GUT MICROBIOTA OF CAPTIVE DEER (*Rusa timorensis*) AT GUNUNG RENG, JELI, KELANTAN

by

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DECLARATION

With the exception of the information provided in the references, I officially declare that this thesis, "GUT MICROBIOTA OF CAPTIVE DEER (*Rusa timorensis*) AT GUNUNG RENG, JELI, KELANTAN" is the product of my own research. The thesis is not being submitted concurrently for the candidature of any other degree, nor has it been accepted for any degree.

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Date : 27/7/2024

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GUT MICROBIOTA OF CAPTIVE DEER (*Rusa timorensis*) AT GUNUNG RENG, JELI, KELANTAN

ABSTRACT

This study investigates the gut microbiota of captive deer (*Rusa timorensis*) at Gunung Reng, Jeli, Kelantan, with a focus on identifying and comparing the microbial communities present in captive and wild deer populations. *Rusa timorensis*, a species native to the Indonesian archipelago, has been introduced to various regions, including Malaysia, where it is valued for its adaptability and resistance to disease. The research aims to classify gut microorganisms using simple staining techniques and compare microbial classes via secondary data. Captive conditions, including diet and habitat, significantly influence gut microbiota, impacting health and adaptability. This study collected faecal samples from deer at Gunung Reng and conducted microbial analysis using various agar media and microscopy techniques. Results indicated distinct differences in microbial composition between male and female deer, with notable implications for health management and conservation efforts. Understanding gut microbiota dynamics in captivity can inform strategies to enhance animal welfare and ensure the sustainable management of captive populations.

MICROBIOTA USUS RUSA TAWANAN (*Rusa timorensis*) DI GUNUNG RENG, JELI, KELANTAN

ABSTRAK

Kajian ini menyiasat mikrobiota usus rusa tawanan (*Rusa timorensis*) di Gunung Reng, Jeli, Kelantan, dengan tumpuan untuk mengenal pasti dan membandingkan komuniti mikrob yang terdapat dalam populasi rusa tawanan dan liar. *Rusa timorensis*, spesies yang berasal dari kepulauan Indonesia, telah diperkenalkan ke pelbagai wilayah, termasuk Malaysia, di mana ia dihargai kerana kebolehsuaian dan ketahanannya terhadap penyakit. Penyelidikan ini bertujuan untuk mengklasifikasikan mikroorganisma usus menggunakan teknik pewarnaan mudah dan membandingkan kelas mikrob melalui data sekunder. Keadaan kurungan, termasuk diet dan habitat, mempengaruhi mikrobiota usus dengan ketara, memberi kesan kepada kesihatan dan kebolehsuaian. Kajian ini mengumpul sampel najis daripada rusa di Gunung Reng dan menjalankan analisis mikrob menggunakan pelbagai media agar dan teknik mikroskopi. Keputusan menunjukkan perbezaan yang berbeza dalam komposisi mikrob antara rusa jantan dan betina, dengan implikasi yang ketara untuk pengurusan kesihatan dan usaha pemuliharaan. Memahami dinamik mikrobiota usus dalam kurungan boleh memaklumkan strategi untuk meningkatkan kebajikan haiwan dan memastikan pengurusan mampan populasi kurungan.

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

The largest species of deer is *Rusa timorensis,* which is medium-sized and rough-coated (Ali et al., 2021). Human activity related to *R. timorensis* is influenced by habitat, human hunting, and translocation. The criteria that influence the anthropogenic activities of this deer species have a significant impact on the genetic diversity and long-term conservation of the local population of this species. The *R. timorensis* is indigenous to the islands of Java, Bali, and Timor in Indonesia. The species has been introduced to various regions, including Irian Jaya, Borneo, the Lesser Sunda Islands, Maluku, Sulawesi, Pohnpei, Mauritius, Reunion, Fiji, Tonga, Samoa, Vanuatu, the Solomon Islands, the Christmas Islands, the Cocos Islands, Nauru, Australia, New Caledonia, New Zealand, Papua New Guinea, New Britain, and New Ireland (Hedges et al., 2015; Long, 2003; Sutrisno, 1993). According to Abdul Kadir (2003), some of breeders in Malaysia choose to rear deer from the species *R. timorensis* due to its high adaptability to the local environment and its resistance to diseases. Hence, *R. timorensis* possesses the ability to meet the demand for meat and is vulnerable to legal or illegal hunting. As a result of these significant activities, the population of *R. timorensis* is expected to decline. Consequently, it is imperative to conduct systematic and continuous monitoring of this species to ensure its optimal development as a valuable biological resource (Zein and Saim, 2001).

