# ANALYSIS OF MICROBIAL COMMUNITY DIVERSITY IN THE REGURGITATED PELLETS OF BARN OWLS (*Tyto javanica javanica*)

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**UNIVERSITI SAINS MALAYSIA**

**2025**

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**by**

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**Thesis submitted in fulfilment of the requirements**
**for the Degree of**
**Master of Science**

**August 2025**

## ACKNOWLEDGEMENT

Alhamdulillah, first of all, I would like to thank and be grateful to Allah the Almighty for giving me a peace of mind, good health, strength, courage and patience to me on completing this master project. Besides, a very special gratitude goes to my supervisor, Dr Kamarul Zaman bin Zarkasi for his consistent guidance and invaluable support given to me in completing this project until the end. His idea, knowledge and experience were essential on completing this project completely and indeed, all those lessons and experiences were extremely useful to my current and future knowledges and techniques about research. In addition, my thanks also go to staffs of School Biological Sciences, Puan Nurul, Mr Faisal and Mahendra who provide guidance, assistance and helpful on lending apparatus to me throughout my Master project in microbiology lab and in lab 414. A special thanks from me was also given to Dr Hasber and his postgraduates and undergraduates students Syafiq, Sakinah and Syafaat who guiding and mentoring me during the sampling session. Without their help, it would be difficult for me to complete this project successfully. Moreover, I also would like to express my gratitude to some postgraduate student in Lab 414, Syauqi and Afina for their guidance, knowledge and their sacrificed time to mentor me in molecular test in my project and also been helpful in lending some apparatus to me throughout my whole project. Furthermore, to all my fellow degree students, thank you for all the help and cooperation given. The memories doing the lab work together will be remembered. May our future go well, Amin. Last but not least, I would like to express my appreciation to my family especially both of my parents for their morals and support given to me. Without their prayers, I could never complete this project successfully and reach until this moment.

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## LIST OF SYMBOL AND ABBREVIATION

| | |
|---|---|
| % | Percentage |
| ˚C | Degree Celsius |
| ∞ | Infinite |
| µl | microlitre |
| cm | centimetre |
| g | gram |
| g | relative centrifugal force |
| L | litre |
| lbs | pound (mass) |
| log | Logarithm |
| M | mol/l |
| mA | miliampere |
| mg | miligram |
| min | minute |
| ml | mililitre |
| mol | mole |
| pH | Potential of Hydrogen |
| rpm | revolution per minute |
| s | second |
| V | Volt |
| A/α | alpha |
| B/β | beta |
| 27F | Forward primer starting at position 27 |
| 1492R | Reverse primer starting at position 1492 |

| | |
|---|---|
| A | Acidic |
| AM1…AM10 | Bacteria 1(Aviari)…Bacteria 10(Aviari) |
| ANOVA | Analysis Of Variance |
| APEC | Avian Pathogenic Escherichia coli |
| API | Analytical Profile Index |
| ASV | Amplicon Sequence Variance |
| BB | Breeder flocks |
| BBDuk | BBMap Discard Utility Kit |
| BBTool | Big Data Toolbox |
| BL | Bumbung Lima |
| BLAST | Basic Local Alignment Search Tool |
| BM1…BM10 | Bacteria 1(BL) …Bacteria 10(BL) |
| bp | base pairs |
| C | Cytosine |
| CB | broiler chickens |
| CFU | Colony Forming Unit |
| CL | commercial layers |
| D | Menhinick Index |
| $D_M$ | Margalef Index |
| DADA2 | Divisive Amplicon Denoising Algorithm 2 |
| DNA | Deoxyribonucleic acid |
| E | Species Evenness |
| EDTA | Ethylenediaminetetraacetic acid |
| EtBr | Ethidium Bromide |
| FASTQ | Fast Quality |
| JM1…JM10 | Bacteria 1(Jengka)…Bacteria 10(Jengka) |

| | |
|---|---|
| FT-IR | Fourier-transform infrared |
| G | Guanine |
| gDNA | genomic DNA |
| H’ | Shannon Index |
| HCN | Hydrocyanic acid |
| HFD | High Fat Diet |
| $H_2O_2$ | Hydrogen Peroxide |
| $H_2S$ | Hydrogen Sulphide |
| IAA | Indole Acetic acid |
| IBM | International Business Machines |
| ITS | Internal Transcribed Spacer |
| J’ | Equitability |
| KOH | Potassium Hydroxide |
| km | kilometre |
| LAB | lactic acid bacteria |
| LA-MRSA | Livestock Associated Methicillin Resistant *Staphylococcus aureus* |
| LipA | Lipase A |
| M | Marker |
| MALDI-TOF | Matrix-Assisted Laser Desorption/Ionization Time-of-Flight |
| MEGAN | Metagenome Analyzer |
| MG-RAST | Metagenomic Rapid Annotations using Subsystems Technology |
| MiSeq | Mixture Sequencing |
| MK-7 | major isoprenoid quinone |
| Mn | Manganese |
| MPN | Microbial Probability Number |
| MR | Methyl red |

