteri* and *Lactobacillus thermotolerans* can be found in intestinal tract of poultry. Among them, *L. salivarius* and *L. reuteri* were the most abundant *Lactobacillus* species (Gong et al., 2007; Hilmi et al., 2007).

Earlier studies on poultry intestinal bacterial microbiota have been conducted by conventional culture-based methods (Apajalahti et al., 2004). Recent studies with participation of molecular techniques have increased our knowledge of chicken intestinal microbiota (Gong et al., 2002; Lan et al., 2005; Gong et al., 2007). However, as evidenced from molecular studies, identified bacterial species with 16S rRNA gene sequences represent approximately 10% of previously known bacterial species constituting cecal microbiota, therefore, many species still remain to be identified for elucidating complexity and bacterial diversity of microbiota. Although culture-dependent methods provide limited information, they are still valuable for addressing impact of dietary changes on bacterial composition and densities in different segments of intestinal tract. The aim of this study was to track cultivable LAB composition in proventriculus and small intestine from Japanese quails in response to different diets and determining the best method of accurate monitoring for diet-dependent alteration in cultivable LAB species.

Materials and Methods

Feeding experiment and sampling

Twenty quails were divided into four experimental groups having two male and three female quails. Animals in the control group was fed *ad libitum* with basal diet (CD), while quails in three treatments were fed with 50% basal diet plus 50% barley, wheat or corn (BAD, WAD, CAD respectively), for 12 days. The ingredients and nutrient composition of basal diet are presented in Table 1. At the end of the experiment, all animals were sacrificed. The Faculty of Agriculture, Animal Policy and Welfare Committee of the Kahramanmaras Sutcu Imam University approved the experimental protocol (2015/2-2). Proventriculus and small intestine samples were collected aseptically from each animal and weighed following to transfer into sterile tubes containing saline solution (0.85% NaCl). Tubes were gently mixed and carried to laboratory for bacteriological analyses.

Enumeration of bacteria

Weighed samples were serially diluted in sterile saline solution. One-hundred µl of each dilution was spread on plates containing DeMan-Rogosa-Sharpe (MRS) agar and CHROMagarECC for enumeration of Lactic Acid Bacteria (LAB) and Enterobacteriaceae. Subsequently, plates were incubated at 35^{o}C for 2 days. Incubations were done anaerobically for MRS plates. Colonies were counted from all plates with bacterial growth. LAB colonies were studied further after re-streaking onto MRS agar. At this stage, LAB isolates were preliminary identified by using Gram-staining and

Table 1: Ingredient and nutrient compositions of basal diet

Ingredient	% of DM
Corn	54.8
Soybean meal	26.8
Boncalite	6.3
Poultry meal	3.5
Corn gluten feed	3.5
Soybean oil	2.6
Dicalcium phosphate	1.0
Trace mineral and vitamin premix	0.5
Sodium bicarbonate	0.5
Salt	0.3
DL-methionine	0.1
L-lysine	0.1
Nutrient	
Crude protein	23.0
Ether extract	6.2
Ash	6.2
Crude cellulose	3.9
Calcium	1.3
Lysine	1.3
Methionine	0.6
Phosphorus	0.6
Sodium	0.2

catalase tests. Five randomly selected and well-isolated colonies with distinct colony morphology from each plate were transferred to MRS broth and grown anaerobically at 35°C for 2 days. Growing cultures were used for extraction of whole cellular DNA and for preparation of long-term bacterial stocks containing 20% glycerol. Among the stored bacterial isolates (around 130) at -80°C, randomly selected 66 were used for molecular analyses.

DNA extraction

Whole cellular DNA samples were obtained from isolates as described previously with slight modifications (Bal et al., 2010). Briefly, 0.5 ml of 2 ml anaerobically grown culture at 35°C for 2 days was transferred into a sterile eppendorf tube and centrifuged at 10.000 rpm for 2 min. After removing of the supernatant, the pellet was washed with 0.75 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and re-centrifuged. The supernatant was removed again and the pellet was washed with 100 µl of TE buffer. Then, the suspension was incubated at 95°C for 20 min. Following the incubation, a final centrifugation at 4°C was performed for 2 min. Finally, the supernatant was collected and maintained at -20°C until PCR amplifications.

