IJAS Iranian Journal of Applied Animal Science

Study of Bacterial Diversity in Poultry Gut Using Denaturing Gradient Gel Electrophoresis

Research Article

V.B. Ahir[1*], K.M. Singh[1], A.K. Tripathi[1], R.A. Mathakiya[2], S.J. Jakhesara[1], P.G. Koringa[1], D.N. Rank[3], M.K. Jhala[2] and C.G. Joshi[1]

[1] Department of Animal Biotechnology, College of Veterinary Science and Animal Science, Anand Agricultural University, 388001, Anand, India
[2] Department of Veterinary Microbiology. College of Veterinary Science and Animal Science, Anand Agricultural University, 388001, Anand, India
[3] Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Science, Anand Agricultural University, 388001, Anand, India

Received on: 22 Aug 2011
Revised on: 20 Oct 2011
Accepted on: 21 Oct 2011
Online Published on: Sep 2012

*Correspondence E-mail: ahirviral@gmail.com

Online version is available on: www.ijas.ir

ABSTRACT

Bacteria play an important role in the initiation and progression of diseases (not only in disease but affect health status of individual). Populations of digestive microflora in chicken gut change with age and also affected by diet, stressors, and performance enhancers as well as in disease conditions. The aim of the present study was to know bacterial makeup in chicken gut, using denaturing gradient gel electrophoresis (DGGE). DGGE results showed 11 different band patterns from the six birds (P1-P6). A total of 11 bands were cloned and sequenced and similarities to known 16S rDNA sequences were examined. All the sequences had 93 to 100% similarity to 16S rDNA database sequences. In P1 birds, total 8 bands were observed; which had varying degree of homology with *Proteus mirabilis*, *Gallibacterium anatis*, uncultured bacterium, bacteroidetes bacterium, rumen bacterium and *Bacillus subtilis*. Similarly in second bird (P2), one clone was showing 98% similarities with uncultured bacterium. Further one clone from P3 was similar to uncultured prokaryote clone (93%). Likewise in P4, one clone was similar (100%) to *Escherichia* species. Moreover, in P5 and P6 birds, DGGE bands were having some common migration pattern to those that were observed in other four birds (P1-P4). These results may help poultry producers to develop more cost-effective health management strategies as well as differences in the composition of the gut microbial community with improved performance, which implies that the presence of specific beneficial and / or absence of specific detrimental bacterial species may contribute to the improved performance of the birds.

KEY WORDS bacterial diversity, electrophoresis, poultry gut.

INTRODUCTION

The gut microbiota has an important role in poultry health and production, which generally affects the health of the host by influencing digestion and nutrient absorption, intestinal morphology, and defence of the host against infection (Abrams *et al*. 1963; Mead, 2000). Moreover, gut microbiota positively influence the host's gastrointestinal development, biochemistry, immunology, physiology, and nonspecific resistance to infection (Gordon and Pesti, 1971; Klasing *et al*. 1999). In poultry, the microorganisms that colonize the gastrointestinal tract during the early post-hatch period form a synergistic relationship with their host. Gastro-intestinal microorganisms have a highly significant impact on the uptake and utilization of energy (Muyzer *et al*. 1998) and other nutrients (Steenfeldt *et al*. 1995; Smits

et al. 1997) and on the response of poultry to anti-nutritional factors (such as non-starch polysaccharides [NSP]), pre and probiotic feed additives and feed enzymes (Lerman *et al*. 1984). Bacteria within the gut microbial community also interact with each other (Swift *et al*. 2000; De Angelis *et al*. 2006) as well as with their host (Van Leeuvan *et al*. 2004; Kelly *et al*. 2005).

The gastrointestinal microbiota has one of the highest cell densities for any ecosystem and in poultry ranges from 10^7 to 10^{11} bacteria per gm of gut content (Apajalahti *et al*. 2004). Earlier studies have predominantly used culture-dependent approaches for identifying the composition of the poultry gut microbiota (Barnes, 1979; Mead, 1989).

