UNIVERSITI PUTRA MALAYSIA

INTESTINAL NORMAL FLORA OF SELECTED WATERBIRDS IN PUTRAJAYA WETLAND PARK

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INTESTINAL NORMAL FLORA OF SELECTED WATERBIRDS IN PUTRAJAYA WETLAND PARK

NUR ABIR BINTI DZULKIFLI

Dissertation submitted in partial fulfilment of the requirement for the course

VPD4999 - Final Year Project in the Department of Veterinary Clinical Studies

Universiti Putra Malaysia

OCTOBER 2022

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CERTIFICATION

It is hereby certified that I/we* have read this project paper entitled "Intestinal Normal Flora of Selected Waterbirds in Putrajaya Wetland Park" by Nur Abir binti Dzulkifli, and in my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 - Project.

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ACKNOWLEDGEMENT

First and foremost, all praises to God, the Almighty, who has given me innumerable blessings, knowledge, and opportunities so I can finally accomplish this thesis.

I would like to express my deepest gratitude to my supervisor, Professor Dr. Zunita Zakaria, Director of Institute of Bioscience. I am genuinely thankful for her feedback, patience and enthusiasm throughout all the matters. I also could not undertake this journey without my co-supervisor, Professor Dr. Jalila Abu for the generous support and for sharing her knowledge, particularly during the sample collection intended for this thesis.

I would be remiss in acknowledging all of the bacteriology staff that has guided me on laboratory procedures and protocol and assisted me throughout my project. My completion of this project could not have been accompanied without the support of my bacteriology mates, and cohort members; Husna, Ravenia, Maria, Khaiyal and Amir for giving a helping hand. I almost shed a tear when I thought I could not make it on time. I am genuinely grateful for their constant support and help.

Finally, I'm incredibly grateful to my caring mother and sisters for their unwavering love and support. Their belief and valuable prayers for me have carried me this far.

Thank you, all.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek

FLORA NORMAL USUS BURUNG AIR TERPILIH DI TAMAN WETLAND PUTRAJAYA

Oleh

NUR ABIR BINTI DZULKIFLI

2022

Penyelia: Profesor Dr. Zunita Zakaria

Penyelia bersama: Profesor Dr. Jalila Abu

Sistem gastrousus haiwan merangkumi flora normal usus yang pelbagai yang mengekalkan keseimbangan dinamik dengan perumah melalui simbiosis mutualisme dan evolusi bersama. Dalam beberapa dekad yang lalu, terdapat minat yang semakin meningkat dalam komuniti saintifik terhadap flora normal usus burung. Walau bagaimanapun, kebanyakan penyelidikan didominasi oleh ayam domestik, yang tidak mungkin mewakili semua spesies burung dengan fisiologi, tingkah laku dan keutamaan alam sekitar yang berbeza-beza. Oleh itu, kajian ini bertujuan untuk menentukan mikrobiota usus dalam burung air terpilih di Taman Wetland Putrajaya. Tiga puluh (30) ekor burung air terpilih telah digunakan untuk menilai flora normal dalam sistem gastrousus. Swab kloaka diperoleh daripada dua spesies burung air, Burung Angsa Mesir (*Alopochen aegyptiaca*) (n=15) dan Itik Domestik (*Anas platyrhynchos*

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domesticus) (n=15), yang kelihatan sihat dengan pelbagai umur, berat dan jantina. Sampel kemudiannya tertakluk kepada pengasingan dan pengenalpastian bakteria menggunakan kaedah konvensional standard. Dua belas spesies bakteria telah diasingkan dan dikenal pasti, yang terdiri daripada *Escherichia coli* (80%), *Staphylococcus* sp. (77%), *Klebsiella pneumoniae* (43%), *Stenotrophomonas maltophilia* (33%), *Aeromonas* sp. (23%), *Plesiomonas shigelloides* (17%), *Pseudomonas aeruginosa* (10%), *Pantoea agglomerans* (10%), *Salmonella* sp. (<3%), *Acinetobacter lwofii* (<3%), *Hafnia alvei* (<3%), dan *Bacillus* sp. (<3%). Kajian mendedahkan bahawa kebanyakan bakteria yang telah dikenalpasti ini ini adalah mikrobiom usus normal burung air, yang boleh menjadi oportunistik, serta mikroorganisma yang berpotensi patogen. Kesimpulannya, kajian ini menjadi asas untuk penyelidikan masa depan mengenai flora usus normal dan potensi zoonosis jangkitan bakteria burung air, yang akhirnya menyumbang kepada strategi pengurusan dan pemuliharaan masa depan di Taman Wetland Putrajaya**.**

Kata Kunci: simbiosis mutualisme, mikrobiota usus, mikrobiom usus, Burung Angsa Mesir (*Alopochen aegyptiaca*), Itik Domestik (*Anas platyrhynchos domesticus*).

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999- Project.

INTESTINAL NORMAL FLORA OF SELECTED WATERBIRDS IN PUTRAJAYA WETLAND PARK

by

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2022

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The animal gastrointestinal system encompasses diverse intestinal normal flora that maintains a dynamic equilibrium with the host through mutual symbiosis and co-evolution. In the last few decades, there has been a growing interest within the scientific community in the avian gut normal flora. However, most research is dominated by domestic poultry, which is unlikely to represent all bird species with varying physiology, behavior, and environmental preferences. Therefore, this study aimed to determine the gut microbiota in selected waterbirds in Putrajaya Wetland Park. Thirty (30) waterbirds were selected to assess the normal flora in the gastrointestinal system. Cloacal swabs were obtained from two waterbird species, Egyptian Goose (*Alopochen aegyptiaca*) (n=15) and Domestic Ducks (*Anas platyrhynchos domesticus*) (n=15), that appear healthy with various ages, weights, and sex. The samples were then subjected to bacterial isolation and identification using standard conventional methods. Twelve species of bacteria were isolated and identified, which comprised of *Escherichia coli* (80%), *Staphylococcus* sp. (77%)*, Klebsiella pneumoniae* (43%), *Stenotrophomonas maltophilia* (33%), *Aeromonas* sp. (23%), *Plesiomonas shigelloides* (17%), *Pseudomonas aeruginosa* (10%), *Pantoea agglomerans* (10%), *Salmonella* sp*. (<3%), Acinetobacter lwofii (<3%)*, *Hafnia alvei (<3%)*, and *Bacillus* sp. (<3%). The study revealed that most of these isolated bacteria are the normal gut microbiomes of waterbirds, which can be opportunistic, as well as potentially pathogenic microorganisms. In conclusion, this study serves as a basis for future research on the normal intestinal flora and zoonotic potential of bacterial infections of waterbirds, which eventually contributes to future management and conservation strategies in Putrajaya Wetland Park.

