ISSN (Print) 0975-685X
ISSN (Online) 0975-864X

J. Appl. Biosci., 50(2): 160-164, December, 2024
doi: 10.61081/joab/50v2i112

ORIGINAL ARTICLE

Metagenomic of Gut Microbiota of Purple Moorhen (*Porphyrio poliocephalus*) from the Wetlands of Kerala: Implications for Avian Ecology and Crop Pest Management

Mani Chellappan[1], M.T. Ranjith[1], G.R. Bindu[1], P.D. Divya[1] and V. Choudhary[2]
[1]AINP on Vertebrate Pest Management, Department of Agricultural Entomology, College of Agriculture, Vellanikkara, KAU, Thrissur, Kerala, India-680 656
[2]Network Co-ordinating Centre, AINP on Vertebrate Pest Management, ICAR-Central Arid Zone Research Institute, Jodhpur, Rajasthan, India-342 003

ABSTRACT

Wetlands are critical ecosystems known for their high biodiversity, supporting complex interactions among various species, including plants, birds, fish, and mollusks. They provide essential resources and ecosystem services that benefit local communities. The kole lands in Kerala serve as vital habitats for both, migratory and resident birds, including the Purple moorhen (*Porphyrio poliocephalus*), which is crucial for maintaining ecological balance. Understanding the gut microbiome of these birds is essential for insights into their health, ecology, and behavior. While substantial research subsists on mammalian and avian microbiomes remain less explored, especially in non-domesticated species like the purple Moorhen, hence the present study exploits the metagenomic analysis of fecal pellets from Purple Moorhens to investigate the relationships between gut microbiota, avian health, and ecological roles. Fecal samples were collected, and metagenomic DNA was isolated and sequenced using next-generation Illumina sequencing. The analysis revealed a diverse bacterial community, with Proteobacteria as the dominant phylum followed by Firmicutes and Actinobacteria. The predominant bacterial species in the fecal pellet were *Pantoea* and *Microbacterium thalassium*, which play significant roles in digestion and pathogen resistance. The study helps to understand the avian microbiome and suggests the potential eco-friendly pest management practices, leveraging the natural behaviors and microbiota of the Purple moorhen to support sustainable agriculture.

KEY WORDS: Purple Moorhen, fecal pellet, Microbiota, 16sRrna gene, Metagenomics, *Porphyrio poliocephalus*

INTRODUCTION

Wetlands are renowned for having one of the richest biodiversity areas, characterized by intricate interactions among aquatic and semi-aquatic plants, water birds, fish, mollusks, and various other species. They also provide essential resources and ecosystem services directly or indirectly and support local communities (Pattanaik & Reddy 2007; Mahanti & Kumar 2016; Mahanti & Kumar 2017).Wetlands being a highly productive area attract various birdspecies. Kole lands serve as an ideal habitat for both migratory and local avian species and also provide resources essential for numerous avians, especially during their life cycle, such as breeding and migration. One of the most striking bird species inhabiting the wetlands of Kerala is the Purple moorhen (*Porphyrio poliocephalu*), which plays a crucial role in maintaining the ecological balance of these ecosystems. Studying wetlands and their biological diversity is necessary to comprehend the connection between ecological balance and sustainable development. The impact of the gut microbiome on the immune system (Brisbin *et al.,* 2008; Ruiz-Rodríguez *et al.,* 2009b; Yang *et al.,* 2012) digestion (Dewar *et al.,* 2013; Godoy-Vitorino; Ruiz-Rodríguez *et al.,* 2009a), growth (Barbosa *et al.,* 2016; Teyssier *et al.,* 2018; Torok *et al.,* 2011; Videvall *et al.,* 2019) and behavior (Cryan & Dinan, 2012) makes it crucial for host health. However, despite extensive research on the gut microbiome of mammals, the origins and effects of microbiome variation in birds remain less understood. The functional significance of avian