However, a large number of bacteria remain unidentified due to lack in knowledge of appropriate culturing conditions. Furthermore, culturing and biochemical techniques have resulted in the misclassification of some bacteria (Tellez, 2006). Recent molecular studies targeting the bacterial DNA in poultry guts have yielded more detailed insight into the composition of the microbial community (Apajalahti *et al*. 1998; Gong *et al*. 2002; Lan *et al*. 2002; Zhu *et al*. 2002; Hume *et al*. 2003; Zhu and Joerger, 2003; Amit-Romach *et al*. 2004). The presence of specific beneficial or absence of specific detrimental bacterial species may contribute to the improved performance of birds (Torok *et al*. 2008). From molecular studies it is estimated that the cecal microbiota consists of at least 640 species from 140 genera, of which 10% of the identified bacterial 16S rDNA gene sequences represent previously known bacterial species, and the remaining sequences belong to new species or even new genera.

Denaturing gradient gel electrophoresis (DGGE) present an approach for directly determining the genetic diversity of complex microbial populations, which is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. In DNA fragments of the same length but with different base-pair sequence composition can be separated on the basis of electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels, which is lesser compared with that of the completely helical form of the molecule (Fischer and Lerman, 1979). The melting of fragments proceeds in discrete so-called melting domains.

Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially melted molecules occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes their melting temperatures to differ. Sequence variants of particular fragments will therefore stop migrating at different positions in the denaturing gradient and hence can be separated effectively by DGGE (Lerman *et al*. 1984). DGGE has the potential advantage of detecting multiple species simultaneously on a large scale but it should be also supported with other reliable techniques like qPCR and high throughput sequencing. However, there is no agreement that DGGE profiles are a representative fingerprint of the population under study (Muyzer *et al*. 1993; Kisand and Wikner, 2003) and it is uncertain whether clinically relevant species are present in DGGE profiles.

A more comprehensive overview of the intestinal microbiota of poultry would not only be of interest to basic microbiological ecology but might also be of practical importance. For example, further development of effective competitive exclusion products might be aided by a better understanding of the intestinal microbiota composition (Wang *et al*. 2009). The aim of the present study was to investigate the potential of DGGE as a tool for the bacterial community identification in poultry gut. At present, DGGE analysis is one of the most suitable and widely used methods for studying complex bacterial communities in various environmental habitats (Muyzer and Smalla, 1998). The present study was carried out with the objective of investigating the microbial flora of the poultry intestinal tract applying DGGE technique to know the microbial makeup of poultry gut.

MATERIALS AND METHODS

Intestinal sample collection

Samples for the present study were collected during post-mortem examination from six birds (P1-P6) of 4-6 weeks of age at the Department of Pathology, Veterinary College, AAU, Anand.

The gastrointestinal tracts from the base of the gizzard down to the rectum were dissected, and intestinal scrapping from the mid regions of the caeca was collected using sterile scissors and forceps in a 2 mL tubes and finally stored at -20 ˚C until DNA extraction.

DNA extraction for 16S ribosomal DNA (rDNA) analysis

Total DNA was extracted as described by Wright *et al*. (1997) with few modifications. The quantity and quality of the genomic DNA was checked by nanodrop spectrophotometer as well as integrity of total DNA was confirmed by electrophoresis with 0.8% agarose gel in 0.5 × TBE. All the samples were treated with RNAse-A to remove contaminating RNA.

Primers and PCR amplification

To amplify V3 region of the bacterial 16S rDNA domain, the primers with GC-clamp- BA338fCG (5′CGC CCG CGC GCG GCG GGC GGG GCG GGG GCA GCA CGG

GGG GAC TCC TAC GGG 3′) and UN518r (5′ATT ACC GCG GCT GCT GG 3′) (Ovreas *et al*. 1997), were used.

PCRs were done in 25-µL volumes containing 2 × PCR mastermix (Fermentas, USA), 0.1 µM concentrations of each primer and 100 ng of total DNA. Thermocycling was carried out in 9700 (ABI, CA) with the following amplification conditions: initial denaturation at 95 ˚C for 5 min followed by 30 cycles of denaturation at 95 ˚C for 1 min, annealing at 58 ˚C for 1 min, and extension at 72 ˚C for 3 min, with a final extension step at 72 ˚C for 10 min. The specificity of PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel electrophoresis and visualized after staining with ethidium bromide.