ARDRA of LAB isolates

The primers used for ARDRA are listed in Table 2. Three primer pairs; namely Lb16a/23-1B, 27F/1492R, Lab 159f/Lab 677r, were tested for determining amplification efficiencies. All PCR amplifications were performed as described previously (Tannock et al., 1999; Guan et al., 2003, Bal et al., 2010; Han et al., 2012). Amplicons that were generated with either Lab 159f/Lab677r or 27F/1492R primers were digested with *Hae*III (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. The resulting ARDRA fragments were separated by electrophoresis on 1.5% (wt/vol) agarose gels. Profiles were then visualized by using a gel documentation system following to ethidium bromide staining (Vilber Lourmat, France).

ERIC-PCR genotyping

The primers used in ERIC-PCR genotyping are outlined in Table 2. Each PCR mixture (25 µl) contained 1µl of template DNA (about 25 ng), 2.5 µl of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 1.5 mM $MgCl_2$, 200 µM of deoxynucleotide triphosphates (Fermentas), 1 U Taq DNA polymerase (Thermo Scientific), and 20 pmol of each primer (EllaTech, Germany). The ERIC-PCR amplification program consisted of an initial denaturation step at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 60 sec, and elongation at 72°C for 5 min; a final extension step at 72°C for 5 min. All amplifications were conducted using Eppendorf thermal cycler. Amplicons were separated by electrophoresis on 1.5% (wt/vol) agarose gels along with Gene Ruler 100 bp DNA ladder (Thermo Scientific) in 1XTBE buffer and visualized by using a gel documentation system following ethidium bromide staining (Vilber Lourmat, France).

Bacterial identification by sequencing of 16S rRNA gene fragment

Sequencing of 16S rRNA gene fragment was performed after PCR amplification using Lab 159f/Lab 677r primers (Table 2). The PCR products were cleaned and sequenced by the Refgen Company (Turkey). Nucleotide sequences were compared with those in the GenBank database using the BLAST search tool. Sequence identities of ≥96% were used as criteria for species and genus identification.

Results

Total viable LAB and Enterobacteriaceae counts from different treatment groups

The viable LAB and Enterobacteriaceae counts in proventriculus and small intestine samples of quails are presented in Table 3. The highest LAB counts were observed in proventriculus of quails fed with either CD or WAD diets averaging 5.45 cfu/g. However, the highest LAB was counted in small intestine of broilers fed with CAD (6.28 cfu/g).

Table 2: Primers used in this study

Primer name	Primer sequence (5′ to 3′)	Reference
Lb16a	GTGCCTAATACATGCAAGTCG	Guan et al., (2003)
23-1B	GGGTTCCCCCATTCGGA	Tannock et al., (1999)
27F	AGAGTTTGATCCTGGCTCAG	Lane (1991)
1492R	GGTTACCTTGTTACGACTT	Lane (1991)
Lab 159f	GGAAACAG(A⁄G)TGCTAATACCG	Han et al., (2012)
Lab 677r	CACCGCTACACATGGAG	Han et al., (2012)
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	Versalovic et al. (1991)
ERIC2	AAGTAAGTGACTGGGGTGAGCG	Versalovic et al. (1991)

Table 3: Dietary treatment effects on viable LAB and Enterobacteriaceae counts (log_{10} cfu/g of digesta) from different digestive tracts of quails on d 12

Treatment	LAB		Enterobacteriaceae	
	Proventriculus	Small intestine	Proventriculus	Small intestine
Control	5.43±0.93	4.51±1.00	4.82±0.78	3.89±2.25
BAD	4.04±0.62	4.65±1.07	3.53±0.00	5.17±1.37
WAD	5.47±1.36	5.04±0.51	6.26±0.94	4.45±0.20
CAD	4.68±1.35	6.28±0.43	5.45±0.88	6.11±1.02