*Corresponding author email: ranjith.mt@kau.in

microbiome studies has long been recognized in the poultry industry (Oakley *et al.,* 2014), but there is increasing potential for avian microbiome research to contribute to the study of avian ecology, evolution, and conservation, studies. The intricate relationship between birds and their microbiota likely has profound implications across all aspects of avian biology (Hird, 2017; Trevelline *et al.,* 2019). Taking advantage of recent advancements, an increasing number of avian researchers are investigating the link between host life-history traits, ecology, and gut microbiota (Van Dongen *et al.,* 2013; Videvall *et al.,* 2019; Grond *et al.,* 2018; Kohl, 2012; Teyssier *et al.,* 2018; Trevelline *et al.,* 2019). Fecal sampling is commonly employed to study gut microbiota due to its non-invasive nature. However, the chemical composition of bird feces, where digestive excretions mix with urine products like uric acid, complicates DNA extraction and molecular analysis (Eriksson *et al.,*2017; Regnaut *et al.,*2006). Despite increased interest in avian microbiome research, there is limited understanding of the microbiota in non-domesticated, wetland-dwelling birds like the Grey-headed Swamphen (*P. poliocephalus*) and their role in supporting sustainable agricultural practices, such as natural pest control, remains largely unexplored. Hence, the present study, metagenomic analysis of fecal pellet (*P. poliocephalus*)bridges the gap in understanding the complex relationships between bird microbiome, their health, and ecological roles. This research also sets the foundation for future studies on the avian microbiome's role in ecological balance, evolution, and eco-friendly pest management practices.

MATERIALS AND METHODS

Field Collection of FaecalPellet Samples

The fecal pellet was collected from the fields of Pazhuvil, Thrissur district, and the samples were preserved in 70% ethanol and stored at -20ºC for further studies.

Isolation of DNA for Characterization of the Faecal Microbiota of Purple Moorhen

The isolation of metagenomic DNA from the faecal pellet was done to study the complex microbiome of purple moorhen in the Kerala wetlands. Zhou *et al*. (1996) developed a direct method for metagenomic DNA isolation which was modified and used to isolate metagenomic DNA from thefecal pellet. A 50 mg pellet weighted and homogenized in 400 µL of extraction buffer [200 mM Tris-HCl (pH-8.0), 25 mM EDTA (pH-8.0), 250 mM NaCl, SDS (0.5%)] in a 1.5 ml microcentrifuge tube and spun at 6,000 rpm for 10 to 15 min in a centrifuge (Eppendorf®). The homogenized sample was incubated at room temperature for 1-h. The supernatant was collected in a new microcentrifuge tube after centrifugation at 12,000 rpm for 5 minutes. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and centrifuged at 10,000 rpm for 20 min at 4°C. Later, the aqueous phase was pipetted out into a fresh microcentrifuge tube and was incubated with 1-µL of proteinase k and 0.5 µL RNase at 37°C for 30and 15 minutes, respectively. The mixture was then incubated at 65°C for 15 minutes to degrade the enzymes.To that, an equal volume of chloroform: isoamyl alcohol (24:1) was added to the mixture and centrifuged at 10,000 rpm for 10 min at 4°C. Again the aqueous phase was pipetted out into a fresh microcentrifuge tube. An equal volume of isopropanol was added to the mixture and was incubated at room temperature for 15 min, centrifuged at 13,000 rpm for 5 min at room temperature and the metagenomic DNA pellet was precipitated out. The DNA pellet was washed with ethanol (95%) by centrifugation at 10,000 rpm for 10 min. The DNA pellet was air-dried, dissolved in 25 µL of sterile distilled water, and stored in a deep freezer (-80°C) for future use.

Amplification of 16 S rRNA Genes

The 16S rRNA fragment from the fecal pellet DNA of the purple moorhen was amplified through polymerase chain reaction (PCR) using universal 16S rRNA primers (F-5' GAGTTTGATCCTGGCTCAG-3', R-5' ACGGCTACCTTGTTACGACTT-3') in a Thermal Cycler (Applied Biosystems®). The PCR mixture included 1-µL of template DNA, 0.6 µL each of forward and reverse primers, 10 µL of EmeraldAmp® GT PCR Master Mix, and 7.8 µL of Millipore® water. The PCR conditions were performed with a lid temperature of 105°C, initial denaturation at 95°C for 1-minute, followed by 35 cycles of denaturation at 95°C for 15 seconds, primer annealing at 49°C for 15 seconds, and primer extension at 72°C for 30 seconds. A final extension step at 72°C for 7 minutes was followed by storage at 4°C. The amplified products were separated using 1.2% agarose gel along with a suitable ladderand visualized with the Invitrogen E-Gel® Imager, E-Gel Imager software, and Gel Quant Express software (Thermo Fisher Scientific).

