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Full Length Research Paper

Identification of microbial diversity in caecal content of broiler chicken

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The intestinal microbiota plays a major role in growth as well as maintenance of the health of animals. Traditionally, the microorganisms present in the intestine of animals were identified by bacteriological culture methods. In this study, the microbial community of caecal content of chicken was identified by sequencing genomic material directly from the caecum. Deoxyribonucleic acid (DNA) was extracted directly from the caecal microbial content of broiler chicken. 16S recombinant deoxyribonucleic acid (DNA) sequence is a hyper-variable region which is conserved in almost all prokaryotes. Using universal primers, the 16S rDNA sequence was amplified, cloned into PCR(4) TOPO vector and transformed in *Escherichia coli* DH_5 α cells, the plasmid from clones containing target 16S rDNA sequence was sequenced. All the sequences were subjected to BLAST search against prokaryote genomic database to identify similar sequences. Cloned sequences exhibited high level of sequence similarity (> 95%) with *Stenotrophomonas maltophilia* (strain D1, AUX077, CCF00024, 776), uncultured bacterium P4D1-70, 87% with uncultured eubacterium clone GL178.1, *S. maltophilia* (strain HB, B2A, 776), *Nanobacterium* spp*. nanoU,* 94% with *S. maltophilia* (strain D1, AUX077), uncultured bacterium clone P4D7-617. Based on the similarity search and phylogenetic tree construction it was identified that the bacterial community present in the caecal content of broiler chicken belongs to *Bacillus* spp group and it exhibits the evolutionary relationship with *Nanobacterium* spp, *Stenotrophomonas* spp and unculturable microorganism.

Key words: 16S rDNA, cecal content, broiler chicken, universal primers.

INTRODUCTION

The caecum of chicken is colonized by a complex microbial community, which is often referred to as the chicken intestinal flora (Pham et al., 2002). Microscopically visualized cells which are viable do not form visible colonies on culture plates (Roszak and Colwell, 1987).

To overcome the above reason, new culture-independent molecular techniques were applied to investigate the intestinal microbiota and to identify the diversity of microorganisms (Sullivan, 1999, Suau et al., 1999). The microbial community present in the gastro-intestinal tract is characterized by high population density and complexity of interactions. Microbial populations present in herbivores, omnivores, carnivores, and in a wide range of zoological classes where they contribute to the nutrition, physiology, immunology, and protection of the host. Despite this vast amount of knowledge, the basic prerequisites for ecological studies, namely enumeration and identification of all community members have tremendous limitations. These limitations can be overcome by suffusing molecular ecology techniques based on sequence comparisons of nucleic acids

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(Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and can be used to provide a molecular characterization, while at the same time providing a classification scheme that predicts phylogenetic relationships (Pace et al., 1985). Nucleic acid probes and the polymerase chain reaction (PCR) were used for complete description of an organism's genetic composition, gene expression as well as taxonomic information.

The direct amplification and analysis of 16S rRNA genes (16S rDNA) sequences are rapidly replacing cultivation to compare the composition, richness and structure of microbial communities such method include 16S rDNA cloning (DeLong et al., 1993; Fuhrman et al., 1993; Pedersen et al., 1996). Like cultivation amplification of rDNA can distort the apparent structure of a community as a result of biases in cellular rDNA, DNA extraction (More et al., 1994; Pederson et al., 1996) and PCR amplification (Reysenbach et al., 1992; Suzuki and Govannon, 1996) all these methods provide meaningful comparisons of bacterial communities. The aim of the study was to identify the microbial community in the caecal content to improve animal husbandry and promote animal health.

MATERIALS AND METHODS

Chemical compounds

All the chemicals used in the present study were of molecular biology grade and were purchased from sigma (USA), Bangalore Genei (India), Himedia (India), Invitrogen (USA) and other reputed firms. Bacteriological media and supplements were obtained from Hi media (India).

ZR Soil Microbe DNA kit ™ was obtained from Zymo Research, Pure Link ™ Quick Gel Extraction Kit (*Invitrogen*, USA), Pure link ™ Quick plasmid Mini preparation kit (*Invitrogen*, USA), TOPO TA cloning ® kit for sequencing (*Invitrogen*, USA).