| | |
|---|---|
| MRS | Methicillin Resistant Staphylococcus |
| MSA | Mannitol Salt Agar |
| MUSCLE | MUltiple Sequence Comparison by Log-Expectation |
| n | sample size |
| NA | Not Applicable |
| NA | Nutrient Agar |
| NAFLD | non-alcoholic fatty liver disease |
| NC | No Change |
| NCBI | Nucleotide Center Biotechnology Information |
| NGS | Next Generation Sequencing |
| NIF | Normal Illeal Feces |
| OTU | Operation Taxa Unit |
| P | p-value(probability) |
| PCR | Polymerase Chain Reaction |
| PE | Paired-End |
| POME | palm oil mill effluent |
| PPPTR | Pusat Penyelidikan Pertanian Tun Razak |
| PR | Province |
| PubMed | Public Medline |
| QC | Quality Control |
| QHD | Qushi Huayu decotion |
| QIIME2 | Quantitative Insights Into Microbial Ecology 2 |
| qPCR | quantitative polymerase chain reaction |
| R | Red |
| R1 | Forward read |
| R2 | Reverse read |

| | |
|---|---|
| rDNA | Ribosomal deoxyribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| SCA | Simmons' Citrate Agar |
| SD | Sprague-Dawley |
| SF | Spin Filter |
| SPSS | Statistical Package for Social Sciences |
| SRI | system of rice intensification |
| STEC | Shigella Toxin *Escherichia coli* |
| STREP/Strep | Streptococcus |
| TAE | Tris-acetate-EDTA |
| TCBS | Thiosulfate Citrate Bile Sucrose |
| TSI | Triple Sugar Iron |
| TVC | Total Viable Count |
| UAB | Urea Agar Base |
| UNITE | Unified System for the DNA-based Fungal Species Linked to the Classification Database |
| UPARSE | Ultra-fast Pipeline for OTU (Operational Taxonomic Unit) Analysis |
| USEARCH | Ultra Fast Sequence Analysis Software |
| UV | Ultraviolet |
| V3 | variable region 3 |
| V4 | variable region 4 |
| V group | Vitacoenzyme group |
| VP | Voges-Proskauer |
| VSEARCH | Verstile Search Tool |
| VTEC | Verocytotoxigenic *Escherichia coli* |
| W/V | Weight/Volume |

| | |
|---|---|
| Y | Yellow |
| YB | Yeast Beads |
| YG | Yellow with gas |
| YSI | Yolk Sac Infection |

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**ANALISIS KEPELBAGAIAN KOMUNITI MIKROB DI DALAM PELET MUNTAHAN BURUNG PUNGGUK JELAPANG (*Tyto javanica javanica*)**

## ABSTRAK

Kepelbagaian mikroorganisma yang dikaitkan dengan burung pungguk jelapang ternakan dan liar sememangnya pelbagai secara amnya. Memandangkan burung pungguk boleh menjadi perumah kepada mikroorganisma yang bermanfaat dan juga yang berbahaya, pemahaman tentang kepelbagaian mikrob burung itu adalah penting untuk mengenalpasti potensi penyebaran penyakit berjangkit sama ada kepada manusia, tumbuhan mahupun tanaman. Kajian ini bertujuan untuk menentukan kepelbagaian dan longgokan komuniti mikroorganisma yang dikaitkan dengan burung pungguk jelapang ternakan dan liar, menyiasat kesan komponen diet serta lokasi geografi burung pungguk itu terhadap populasi mikrob ini, dan mengenalpasti spesies mikrob utama yang terdapat di dalam burung pungguk tersebut. Semua pelet regurgitasi segar telah dikumpul daripada setiap burung pungguk dari lokasi masing-masing iaitu Aviari USM, Bumbung Lima dan Jengka. Kaedah kiraan dan pengkulturan mikrob, aplikasi pendekatan molekul serta penilaian kepelbagaian mikrob melalui analisis statistik telah digunakan dalam kajian ini. Hasil kajian menunjukkan bahawa pelbagai kepelbagaian mikrob dapat ditemui daripada kedua-dua jenis burung hantu tersebut, di mana *Escherichia* sp. dikenalpasti sebagai bakteria paling dominan yang ditemui daripada burung pungguk jelapang ternakan, manakala *Sporosarcina* sp. merupakan bakteria paling dominan yang ditemui daripada burung pungguk jelapang liar. Kajian ini memberikan maklumat yang berguna tentang kepelbagaian komuniti mikrob di dalam burung hantu, di mana pelbagai mikrob yang berbahaya dan bermanfaat boleh dikenalpasti hanya daripada burung pungguk

jelapang, justeru itu pendekatan yang betul serta beberapa langkah pencegahan perlu dilaksanakan kepada masyarakat kerana penemuan kepelbagaian mikrob daripada burung pungguk adalah kompleks.

**ANALYSIS OF MICROBIAL COMMUNITY DIVERSITY IN THE REGURGITATED PELLETS OF BARN OWLS (*Tyto javanica javanica*)**

## ABSTRACT

The diversity of microbe associated with captive and wild tropical barn owl is indeed diverse in general. Given that owls can harbour both beneficial and harmful microbes, understanding their microbial diversity is crucial for identifying the potential of infectious diseases spreading either to human, plants and crops. The aim of this study is to determine the diversity and abundance of microbial communities associated with captive and wild barn owls, investigate the effects of dietary components and geographical locations of the owl on these microbial populations, and discover key microbial species through the owl. All fresh regurgitated pellets were retrieved from each owl from their own site which are Aviari USM, Bumbung Lima and Jengka. Microbial enumeration & cultivation method, molecular approach application and microbe diversity evaluation through statistical analysis were applied in this study. Result of this study showed that various microbial diversity was able to be discovered from both type of the owl and *Escherichia* sp. is known as the most dominant bacteria discovered from captive barn owl whereas *Sporosarcina* sp. is known as the most dominant bacteria discovered from wild barn owl. This study provides some valuable information about the diversity of microbial community in the owl. Various harmful and beneficial microbe can be discovered just from the owl therefore an essential approach and several preventative measures need to be implemented in a correct way to the public as discovery of microbial diversity from the owl is complex.