BAD= barley added diet, WAD= wheat added diet, CAD= corn added diet.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Although ARDRA is powerful method for differentiation of *Lactobacillus* species, success of PCR amplifications are crucial for the analysis. In the present study, three different primer sets were tested on DNA samples of all isolates for availability in ARDRA (Table 2). Amplifications with 27F and 1492R primers failed for DNA samples from most of the isolates. Three different ARDRA profiles have been observed for only seventeen isolates (Fig. 1). One of those profiles was identical and detected from fifteen isolates, while two distinct profiles were obtained for two isolates (7-7 and 8-4). Similarly, ARDRA profiles could not be obtained with Lb16a and 23-1B primers except for a single isolate (data not shown). Amplifications were acquired for all isolates with Lab 159f and Lab 677r primers (Fig. 1). In ARDRA profiles, two main bands were present for all isolates. Similar to *Hae*III-ARDRA profiles of isolates after amplification with 27F and 1492R primers, three different *Hae*III-ARDRA profiles were observed. One profile was common for most of the isolates; however, the other two profiles were unique and observed for two isolates (7-7 and 8-4). Sizes of upper band were similar for all isolates. However, sizes of lower band were different for only two isolates (7-7 and 8-4). Size of this band for 7-7 isolate was smaller, but size of this band for 8-4 isolate was larger than all other isolates.

Although sizes of PCR amplicons generated with Lab 159f and Lab 677r primers were shorter compared to those generated with the other tested primers, it was efficiently used for the species and/or genus level identification based on 16S rRNA gene sequencing (Table 4). However, the amplicon size and region within 16S rRNA gene were only allowed genus level identification as *Enterococcus* spp.

Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) Analysis and 16S rRNA gene sequencing of LAB

As opposed to ARDRA profiles, higher numbers of bands (averaging 2-6) with the size range from 50bp to 2.0 kb were observed in ERIC-PCR profiles of all isolates (Fig. 2). Moreover, more variation was detected in ERIC-PCR profiles when compared to ARDRA profiles. In ERIC-PCR profiles, bands detected at 150 and 250 bp region were common for most of those isolates. Similar to ARDRA results, two isolates (7-7 and 8-4) had distinct ERIC-PCR profiles compared to profiles from rest of the other isolates. As detected from ERIC-PCR profiles, some isolates with the same ARDRA profiles had different ERIC-PCR profiles (Fig. 1 and 2). Therefore, ERIC-PCR might be more potent for discrimination of strains from different segments of digestive tract. In order to test this, amplification products generated with Lab 159f and Lab 677r primers were sequenced from isolates possessing distinct ERIC-profiles.

Discussion

Wheat is slowly degradable and fermentable in digestive tract of birds due to its starch structure. In addition, barley is the least degradable in digestive system of birds due to its cellulosic nature. The highest LAB count in proventriculus of WAD received quails could be explained with this nature of wheat. In addition, the highest LAB count in small intestine of quails fed with CAD could be due to overwhelmed of corn coming into this diet. This might have caused greater starch fermentation in the small intestine and resulted in the highest LAB presence. The highest Enterobacteriaceae numbers in small intestine of quails

Table 4: Bacterial identification of LAB isolates as concluded from ARDRA, ERIC-PCR and 16S rRNA gene partial sequencing