Keywords: mutual symbiosis, gut microbiota, gut microbiome, Egyptian Goose (*Alopochen aegyptiaca*), Domestic Ducks (*Anas platyrhynchos domesticus*)

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1.0 INTRODUCTION

Wetlands in Malaysia are blessed with a diverse and numerous ranges of life-sustaining ecosystems. One of the common species that can be found in wetland are waterbirds, a group of birds that spend most of their lifetime in aquatic ecosystems (Rahman & Ismail, 2018). Waterbird populations tend to be highest in wetlands with the most diverse vegetation or those with persistent sources of water. Each water bird adapted based on feeding habits, food resources, defence against predators and severe weather with the anatomical adaptation such as various long beak shapes and webbed toes. Examples of waterbird species present in wetlands of Malaysia are Egret, Heron, swamphen, moorhen, watercock, sandpipers, lapwings, and kingfishers (Baset et al., 2018).

Normal flora or microbiome is a microorganism that resides in animals without causing disease. Typically, normal flora is stable with different genera inhabit in different parts of the body. On a few occasions, microorganisms in normal flora may harm the host causing infection. Infections can occur from immunosuppression due to poor nutrition, an unhygienic environment and stress, allowing more growth of pathogenic bacteria (Sarker et al., 2013).

Wild bird, especially migratory species are prone to become reservoir, mechanical vector or both of zoonotic pathogens, such as enteric pathogens *E. coli* and *Salmonella spp, Yersinia spp., Campylobacter jejuni, Vibrio spp*, influenza A viruses, and West Nile virus (Konicek et al., 2016).

Two types of waterbirds in Putrajaya wetlands park are selected for this study which are the Egyptian Goose *(Alopochen aegyptiaca)* and Domestic Duck (*Anas* *platyrhynchos domesticus*). At present, information on intestinal aerobic normal flora in waterbirds is limited and most of the studies in Malaysia are related to poultry. Few articles on waterbirds in Malaysia mostly focused on obtaining species diversity and distribution using inventory and checklist activity, and lack of information relating to the species biology and ecology (Rahman et al,. 2018). In the recent years, there has been a growing popularity of outdoor leisure activities and has resulted in increased connection with waterbird colonies. Hence, the objectives of this study were:

1. to isolate and identify the gut aerobic microbiome in selected waterbirds in Putrajaya Wetland Parks.

2. to identify gut microbiome that may harbour possible zoonotic diseases transmitted from waterbird to human.

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2.0 LITERATURE REVIEW

2.1 Waterbird

Waterbird is made of a family of *Anatidae* that consists of swan, goose, and duck. The family has wide distribution all around the world with only exception in Antarctica. There are approximately 878 waterbird species representing 33 families that are ecologically dependent on wetland habitats, 815 of which are found in Asia *(Delany & Scott 2006).* George and Zack (2001), explained that waterbirds utilised different types of wetland habitats for their natural need in terms of shelter, foraging, breeding and rearing purposes. Habitat selection also takes into account the availability of prey, characteristics of the beak and foraging behaviour.

Egyptian goose is one of the waterbirds that are taxonomically placed in the subfamily Tadorninae, the shelduck-sheldgoose subfamily of Anatidae. From its morphology this family is intermediate between geese and dabbling duck, and from molecular data, the subfamily of Tadorninae evolved closer to duck-like morphology. In the genus of *Alopochen*, this genus consisted of Egyptian geese and two other extinct species, and the scientific name of Egyptian goose is Alopochen *aegyptiaca*.

Egyptian Goose is a species of goose originating from Egypt and reared in ancient Egypt for ornamental purposes in temples and palaces. Now, the Egyptian goose is not widely in Egypt, but common in the rest of Africa and introduced to Europe due to its capability to withstand hot and cold climate conditions. According to new research from Hohmann and Woog, (2021), Egyptian geese are not migratory birds. In terms of behaviour, Egyptian geese are well known for their aggressive behaviour toward their

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territories especially during breeding seasons. Egyptian goose is aggressive to their own species and other birds, by the means of chasing away and competing for nests. The Egyptian Goose has been classified as a non-native invasive species by the European Union (EU) because its aggressive behaviour and competitive abilities appear to pose a threat to native species (European Commission, 2017).

Duck is the common name for several species of waterfowl in the Anatidae family. Several domestic ducks (*Anas platyrhynchos domesticus*) that commercially used such as Rouen, Ancona, Call, Indian Runner, Khaki Campbell and Muscovy duck (Babington & Campbell, 2022). Domestic ducks can be found in wetland and agricultural areas around the world. Wild ducks are the ancestors of domestic ducks where the earliest record took place around 1 000–2 500 years ago in China, and once domesticated, domestic ducks had modifications to their size, behaviour, and coat of plumage. (Museum, 1979). Domestic ducks are known as the most economically significant species of waterfowl, which are also a major source of high-quality protein for many people (Huang et al., 2013).

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2.2 Wetland area in Malaysia

Wetlands is defined as an area of transitional regions between the aquatic and terrestrial zones with highly productive ecosystems with a varied flora and fauna such as mammals, reptiles, birds, sea creatures and amphibians (Martins et al,. 2017). Wetlands are a dynamic and transitional ecosystem environment that is affected by natural disturbance, distinct from several other habitats. In the last century, half of the world's wetlands have been destroyed as a result of redirecting and dampening of stream streams, conversion of ditches, swamps, lakes, and floodplains into farming fields and aquaculture lakes, eutrophication, contamination of water from agricultural fields and ventures (Berry et at,. 2008, as cited in Onwuka et al,. 2017). At the moment, there are 91 wetlands in Malaysia, 55 in peninsular Malaysia and 18 each in Sabah and Sarawak (Rahman & Ismail, 2018).