# CHAPTER 1 INTRODUCTION

## 1.1 Background of research

In the Southeast Asian region, the most significant pest among rodents is the rice field rat, *Rattus argentiventer* (Bari *et al*., 2020, 2021). Other rodent species that commonly found in Malaysia such as black rat (*Rattus rattus*) and brown rat (*Rattus norvegicus*) (Lim, 2015). The great bandicoot rat, *Bandicota indica*, is an invasive rodent species limited to the northern regions of Peninsular Malaysia, specifically Kedah, Perlis, and Penang. Recent observations by Chung (2018) in Sabah's oil palm plantations have recorded new invasions of *B. indica*. This species has also been introduced to Java Island in Indonesia and Taiwan. The incursion of bandicoot rats raises concerns due to their large size, (Aplin *et al*, 2003); adaptability across varied landscapes (Bodlah *et al*., 2011; Pacheco, 2019); high reproductive rates, role as disease carriers (Böge *et al*., 2021; Krairojananan *et al*., 2020); and capable for causing serious damage to crops, stored goods, or infrastructure (Noor *et al*., 2023; Phukon & Borah, 2019).

Various strategies have been suggested for managing rodent pests, with chemical control being the fastest and most effective method (Salim, 2010)**.** Another commonly adopted approach among farmers involves utilizing natural biocontrol agents such as owls (Noor *et al*., 2023).

Owls belong to the group known as true owls, characterized by their circular facial structure, prominent craniums, distinct feather designs, and abbreviated tails. Barn owls, in contrast, possess facial shapes resembling hearts, along with modest

dimensions and elongated limbs equipped with formidable talons. Researchers have identified a total of 28 owl genera, with species like *Tyto alba, Tyto furcate, Otus gurneyi*, and *Otus balli* dispersed globally (Konig *et al*., 2009). Owls inhabit a wide range of environments across the world, excluding Antarctica and most isolated islands. Each owl species favors specific habitats, including evergreen forests, cultivated fields, shorelines, arid landscapes, and icy tundras. Unlike diurnal predators like hawks that hunt by day, owls are nocturnal hunters, relying on stealth during their nightly forays. Some owls possess dark plumage, enhancing their stealth capabilities against unsuspecting prey. Their flight is nearly silent, complemented by large, spherical eyes. Overall, owls are esteemed as adept nocturnal hunters (Lynch, 2007).

Owls ingest their prey whole, encompassing small mammals, birds, insects, and diminutive reptiles, given their absence of teeth. Because they cannot thoroughly chew their meals, indigestible remnants such as feathers, fur, and bones are regurgitated as pellets (solid waste material). Researchers gain insights into owl diets and microbial diversity by studying these pellets.

The subject of this research is *Tyto javanica javanica*, commonly known as the barn owl. These owls typically feature a pale facial disc rimmed in brown, with brownish-black eyes and a tail adorned with dark bars and feathers tipped in white (James, 2003). They measure between 29 to 44 centimetres in length and weigh 250 to 480 grams, displaying primarily nocturnal behaviour. Barn owls navigate the skies silently, often gliding between wingbeats. They thrive in diverse habitats such as open grasslands, forests, heaths, and rice fields (often kept as pets for pest control). Nesting

preferences include tree hollows, old structures, and caves. Despite their widespread presence in regions spanning from Britain and Europe to Africa and the Middle East, barn owls have relatively short lifespans.

In terms of diet, barn owls specialize in hunting small mammals like field mice, rats, bats, and shrews. They breed prolifically in response to fluctuations in mouse populations, frequently hunting from low perches. Additionally, they show interest in insects, small reptiles, birds, and frogs (Heimo, 2012).

The diversity of owl species, alongside their behaviors, movements, and habitats, plays a role in shaping the microbial diversity within their digestive systems and it can be simply determined by having a proper identification of isolated microbes from their regurgitated pellet since the content of the pellets is made up from what the owls eat in their daily life. The microbial composition of captive barn owls differs from that of their wild counterparts. A study by Corl *et al*., (2020) focused on movement ecology and sex in barn owls, revealing that dominant bacterial phyla including Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes were discovered in barn owl guts. However, comprehensive research on microbial diversity within regurgitated pellets of the owl is still limited.