Collection of caecal content

Caecal content was collected from the caecum of broiler chicken in a sterile condition from slaughter house in Purasaivakam, Chennai. The broiler chicken was 90 days old, fed with poultry nutritional balancer and healthy.

DNA extraction

The DNA was extracted from the caecal microbial content using ZR soil Microbe DNA kit TM according to the manufacturer's instructions.

Polymerase chain reaction

Amplification of 16S rDNA sequence

The primers used for PCR amplification of the 16S rDNA sequences were 27f (5'-GTG CTG CAG AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-CAC GGA TCC TAC GGG TAC CTT GTT ACG ACT T-3') according to the method of Pham et al., 2002 with minor modifications.

The reaction mixtures were amplified in a Thermal cycler (Eppendorf, USA) by use of the following program: 95°C for 3 min, followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 90 s. The final extension Step was 72°C for 7 min.

The amplification of 16S rDNA sequence was also carried out using Primers P616V (5'-AGA GTT TGA T (CT) (AC) TGG CTC-3') and P630R (5'-CA (GT) AAA GGA GGT GAT CC-3') according to the method of Berezina et al. (2000). PCR carried out in a 25 µl reaction volume mix. Amplification was performed for 30 cycles at 94°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 44°C for 1 min, extension at 72°C for 1 min. The final extension step was 72°C for 10 min.

The amplification of 16S rDNA was carried out by another set of Primers 1Af (5' – TC (CT) G (GT) T TGA TCC (CT) G (CG) C (AG) G AG-3') and 1100 Ar (5'-TGG GTC TCG CTC GTT G-3') according to the method of Whitford et al. (2001). Amplification was performed for 30 cycles at 95°C for 40 s, 55°C for 1 min, 72°C for 2 min and a final extension step at 72°C for 3.5 min.

Detection

Electrophoresis was done using 2% agarose gel. First 2 gm of agarose was weighed and dissolved in 100 µl of 1x Tris-acetate-EDTA (TAE) buffer. It was then allowed to cool and 3 µl of ethidium bromide was added. This was then powered into casting tray containing a sample comb and was allowed to solidify at room temperature. 8 µl of amplified samples were loaded in to the wells. It was then run at 100 volts in a Submarine tank. Finally bands were visualized under UV.

Purification of the amplified 16S rDNA gene

Purification of the PCR product was carried out according to the manufacturer's protocol in pure link TM quick gel extraction kit.

Competent cell preparation (E. coli DH_5 α Cells)

A protocol according to Sambrook et al. (1989) was followed for the competent cell preparation. *E coli* were streaked on to LB agar plate from stock incubated at 37°C for overnight. Single colony was inoculated into 5 ml of LB broth and incubated at 37°C overnight with rotary shaking at the speed of 20 rpm. The OD at 600 nm was observed to arrest the growth by chilling on ice. The flask was chilled for 10 min. It was centrifuged at 5000 rpm for 10 min at 4°C, the supernatant was discarded. The tube was kept on ice and the pellet re-suspended in 30 ml of ice cold 50 mM calcium chloride. It was kept on ice for 25 min. It was centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet re-suspended in 2 ml ice cold 50 mM $CaCl_2$ containing 15% glycerol. The suspension in 100 µl volume was aliquot in cold micro centrifuge tubes and store at –70°C.

Cloning of 16S rDNA sequence

Purified DNA (amplified by 27 f and 1492 r Primers) was cloned in a pCR(4) TOPO Vector provided in the TA cloning kit (Invitrogen, USA). The purified DNA was approximately 1500 bp.