DGGE analysis

Amplicons of 16S rDNA V3 region were used for sequence-specific separation by DGGE according to the specifications of Muyzer *et al*. (1993), using DGGE Assembly (Cleaver Scientific, UK). DGGE was performed in 6.5% polyacrylamide gels (37.5: 1 acrylamide-bisacrylamide) in a denaturing gradient of 40% to 60% of urea. The electrophoresis was initiated by pre-running for 10 min at 200 V and subsequently at a fixed voltage of 85 V for 12 h at 60 ˚C. After electrophoresis bands were visualized by conventional silver staining. The fragments of interest were excised from the gel, extracted ('Crush and Soak' method), and re-amplified for further analysis.

Sequencing of DGGE fragments

The representative bands of all the birds from the DGGE gel of 16S rDNA fragments were excised with a razor blade and put in 2ml sterile Eppendorf tubes. The gel was crushed with a homogenizer (Pro Scientific, USA) and DNA was retrieved by heating the tubes in boiling water bath for 15-20 min. Tubes were centrifuged at maximum speed and each supernatant containing DNA was used for re-amplification with same primers (BA338fCG and UN518r). The desired purified fragments were cloned into pTZ57R vector (InsT/Aclone™, Fermentas, USA). The vector containing inserts were propagated in *E.coli* DH5-α following manufacturer's instructions, and colonies were selected by blue white screening. The recombinant plasmids, carrying desired insert, were isolated from the representative clone using QIAprep® Spin Miniprep kit (QIAGEN, CA), and subjected to BigDye® Terminator v3.1 Cycle Sequencing reaction (Applied Biosystems, CA). These purified products were resolved on automated ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, CA), and the sequences were analyzed using Sequencing Analysis Software v5.2 (Applied Biosystems, CA). The similarity was looked in to the non-redundant database of GenBank with BLAST algorithms.

RESULTS AND DISCUSSION

It has previously been shown that denaturing gradients have the capability of separating mixed DNA fragments of the same length that differ by only a single base (Fischer and Lerman, 1983; Myers *et al*. 1985; Noll and Collins, 1987).

These findings support the argument that DGGE has the ability to resolve PCR products derived from a marker gene down to a species or even subspecies level. Therefore, the number of bands revealed by DGGE directly reflects upon the overall degree of microbial diversity within a sample, making DGGE such an effective tool in assessing environmental and intestinal microbiomes.

PCR amplification of the V3 region in caecal samples generated a single product on agarose gel electrophoresis. A total of 11 bands (Figure 1) were analysed from the 6 samples of poultry birds (i.e P1-P6), and sequences had 93% to 100% similarity to 16S rDNA database sequence (Table1).

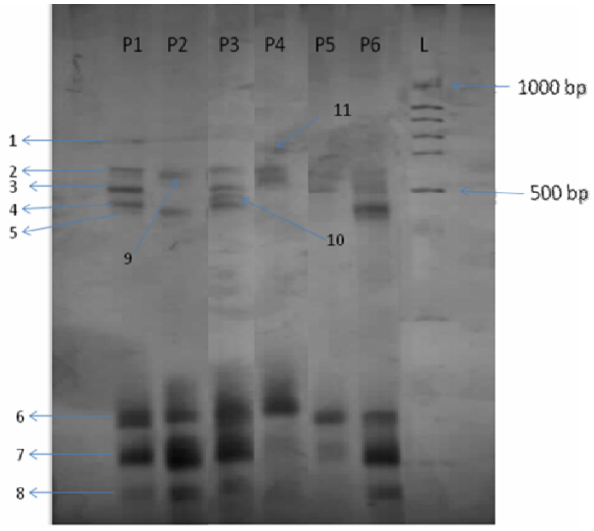

Figure 1 PCR-DGGE pattern of poutry gut bacteria (i.e. P1- P6 Bird no. and L-Ladder)

Further, there were some common bands present in all sample. The intensity of the bands was variable, which meant that there were common microorganisms in six birds, but the abundance of the microorganisms was different.