### 1.2 Problem Statement

There is a notable scarcity of research on the microbial diversity within tropical barn owls, making reliable data on their microbial composition uncertain. Researchers

are increasingly concerned with how different habitats and diets affect owl behaviour. Given that owls can harbour both beneficial and harmful microbes, understanding their microbial diversity is crucial as well as their role as reservoir of various microbe. This knowledge will help determine if owls can act as a good reservoir for harmful microbes or not, which is vital for preventing infectious diseases, especially among farmers. Previous studies have found *Micrococcus* sp. in various birds, such as the red-tailed hawk (*Buteo jamaicensis*), hoopoe (*Upupa epops*), houbara bustards (*Chlamydotis undulata*), and goshawk (*Accipiter gentilis*), suggesting the potential for owls to carry harmful microbes as the bacteria capable to become pathogen to human being (Silvanose *et al*., 2001). The diversity of microbial communities in barn owls is also influenced by their habitats, including coastal areas, paddy fields, and palm oil estates (Corl *et al*., 2020). Meanwhile, metagenomics, an advanced technique, allows for comprehensive and efficient study of microbial populations, offering a deeper understanding of microbial diversity and functionality. Employing metagenomics will fill knowledge gaps and ensure optimal data acquisition on barn owl microbiology (Felczykowska *et al*., 2015).

### 1.3 Limitations of the study

In this research, the sampling process for this study depends on the availability of collaborator of the sampling site. No sampling of wild owl pellets can be done if rains or monsoon season occur due to weather condition and flood that will disrupt the process of retrieving the sample. Besides, due to habit of the owl in which they favour to live in already made artificial nest, the study area was focus to only the Aviari USM, Kompleks Bumbung Lima, Bertam and Pusat Penyelidikan Pertanian Tun Razak

Jengka, Pahang since the site has abundance species of the owl for this study. Moreover, the findings are derived solely from this individual species of tropical barn owl, *Tyto javanica javanica*.

### 1.4 Objective

1) To determine the diversity and key most abundance of microbial community associated with barn owl
2) To determine effects of different and various dietary components on populations of microbe toward barn owls via their regurgitated pellets
3) To determine the diverse microbe population in barn owl through their regurgitated pellets based on various geographical locations

# CHAPTER 2 LITERATURE REVIEW

## 2.1 Owls

The Strigiformes order includes the owl, which is a predator primarily active at night and can be found all over the world. This order is divided into two families: Tytonidae and Strigidae. Strigidae is dominant in number with 159 species across 20 genera found almost everywhere, ranging from 13 to 70 cm in length. Tytonidae, on the other hand, has only 21 species across two genera found in tropical to temperate regions, with a body length of 30 to 54 cm. Owls are difficult to study due to their nocturnal habits, and so our understanding of them is limited. They are carnivorous and typically prey on small rodents. Owls generally have similar physical features, such as a flat face, small hooked beak, and large forward-facing eyes. They have rounded wings and short tails, and their feet are large with powerful talons. These features make them one of the best nocturnal predators in the world. Owls can be found on every continent except Antarctica and some isolated islands. While the barn owl (*Tyto alba*) and the short-eared owl (*Asio flammeus*) are widely distributed, the Palau owl (*Pyrroglaux podargina*) and Seychelles owl (*Otus insularis*) are species found only on certain islands with small populations, as both are endemic (Gill, 2006)

### 2.1.1 Taxonomic classification of *Tyto javanica javanica*

A particular taxonomic classification of barn owl (*Tyto javanica javanica*) (Fig 2.1) was based on Gill & Wright (2006). The classification is as Table 2.1 below:

Table 2.1 Detail taxonomic classification of *Tyto javanica javanica*

| Taxa level | Description |
|---|---|
| Kingdom | Animalia |
| Subkingdom | Bilateria |
| Infrakingdom | Deuterostomia |
| Phylum | Chordata |
| Subphylum | Vertebrata |
| Infraphylum | Gnathostomata |
| Superclass | Tetrapoda |
| Class | Aves |
| Order | Strigiformers |
| Family | Tytonidae |
| Subfamily | Tytoninae |
| Genus | Tyto |
| Species | *Tyto javanica javanica* |

Figure 2.1: *Tyto javanica javanica*

### 2.1.2 The Genus *Tyto javanica javanica*

#### 2.1.2(a) Morphology

The unique structure of *Tyto javanica javanica* sets it apart from other commonly found birds. Specifically, its heart-shaped face with small, stiff feathers around the edge distinguishes it as a barn owl. These owls are renowned for their nocturnal prowess, possessing excellent eyesight, extremely sensitive hearing, and a flexible neck that enables them to turn their heads 180 degrees in both directions. Their light bodies feature big, broad wings made of soft feathers that allow them to fly very quietly, while their long legs help them reach into long grass or bushes when hunting. The owl's long toes, sharp claws, and powerful feet make it adept at catching prey (Bruce, 1999).

#### 2.1.2(b) Distribution and habitat

*Tyto javanica javanica* is present in all continents except Antarctica due to the extreme cold climate that makes it difficult for the owl to store enough energy reserves. Also, mountainous areas and the Sahara Desert are not suitable habitats for them. Their preferred living areas are oil palm plantations, which offer abundant food resources. In Singapore, they have been found mostly in the southern and eastern regions of the island, inhabiting tree holes and man-made structures like roofs of houses and bridges (Bruce, 1999)

### 2.1.2(c) Diet and feeding behaviour of the owl

The Barn Owl (*Tyto javanica javanica*) is a nocturnal raptor renowned for its adaptability in diet and feeding behavior, primarily preying on small mammals such as rodents. This dietary preference positions the Barn Owl as a valuable biological control agent in various ecosystems. For instance, in Peninsular Malaysia's oil palm plantations, Barn Owls predominantly consume the Malayan wood rat (*Rattus tiomanicus*), which made up 99.5% of their prey biomass, highlighting their significance in agricultural pest management (Lenton, 2008). In urban settings, Barn Owls exhibit similar dietary flexibility. A study conducted at Universiti Sains Malaysia revealed that introduced Barn Owls primarily fed on Norway rats (*Rattus norvegicus*), constituting 65.37% of the diet, followed by the common shrew (*Suncus murinus*) at 30.12%, showcasing their adaptability to human-modified habitats (Saufi et al., 2020).