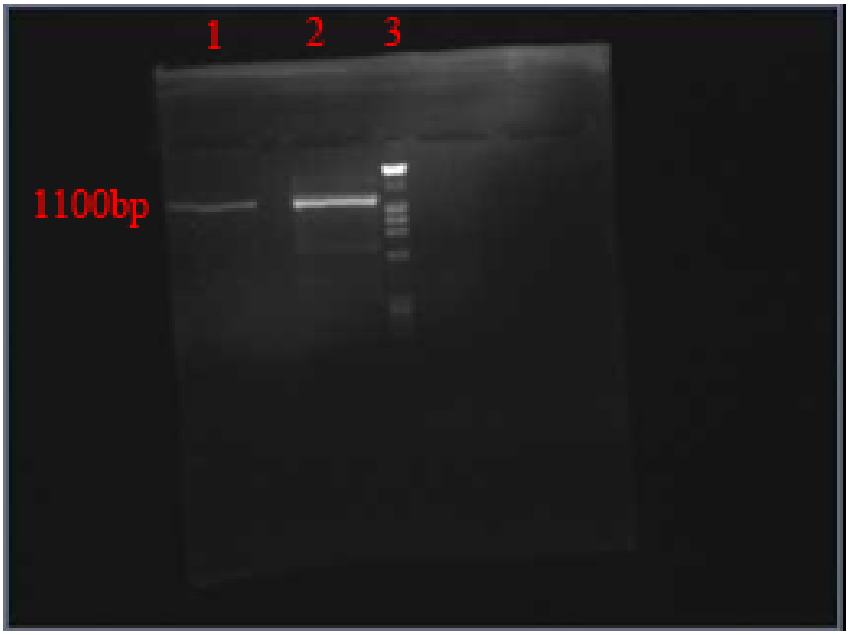

Figure 1. 16S rDNA sequence amplified by primers (1Af, 1100Ar). Lane 1- SAMPLE 1 DNA. Lane 2- SAMPLE 2 DNA. Lane 3- λ DNA – Hind III & ϕx174- Hae III Digest Marker. The amplified product size was 1100 bp.

Transformation

The recombinant plasmids were transformed in to the competent cells (Suau et al., 1999). The ligation mixture was added into the competent cells. It was kept on ice for 30 min. Then the mixture was kept in 42°C water bath for 1 to 2 min then kept on ice for 2 min. 1 ml of LB medium was added to the tubes and kept under 37°C for 1 h in orbital shaker. It was centrifuged at 6000 rpm for 10 min in room temperature. The supernatant was discarded and 500 µl of LB medium was added and it was mixed thoroughly with pellet. 250 µl of re-suspended pellet was taken and added into LB agar plate with 25 mg/ml of amplicillin and streaked with spreader kept overnight at 37°C.

Identification of the recombinants

Colony PCR (Mezei et al., 1994)

Individual colonies were picked and streaked onto a new ampicillin LB plate and the colonies were allowed to grow overnight. Colony PCR was performed by picking a few cells from the streaked plate with a sterile microtip and suspending the cells on to the PCR reaction mixture. The primers used for performing the PCR consisted of the M13 forward or reverse gene specific primers. The Amplification was performed for 30 cycles by use of the following program, initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final extension step was 72°C for 7 min.

Plasmid isolation from DH_5 α cells

Plasmid was isolated from DH_5 α Cells using pure link TM quick plasmid Mini preparation kit as per the manufacturer's instruction.

Sequencing

Plasmids isolated from five clones were sequenced in 3130 model genetic analyzer, (*Invitrogen*, USA).

Sequence analysis

The sequences were compared with the BLAST program available at the National Centre for Biotechnology Information (NCBI), (http/www.mbio.ncsu.edu/Bioedit/bioedit.html). Sequences were aligned using Clustal W and the sequence identities were calculated and the phylogenetic tree were constructed from the Clustal W aligned sequences.

RESULTS

Approximately 50 mg of caecal content was collected from the caecum of broiler chicken which is 90 days old. DNA was extracted directly from the caecal microbial content. The quality of the DNA obtained from the sample was assessed by Agarose gel electrophoresis. Specific PCR amplification of the DNA extracts with primers: 1Af and 1100Ar; 27f and 1492r; P616V and P630R was carried out. The product size of the 16S rDNA sequence amplified by primer 1Af and 1100Ar was found to be 1100 bp which was detected in Agarose gel eletrophoresis (Figure 1) and the product size of the 16S rDNA sequence amplified by primers 27f and 1492r; P616Vand P630R was found to be 1500 bp(Figure 2).