LAB species	Diets							
	BAD		WAD		CAD		CD	
	Parts of intestinal tract							
	Proventriculus	Small intestine	Proventriculus	Small intestine	Proventriculus	Small intestine	Proventriculus	Small intestine
	Isolates							
Enterococcus sp. *Enterococcus faecium* *Enterococcus durans* *Enterococcus hirae* *Enterococcus thailandicus*	**7-1** (F2) KT751315 (99%)	**2-1** (M1) NR_113904 (99%) 2-2 (M1)		**12-4** (M1) KT751315 (98%)	**25-2** (F2) KT725826 (100%) **25-3** (F2) FJ917736 (99%)	**24-3** (F1) KJ560966 (99%)		
					27-7 (F3)	26-4 (F2)		
Number of isolates	1	2	0	1	3	2	0	0
Lactobacillus salivarius	5-2 (F1)	6-4 (F1)	**11-4** (M1) KU295181 (99%)	**12-2** (M1) KU295181 (100%)	19-1 (M1)	**24-7** (F1) KU295181 (100%)	29-1 (M1)	30-3 (M1)
	5-6 (F1)	6-8 (F1)	11-8 (M1)	**14-1** (F1)	21-1 (M2)	26-1 (F2)	29-2 (M1)	30-4 (M1)
	9-1 (F3)	**8-2** (F2) KU295181 (100%)	13-4 (F1)	**14-4** (F1) KU295181 (99%)	21-2 (M2)	28-4 (F3)	31-2 (M2)	32-4 (M2)
	9-3 (F3)	10-2 (F3)	15-1 (F2)	**16-3** (F2)	23-2 (F1)		31-3 (M2)	32-7 (M2)
		10-4 (F3) KU295181 (99%)	**15-2** (F2) KU295181 (99%)	**16-4** (F2)	23-3 (F1)		33-1 (F1)	34-2 (F1)
			17-1 (F3)		27-3 (F3)		33-3 (F1)	34-4 (F1)
			17-3 (F3)				**35-1** (F2) KU295181 (99%)	36-1 (F2)
							35-2 (F2) KU295181 (99%)	36-4 (F2)
							37-1 (F3)	38-3 (F3)
							37-2 (F3) KU295181 (100%)	**38-4** (F3) KP090131 (96%)
Number of isolates	4	5	7	5	6	3	10	10
Lactobacillus reuteri	**7-7** (F2) NR_113820 (99%)							
Number of isolates	1	0	0	0	0	0	0	0
Lactobacillus oris		**8-4** (F2) AB596981 (99%)						
Number of isolates	0	1	0	0	0	0	0	0
Total number of isolates	6	8	7	6	9	5	10	10

BAD= barley added died, WAD= wheat added diet, CAD= corn added died. Bold letters indicate sequenced isolates; one representative accession number among closest matching sequences and identity percentages given in parenthesis is indicated below the isolate number. Sexes of animals are indicated as F or M in parenthesis and numbers indicate specific animal in the each treatment group.

fed with both BAD (5.17 cfu/g) and CAD (6.11 cfu/g) also verified the result that the quails struggled with cellulose rich barley and much of corn brought into the diet.

Corn, wheat, and soybean meal are the most widely used feed ingredients in global poultry production. Barley can also be used in diets with some extent; it cannot be digested easily due to its non-starch polysaccharide (NSP) nature. The presence of non-preferred NSPs, such as beta-glucans is defined as anti-nutritional factors. With the presence of phytic acid, they cause problems for gut health, nutrition and growth. These NSPs in cereal-based diets increase viscosity within the intestinal lumen (Choct et al.,

1996). They are associated with low apparent metabolizable energy, poor nutrient absorption, and cause formation of wet and sticky droppings. Another problem of NSP containing grains is the low energy recovery in poultry. Although there is NSP degradation in some extent due to gut microbial enzyme activity, short digesta transit time limits the extensive fermentation.

The NSP degrading enzymes (NSPases), such as xylanases and glucanases can reduce anti-nutritional effects of most cereals. The use of NSPases appear to influence intestinal microbiota through two main mechanisms; removal of fermentable starch and protein through accelerated digestion, and provision of soluble, fermentable oligosaccharides because of de-polymerization of insoluble fiber (Choct et al., 1996). Oligosaccharides or even monosaccharides that are released by enzyme activity could either be directly absorbed or degraded by the intestinal microflora to provide volatile fatty acids (VFAs) for the animal. This is beneficial to intestinal health and animal performance. The short chain fatty acids (SCFAs) produced by autochthonous bacteria may provide protection against pathogenic bacteria *Salmonella*, coliforms, and *Campylobacter* (van Der Wielen et al., 2000). In the present study, BAD fed group was not supplemented with any exogenous enzyme, and therefore Enterobacteriaceae count in small intestine was higher than WAD and Control groups. This may be explained with the highest insoluble fiber content of barley compared to wheat and corn. Our results are in agreement with Vahjen et al. (1998), where barley based and exogenous NSP-degrading enzyme supplemented diets altered broiler gut microbiota by lowering Enterobacteriaceae and total Gram-positive cocci. Exogenous enzyme supplementation to a barley-based diet also caused alteration in overall gut bacterial community suggesting the presence of specific beneficial and/or absence of harmful bacterial species may contribute to improvement in broiler performance (Torok et al., 2008). Other studies also showed that xylanase supplementation of a wheat based broiler diet resulted in rapid removal of starch from the jejunum and ileum, with a concomitant reduction in ileal SCFA's (Choct et al., 1999) and LAB numbers (Jia et al., 2009). In the present study, the highest LAB counts were observed in small intestinal samples of quails fed CAD and WAD, respectively. In accordance with our results, Engberg et al. (2004) showed that whole wheat feeding increased intestinal counts of *Lactobacillus* species. Among small grains that are available in this study, wheat can have slower digestion but higher protein content. Therefore, this would explain higher intestinal LAB count for CAD group than WAD group. Detection of lower LAB count for intestinal samples in BAD group compared to WAD and CAD group might not be due to the limitation of nutrients available for microbes. If this was the case, both population size and diversity should have been reduced. However, the highest diversity was detected in BAD fed compared to other groups. Moreover, our data agreed with earlier reports that birds fed on diets exhibiting significantly low performance generally showed more variation in gut microbial community between birds (Torok et al., 2011).