This finding supports our hypothesis that the different microbial communities of poultry gut play an important role in health status of the bird.

In P1 birds, total 8 bands were observed; band 1 was showing 100% similarity with *Proteus mirabilis*, and other clones were also similar to *Gallibacterium anatis*, uncultured bacterium, bacteroidetes bacterium, rumen bacterium and *Bacillus subtilis*. Similarly in second bird (P2), one clone was showing 98% similarities with uncultured bacterium.

Table 1 Taxonomic distribution of clones sequenced isolated from all the birds

Band no.	Nearest taxa	Accession No. of nearest taxa	Our accession no.	Identity
1	*Proteus mirabilis*	JN162422	JF308634	100%
2	*Gallibacterium anatis*	HQ629808	JF308635	98%
3	Uncultured bacterium	FN659203	JF308636	98%
4	Bacteroidetes bacterium culture	HM346213	JF308637	100%
5	Rumen bacterium	HM346206	JF308647	98%
6	*Gallibacterium anatis*	HQ629810	JF308638	96%
7	*Bacillus subtilis*	JN092585	JF308639	96%
8	Rumen bacterium	HM346200	JF308640	100%
9	Uncultured bacterium	JF115112	JF308646	98%
10	Uncultured prokaryote	HQ155045	JF308642	93%
11	*Escherichia* sp.	HM346190	JF308643	100%

Further one clone from P3 was similar to uncultured prokaryote (93%) which is having least similarity to the 16 sRNA sequence. In P4, one clone (band no. 11) was similar to *Escherichia* species (100%). In P5 and P6 birds, DGGE bands were common to those observed in other birds (P1-P4) having same migration rate. It was noted that few fragments displayed high sequence homology to unculturable microbes but viable bacterial species (Table 1).

This is not unusual, as other investigators have found similar results. Zhou *et al*. (2007) reported that most of clones out of 13 sequenced were closely related to uncultured bacteria without specific species information. The closest relatives of cultivable bacteria to which some clones corresponded included *Klebsiella granulomatis* at day 3 from the ceca, *Enterococcus* sp. AK61 at day 3 from the ileum, and *L. salivarius* at day 14 from the ileum. There are also reports describing only 10 to 60% of the total bacteria in the caecum of chickens were detected by culture (Barnes, 1979; Mead, 1989; Salanitro *et al*. 1974). Thi Ngoc Lan *et al*. (2002) identified 90% of bacteria present in the caecum of chickens were unculturable. The culturable species identified in our study included those from the genera *Bacillus subtilis*, *Proteus mirabilis*, bacteroidetes bacteria, *Gallibacterium anatis*, uncultured rumen bacterium and uncultured prokaryote clones. These results concur with those from another study, investigating the diversity of intestinal bacterial community of the broiler chicken (Lu *et al*. 2003). Using same techniques in other closely related species by Matsui *et al*. (2010) reported that 39.4% of sequences were affiliated with Bacteroidetes in the ostrich caecum and the abundance of Bacteroidetes species is an important factor for the fibrolytic and actively fermenting microbial ecosystem in the GI tracts and Bacteriodetes were also present in our study. More recently Liu *et al*. (2011) presented that goose caecum were dominantly occupied by Clostridia-related species (58.7%) with other abundant sequences being related to Bacteroidetes (26.9%) and Erysipelotrichi (11.2%). Gammaproteobacteria (59.6%) and Clostridia (20.1%) were predominant in the mucosa of goose caecum.

Simpson *et al*. (1999) proposed that it is unlikely for bacteria numbering less than 10^8 cfu/g in a total digestive population to be visualized by DGGE. Zhu *et al*. (2002), using temporal temperature gradient gel electrophoresis (TTGE) as the separation technique, identified 243 different 16S rDNA sequences in cloned amplicons from cecal bacteria of broiler chicks 1 day and 1, 2, 4, and 6 wk old.