16S ribosomal RNA Amplicon Sequencing using Next Generation Illumina MiSeq

The 16S V3-V4 gene regions were amplified using specific primers. Each PCR reaction was performed in a 50 µL volume, containing 25 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.4 µM of both forward and reverse primers, and approximately 20 ng of template DNA. The thermal cycling conditions began with an initial denaturation at 98°C for 1-minute, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 60

seconds, with a final extension step at 72°C for 5 minutes.

The PCR products were electrophoresed on a 1.2% agarose gel. The samples with one bright main strip between 300 and 600bp were taken for library preparation.

Library Preparation and Sequencing

Following the manufacturer's instructions, sequencing libraries were created using the NEB Next® UltraTM DNA Library Prep Kit for Illumina (NEB, USA), and index codes were added. The library quality was assessed on the Qubit@ 4.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina Mi Seq™ sequencer.

Analysis of NGS Data

Quality parameters such as base quality parameters, base composition distribution, and GC distribution were evaluated on all raw sequencing reads acquired from the sequencer. MG-RAST programme was used for downstream analysis. For each OTU, representative sequences were found and compared to the Greengenes core set of sequences.

RESULTS AND DISCUSSION

Metagenomic DNA was isolated from the fecal pellet of purple moorhen and confirmed the presence of 16S rRNA fragments in the isolated product by amplification with universal 16S rRNA primers.When the product was resolved on a 1.2% agarose gel, a distinct band of 400-500 bp was observed. The metagenomic DNA was quantified using a JENWAY® Genova Nano Micro-volume Life Science Spectrophotometer. The hypervariable (V3-V4) regions of the 16S rRNA were amplified using specific primers, and 16S rRNA library preparation.

The next generation Illumina sequencing of 16S rRNA region revealed the bacterial communities associated with the fecal pellet of purple moorhen. A paired-end sequence from V3 metagenomics typically includes conserved region, spacer, and V3 region. The initial step involves removing the spacer and conserved regions from the paired-end reads. After trimming these sequences from the original data, a consensus V3 region sequence is created using the Clustal Omega program. Various filters, such as conserved region, spacer, and mismatch filters, are applied to retain only high-quality V3 region sequences for downstream analyses, which resulted in 97062 sequences with GC content of 53.16%. Rarefaction analysis was also carried out to determine the bacterial diversity and revealed the highest number of bacterial diversity present in the purple moorhen (47.21) and theIllumina sequencing data have been submitted to the Sequence Read Archive with the SRA number SRR16146720.

Composition of Bacteria Present in the Fecal Pellet of Purple Moorhen

The analysis revealed the composition of bacteria present in the fecal pellet of purple moorhen and grouped them into each taxonomic category from phyla to species level. The abundance of 10 major bacterial groups in each taxonomic category is presented in Table 1. A total of 12 bacterial phyla were recorded in the fecal pellet of purple moorhen. Among them, Proteobacteria was the most dominant phyla (23%)followed by Firmicutes (14%), and Actinobacteria (3%). The other phyla recorded in the sample include Chloroflexi, Flavobacteria, Nitrospira,

Table 1: Abundance of top 10 bacterial communities with taxonomic categories from phyla to species level

Phylum	Class	Order	Family	Genus	Species
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	*Pantoea* sp. P0356
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	*Clostridium disporicum*
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	*M. thalassium*
Chloroflexi	Chloroflexi	Chloroflexales	Chloroflexaceae	Chloroflexus	*C. aurantiacus*
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	*C. soldanellicola*
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Thermodesulfovibrio	*T. Islandicus*
Deinococcus thermos	Deinococci	Thermales	Thermaceae	Thermus	*T. scotoductus*
Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Pirellula	*P. staleyi*
Chlamydiae	Chlamydiae	Chlamydiales	Parachlamydiaceae	Parachlamydia	*Parachlamydia acanthamoebae*
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	*Mycoplasma caviae*