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Moreover, a study in Cyprus indicated that Barn Owls consistently relied on small mammals, yet their diet varied seasonally depending on prey availability (Moysi et al., 2018). Likewise, a regional study in Southern Punjab, Pakistan, showed that house shrews (*Suncus murinus*) dominated the diet at 60.2%, followed by birds (24.1%) and rodents (12.7%), revealing distinct variations in prey preference across geographic regions (A. Mohamed Samsoor Ali & R. Santhanakrishnan, 2012). In addition, Kross et al. (2016) emphasized the Barn Owl's contribution to pest control, stating that "Barn Owls nesting in agricultural landscapes consumed a high proportion of pest species, especially rodents," suggesting their effectiveness as a natural solution for integrated pest management.

These findings collectively highlight the Barn Owl's opportunistic feeding behavior and ecological importance in maintaining prey population balance in both natural and human-dominated environments.

### 2.1.3 Barn Owl Pellets

Owl pellets is a waste organic material that came from owl through regurgitation when owls swallow their prey as a whole and expel undigested remains, such as bones, compacted in hair and feathers (Andrade et al., 2016). Through proper observation and examination of the pellets, we can have various knowledge access about diet of owl, who is their common prey of and even microbial species inhabit in the owl. The study of 'Are owl pellets good estimators of prey abundance?', state that Barn owl pellets' contents are a useful method to estimate relative abundances of its preys (Andrade et al., 2016). It is also known that by just observing the detail of the pellets, the common diet of the owl is mainly consist of rat and it is supported by previous researcher (Alasdair et al., 2000; Avery et al., 2002; Lyman, 2012). Past researcher also state that they also able to discover various of microbial spesies within the owl pellets such as *Escherichia coli*, *Enterobacter ludwigii*, *Pseudomonas fragi, Malbranchea albolutea* and *Kocuria tytonicola* (Braun et al., 2019; Caprari et al., 2024)

Figure 2.2: Barn owl pellets

## 2.2 Owl as biocontrol

Utilization of natural predators to control pest numbers in crops is known as biocontrol. This method is achieved through the introduction of interspecific competitors, parasites, or natural predators. Rats are a common pest in palm oil plantations and can cause significant damage to the crops. Previous research in the bio-control of rats has shown that the use of barn owls (*Tyto javanica*), grass owls (*Tyto longimembris*), and Black-shouldered Kites (*Elanus caeruleus*) is an effective way of reducing rat populations in oil palm plantations through biological approaches. This research was conducted on the shore areas of Felda oil palm plantations in Borneo, Malaysia (Cik *et al.,* 2014)

*Tyto javanica* is a species of bird that naturally preys on many types of agricultural pests like rodents. Therefore, the owl can offer farmers pest control services that are economically valuable. The owl populations can be easily established by providing nest boxes in the target area and with low maintenance requirements annually. Traditional pesticides that are used to control rodents could be less effective as the pests can become immune to certain chemical compounds. Moreover, the use of pesticides can also lead to the unwanted poisoning of other wildlife species in the targeted area. Additionally, using traps to control rodents requires continuous effort and staffing costs, which can result in high initial inputs for purchasing traps (Kross *et al.*, 2016)

Furthermore, previous studies have suggested that using owls as a biocontrol agent in vegetation crops can be advantageous. According to research comparing the

biocontrol of common vole populations by avian predators versus rodenticide application, the most common methods for controlling *Microtus arvalis* in agricultural landscapes include habitat management, farming practices, and rodenticide application. However, various biocontrol methods are increasingly being explored and promoted. There are two primary methods for biocontrol of voles in certain agricultural landscapes: installing artificial nest boxes in the agricultural landscapes during their nesting period or installing perches in the agricultural landscapes (MacHar *et al.*, 2017). Perches serve as an elevated lookout for prey and resting places for owls. The research also suggested that the financial cost of rodenticide application was roughly twice that of biological control on common vole populations. This indicates that using owls as a biocontrol agent on certain vegetation crops is useful, efficient, and profitable for farmers (MacHar *et al.*, 2017)

### 2.2.1 Nest box of barn owl

Wild barn owls (*Tyto javanica javanica*) often utilize artificial nest boxes, especially when natural nesting sites are scarce. Numerous studies since 2000 have explored their preferences and the effectiveness of these structures. A study in the Hula Valley, Palestine, monitored 145 nest boxes over eight years and found that barn owls preferred boxes located on trees (59.8% occupancy) over those on poles in the shade (48.0%) or in the sun (29.2%). The placement significantly affected both occupancy and breeding success. In Madurai District, Tamil Nadu, India, barn owls utilized various man-made structures for roosting, including artificial wooden nest boxes. Of the 17 nest boxes installed, 41% were used by barn owls for roosting and nesting, suggesting that nest boxes can be effective in increasing barn owl

populations in agricultural areas (Charter & Rozman, 2022; Santhanakrishnan et al., 2011). With the nestbox of barn owl were installed at agricultural areas, all the regurgitated pellets produce from there is from the owl and from the pellets, various study can be conducted from the pellets such as microbial species identification