However, because only a few bands (10 to 20) were clearly visible on gel images presented by Zhu *et al*. (2002), the majority of sequences identified were likely those from amplicons present in concentrations below the sensitivity level of the silver staining technique used in that study. This indicates that amplicons present in low concentration and not visible following staining or obscured by co-migration in the gradient could successfully form vector inserts and transformed colonies during cloning. Additionally, visualization of some bands (in high or low concentration) might have been obscured by band comigration (Wintzingerode *et al*. 1997; Muyzer and Smalla, 1998). Hong *et al*. (2011) reported that due to the short length of the amplified sequences several organisms can not clearly be identified using PCR-DGGE also. In spite of having different GC content and primary sequences, multiple amplicons may migrate to the same denaturant level resulting in an inaccurate indication of genotypic diversity and abundance.

Early studies involved both culture-dependent and culture-independent methods to show an increase in the diversity of the bacterial community in the ileum and cecum as a bird ages (Gong *et al*. 2008), so the molecular techniques such as DGGE should be combined with culture-based approaches to analyse the microbiota (Xiao *et al*. 2007). Applications of DGGE are widespread in many different ecosystems, but they still have some limitations. They need expensive equipment, different DNA may have similar melting properties, so the addition of a 30-40 bp GC clamp to one of the PCR primers is needed (Reeson *et al*. 2003), while the use of different regions of the 16S rDNA and different DGGE conditions might result in different resolutions of separation.

Yu and Morrison (2004) compared and established a preferred choice of vregion (s) to examine by DGGE, and found that DGGE profiles of the V3 region were the best, and the V3 to V5 and V6 to V8 regions produced better DGGE profiles than did other multiple V-region amplicons. Other factors than DGGE conditions may also affect the sequence information and PCR amplification, and this implies to optimize the experimental conditions.

CONCLUSION

PCR-DGGE analysis of bacterial isolates from intestinal content is not always suitable for the identification of all species, but can be used for screening and grouping the isolates and reducing the number of cultures to identify by molecular or biochemical methods. DGGE offers an opportunity to detect multiple species simultaneously and to distinguish between different serotypes and can easily be extended to other species of interest. Moreover, the use of DGGE offers the opportunity to study the presence of these pathogens in relation to the presence of other species. The data will be useful for future studies related to competitive exclusion and for experiments evaluating the impact of, for example, growth promoters, anti-coccidial compounds or stress on the intestinal microbiota.

REFERENCES

Abrams G.D., Bauer H. and Sprinz H. (1963). Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germfree and conventional mice. *Lab. Invest.* **12,** 355-364.

Amit-Romach E., Sklan D. and Uni Z. (2004). Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poult. Sci.* **83,** 1093-1098.

Apajalahti J., Kettunen A. and 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.H.A., Sarkilahti L.K., Maki B.R.E., Heikkinen J.P., Nurminen P.H. and Holben W.E. (1998). Effective recovery of bacterial DNA and percent-guanine-plus-cytosine-based analysis of community structure in the gastrointestinal tract of broiler chickens. *Appl. Environ. Microbiol.* **64,** 4084-4088.

Barnes E.M. (1979) The intestinal microflora of poultry and game birds during life and after storage. *J. Appl. Bacteriol.* **46,** 407-419.

De Angelis M., Siragusa S., Berloco M., Caputo L., Settanni L., Alfonsi G., Amerio M., Grandi A., Ragni A. and Gobbetti M. (2006). Selection of potential probiotic lactobacilli from pig faeces to be used as additives in pelleted feeding. *Res. Microbiol.* **157,** 792-801.

Fischer S.G. and Lerman L.S. (1979). Length-independent separation of DNA restriction fragments in two dimensional gel electrophoresis. *Cell*. **16,** 191-200.

Fischer S.G. and Lerman L.S. (1983). DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proc. Natl. Acad. Sci.* **80,** 1579-1583.

Gong J., Forster R.J., Yu H., Chambers J.R., Sabour P.M., Wheatcroft R. and Chen S. (2002). Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. *FEMS Microbiol. Lett.* **208,** 1-7.