The 50 µl of PCR reaction mixture was run in agarose

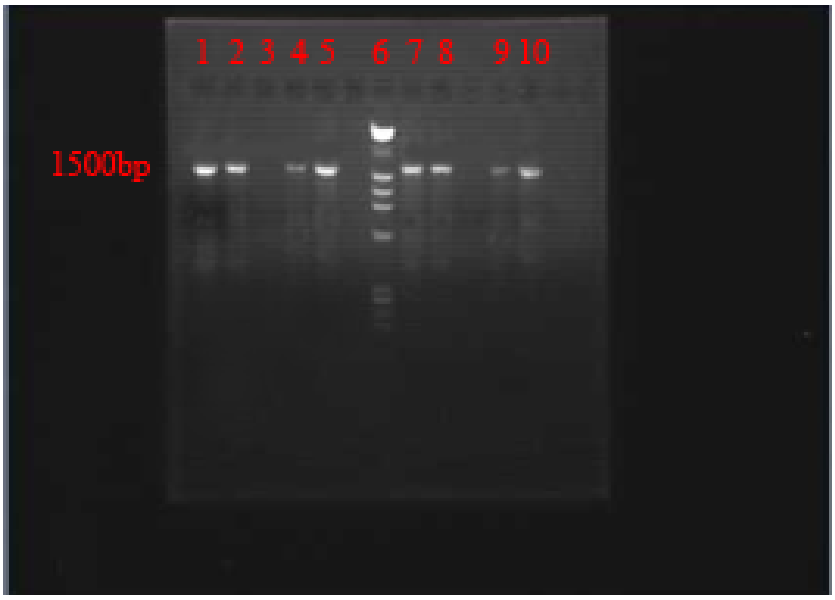

Figure 2. 16S rDNA sequence amplified by the primers (p616v, p630r) and (27f, 1492r). Lane 1-5- cecal microbial content DNA amplified by (p616V, p630R). Lane 6- λ DNA – Hind III & ϕx174- Hae III Digest Marker. Lane 7-10 - cecal microbial content DNA Amplified by (27F, 1492R).The amplified product size was 1500 bp.

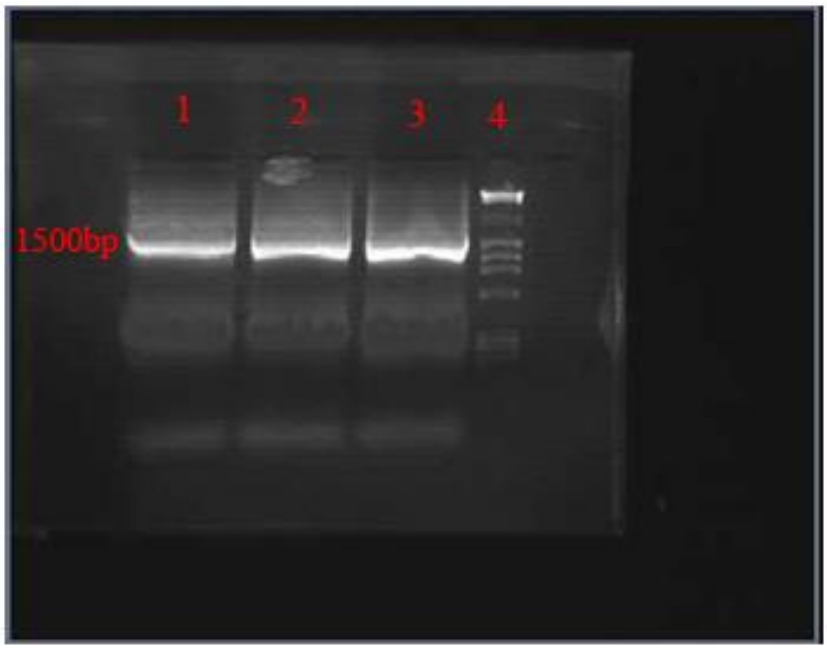

Figure 3. GEL ELUTED PCR product. Lane 1, 2, 3- Gel eluted PCR product, primer used (27F, 1492R). Lane 4- λ DNA – Hind III and ϕx174- Hae III Digest Marker.

gel electrophoresis (Figure 3.) and the product was purified and ligated into pCR(4) TOPO TA vector. The 16S rDNA sequence amplified by 27f and 1492r primer was purified and cloned into pCR(4) TOPO vector. Between 100 to 200 transformants were obtained (Figure 4).