Putrajaya Wetland Park (Figure 1) is a man-made marsh and lake located about 26 km south of Kuala Lumpur, with coordinates of 2° 57’ 43” latitude and 101° 41’ 47” longitude (Zakaria & Rajpar, 2013). The land size covers around 200 ha, where 77.70 ha are covered with planted area, 76.80 ha open water bodies, 9.60 ha islands, 23.70 ha inundation area and 9.40 ha tracks and consisted of five arms; upper west arm, upper north arm, upper east arm, lower east arm and upper bisa (Sim et al. 2008). A large proportion of the wetland catchment is obtained from cultivation of palm oil, rubber plantation and developments surrounding Putrajaya. Concurrently, collects outstream sources primarily from Chua River, Bisa River and three tributaries into Putrajaya Lake.

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Figure 1: Map of Putrajaya Lake and wetlands

The architecture of Putrajaya Wetland Park is based on imitating natural wetland that act by cleansing contaminated water in the existing landscape, where the process includes a sequence of biochemical mechanisms of cleansing and filtration, hydrological function as flood control, ecological, erosion, and coastal protection, with social relevance as ecotourism, research, and recreational purpose. The significance of Putrajaya Wetland Park is treating urban waste through a natural filtration system by intercepting flow of water, trapping particulates and pollutants, removing toxic materials, and absorbing energy and nutrition from the upstream catchment region (Putrajaya Wetlands, 1999).

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2.3 Normal flora

Normal flora is defined as a microorganism that resides and colonizes inside or on the surface of a complex living organism without harming the host. The microorganism comprised not only bacteria but also fungus, viruses, and protozoans (Serikov et al,. 2010, as cited in Jandhyala, 2015). Benskin et al., (2009) noted that the gut microbiome is regarded as commensal, some are able to alter physiology and few could lead to pathogenic conditions and zoonotic transmitted disease. Several important contributions of gut microbiome, as stated in Grond et al., (2018) are facilitate breakdown and metabolism of nutrient, protection against pathogen through competitive exclusion, play role in immune function, and maintain health through sustaining structural integrity of the gut mucosal barrier.

Understanding of the intestinal microbiome of free-living birds, especially waterbirds, are still scarce for large amounts of studies focused on mammals, where publications are outnumbered by 10:1. However, over the last few decades, there has been a growing interest in avian gut microbial populations within the scientific community (Grond et al., 2018).

Most of the study related intestinal normal flora in birds and reptiles are non-invasive and collected from cloacal swabs. Cloacal swab is chosen as a method of sampling for its repeatability, directness, and ability to collect individual samples in one cycle. This clearly has the upper hand from faecal collection as it may be inaccurate from contamination where it is difficult to identify faeces ownership to and waiting time of defecation (Videvall et al,.2017).

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Bodawatta et al., (2022) stated that interaction between avian and microbes are formed by the host immune system, physiology of the gut, as well the development and formation of bacterial symbionts in the gut. In terms of behaviour and ecological factors, variation of gut normal flora can be due to vertical microbial transmission (parent-to-infant), diet, and socialisation. Environmental changes can affect gut microbiomes, both natural and manmade, including parasite infestations, and disease status of the host. Videvall et al,.(2017) also acknowledges that the intestinal microbiome has been linked to disease resistance, behaviour, mating selection, lifespan, and adaptation through a number of ecological and evolutionary mechanisms.

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2.4 Common gut aerobic bacteria and zoonotic potentials

Several studies have been conducted on waterbirds in China (Yang et al, 2018,2020), the research revealed that both geese and ducks exhibit similar major phyla of gut microbiome. The main phyla of bacteria that inhabit the gastrointestinal tract are Proteobacteria, Firmicutes, Bacteroidetes, Cyanobacteria and Actinobacteria.

Sarker et al,. (2012) identified gut normal flora of waterbirds in Bangladesh with a total of 72 samples from a cloacal swab, oral swab and faecal sample were taken. There are five types of bacteria isolated in the study and bacteria that had the highest rate of isolation are *E. coli* by 54.16%. The second highest isolate is *Salmonella sp*. which is around 31%, and the rest listed by order of isolates are *Staphylococcus spp., Bacillus spp*. and *Proteus spp.*

In another study, Stenkat et al., (2014) examined six species of free-living birds by isolation and biochemical identification. From the study it is emphasised that the prevalence of microbiota of healthy birds is mainly affected by the environment and issue in the digestive systems. The writer found 7 family of culturable aerobic bacteria from cloacal swab of free-living birds, which are Enterobacteriaceae, Pseudomonadaceae, Aeromonadacae, Bacillaceae, Staphylococcaceae, Moraxellaceae and Flavobacteriaceae.

According to Sun et al,. (2022), birds are important indicator animals in several ecosystems due to their high spatial dispersion capacity and massive distribution area. Waterbirds, in particular, have been often used as bio-indicators to highlight concerns and other dangers that may have an impact on wetland settings. These responses are essential indications of pollution and a reduction in the quality of the ecosystem. Additional feeding from visitors draws big flocks of ducks and geese and encourages their reliance on leftovers, which do not supply adequate nourishment. However, when humans discard uneaten food and leftovers like bread and chips, the rotting food can contaminate water and trigger bacterial diseases in animals.

Elberg et al,. (2017) noted that there is a risk of transmission of pathogenic disease and outbreaks resulting from waterbirds to the human population. Moreover, there are more herbivorous waterfowl than ever before near to humans, animals, and poultry where this arises recurring issue of waterfowl to be source of infection. Waterbirds can act as biological vectors and mechanical vectors to pathogens with zoonotic potentials. They may be able to carry pathogens that are actively multiplying and carrying infectious disease or becoming fomite, bringing the pathogen physically from one site to another.

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One of the potential gastrointestinal bacterial diseases that can be found in geese and swans is *Salmonella. Salmonella* is globally distributed and consists of various serovars, some may cause alimentary disease in animals, in this case bird and human via faecal-oral route (Benskin et al., 2009). *Escherichia coli* is known as normal flora in the gut where they are usually non-pathogenic and harmless to the host. Nevertheless, some strains of e coli can be pathogenic and resistant to numerous antibiotics, and waterbirds may be vulnerable to *E coli* as well as becoming vectors of dispersions of pathogenic virulence (Nowaczek et al., 2021).