Several studies have addressed the effect of insoluble fiber on the composition and the quantity of gut microflora. Results on this subject have inconstancies. Evidences from some *in vitro* studies showed that populations were altered by different fiber sources fermented with caecal inoculum of chickens (Lan et al., 2005). Another study showed that wheat-based feeding promoted gizzard development and prevented potentially pathogenic bacteria from entering the intestinal tract (Engberg et al., 2004).

The experimental duration may be an important factor for determining the effect of different diets on bacterial composition and final population of digestive tract. In the present study, differences observed in LAB and Enterobacteriaceae counts indicated that duration of experiment was sufficient for detection of differences in LAB composition associated with different diets. Studies conducted on Japanese quails for a longer period showed higher LAB counts than reported in the present study. Siriken at al. (2003) reported higher LAB counts in caecal samples of Japanese quails after 10 week of trial period. Compared to these studies, shorter experimental duration might be resulted in lowest LAB counts in our study. Moreover, this result might be related to the differences in LAB density and abundance residing in various parts of digestive system (Gong et al., 2002; Lu et al., 2003).

The pH profile of digestive tract is also an important factor for both digestive tract functions and establishment of a balanced microbial community. The LAB, such as *Lactobacilli*, can influence the pH of the digestive tract with lactic acid and other VFA production abilities (Siriken et al., 2003). However, no data is available about the pH in the present study.

According to ARDRA results, sizes of the upper two bands appeared in most frequently detected ARDRA profiles after amplification with 27F and 1492R primers were matched with ARDRA profiles of two species that were previously published (Guan et al., 2003). Therefore, those isolates were preliminary identified as either *L. salivarius* or *L. reuteri*.

Variation in the amplification efficiency of three primers is most likely to be related to annealing ability of certain primer sequences to different microbial genomes or PCR amplification conditions. In the present study, testing of different PCR conditions did not improve amplification efficiency. Therefore, the

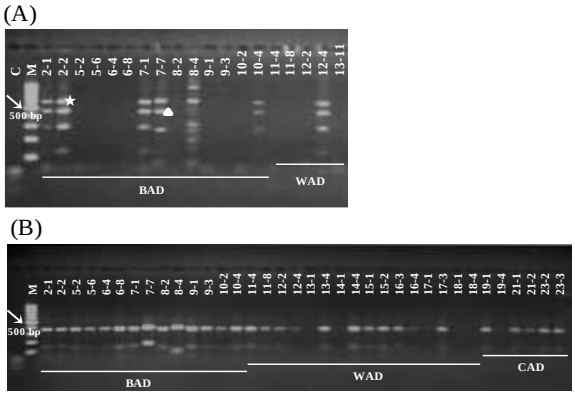

Fig. 1: ARDRA profiles belonging to some portion of isolates. (A) *Hae*III fragmentation patterns of the PCR product following to amplification with 27F and 1492R primers; (B) *Hae*III fragmentation patterns of the PCR product following to amplification with Lab 159f and Lab 677r primers M, Gene Ruler 100 bp DNA ladder within range of 100-1000 bps (Thermo Scientific); C, negative PCR control. Star and rectangular symbols indicate fragments that have similar sizes with the previous reports.