**2.3 Microbiome**

The phrases "microbiome" and "microbiota" are sometimes utilized interchangeably, but they have slight differences. The term "microbiome" refers to the genomes of microorganisms present in the environment, while "microbiota" refers to the microorganisms themselves in the environment (Valdes *et al.*, 2018). The field of microbiome has made significant advancements over the last few decades, mainly because of DNA sequencing technology's progress. This has resulted in a field that has great scientific and public interest (Cullen *et al.*, 2020; Gonzalez & Knight, 2012; Knight, 2016). In this field, research has revealed a vast amount of data, leading to a much better understanding of microbial studies, including the interactions and impacts of microorganisms within the host and external environments. Understanding how these microorganisms' function could have potential benefits in various fields such as ecology, agriculture, forensics, and medicine (Cullen *et al*., 2020; Huttenhower *et al*., 2014)**.**

There are currently two methods for studying microbiomes: the top-to-bottom approach and the bottom-to-top approach. The former entails examining microbial communities from a more comprehensive perspective. Byerley *et al*. (2017) observed that adding walnuts to the diet of Fischer 344 rats caused changes in their gut microbial

communities. This study indicated that walnuts could potentially offer health benefits through a novel mechanism. Meanwhile, Henderson *et al*. (2015) examined the microbial community composition of ruminant livestock across a broad geographical range. Their study provided insights into the dominant methanogens that could be harnessed to develop strategies for mitigating methane emissions. Conversely, the latter approach focuses more on mechanistic studies or the roles of individual microorganisms, metabolites, or genes. Caesar *et al*. (2016) employed this approach in their study to explore how the interaction between the gut microbiota in mice and dietary lipids controls the lipid composition in the liver and plasma, as well as the gene expression in the liver. Currently, there are two approaches to microbiome studies: the top-to-bottom approach and the bottom-to-top approach. The former involves examining microbial communities from a broader perspective. For instance, Byerley *et al*. (2017) found that the inclusion of walnuts in the diet of Fischer 344 rats resulted in changes to their gut microbial communities. Their study suggested that walnuts could potentially provide health benefits through a new mechanism. In another study, Henderson *et al*. (2015) investigated the microbial community composition of ruminant livestock across a wide geographical range. Their study provided insight into the dominant methanogens that could be used to develop strategies for mitigating methane emissions. On the other hand, the latter approach is more focused on mechanistic studies or the roles of individual microorganisms, metabolites, or genes. This approach was used by Caesar *et al*. (2016) in their study to investigate how the interaction between the gut microbiota in mice and dietary lipids controls the lipid composition in the liver and plasma, as well as the gene expression in the liver.

### 2.3.1 Factors affecting microbial communities

In general, all living organisms do possess an abundance and variety of microbes in and out of their body. All living organisms from tiny, microscopic insects to the biggest animals in the world, even the animals that we consume daily as a source of protein do possess some bacteria within their gut. As an example, herbivore animals. Such animals like cows, goats, sheep, deer, etc, surely do possess a lot of beneficial or harmful bacteria within their gut and the bacteria present within them for several reasons/factors. Those several factors are the cause of how the microbial communities in some living organisms are affected (Faniyi *et al*., 2019)**.**. Various intricate factors, including diet, influence the microorganisms residing in the rumen, leading to fluctuations in the pH levels of the rumen. The pH levels of the rumen, in turn, have an impact on both feed consumption and feed digestibility, resulting in a microbial change in the members of the microbial community living in the foregut and hindgut (Faniyi *et al*., 2019)**.**

Aquatic animals are also not excluded too from this condition too. Instead, they are more prone to colonization of several microbe in and out of their body since they live in aquatic environment which is very suitable for variety of microbial existence, growth and shaping their own community in the aquatic animals. The microbe abundancy in these intestines of the aquatic animals was also significantly higher when compared to other animals. The location of the shrimp intestines near the body surface may cause intestinal microbes to be more susceptible to changes in external environments (Rungrassamee *et al*., 2014). This may explain the significant differences in the bacterial populations found in shrimp samples. It has been observed

that changes in dominant microbial taxa occur among different animals when the environment is relatively stable. This suggests that host physiology plays a significant role in shaping the intestinal microbiota. Therefore, the intestinal microbiome can be influenced by various factors (Rungrassamee *et al*., 2014; Fulin Sun *et al*., 2020; Fulin Sun & Xu, 2021) including host genetics, which can determine nutritional levels and subsequently impact the diversity of the microbiome (Benson *et al*., 2010; Ley *et al.*, 2008)

Avian too possesses a diversity of microbiota within or outside of them and they too share almost quite similar factor with other animal on how the microbial communities at them were affected by time to time. There are several factors affecting microbial communities at them such as host genetic, age and sex, diet, environmental factors, behavioural habits, social contact of the avians, health, phylogeny and evolutionary traits of the avians (Bindari & Gerber, 2022; Grond *et al.*, 2019; Lucas & Heeb, 2005; Fengfei Sun *et al*., 2022; Waite & Taylor, 2014)