In addition, *Vibrio cholerae* is a pathogenic bacteria known to infect humans and animals and commonly found in aquatic environments, where the pathogenic strain produces toxins that lead to fatality in humans. According to Buck JD, (1990), cited by Elberg et al,. (2017), *V. cholerae* has been found in fresh faeces of Canada geese, Mute swans, and other aquatic birds in coastal parts of the United States. Other notable gastrointestinal pathogens that may be transmitted in wild birds are *Pasteurella multocida* or fowl cholera in poultry but it has not proven that it causes risk to humans or livestock.

Elberg et al,.(2017) also mentioned that *Campylobacter* are frequently found in the intestinal tract in wild birds which include waterbirds. *Campylobacter jejuni* as well as *Campylobacter coli* has been reported in humans of gastroenteritis in all parts of the world. The association of the infection is from contaminated water or meat and other meat products. Other species of *Campylobacter* have been isolated in faecal and cloacal swabs of healthy geese, yet the role of waterbirds for zoonotic disease is unclear.

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Yersinia is also one of the pathogens being gastrointestinal issues. A study by Niskanen et al,. (2003), conducted in Sweden has highlighted that *Yersinia* have been found in more than 12% of migratory birds. However, many of these belonged to non-pathogenic *Yersinia* sp. or *Y. enterocolitica* strains.

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3.0 MATERIALS AND METHODS

3.1 Sampling

Samplings were carried out at Putrajaya Wetland Parks. Two (2) types of waterbirds, Egyptian Geese *(Alopochen aegyptiaca)* (n=15) and Domestic Duck (*Anas platyrhynchos domesticus*) (n=15), apparently healthy with various age, weight and gender were identified and selected.

Figure 2: Egyptian goose (Alopochen aegyptiaca)

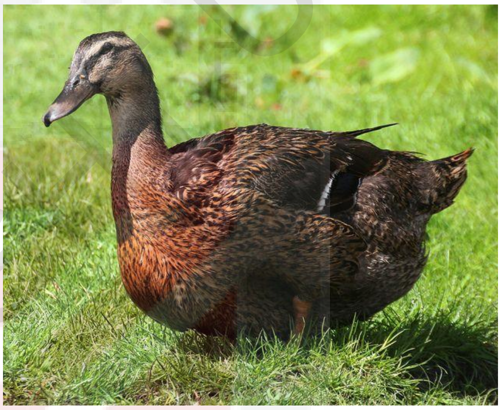

Figure 3: Domestic Duck (Anas platyrhynchos domesticus)

The birds were captured using a net and restrained with their wings. A cloacal swab was taken by inserting a sterile cotton swab gently into the vent and the swab was rubbed and rotated onto the mucosal layer. The swabs were withdrawn aseptically and the faeces must be obtained on the swab. Two cloacal swabs were taken from each bird, one (swab 1) for bacterial isolation and identification; and another (swab 2) for detection of *Salmonella* sp.

Ethical approval to conduct study on live waterbirds was approved by Institutional Animal Care and Use Committee (IACUC), with IACUC approval No: UPM/IACUC/AUP-U010/2022.

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3.2 Transportation of samples

The samples were directly placed into a transport media and labelled according to its species, sample number and duplicate number. Next, the samples were kept chilled in a Styrofoam ice box and transported to Bacteriology Laboratory, Faculty of Veterinary Medicine, University Putra Malaysia for further processing.

3.3 Isolation of bacteria

The two swabs from each sampled bird were processed separately. Swab 1 was streaked on MacConkey agar and blood agar for primary culture, incubated at 37°C for 24 hours. Next, different bacteria colony morphology will be selected and subcultured into nutrient agar at 37°C for another 24 hours to obtain pure culture.

Swab 2 was inoculated in buffered peptone water broth (BPW), pre-enrichment media for *Salmonella* sp., and incubated at 37°C for 24 hours under aerobic conditions. Then, 1 ml of BPW were transferred into Rappaport-Vassiliadis (RV) broth, and enriched media for cultivation of *Salmonella* spp. One loopful of RV broth was then cultured onto Xylose Lysine Deoxycholate (XLD) agar and Brilliant Green Agar (BGA) agar and incubated at 37°C for 24 hours. If there is no growth in the colony, the incubation will be extended for another 24 hours.

3.4 Identification of bacteria

Colonies that were present on Blood agar, MacConkey agar, XLD agar and BGA agar were observed for their morphology and were subjected for gram staining. Further identification of the obtained bacteria were done using biochemical tests based on gram staining results, such as oxidase test, coagulase test, Triple Sugar Iron (TSI) test,

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Sulphur, Indole, Motility (SIM) test, urease test, citrate test, slide agglutination test, and other relevant tests. The cultures that displayed typical *Salmonella* spp. reactions and presented *Salmonella* in biochemical tests were therefore subjected to slide agglutination test (SAT) using Salmonella polyvalent ‘O’ antisera A-S. The procedure and identification were as in the *A Diagnostic Manual of Veterinary Clinical Bacteriology and Mycology* by Jang et al. (2008).

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4.0 RESULTS

A total of 12 different species of aerobic bacteria were isolated and identified from the 30 examined waterbirds. Eight types bacteria were successfully recognised to species level, while another 4 types (*Staphylococcus* sp*., Aeromonas* sp., *Salmonella* sp*.,* and *Bacillus* sp.) were identified to genus. The mean number of isolations from each Egyptian goose was 2.67, while for the domestic duck the mean of isolation was 3.47. The list of isolated bacteria species and frequency are presented in Table 1. The bacteria species were placed in order of frequency, and the most frequent isolates were first-named.