The 50 µl of PCR reaction mixture was run in agarose gel electrophoresis and the product was purified and

Figure 4. Recombinant cells.

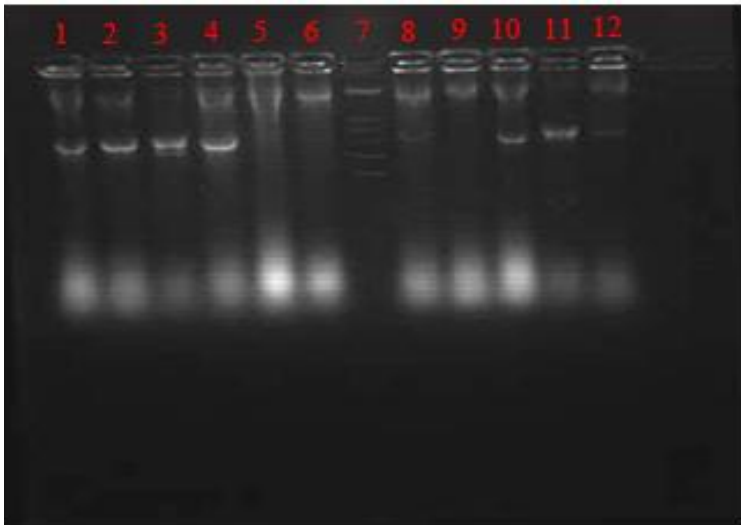

Figure 5. COLONY PCR. Lane 8- λ DNA – Hind III and ϕx174- Hae III Digest Marker. Lane 1, 2, 3, 4, 8, 10, 11, 12 - Positive colonies. Lane 6, 7, 9- Negative colonies.

ligated into pCR 4 TOPO TA vector.By colony PCR, the recombinants were identified, 95% of the colonies exhibited positive result (Figure 5). The plasmids were isolated from the clones and the size of the plasmid detected in agarose gel electrophoresis (AGE), which was found to be 5500 bp. The isolated plasmids was amplified by PCR using M13 forward and reverse primer; 27f and 1492r primer, the presence of target DNA in the plasmid were confirmed.

Identification of the recombinants

It was demonstrated that in BLAST search of five cloned sequences, three clones exhibited high degree of sequence similarity (95%) with the known organisms, one clone exhibited 93% sequence relatedness and another one exhibited 85% sequence similarity.

Multiple sequence alignment was done for the organisms which exhibits close sequence similarity in

BLAST search. From the aligned sequences, phylogenetic tree were constructed, from the tree it was concluded that *Bacillus* species strain XJRML- 1 and *Bacillus flexus* strain KSC- SF9C exhibit close similarity and comes under in same cluster. *Bacillus* spp and *Nanobacterium* spp shares the common evolutionary relationship, which is derived from the *S. maltophilia* species group. The *Bacillus* spp and the *Bacterium* strain 9-gwz3-7 exhibit less similarity.

Based on the similarity search and phylogenetic tree construction it has been identified that the bacterial community present in the caecal content of broiler chicken belongs to *Bacillus* spp group and it exhibits the evolutionary relationship with *Nanobacterium* spp, *Stenotrophomonas* spp and unculturable microorganism.

DISCUSSION

Studies on the analysis of microflora of the alimentary tract in normal chicken have shown that the caecum contains the largest number of bacteria (Timms, 1968). The composition of the gut microbiota remains steady in healthy chickens; but, it may be broken by marked changes in the animal itself, food products contaminated with pathogenic microorganisms or feed additives or by the contact with the pathogenic organisms present in the environment. Thus disturbances of the intestinal flora weaken the immunological resistance and could potentially lead to serious infections. For these reasons, detailed studies of intestinal microbiota are important for an improvement of animalization. Lactic acid bacteria originating from chicken intestine can be selected and used as probiotics for chicken with the aim of restoring the balance of intestinal flora to support digestion, to suppress pathogens such as *Salmonellae* and *Campylobacteria* colonizing in the chicken intestine (Stein et al., 1996) and to improve the immune system (Fuller,1989).