### 2.3.2 Microbiota in owls and their roles

The adaptation mechanisms employed by the bacterial community enable them to thrive in diverse environments. Interactions between bacteria and other organisms, including both bacterial-bacterial and bacterial-host, facilitate this process. Bacteria also interact with other biotic organisms in their surroundings, such as plants and animals (Braga *et al.*, 2016; Ely & Smets, 2019; Kai *et al.*, 2016; Santhanam *et al*., 2014)**.** The functionality of the bacterial communities found in seeds, roots, leaves, stems, tubers, ovules, and fruits varies (Zinniel *et al*., 2002)**.**

The microbiome of the gastrointestinal (GI) tract and its impact on the immune function and physiology of the host are subjects that are gaining importance in medicine, ecology, and microbiology. With the help of advanced molecular techniques and an increasing recognition of the vast effect of avian gut microbiota on host health, there has been a significant rise in studies focused on the microbes present in the avian gut (Grond *et al.*, 2018). Birds, also known by various common names, are a successful and diverse lineage that inhabits a wide range of niches across the globe. Avian gut microbiomes are rich, diverse and play crucial roles in providing nutrition and protection against harmful pathogens. Research on avian microbiology has been uneven, with a few species accounting for many studies conducted. These species include those that are important for agriculture, such as chickens, turkeys, and owls, as well as those that are of evolutionary or conservation interest (Waite & Taylor, 2015).

The content of microbiota in the gut of owls can be influenced by various factors including their gender, foraging behaviour, and movement ecology. The makeup of microorganisms present in the gut of owls is highly associated with their ecological movement. The ecological movement of an individual host can vary due to several aspects such as their foraging range, interactions with different individuals, habitat, size of territory, and displacement from a residential area. These distinct ecological movements can have an impact on the microbiota present in the gut of owls (Corl *et al.*, 2020).

If the distribution of microbial species in the environment is patchy, it is possible that owls who move around and interact with a diverse range of environments

might have a greater diversity of microbes in their system than less active owls. On the other hand, the movement behaviour of owls could be linked indirectly to the microbes in their gut, insofar as the owl's physiology impacts both their movements and the communities of microbes present (Corl *et al.*, 2020). For instance, birds that migrate to a new location may experience temporary gut atrophy, and studies have shown that they tend to have lower microbial diversity compared to populations of non-migratory birds (Risely *et al.*, 2018).

### 2.3.3 Impact and potential of microbiota in Owls

The health of a host could be positively affected by the microbes present in their gut. The theory of hologenome suggests that an organism can increase its fitness parameters, including survival rates, reproductive performances, and phenotypic plasticity, by coevolving with microbes. For example, changes in gut microbiota have resulted in a reduced fecundity rate in termites and honeybees, while the use of antibiotics that change the gut microbiota of sparrows has led to depressed nestling growth (Grond *et al.*, 2018). Additionally, microbes are responsible for adapting birds to a carrion-focused diet. Although the theory argues that an organism can live with its microbe and provide benefits to the organism, such as increasing fitness parameters, these microbes are also useful to other organisms, such as owls, contributing significantly to nutritional uptake, detoxification, and understanding of bird immune function (Grond *et al.*, 2018).

Even if the microbiota within an owl does not have any negative or positive impact on its host, it can still serve as a transmitter agent. A previous study conducted

on Tawny owls (*Strix aluco*) found that they can act as a reservoir and transmitter of Enterobacteriaceae species, which are harmful bacteria for both humans and animals (Grzywaczewski *et al*., 2017). The bacteria were isolated from birds located in areas where there was human habitation or pressure due to the presence of people such as forest service workers, nature protection service, and ornithologists. The study concluded that Tawny owls have the potential to transmit these harmful bacteria to humans and animals (Grzywaczewski *et al*., 2017)

It is interesting to note that certain microbes can be fatal to certain species of owls due to their unsuitability to these microbes. A previous research conducted on the activity of the Usutu virus (USUV) in Austria between 2001-2002 found that the virus, which originated from mosquitoes of the Flaviviridae family, caused the deaths of blackbirds (*Turdus merula*) and great grey owls (*Strix nebulosa*) in the Vienna city and surrounding villages in 2001 (Weissenböck *et al*., 2003)

Thus, the effects of microbes on owls can vary based on previous research that has been conducted on the impact of microbes on owls. Whether the microbe is beneficial or harmful to the owl depends on the natural characteristics of the microbe that inhabit the owl.

**2.4 Metagenomics**

Microbes are present in a wide range of environments, and most of our understanding of microbial life comes from organisms grown in pure cultures.

However, many microbes cannot be cultured in labs, which makes traditional identification methods a major challenge. Less than 1% of the total microbial diversity of an environment can be accounted for through cultural methods (Edet *et al*., 2017; Streit & Schmitz, 2004; Tyson & Banfield, 2005). Metagenomics offers a way to overcome this obstacle by examining DNA samples taken from the environment instead of cultivating the organisms. Handelsman *et al.* (1998), created term "metagenomics" to describe the study of genomes of members of a microbial community. This area of study allows researchers to access, characterize and understand the vast and unknown microbiome (Ghosh *et al.*, 2018)**.**

### 2.4.1 History of metagenomics

In its early stages, pioneering scientists began using solid-phase nutrients to isolate microorganisms for enumeration and visualization. This technique aided their understanding of microbial physiology (Blevins & Bronze, 2010; Escobar-Zepeda *et al*., 2015). Subsequent improvements in staining methods significantly enhanced the resolution of microscopy, becoming the primary means of exploring microbes and their interactions (Beveridge, 2001; Blevins & Bronze, 2010; Escobar-Zepeda *et al*., 2015) This exploration revealed that microbes have specific growth requirements, leading to the development of culture media that simulate their natural habitats. Winogradsky's contributions transformed microbiology by introducing microbial ecology, which examines microorganisms and their functions in ecosystems (Escobar-Zepeda *et al*., 2015; Mcfall-ngai, 2008; Prayogo *et al*., 2020).