Deinococci, Planctomycetacia, Chlamydiae, and Mollicutes. Altogether 32 bacterial classes were identified in the sample, among them, Gammaproteobacteria was the most dominant class followed by Clostridia and Actinobacteria.The sequence analysis at the order and family level revealed 39 and 135 bacterial orders and families, respectively. The dominant families include Enterobacteriaceae, Clostridiaceae, Microbacteriaceae, Chloroflexaceae, Flavobacteriaceae, Nitrospiraceae, Thermaceae, Planctomycetaceae, Parachlamydiaceae, and Mycoplasmataceae. A total of 532 bacterial species were recorded in the fecal pellet of purple moorhen across 248 bacterial genera. Among them, *Pantoea* was the most dominant genus followed by *Clostridium, Microbacterium, Chloroflexus, Chryseobacterium, Thermodesulfovibrio, Thermus, Pirellula, Parachlamydia,* and *Mycoplasma.*

The predominant bacterial species recorded in the fecal pellet of purple moorhen was *Pantoea* sp. Similarly, Gibbs *et al.* (2007) also reported *Pantoea* in the feces of yellow-headed blackbirds (*Xanthocephalus xanthocephalus*). *Pantoea* acts as an antimicrobial agent and has also been developed as a commercial biocontrol agent to control insect pests (Johnson *et al.,*1993, 2000; Johnson & Stock Well, 1998), it also has bioremediation potential, with the ability to degrade herbicides (Pileggi *et al.,*2012), and thus it helps to maintain the health of purple moorhen by preventing pathogens. *M. thalassium* was another dominant bacterial species present in the fecal pellet of purple moorhen. *M. thalassium* is the commonly known bacterial symbiont in mangroves, degraded litter,and breakdown of complex carbohydrates (Andreote *et al.,* 2012), which serves a role in the bird's diet to breakdown complex organic compounds.

Another major bacterial symbiont present in the fecal pellet of purple moorhen was *Chloroflexus aurantiacus*, also known as green non-sulfur bacterium or green gliding bacterium. This bacterium could break down organic material in the food (Bryant & Frigaard, 2006). Thus it plays essential roles in digestion, immunity, and overall health. *Chrysobacterium soldanellicola* was also recorded in the fecal pellet of purple moorhen, Charimba, (2012) also reported this bacteria in the gut of birds. *Chryseobacterium* has shown antagonistic properties against plant pathogens (Mwanza *et al.,* 2022) suggesting that it might protect birds from pathogens. *Thermodesulfovibrio islandicus* and *Thermus scotoductus* were also identified in the sample. These bacteria were not yet reported in the feces and gut of the birds earlier. They are thermophilic and are known for their ability to survive in high-temperature environments (Wilpiszeski *et al.,* 2018). Thus they might play a role in tolerating high temperature conditions to purple moorhen. *Pirellula staleyi* was also identified in the sample and is known to be involved in the degradation of complex organic compounds (Corsaro & Greub, 2006). Thus it plays essential roles in digestion, immunity, and overall health. Most bacterial species present in the fecal pellet of purple moorhen were not yet reported earlier in the gut or feces of birds. Thus, the study of the Metagenomic analysis of fecal microbiota in Purple moorhen (*P. poliocephalus*) from Kerala wetlands provides valuable insights into avian ecology and crop pest management by understanding the microbial communities within the purple moorhen. It also highlights the potential for using natural biological indicators, such as the purple moorhen, to develop eco-friendly pest control methods by leveraging the purple moorhen's natural behaviors and microbiome, which reduces the reliance on chemical pesticides and promotes a more sustainable approach to agriculture.

REFERENCES

Andreote, F.D., Jiménez, D.J., Chaves, D., Dias, A.C.F., Luvizotto, D.M., Dini-Andreote, F., Fasanella, C.C., Lopez, M.V., Baena, S., Taketani, R.G., & De Melo, I.S. (2012). The microbiome of Brazilian mangrove sediments as revealed by metagenomics. *PLoS One,* 7(6): e38600. https://doi.org/10.1371/journal.pone.0038600

Barbosa, A., Balagué, V., Valera, F., Martínez, A., Benzal, J., Motas, M., Diaz, J.I., Mira, A., & Pedrós-Alió, C. (2016). Age-related differences in the gastrointestinal microbiota of chinstrap penguins (Pygoscelis antarctica). *PLOS ONE,* 11(1): e0153215. https://doi.org/10.1371/journal.pone.0153215

Brisbin, J.T., Gong, J., & Sharif, S. (2008). Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Ani. Heal. Res. Rev.,* 9(1): 101-110. https://doi.org/10.1017/S146625230800145X

Bryant, D.A., & Frigaard, N.U. (2006). Prokaryotic photosynthesis and phototrophy illuminated. *Trends in Microbiol.,* 14(11): 488-496.