This study has demonstrated the microbial diversity and the presence of uncultured microorganisms in the caecal content of Broiler chicken. This work has shown that the broad-range of 16S rDNA PCR can detect and identify DNA from a wide range of organisms from caecal content and the valuable information about the microbial diversity and the phylogenetic relationship between the species. The BLAST search and phylogenetic analysis revealed the similarity and diversity of organisms. It is clear that the use of molecular biology techniques will lead to major advances in the description of gastro-intestinal ecosystems. The successful development and application of these methods, promises to provide the first opportunity to link distribution and identity of gastrointestinal microbes in their natural environment with their genetic potential and *in situ* activities. This will result in an increased understanding and a complete description of gastrointestinal community of production animals under different feeding regimes, and lead to new strategies for improving animal growth.

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REFERENCES

Berezina O, Zverlov VV, Rief S, Einspanier R, Schwarz WH (2000). Bacterial diversity in the rumen of cows fed transgenic maize. BMBF-Schwerpunct program. Technische Universitat Munchen.

Delong EF, Franks EG, Alldredge AL (1993). Phylogenetic diversity of aggregate attached Vs free-living marine bacterial assemblages. Limnol. Oceanogr., 38: 924-934.

Fuhrman JA, McCallum KM, Davis AA (1993). Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and pacific oceans. Appl. Environ. Microbiol., 59: 1294-1302.

Fuller R (1989). Probiotic in man and animals. J .Appl. Bacteriol., 66: 131-139.

Mezei CM, Storts DR (1994). Purification of PCR products. In: PCR Tech: Current Innovations, Griffin, I.G. and Griffin, A.M., Eds, CRC Press, Boca Ranton., FL. 21.

More MI, Herrick JB, Silva MC, Ghiorse WC, Madesh EL (1994). Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Alli. Environ. Microbiol., 60: 1572-1580.

Pace NR, Stahl DA, Olsen GJ, Lane DJ (1985). Analyzing natural microbial Populations by rRNA sequences. ASM News, 51: 4-12.

Pedersen K, Arlinger J, Ekendahl S, Hallbeck L (1996). 16S rRNA gene diversity of attached and unattached gound water bacteria along the access tunnel to the AspoHard Rock Laboratory, Sweden. FEMS Microbiol. Ecol., 19: 249-262.

Pham TNL, Hidenori H, Mitsuo S, Yoshimi B (2002). Phylogenetic Analysis of caecal Microbiota in Chicken by the use of 16S rDNA clone libraries. J. Microbiol. Immunol., 46(6): 371-382.

Reysenbach AL, Giver LJ, Wickham GS, Pace NR (1992). Differential amplification of rRNA genes by Polymerase chain reaction. Appl. Environ. Microbiol., 58: 3417-3418.

Roszak DB, Colwell RR (1987). Survival Strategies of bacteria in the natural environment. J. Microbiol., 51: 365-379.

Stein JL, Marsh TL, Wu KY, Shizuya H, Delong EF (1996). Characterization of uncultivated prokaryotes:Isolation and analysis of a 40-Kilobase-Pair genome fragment from a planktonic marine archaeon. J. Bacteriol., 178: 591-599.

Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Dore J (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. J. Appl. Environ. Microbiol., 65: 4799-4807.

Sullivan DJ (1999). Methods for analysis of the intestinal microflora. In Tannock, G.W.(ed), Probiotics: a critical review, Horizon scientific press. Wymondham. UK, 23: 44

Suzuki MT, Govannoni SJ (1996). Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ Microbiol., 62: 625-630.

Timms L (1968). Observations on the bacterial flora of the alimentary tract in three age groups of normal chickens. Br. Vet. J., 124: 470-477.

Whitford MF, Forster RF, Beard CE, Gong J, Teather RM (2001). Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe, 4: 153-163.