Table 1: The list of isolated bacteria species and frequency

Bacteria Species	**Frequency**		
	Egyptian goose *(Alopochen aegyptiaca)* (n=15)	Domestic duck *(Anas platyrhynchos)* (n=15)	Total in waterbird
Escherichia coli	11	13	24
Staphylococcus sp.	11	12	23
Klebsiella pneumoniae	8	5	13
Stenotrophomonas maltophilia	1	9	10
Aeromonas sp.	3	4	7
Plesiomonas shigelloides	0	5	5
Pseudomonas aeruginosa	3	0	3
Pantoea agglomerans	0	3	3
Salmonella sp.	0	1	1
Acinetobacter lwoffii	1	0	1
Hafnia alvei	1	0	1
Bacillus sp.	1	0	1

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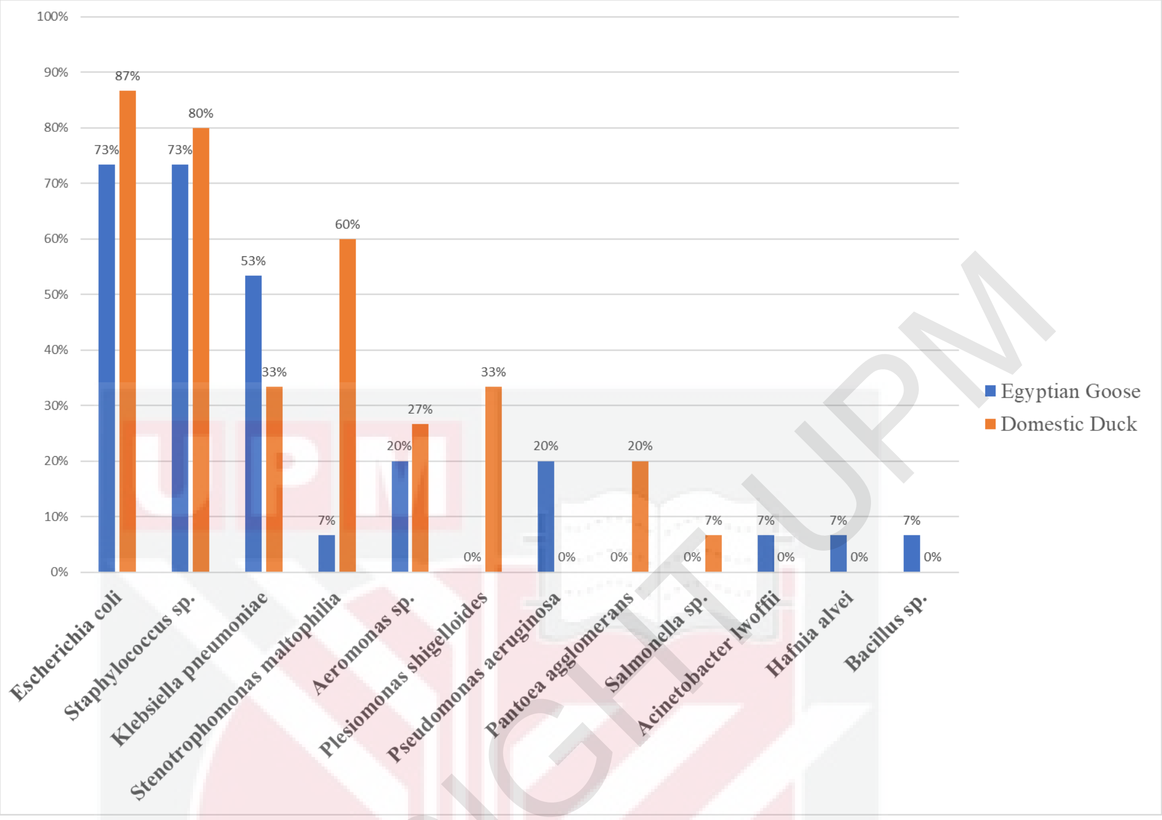

Figure 4: Percentages of isolated bacteria between waterbird species

The percentages of each bacterial species among two selected waterbirds are illustrated in Figure 4. Most bacteria isolates are gram-negative bacteria wih the *Escherichia coli* and *Staphylococcus* sp. being the most prominent bacteria species in Egyptian goose and are *Klebsiella pneumoniae* and *Stenotrophomonas maltophilia* in domestic ducks. *Aeromonas sp.* occurrences in Egyptian goose and domestic duck were 20% and 27%, respectively. *Plesiomonas shigelloides* was only present in domestic ducks by around 33%. Meanwhile, *Pseudomonas aeruginosa* was isolated in 20% of Egyptian geese, while Pantoea *agglomerans* presented in 20% of domestic ducks.

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On the other hand, species of bacteria that have low occurrence are *Salmonella* sp. in domestic duck; as well as *Acinetobacter lwoffii, Hafnia alvei*, and *Bacillus* sp. in Egyptian goose.

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5.0 DISCUSSION

A total of 12 species of aerobic bacteria were found to be normal gut flora of 30 selected waterbirds in Putrajaya wetland parks. Ten out of twelve of the isolated bacteria under phylum Proteobacteria, while the other two, *Bacillus* sp. and *Staphylococcus* sp. are phylum Firmicutes. Proteobacteria that are made of gram-negative bacteria, are expected to be abundant in gut microbiomes of waterbirds, as birds carry a higher proportion of Proteobacteria in GI tract, compared to mammals. While for Firmicutes, it is one of the common phyla that can be found in GI tract, predominantly consisting of gram-positive bacteria. All bacteria present are common bacteria found in soil and water, and are expected to be present as normal flora in animals living in wetland habitats. In addition, most bacteria were considered normal gut flora of waterbirds, which can also be opportunistic.

Escherichia coli are bacteria that are commonly obtained and regarded as normal flora in mammals, humans, and birds (Elberg et al., 2016). *Escherichia coli* was one of the predominant bacteria in this study, with a frequency of 80% of the total waterbird samples. There was a report of the presence of multidrug-resistant *E. coli* bacteria in wild waterfowl where these hosts have been identified as reservoirs of avian pathogenic *E. coli* strains (APEC) and pathogenic human *E. coli* strain serotype H7:O1572. (Nowaczek et al., 2021). Extra-intestinal illnesses mostly from systemic diseases or infections of the respiratory tract are known to be brought on by avian pathogenic *E. coli* (Dho-Moulin & Fairbrother, 1999).

Staphylococcus, the second highest isolates in this study by a frequency of 80% of the total waterbird samples, is a gram-positive bacterium usually inhabiting the cutaneous and mucosal microbiota of animals and humans, can act as opportunistic

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pathogens, particularly in an immunosuppressive host (Stepan et al., 2004). Once the bacterium is inside the host, the bacteria colonize many organs that may cause internal lesions, endocarditis, arthritis, osteomyelitis, and acute septicaemia. On exogenous infection, dermatitis and bumblefoot are common complications (Morishita, 2022).