Gong J., Yu H., Liu T., Gill J.J., Chambers J.R. and Wheatcroft R. (2008). Effects of zinc bacitracin, bird age and access to range on bacterial microbiota in the ileum and caeca of broiler chickens. *J. Appl. Microbiol.* **104,** 1372-1382.

Gordon H.A. and Pesti L. (1971). The gnotobiotic animal as a tool in the study of host-microbial relationships. *Bacteriol. Rev.* **35,** 390-429.

Hong T.T.T., Passoth V. and Lindberg J.E. (2011). Bacterial diversity at different sites of the digestive tract of weaned piglets fed liquid diets. *Asian-Aust. J. Anim. Sci.* **24,** 834-843.

Hume M.E., Kubena L.F., Edrington T.S., Donskey C.J., Moore R.W., Ricke S.C. and Nisbet D.J. (2003). Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. *Poult. Sci.* **82,** 1100-1107.

Kelly D., Conway S. and Aminov R. (2005). Commensal gut bacteria: mechanisms of immune modulation. *Trends. Immunol.* **26,** 326-333.

Kisand V. and Wikner J. (2003). Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. *J. Microbiol. Methods.* **54,**183-191.

Klasing K.C., Johnston B.K. and Benson B.N. (1999). Implications of an immune response on growth and nutrition requirements of chicks, Pp. 35-47 in Recent Developments in Poultry Nutrition, P.C. Garnsworthy and J. Wiseman Ed., vol. 2. Nottingham University Press, Nottingham, United Kingdom.

Lan P.T.N., Hayashi H., Sakamoto M. and Benno Y. (2002). Phylogenetic analysis of cecal microbiota in chicken by the use of 16S rDNA clone libraries. *Microbiol. Immunol.* **46,** 371-382.

Lerman L.S., Fischer S.G., Hurley I., Silverstein K. and Lumelsky N. (1984). Sequence-determined DNA separations. *Annu. Rev. Biophys. Bioeng.* **13,** 399-423.

Liu B.Y., Wang Z.Y., Wang H.R., Hu P., Xu D. and Wang Q. (2011). Molecular profiling of bacterial species in the caecum of geese. *Czech J. Anim. Sci.* **56,** 192-203.

Lu J., Idris U., Harmon B., Hofacre C., Maurer J.J. and Lee M.D. (2003). Diversity and succession of the intestinal bacterial communities of the maturing boiler chicken. *Appl. Environ. Microbiol.* **69,** 6816-6824.

Matsui H., Kato Y., Chikaraishi T., Moritani M., Ban- Tokuda T. and Wakita M. (2010). Microbial diversity in ostrich ceca as revealed by 16S ribosomal RNA gene clone library and detection of novel *Fibrobacter* species. *Anaerobe*. **16,** 83-93.

Mead G.C. (1989). Microbes of the avian cecum: types present and substrates utilized. *J. Exp. Zool.* **3,** 48-54.

Mead G.C. (2000). Prospects for competitive exclusion treatment to control salmonellas and other foodborne pathogens in poultry. *Vet. J.* **159,** 111-123.

Muyzer G., De Waal E.C. and Uitterlinden A.G. (1993). Profiling

of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59,** 695-700.

Muyzer G. and Smalla K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Leeuwenhoek*. **73,** 127-141.

Muyzer G., Brinkhoff T., Nu¨bel U., Santegoeds C., Scha¨fer H. and Wawer C. (1998). Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, Pp. 3441-3442 in Molecular microbial ecology manual, A.D.L. Akkermans, J.D. Van Elsas and F.J. De Bruijn Ed. 3rd Ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Myers R.M., Lumelsky N., Lerman L.S. and Maniatis T. (1985). Detection of single base substitutions in total genomic DNA. *Nature.* **313,** 495-498.

Noll W.W. and Collins M. (1987). Detection of human DNA polymorphism with a simplified denaturing gradient gel electrophoresis technique. *Proc. Natl. Acad. Sci.* **84,** 3339-3343.

Ovreas L., Forney L., Daae F.L. and Torsvik V. (1997). Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* **63,** 3367-3373.