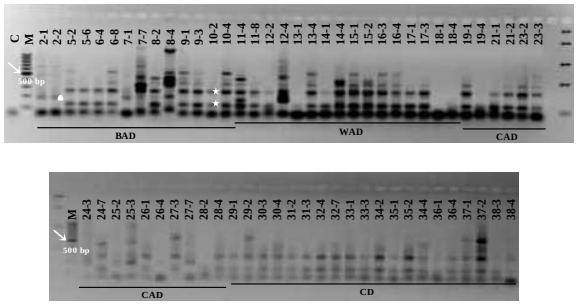

Fig. 2: ERIC-PCR profiles of all isolates. Star symbols indicate species-specific fragments for *L. salivarius* isolates, rectangular symbol indicate genus specific fragments for *Enterococcus* spp. isolates. M, Gene Ruler 100 bp DNA ladder within range of 100-1000 bps (Thermo Scientific); C, the negative PCR control.

failure in PCR amplifications were possibly due to primer sequences. For supporting this explanation, primer set used by Soto et al. (2010) had only one pair base difference in 1492R sequence compared to sequences of primers used in our study and efficiently amplified DNA of LAB isolates from bovine gut. In addition, primers with different sequences have been used successively for ARDRA analyses (Kšicová et al., 2013). Amplification efficiency with universal primer has also been shown to be affected by presence of certain type of bacteria. For example, the 16S rRNA gene of bifidobacteria shows low degree of similarity to the genes of the other microbes. Therefore, its detection is hard with certain universal primers. Similarly, formulation of 27F primer helped in increasing amplification efficiency and specificity.

According to sequencing results, ARDRA was not sufficient to discriminate *Enterococcus* spp. and *L. salivarius* strains in the present study. For instance, isolate 10-4 was identified as *L. salivarius* with 99% identity, whereas 12-4 showed 98% of nucleotide identity to several *Enterococcus* species, namely *E. faecium*, *E. durans*, *E. hirae*, *E. thailandicus*, and *Enterococcus* spp. It was also concluded from the previous study that ERIC-PCR profiles from *Lactobacillus* spp. are not affected by DNA concentrations and purities (Stephenson et al., 2009). This feature might offer an additional advantage for ERIC-PCR analyses.

Based on sequencing results, several bands in ERIC profiles were concluded to be species or genus specific (Fig. 2). Therefore, those could help fast identification of certain species as detected in the present study. All *L. salivarius* isolates had bands at about 150 and 250 bp region, whereas all *Enterococcus* spp. had bands at 200 bp region. Six bands were detected from single *L. reuteri* (7-7) isolate in ERIC profiles of our study and two of those bands were similar in sizes (bands at around 300 and 500 bp region) as detected in the previous studies for *L. reuteri* isolates (Stephenson et al., 2009).

Analysis of isolates by two typing methods (ARDRA and ERIC-PCR) and sequencing showed that LAB composition in terms of diversity and abundance in proventriculus and small intestine samples were quite different among treatment groups. The highest number of different LAB species was isolated from BAD fed group, while single LAB species (*L. salivarius*) was observed for the control group. Interestingly, four different LAB species were isolated from proventriculus and intestinal samples of the single female animal (F2), while all *L. salivarius* isolates were detected from only females in BAD group (Table 4). This might indicate that there may have animal and/or sex variation(s) as consisted with other reports (Torok et al., 2011; Su et al., 2014; Wilkinson et al., 2016).

It was found earlier that gut microbial communities varied significantly among gut sections, except between the duodenum and jejunum and significant diet-associated differences in gut microbial communities were detected within the ileum and cecum only. Moreover, several indicator bacterial species have been identified for contribution to the diet-induced differences in the overall gut microbial community (Torok et al., 2008). In the present study, *L. salivarius* was the most abundant LAB species that was detected from almost all treatment groups. Therefore, this result is congruent with the results of other studies where *L. salivarius* found to be as one of the predominant *Lactobacillus* species in poultry intestinal tract (Gong et al., 2007). However, single *L. reuteri* was observed in BAD group and this was disagreed with a report where

this species was in abundance in chicken's gastrointestinal tract (Hilmi et al., 2007).