Klebsiella pneumoniae is a nonmotile Enterobacteriaceae that acts as an opportunistic pathogen to an immunosuppressive host, found in Egyptian goose and domestic ducks, 53% and 33%, respectively. Gibbs et al. (2007) noted that *Klebsiella pneumoniae* could be isolated from faeces samples and the oropharynx of clinically healthy parrots and passerines.

Stenotrophomonas maltophilia, found in 60% of domestic ducks, is a waterborne organism that commonly appears in water sources, notably rivers, sewage, and lakes, including soil and plants. It's also found in animals, mainly aquatic species. A study in South America by Fernández-Zenoff et al. (2015) showed that *Stenotrophomonas* was found in most lakes, water, and excrement of flamingos in lakes near the Andes mountains. This bacterium is an emerging worldwide multidrug-resistant opportunistic pathogen with an increased incidence of respiratory issues in hospital-acquired infections (Brooke, 2012).

Aeromonas sp. is predominant in water bodies and birds from aquatic ecosystems and is considered a potentially pathogenic bacterium. For example, A. hydrophila was identified from a diarrhea outbreak and a chicken's mortality and is regarded as a cause of enteritis in poultry (Disouky et al., 2022). In previous work by Laviad-Shitrit et al. (2019), *Aeromonas* were isolated in three wild waterbird species; the writer believed that Aeromonas transmission might be attributed to the bird's migration.

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Another family of Enterobacteriaceae that was once considered the family of Vibrionaceae, *Plesiomonas shigelloides*, has recovered 33% in domestic ducks. *P. shigelloides* primary environment is freshwater and seawater, mainly in tropical or subtropical climates, and its waterfowl recorded with the bacterium are cormorants, goldeneyes, gulls, herons, penguins, storks, and swans. *P. shigelloides* is a rare case as it is gaining recognition as a source of gastrointestinal illness linked to traveller's diarrhea and intake of polluted water, raw or undercooked fish, and shellfish. Plesiomonads have been found in many animals, including those that were healthy, ill, or under autopsy. Current research indicates that Plesiomonads can trigger an outbreak or sporadic stress-related infections (Janda et al., 2016).

Pseudomonas aeruginosa, found in 20% of Egyptian geese, is regarded as an opportunistic bacteria and common avian pathogen. This bacterium usually settles in the upper respiratory tract and causes issues in ducks as septicaemia, respiratory symptoms, lameness, conjunctivitis, and diarrhea. According to a study by M.S. et al. (2021), since cloacal swabs had the most significant incidence of isolation, faeces were thought to be the most harmful source of *P. aeruginosa* transmission inside duck farms. *Pseudomonas aeruginosa* also appears in aquatic environments and intestines of apparently healthy grey herons (Fudge, 2001).

Pantoea agglomerans is a family of Enterobacteriaceae commonly found in plant materials and environments. The presence of this bacterium in birds is poorly understood; nevertheless, *Pantoea* strains were linked to terrestrial and aquatic species. There was a report where *P. agglomerans* were isolated from a cloacal swab in an adult grey-beast parakeet (Beleza et al., 2021). In addition, *P. agglomerans* was recovered in

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all free-living bird samples; black cormorants, water rails, spotted crakes, barn swallows, mute swans, and reed warblers (Stenkat et al., 2014).

Salmonella is a foodborne pathogenic bacteria distributed worldwide known to cause gastroenteritis in humans and animals, including birds. Salmonella species have been discovered in both healthy and ill wild birds. This pathogen is generally known for severe sickness and death from septicaemia in little passerines, whereas many other species, such as gulls, carry asymptomatic infection. The presence of infected gulls and passerines has been proposed as a risk factor for salmonellosis in domestic animals. However, wild birds are not considered the primary cause of livestock infection; feed contamination and recycling among farm animals are familiar sources (Elmberg et al., 2017). *Salmonella enterica* serotype *Typhimurium* is the serotype most usually linked with wild birds. (Benskin et al., 2009). Birds infected with *Salmonella* will show symptoms such as lethargy, fluffed-up plumage, and an inclination to stay near feeding sites; despite these apparent swallowing problems, they feed until shortly before death (Kirkwood et al., 1995).

Acinetobacter lwofii only recovered one isolate, around 7% in Egyptian geese. *Acinetobacter lwofii* is an opportunistic organism that predominates in the environment and human skin (Yang, 2014). The bacterium is one of the most common *Acinetobacter* species found in commercially significant spoilage foods such as poultry, eggs, and fish, even stored under refrigeration or after irradiation treatment (Doughari et al., 2011). Until recently, no study has proven that A. lwofii recovered in a cloacal swab of birds.

Another family of Enterobacteriaceae, *Hafnia alvei*, was only detected for 7% of Egyptian geese. *H. alvei* can be found in natural settings, including sewage, water, and

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soil. Given its widespread occurrence in nature, it is a common resident of faeces and digestive contents of birds, mammals, reptiles, fish, and insects. However, there has been a report by Simpraga et al. (2005) where co-infection of *Hafnia alvei* and *Salmonella typhimurium* were isolated from various organs in necropsy and found to cause myocardial and renal degeneration, splenomegaly, liver dystrophy with white yellow foci, destruction of pancreatic tissue and catarrhal enteritis. (Padilla et al., 2014).

Identification of Bacillus in birds usually occurs until genus due to the difficulty in differentiation. *Bacillus* sp. was regularly obtained from normal and healthy birds; thus, they are considered normal host flora. Additionally, bacillus modifies the gut microbiota by promoting probiotic bacteria, lowers nutritional competition between microorganisms and the host, and promotes GI health (Grant et al., 2018).

There are several factors that accounted for the component of gut microbiota obtained from this study. Cloacal swabs were less precise at acquiring gut microbiota. The most accurate method to evaluate occurrence of gut microflora is via intestinal necropsy which is destructive and not practical for conservation of birds (Kohl et al., 2016). Multiple study from has proved that other non-invasive technique, faecal sample has better representation of colon microflora than cloacal swab (Videvall et al,.2018;Yan et al., 2019), but this method still not able represent the bacterial communities in the ileum and caecum. In this study, cloacal swab was conducted due to lack of time to wait for fresh faeces, higher risk of contamination and difficulty to identify faeces ownership.