In the late 1970s, Carl Woese proposed a groundbreaking concept: utilizing ribosomal RNA genes as distinctive markers for microbial classification (Woese & Fox, 1977). This innovative approach, in conjunction with the introduction of Sanger's automated sequencing technique, had a profound impact on the landscape of microbial research and classification. Over subsequent decades, a plethora of molecular techniques emerged, among them the polymerase chain reaction (PCR) pioneered by Kary B. Mullis and Fred A. Faloona (Mullis & Faloona, 1993). Progress also led to the development of systems employing microorganisms to explore gene functions and roles within microbial communities, uncovering new genes, functions, and metabolic products that propelled biotechnology forward (Escobar-Zepeda *et al*., 2015). These advancements laid the foundation for the era of metagenomic analysis. To this day, these methodologies continue to reveal previously unknown members of microbial communities, novel molecules, and diverse microbial functions (Escobar-Zepeda *et al*., 2015; Jünemann *et al.*, 2017)**.**

The initial Next Generation Sequencing (NGS) tool, the GS20 sequencing-by-synthesis (SBS) pyrosequencing instrument, was pioneered by 454 Life Science in 2004 (Kulski, 2009; Slatko *et al*., 2018). This system was subsequently succeeded by the 454 GS GLX, providing read lengths of 100 - 150 base pairs (bp) and achieving a throughput of 20 Megabytes (Mb) per run. The platform underwent continuous enhancements, culminating in the 454 GX FLX+ in 2009, capable of generating read lengths up to 1 kbp and exceeding 600 Mb in a single run (Kulski, 2009; Slatko *et al.*, 2018)**.** Another significant competitor in the market was the Genome Analyzer, introduced in 2006 by Solexa (Kulski, 2009; Slatko *et al*., 2018). In 2007, Illumina acquired Solexa, leading to substantial advancements and the development of

instruments such as the HiSeq and MiSeq families in subsequent years. While there are several other platforms available, Illumina's sequencing platform remains the most widely adopted due to its exceptional per-base cost efficiency and great sequencing accuracy (Goodwin *et al.*, 2016; Hodkinson & Grice, 2015; Jünemann *et al*., 2017; Kulski, 2009; Slatko *et al.*, 2018).

### 2.4.2 Amplicon sequencing

In this research, amplicon sequencing was employed and will be outlined here. The amplicon sequencing method is widely used for its convenience in performing taxonomic and phylogenetic analysis of diverse, intricate samples (Clarridge, 2004; Oulas *et al*., 2015). It provides a swift and cost-effective means to characterize bacterial communities or taxonomic profiles (Clarridge, 2004; Oulas *et al*., 2015). This technique utilizes DNA as a template for polymerase chain reaction (PCR) to amplify a target marker or gene, like 16S ribosomal RNA gene for bacteria, internal transcribed spacer (ITS) of fungi, and 18S rRNA gene of eukaryotes (Clarridge, 2004; Mitra, 2019; Oulas *et al.*, 2015).

In studies of bacterial communities, DNA extraction is initially performed on environmental samples. Subsequently, specific conserved regions of the bacterial 16S rRNA gene are targeted for amplification using universal primers complementary to those regions of interest. The resulting PCR products are purified before undergoing sequencing. The sequencing platform commonly chosen for this purpose is the Illumina MiSeq, renowned for its high read capacity and accuracy (Lawley & Tannock, 2017).

Post-sequencing, various software packages are available for data analysis, such as Mothur (https://mothur.org/) and QIIME2 (https://qiime2.org/). Despite the abundance of software options, no single "best" software exists. Raw sequence data undergoes preprocessing to assess sequence quality and remove adapters and primers. Sequences meeting quality criteria are merged to form contigs and aligned against selected reference databases like SILVA (Quast *et al*., 2013), Greengenes (DeSantis *et al*., 2006), or the Ribosomal Database Project (RDP) (Cole *et al.*, 2007; Maidak *et al.*, 1996).

Chimeric sequences are identified and removed before clustering and assignment into operational taxonomic units (OTUs). For bacteria, OTUs sharing over 97% similarity are typically grouped into the same species (Fox *et al*., 1992; Lozupone & Knight, 2008; Martin, 2002)**.** Taxonomic classification of OTUs relies on the chosen reference database.

α-diversity and β-diversity metrics are then calculated and visualized using preferred software tools such as RStudio (https://rstudio.com/). A-diversity assesses community diversity and species richness, while B-diversity reveals differences in species composition across groups (Escobar-Zepeda *et al*., 2015; Lawley & Tannock, 2017; Lozupone & Knight, 2008; Mitra, 2019; Oulas *et al*., 2015: Whittaker, 1972).