Reeson A.F., Jankovic T., Kasper M.L., Rogers S. and Austin A.D. (2003). Application of 16S rDNA-DGGE to examine the microbial ecology associated with a social wasp *Vespula germanica*. *Insect. Mol. Biol.* **12,** 85-91.

Salanitro J.P., Fairchild I.G. and Zgornicki Y.D. (1974). Isolation, culture characteristics and identification of anaerobic bacteria from the chicken caecum. *Appl. Microbiol.* **27,** 678-687.

Simpson J.M., McCracken V.J., Gaskins H.R., White B.A. and Mackie R.I. (1999). Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J. Microbiol. Methods.* **36,** 167-179.

Smits C.H., Veldman A., Verstegen M.W. and Beynen A.C. (1997). Dietary carboxymethyl cellulose with high instead of low viscosity reduces macronutrient digestion in broiler chickens. *J. Nutr.* **127,** 483-487.

Steenfeldt S., Bach Knudsen K.E., Børsting C.F. and Eggum B.O. (1995). The nutritive value of decorticated mill fractions of wheat. 2. Evaluation with raw and enzyme treated fractions using adult cockerels. *Anim. Feed Sci. Technol.* **54,** 249-265.

Swift S., Vaughan E.E. and De Vos W.M. (2000). Quorum sensing within the gut ecosystem. *Microb. Ecol. Health Dis.* **2,** 81-92.

Tellez G., Higgins S.E., Donoghue A.M. and Hargin B.M. (2006). Digestive physiology and the role of microorganisms. *J. Appl. Poult. Res.* **15,** 136-144.

Thi Ngoc Lan P., Hayashi H., Sakamoto M. and Benno Y. (2002). Phylogenetic analysis of cecal microbiota in chicken by the use of 16S rDNA clone libraries. *Microbiol. Immunol.* **46,** 371-382.

Torok V.A., Ophel-Keller K., Loo M. and Hughes R.J. (2008). Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Appl. Environ. Microbiol.* **74,** 783-791.

Van Leeuwen P., Mouwen J.M., Van Der Klis J.D. and Verstegen M.W. (2004). Morphology of the small intestinal mucosal surface of broilers in relation to age, diet formulation, small intestinal microflora and performance. *Br. Poult. Sci.* **45,** 41-48.

Wang Z.Y., Shi S.R., Xu M.J. and Yang H.M. (2009). 16S rRNA-based analysis of bacterial diversity in the microbial flora of the goose intestinal tract. *Anim. Feed Sci. Technol.* **18,** 531-540.

Wintzingerode F.V., Gobel U.B. and Stackebrandt E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analyses. *FEMS Microbiol. Rev.* **21,** 213-229.

Wright A.D.G., Dehority B.A. and Lynn D.H. (1997). Phylogeny of the rumen ciliates Entodinium, Epidinium and Polyplastron (Litostomatea: Entodiniomorphida) inferred from small subunit ribosomal RNA sequences. *J. Eukaryot. Microbiol.* **44,** 61-67.

Xiao W., Peng Q., Liu H.W., Wen M.L. and Cui X.L. (2007). Prokaryotic microbial diversity of the ancient salt deposits in the Kunming Salt Mine, P.R. China. *Acta. Microbiol. Sinica.* **47,** 295-300.

Yu Z.T. and Morrison M. (2004). Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **70,** 4800-4806.

Zhou H., Gong J., Brisbin J.T., Yu H., Sanei B., Sabour P. and Sharif S. (2007). Appropriate chicken sample size for identifying the composition of broiler intestinal microbiota affected by dietary antibiotics, using the polymerase chain reaction-denaturing gradient gel electrophoresis technique. *Poult. Sci.* **86,** 2541-2549.

Zhu X.Y. and Joerger R.D. (2003). Composition of microbiota in content and mucus from caeca of broiler chickens as measured by fluorescent in situ hybridization with group-specific, 16S rRNA-targeted oligonucleotide probes. *Poult. Sci.* **82,** 1242-1249.

Zhu X.Y., Zhong T.Z., Pandya Y. and Joerger R.D. (2002). 16S rRNAbased analysis of microbiota from the cecum of boiler chickens. *Appl. Environ. Microbiol.* **68,** 124-137.