The composition of avian gut microflora is affected by intrinsic and extrinsic factors, discussed by Sun et al, (2022) and Grond et al., (2018). The intrinsic factor of the bird's role in intestinal normal flora such as genetic composition. It is derived from

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evolution between host-microbe and vertical transmission, so even while living in the same or comparable environment, various species' gut microbiota might differ dramatically. For example, both avian residues in Delaware Bay, United States, red knot and ruddy turnstone showed different specific gut microflora components (Grond et al., 2014). Another intrinsic factor are ages that contributing to the striking variations between individuals for adult having more complex and stable bacterial diversity, as well as variation of sex but even so, studies were still limited (Sun et al., 2022).

Next, extrinsic factors also affect gut microflora in birds. Differences in diets of each species of bird lead to significant changes in the microbial diversity and richness of the host gut, and this resulted in colonisation of new gut flora with permanent change of diet (Gubert et al., 2020). From this study, the waterbird lives in a quarantine area and feeds with commercial feed and roughage. Captive waterbirds live a very different lifestyle than their free-living species. This resulted in permanent changes in the diet that eventually may induce the colonization of new gut microbes.

Moreover, gut normal flora in birds are strongly associated with environmental features rather than genetic factors (Grond et al., 2018). Changes of habitat, climate, and environmental biota have a significant impact on the host's gut flora. Distinct hosts could mutually transmit intestinal microbes in a shared environment through physical contact, air, water, soil, food, or other media surround them. Therefore, the different species of bird could easily transmit their gut microbes to other organisms in a shared environment, so there would be similarities in gut microbial components of waterbirds species.

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In addition to extensive factors, behavioural habits of birds alter composition of normal flora. There are differences of diversity of gut microbiome in migratory and resident or non-migratory birds. For this study, both species of waterbird are non-migratory. Resident birds have more microbiome community for its foraging behaviour, while migratory birds acquire fewer microbial diversity for only utilising one habitat and area (Bisson et al., 2009).

The last external factor is social contact that can facilitate the interchange of microflora between individuals of the same species and those of other species. In animals such as birds, reptiles and amphibians, cloaca is a single opening for the digestive tract, reproductive tract and urinary tract. Gut microbiome was directly transferred during mating. For instance, during copulation many bird species share cloacal germs as well as sperm in a process known as a "cloacal kiss" (White et al., 2010). The process of mating touches their cloacae together, alternately allowing transmission of gut microbes of their partners. Parental care does have a considerable influence on the gut microbial community of their offspring. Notably, parental saliva also plays an essential role during feeding, transmitting microbiota to the chick.

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6.0 CONCLUSION

In total, 12 aerobic bacteria were successfully isolated from cloacal swabs of selected waterbirds, consisting of *Escherichia coli, Staphylococcus* sp.*, Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Aeromonas* sp., *Plesiomonas shigelloides*, *Pseudomonas aeruginosa*, *Pantoea agglomerans*, *Salmonella* sp*., Acinetobacter lwofii*, *Hafnia alvei*, and *Bacillus* sp. The bacteria isolates are considered as gut normal flora of birds, however they are also able to pose threat to immunosuppressive hosts. Two pathogenic bacteria with zoonotic potential were identified, *Escherichia coli* and *Salmonella* sp. Factors that may affect composition of avian gut microflora are, genetic composition, age and sex, diet, environment, behavioural habit and social contact.

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7.0 RECOMMENDATION

For future studies, it is recommended to increase sample size so that the identified microorganism can be the actual representative of the composition of gut microbiome of waterbirds. Use of multiple sampling methods in birds, such as combination of oral swab, cloacal swab and faecal sample for better understanding of the diversity of avian normal flora, as well as to include their local environment in a study. For better profiling and identification of bacteria, molecular methods of PCR or high-throughput sequencing can be used.

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REFERENCES

Benskin, C. M. H., Wilson, K., Jones, K., & Hartley, I. R. (2009). Bacterial pathogens in wild birds: a review of the frequency and effects of infection. *Biological Reviews*, *84*(3), 349–373.

Beleza, A. J. F., Maciel, W. C., Carreira, A. S., Marques, A. R., Nunes, F. P., Raso, T. F., Vasconcelos, R. H., & Teixeira, R. S. (2021). Antimicrobial susceptibility profile of enterobacteria isolated from wild grey-breasted parakeets (Pyrrhura griseipectus). *Pesquisa Veterinária Brasileira*, *41*.

Bisson, I.-A., Marra, P. P., Burtt, E. H. Jr., Sikaroodi, M., and Gillevet, P. M. (2009). Variation in Plumage Microbiota Depends on Season and Migration. *Microb. Ecol*. 58, 212–220.

Brooke, J. S. (2012). Stenotrophomonas maltophilia: an Emerging Global Opportunistic Pathogen. *Clinical Microbiology Reviews*, *25*(1), 2–41.

Doughari, H. J., Ndakidemi, P. A., Human, I. S., & Benade, S. (2011). The Ecology, Biology and Pathogenesis of Acinetobacter spp.: An Overview. *Microbes and Environments*, *26*(2), 101–112.

Fernández-Zenoff, M. V., Dib, J. R., Neumann, A., Estévez, M. C., & Farías, M. E. (2015). Water and Flamingo Feces Bacterial Communities from High- Altitude Andean Lakes under Selective Antibiotic Pressure Studied by PCR-DGGE Analyses. *Antimicrobial Resistance - an Open Challenge*.

Fudge, A. M. (2001). Diagnosis and treatment of avian bacterial disease. Seminars in Avian and Exotic Pet Medicine, 10(1), 3–11.

Grant, A., Gay, C. G., & Lillehoj, H. S. (2018). *Bacillus sp.* as direct-fed microbial antibiotic alternatives to enhance growth, immunity, and gut health in poultry. *Avian Pathology*, *47*(4), 339–351.