Even though the lowest LAB count was detected in control group in the present study, *L. salivarius* was the only major *Lactobacillus* species and almost equally detected in both sexes. For instance, twenty isolates recovered from the control group were distributed as 12 from females and 8 from males. Except in control group, *L. salivarius* was isolated mainly from female animals. In accordance to our findings, significant differences in microbial communities were also reported between the sexes for across all dietary treatments in another study (Torok et al., 2011). Moreover, consisted detection of certain species was shown to be related to certain diets in several studies. Torok et al. (2011) identified three operational taxonomic units (OTUs) that potentially represent three *Lactobacillus* spp. (*L. salivarius*, *L. aviarius*, and *L. crispatus*) within the ileum as being associated with decreased performance. It was also found that *L. salivarius* populations were reduced in broilers fed antimicrobials relative to untreated ones and bile salt deconjugation in the ileum by *L. salivarius* was linked to the growth depression in chickens (Harrow et al., 2007). It was also reported that several gut bacteria isolated from chickens have various biochemical properties. For instance, several bacterial species including *Clostridium perfringens*, *Enterococcus faecium*, *Streptococcus bovis* and *Bacteroides* spp. were shown to have polysaccharide degrading activity against grain NSPs (Beckmann et al., 2006). Considering biochemical activities of certain bacterial species in gastrointestinal tract, detection of more *Enterococcus* spp. in small intestine of BAD, WAD and CAD fed quails are an indication of their possible roles in grain digestion.

In conclusion, ERIC-PCR alone was highly efficient for molecular tracking of cultivable LAB in gastrointestinal tract of quails fed with different diets. This approach can be applied for determination of bacterial indicators linked to performance parameters, such as feed efficiency and nutrient digestibility.

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References

Apajalahti J, Kettunen A, Graham H (2004) Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. World's Poult Sci J 60:223-232.

Apajalahti J, Kettunen A (2006) Rational development of novel microbial modulators. In: Barug D, de Jong J, Kies AK, Verstegen MWA (editors), antimicrobial growth promoters. where do we go from here? Wageningen Academic Publishers, pp: 165-181.

Bal EBB, Bayar S, Bal MA (2010) Antimicrobial susceptibilities of coagulase-negative Staphylococci (CNS) and Streptococci from bovine subclinical mastitis cases. J Microbiol 48: 267-274.

Bedford MR, Apajalahti J (2000) Microbial interaction in the response to exogenous enzyme utilization. In: Bedford MR, Partridge GG (editors), enzymes in farm animal nutrition. CABI Publishing, Wallingford, pp: 299-315.

Beckmann L, Simon O, Vahjen W (2006) Isolation and identification of mixed linked beta-glucan degrading bacteria in the intestine of broiler chickens and partial characterization of respective 1,3-1,4-glucanase activities. J Basic Microbiol 46**:**175-185.

Choct M, Hughes RJ, Wang J, Bedford MR, Morgan AJ, Annison G (1996) Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. Br Poult Sci 37: 609-621.

Choct M, Hughes RJ, Bedford MR (1999) Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. Br Poult Sci 40: 419-422.

Engberg RM, Hedemann MS, Steenfeldt S, Jensen BB (2004) Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poult Sci 83: 925-938.

Feighner SD, Dashkevicz MP (1987) Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. Appl Environ Microbiol 53: 331-336.

Gong J, Forster RJ, Yu H, Chambers JR, Wheatcroft R,Sabour PM, Chen S (2002) Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. FEMS Microbiol Ecol 41: 171-179

Gong J, Si W, Forster RJ, Huang R, Yu H, Yin Y, Yang C, Han Y (2007) 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. FEMS Microbiol Ecol 59:147-157.

Guan LL, Hagen KE, Tannock GW, Korver DR, Fasenko GM, Allison GE (2003) Detection and identification of *Lactobacillus* species in crops of broilers of different ages by using PCR-denaturing

gradient gel electrophoresis and amplified ribosomal DNA restriction analysis. Appl Environ Microbiol 69:6750-6757.

Han H, Takase S, Nishino N (2012) Survival of silage lactic acid bacteria in the goat gastrointestinal tract as determined by denaturing gradient gel electrophoresis. Lett Appl Microbiol 55:384-389.

Harrow SA, Ravindran V, Butler RC, Marshall JW, Tannock GW (2007) Real-time quantitative PCR measurement of ileal *Lactobacillus salivarius* populations from broiler chickens to determine the influence of farming practices. Appl Environ Microbiol 73:7123-7127.