Charimba, G. (2012). The taxonomy and significance of Chryseobacterium isolates from poultry (Doctoral dissertation, University of the Free State).

Corsaro, D., & Greub, G. (2006). Pathogenic Potential of Novel Chlamydiae and Diagnostic Approaches to Infections Due to These Obligate Intracellular Bacteria. *Clin. Microbiol. Rev.,* 19(2): 283-297. https://doi.org/10.1128/CMR.19.2.283-297.2006

Cryan, J.F., & Dinan, T.G. (2012). Mind-altering microorganisms: The impact of the gut microbiota on brain and behavior. *Nat. Rev. Neurosci.*, 13(10): 701-712. https://doi.org/10.1038/nrn3346

Dewar, M.L., Arnould, J.P.Y., Dann, P., Trathan, P., Groscolas, R., & Smith, S. (2013). Interspecific variations in the gastrointestinal microbiota in penguins. *Microbiol. Open,* 2(1): 195-204. https://doi.org/10.1002/mbo3.57

Eriksson, P., Mourkas, E., González-Acuna, D., Olsen, B., & Ellström, P. (2017). Evaluation and optimization of microbial DNA extraction from fecal samples of wild Antarctic bird species. *Infec. Ecol. Epidemiol.,* 7: Article 1386536. https://doi.org/10.1080/20008686.2017.1386536

Gibbs, P.S., Kasa, R., Newbrey, J.L., Petermann, S.R., Wooley, R.E., Vinson, H.M., & Reed, W. (2007). Identification, antimicrobial resistance profiles, and virulence of members from the family Enterobacteriaceae from the feces of yellow-headed blackbirds (*Xanthocephalus xanthocephalus*) in North Dakota. *Avian Dis.,* 51(3): 649-655.

Godoy-Vitorino,F., Goldfarb, K.C., Brodie, E.L., Garcia-Amado, M. A., Michelangeli, F., & Dominguez-Bello, M.G. (2010). Developmental microbial ecology of the crop of the folivorous hoatzin (*Opisthocomus hoazin*). *The ISME J.,* 4(5): 611-620. https://doi.org/10.1038/ismej.2010.9

Grond, K., Sandercock, B.K., Jumpponen, A., & Zeglin, L.H. (2018). The avian gut microbiota: community, physiology and function in wild birds. *J. Avian Biol.,* 49: e01788. https://doi.org/10.1111/jav.01788

Hird, S.M. (2017). Evolutionary biology needs wild microbiomes. *Front. Microbiol.,* 8: 1-10. https://doi.org/10.3389/fmicb.2017.00725

Johnson, K.B., Stockwell, V.O., & Sawyer, T.L. (1993). Mechanisms of antagonism of the fire blight pathogen *Erwinia amylovora* by the bacterial biocontrol agents *Pseudomonas fluorescens* and Pantoea agglomerans. *Phytopathol.,* 83(9): 982-990. https://doi.org/10.1094/Phyto-83-982

Johnson, K.B., & Stockwell, V.O. (1998). Biological control of fire blight: Understanding mechanisms and improving efficacy. *Phytopathol.,* 88(6): 475-479. https://doi.org/10.1094/PHYTO.1998.88.6.475

Johnson, K.B., Temple, T.N., & Stockwell, V.O. (2000). Preliminary results on the interaction of biological agents with oxytetracycline in the management of fire blight. *Acta Horticul.*, 551: 159-165.

Kohl, K.D. (2012). Diversity and function of the avian gut microbiota. *J. Avian Biol.*, 43: 154-162. https://doi.org/10.1111/j.1600-048X.2012.05578.x

Mwanza, E.P., Hugo, A., Charimba, G., & Hugo, C.J. (2022). Pathogenic potential and control of *Chryseobacterium* species from clinical, fish, food, and environmental sources. *Microorgan.,* 10(5): 895. https://doi.org/10.3390/microorganisms10050895

Oakley, B.B., Lillehoj, H.S., Kogut, M.H., Kim, W.K., Maurer, J.J., Pedroso, A., Lee, M.D., Collett, S.R., Johnson, T.J., & Cox, N.A. (2014). The poultry gut microbiome and its relationship to health and disease. *Front. Veterin. Sci.,* 1: 10. https://doi.org/10.3389/fvets.2014.00010