Grond K., Ryu H., Baker A. J., Domingo J. W. S., Buehler D. M. (2014). Gastro-intestinal microbiota of two migratory shorebird species during spring migration staging in Delaware Bay USA. J. Ornithol. 155 969–977.

Grond, K., Sandercock, B. K., Jumpponen, A., & Zeglin, L. H. (2018). The avian gut microbiota: community, physiology and function in wild birds. *Journal of Avian Biology*, *49*(11), e01788.

Gubert, C., Kong, G., Renoir, T., & Hannan, A. J. (2020b). Exercise, diet and stress as modulators of gut microbiota: Implications for neurodegenerative diseases. *Neurobiology of Disease*, *134*, 104621.

Huang, Y., Li, Y., Burt, D. W., Chen, H., Zhang, Y., Qian, W., Kim, H., Gan, S., Zhao, Y., Li, J., Yi, K., Feng, H., Zhu, P., Li, B., Liu, Q., Fairley, S., Magor, K. E., Du, Z., Hu, X., . . . Li, N. (2013). The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nature Genetics*, *45*(7), 776–783.

©COPYRIGHT UPM

Janda, J. M., Abbott, S. L., & McIver, C. J. (2016). Plesiomonas shigelloides Revisited. *Clinical Microbiology Reviews*, *29*(2), 349–374.

Jandhyala, S. M. (2015). Role of the normal gut microbiota. *World Journal of Gastroenterology*, *21*(29), 8787.

Laviad-Shitrit, S., Izhaki, I., Lalzar, M., & Halpern, M. (2019). Comparative Analysis of Intestine Microbiota of Four Wild Waterbird Species. *Frontiers in Microbiology*, 10.

Marchant, J. (2022). Alopochen aegyptiaca (Egyptian goose). *CABI Compendium*, *CABI Compendium*. https://doi.org/10.1079/cabicompendium.94205

Martins, C.O., Rajpar, M,N., Nurhidayu, S., Zakaria, M. (2017). Habitat Selection of Dendrocygna javanica in Heterogeneous Lakes of Malaysia*. J Biodivers Manage Forestry 6:3*

Morishita, T. Y. (2022). Streptococcosis in Poultry. MSD Veterinary Manual. Retrieved October 6, 2022, from https://www.msdvetmanual.com/poultry/streptococcosis/streptococcosis-in-poultry.

M.S., A., A.Salim, A., & M.F., E. D. (2021). Virulence Of Isolated Pseudomonas Aeruginosa Infecting Duckling And Antibiotic Resistance With An Experimental Treatment Trial. *Assiut Veterinary Medical Journal*, *67*(169), 74–90

Museum, Z. C. (1979), The excavation of the mound tomb at Guoyuan of Fushan, Jurong County, Jiangsu Province. *Kaogu.* 1979(2):113.

Niskanen, T., Waldenström, J., Fredriksson-Ahomaa, M., Olsen, B., & Korkeala, H. (2003). *virF* -Positive *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* Found in Migratory Birds in Sweden. *Applied and Environmental Microbiology*, *69*(8), 4670–4675.

Onwuka, Martins & Zakaria, Mohamed & Ismail, Mohd Hasmadi & Nurhidayu, Siti & Olaniyi, Oluwatobi. (2019). Population and Occupancy Estimates of Avian Species in Paya Indah and Putrajaya Wetlands, Peninsular Malaysia. 8. 1 - 9.

Padilla, D., Acosta, F., Ramos-Vivas, J., Grasso, V., Bravo, J., El Aamri, F., & Real, F. (2014). The pathogen Hafnia alvei in veterinary medicine: a review. *Journal of Applied Animal Research*, 43(2), 231–235.

Rieke Hohmann, & Friederike Woog. (2021). How aggressive are Egyptian Geese Alopochen aegyptiaca? Interactions with Greylag Geese Anser anser and other birds in an urban environment. *Wildfowl, 71(71), 234–243.*

Sun, F., Chen, J., Liu, K., Tang, M., & Yang, Y. (2022). The avian gut microbiota: Diversity, influencing factors, and future directions. *Frontiers in Microbiology*, *13*.

Stenkat, J., Krautwald-Junghanns, M. E., Schmitz Ornés, A., Eilers, A., & Schmidt, V. (2014). Aerobic cloacal and pharyngeal bacterial flora in six species of free-living birds. *Journal of Applied Microbiology*, 117(6), 1564–1571.

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Stepan, J., Pantucek, R. and Doskar, J. (2004) Molecular diagnostics of clinically important Staphylococci. *Folia Microbiol,* 49, 353–386.

Videvall, E., Strandh, M., Engelbrecht, A., Cloete, S., & Cornwallis, C. K. (2017). Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. *Molecular Ecology Resources,* 18(3), 424–434.

White, J., Mirleau, P., Danchin, E., Mulard, H., Hatch, S. A., Heeb, P., & Wagner, R. H. (2010). Sexually transmitted bacteria affect female cloacal assemblages in a wild bird. *Ecology Letters*, 13, 1515–1524.

Yang, H., Xiao, Y., Gui, G., Li, J., Wang, J., & Li, D. (2018). Microbial community and short-chain fatty acid profile in gastrointestinal tract of goose. *Poultry Science*, *97*(4), 1420–1428.

Yang, H., Lyu, W., Lu, L., Shi, X., Li, N., Wang, W., & Xiao, Y. (2020). Biogeography of microbiome and short-chain fatty acids in the gastrointestinal tract of duck. *Poultry Science*, *99*(8), 4016–4027.

Yang, X. (2014). Moraxellaceae. *Encyclopedia of Food Microbiology*, 826–833.

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APPENDIX

Flowchart for sample collection (cloacal swab)

Two (2) types of waterbirds, Greater Flamingo (N=15) and Domestic duck (N=15) from the Putrajaya Wetland Park area will be identified.

↓

The birds will be restrained with their wings tucked in.

↓

Cloacal swab technique taken for detection of intestinal normal flora. Technique of cloacal swab is by inserting swab into the vent and the swab rubbed onto the mucosal layer. (2 replicates)

Group	Egyptian Goose *[n=15]*	Domestic Duck *[n=15]*
Cloacal sample	**15 X 2 = 30**	**15 X 2 = 30**

↓

The birds will be released back to the population.
(Capture and release method)