Hilmi HTA, Surakka A, Apajalahti J, Saris PEJ (2007) Identification of the most abundant *Lactobacillus* species in the crop of 1- and 5-week-old broiler chickens. Appl Environ Microbiol 73:7867-7873.

Itoh N, Kikuchi N, Niwa K, Makita T, Hiramune T (1997) Bacterial flora in the feces and intestine of the quail. Nippon Juishikai Zasshi 50:593-595.

Jia W, Slominski BA, Bruce HL, Blank G, Crow G, Jones O (2009) Effects of diet type and enzyme addition on growth performance and gut health of broiler chickens during subclinical *Clostridium perfringens* challenge. Poult Sci 88:132-140.

Kšicová K, Dušková M, Karpíšková R (2013) Differentiation of *Lactobacillus* species by ARDRA. Czech J Food Sci 31:180-188.

Lan Y, Williams BA, Tamminga S, Boer H, Akkermans A, Erdi G, Verstegen MWA (2005) In vitro fermentation kinetics of some non-digestible carbohydrates by the caecal microbial community of broilers. Anim Feed Sci Tech 123-124: 687-702.

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (editors), nucleic acid techniques in bacterial systematic. Wiley, Chichester, pp. 115-175.

Liu S, Bennett DC, Tun HM, Kim JE, Cheng KM, Zhang H, Leung FC (2015) The effect of diet and host genotype on ceca microbiota of Japanese quail fed a cholesterol enriched diet. Front Microbiol 6:1-14.

Lu J, Idris U, Harmon B, Hofacre C, Mauer JJ, Lee MD (2003) Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl Environ Microbiol 69: 6816-6824.

Niba AT, Beal JD, Kudi AC, Brooks PH (2009) Bacterial fermentation in the gastrointestinal tract of non-ruminants: influence of fermented feeds and fermentable carbohydrates. Trop Anim Health Prod 41:1393-1407.

Pan D, Yu Z (2014) Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5:108-119.

Siriken B, Bayram I, Onal AG (2003) Effects of probiotics: Alone and in a mixture of Biosacc® plus Zinc Bacitracin on the caecal microflora of Japanese quail. Res Vet Sci 75:9-14.

Stephenson DP, Moore RJ, Allison GE (2009) Comparison and utilization of repetitive-element PCR Techniques for typing *Lactobacillus* isolates from the chicken gastrointestinal tract. Appl Environ Microbiol 75:6764-6776.

Soto LP, Frizzo LS, Bertozzi E, Avataneo E, Sequeira GJ, Rosmini MR (2010) Molecular microbial analysis of *Lactobacillus* strains isolated from the gut of calves for potential probiotic use. Vet Med Int 1-7.

Su H, McKelvey J, Rollins D, Zhang M, Brightsmith DJ, Derr J, Zhang S (2014) Cultivable bacterial microbiota of Northern Bobwhite (*Colinus virginianus*): A new reservoir of antimicrobial Resistance? PLoS ONE 9: 1-11.

Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Ng J, Munro K, Alatossava T (1999) Identification of Lactobacillus isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. Appl Environ Microbiol 65: 4264-4267.

Torok VA, Ophel-Keller K, Loo M, Hughes RJ (2008) Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. Appl Environ Microbiol 74:783-791.

Torok VA, Hughes RJ, Mikkelsen LL, Perez-Maldonado R, Balding K, MacAlpine R, Percy NJ, Ophel-Kelleretal K (2011) Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. Appl Environ Microbiol **77**:5868-5878.

Vahjen W, Glaser K, Schafer K, Simon O (1998) Influence of xylanase-supplemented feed on the development of selected bacterial groups in the intestinal tract of broiler chicks. J Agric Sci 130:489-500.

Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19: 6823-6831.

van Der Wielen PW, Biesterveld S, Notermans S, Hofstra H, Urlings BA, van Knapen F (2000) Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl Environ Microbiol 66:2536-2540.

Wilkinson N, Hughes RJ, Aspden WJ, Chapman J, Moore RJ, Stanley D (2016) The gastrointestinal tract microbiota of the Japanese quail, *Coturnix japonica*. Appl Microbiol Biotechnol 100: 4201-4209.