Pileggi, S.A.V., Pileggi, M., Olchanheski, L.R., Pileggi, M.E.V., & de Souza, M.M. (2012). Biodegradation of herbicides 2,4-D and picloram by *Pantoea ananatis* strain MMB-1. *Brazilian J. Microbiol.,* 43(1): 177-183. https://doi.org/10.1590/S1517-83822012000100020

Regnaut, S., Lucas, F.S., & Fumagalli, L. (2006). DNA degradation in avian faecal samples and feasibility of non-invasive genetic studies of threatened capercaillie populations. *Conserv. Genet.,* 7(3): 449-453. https://doi.org/10.1007/s10592-005-9046-6

Ruiz-Rodríguez, M., Lucas, S., Heeb, P., De Neve, L., Martý, J.G., & Soler, M. (2009a). Bacterial diversity at the cloaca relates to an immune response in magpie Pica pica and to the body condition of great spotted cuckoo *Clamator glandarius* nestlings. *J. Avian Biol.,* 40(4): 452-460. https://doi.org/10.1111/j.1600-048X.2009.04800.x

Ruiz-Rodríguez, M., Lucas, S., Heeb, P., & Soler, J.J. (2009b). Body condition and immune response of great spotted cuckoo and magpie host nestlings. *J. Avian Biol.,* 40(3): 306-311. https://doi.org/10.1111/j.1600-048X.2008.04623.x

Teyssier, A., Rouffaer, L.O., Saleh Hudin, N.S., Strubbe, D., Matthysen, E., Lens, L., & White, J. (2018). Inside the guts of the city: Urban-induced alterations of the gut microbiota in a wild passerine. *Scient. Rep.,* 8(1): 11777. https://doi.org/10.1038/s41598-018-29988-9

Teyssier, A., Tolsma, S., Katsis, A.C., Holveck, M.J., & Dechaume-Moncharmont, F.X. (2018). Changes in gut microbiota do not correspond to variation in mating behavior in an avian mutualist. *Microb. Ecol.,* 76(3): 709-720. https://doi.org/10.1007/s00248-018-1178-8

Torok, V.A., Allison, G.E., Percy, N.J., Ophel-Keller, K., & Hughes, R.J. (2011). Influence of antimicrobial feed additives on broiler commensal post hatch gut microbiota development and performance. *Appl. Environ. Microbiol.,* 77(10): 3380-3390. https://doi.org/10.1128/AEM.02727-10

Trevelline, B.K., Fontaine, S.S., Hartup, B.K., & Kohl, K.D. (2019). Conservation biology needs a microbial renaissance: A call for the consideration of host-associated microbiota in wildlife management practices. Proceedings of the Royal Society B: Biological Sciences, 286: Article 20182448. https://doi.org/10.1098/rspb.2018.2448

Van Dongen, W.F., White, J., Brandl, H.B., Moodley, Y., Merkling, T., Leclaire, S., Blanchard, P., Danchin, É., Hatch, S.A., & Wagner, R.H. (2013). Age-related differences in the cloacal microbiota of a wild bird species. *BMC Ecol.,* 13: 11. https://doi.org/10.1186/1472-6785-13-11

Videvall, E., Song, S.J., Bensch, H.M., Strandh, M., Engelbrecht, A., Serfontein, N., Hellgren, O., Olivier, A., Cloete, S., Knight, R., & Cornwallis, C.K. (2019). Major shifts in gut microbiota during development and its relationship to growth in ostriches. *Mole. Ecol.*, 28(12): 2656-2672. https://doi.org/10.1111/mec.15087

Wilpiszeski, R.L., Zhang, Z., & House, C.H. (2018). Biogeography of thermophiles and predominance of *Thermus scotoductus* in domestic water heaters. *Extremo.,* 23(1): 119-132. https://doi.org/10.1007/s00792-018-1066-z

Yang, C.M., Cao, G.T., Ferket, P.R., Liu, T.T., Zhou, L., Zhang, L., & Xiao, Y.P. (2012). Effects of probiotic, *Clostridium butyricum*, on growth performance, immune function, and cecal microflora in broiler chickens. *Poul. Sci.,* 91(9): 2121-2129. https://doi.org/10.3382/ps.2012-02131