The gut microbiota in animal hosts plays a significant role in various elements such as nutrition metabolism, intestinal health, immunity, and other relevant factors.

This influence is particularly notable in caged animals, where adaptability is of utmost importance. After giving birth, animals experience colonization of their gut by bacteria, and the gut microbiota have a significant impact on the maintenance of host homeostasis (de Muinck & Trosvik, 2018). The dynamic fluctuation of gut microbiota composition and diversity throughout different growth stages is a well-established phenomenon (Jami et al., 2013). Several studies have demonstrated that the composition of gut microbiota undergoes constant changes during the process of growth and development (Zhu et al., 2021). Furthermore, it has been observed that the gut microbiota tends to become increasingly similar among individuals as they age. The health status of animals can be reflected by changes in intestinal microbiota during different growth stages, which can be influenced by various diets and physiological factors (Jeffery & O'Toole, 2013). Moreover, substantial variations in the composition of gut microbiota have been seen across several animal species, and the diversity of gut bacteria plays a crucial role in the development and well-being of animals (Wang et al., 2019).

Captive breeding and rearing strategies have been employed for various deer species that are currently under the threat of extinction. The composition of feed fed to captive deer species typically exhibits significant differences when compared to food available in the natural habitat (Li et al., 2017). Therefore, there exists a notable difference in the composition of gut bacteria between captive and wild deer populations (Guan et al., 2017). The degradation of lignin and other compounds by gut microbes of the Proteobacteria phylum has been documented (Fang et al., 2012), providing additional evidence for the impact of artificially designed diets on the structure of gut microbiota in captive deer species. Nevertheless, the rise in Proteobacteria in captive deer species may suggest a heightened susceptibility to digestive disorders, as some intestinal bacteria linked to Proteobacteria have been identified as pathogenic or potentially pathogenic (Joat et al., 2021). The gut microbiota exhibits a degree of adaptability that enables the host to adjust to the transition from a natural food source to captivity. However, it is important to acknowledge certain health risks associated with captive populations, including reduced efficiency in nutrient absorption and an elevated presence of potentially pathogenic bacteria (Gogarten et al., 2012). Hence, it is essential to closely observe the gastrointestinal system of captive deer species and evaluate their ability to acclimate to synthetic foods and new habitats, since these factors hold significant implications for the preservation of wildlife.

1.2 Problem Statement

The gut microbiota of various animal species, including deer, wildlife, and companion animals, plays an important role in their physiological state, production, and general welfare. However, there is a correlation between disruptions in the structure and operation of the gut microbiota and various health problems, including digestive issues, reduced growth and increased susceptibility to disease. An irregular drainage system can contribute to the growth of the microbiome in the digestive system. This is because when water stagnates in one place, *R. timorensis* will drink in the contaminated stagnant water area. However, it is important to understand the influence of environmental factors, including diet and habitat, on the structure and resilience of the gut microbiota in wild animal populations to assess the well-being and adaptability of these species in response to environmental change. This can be linked to the problem of the captive occupied by *R. timorensis* in Gunung Reng. A small captive limited its movement, when this happens it will put *R. timorensis* on stress.

1.4 Objectives

- To classify captive *R. timorensis* gut microbiota through simple staining technique.
- To compare gut microorganism classes of *R. timorensis* in captive and in wild via secondary data acquisition.

1.5 Scope of Study

The microscope analysis were conducted at the Microbiology and Biochemistry Laboratory, Faculty of Earth Science (FSB) at Universiti Malaysia Kelantan (UMK), Jeli campus. However, deer faecal sample will be collected at Gunung Reng, Jeli District, Kelantan. These sample were collected as soon as possible after defecation for the most accurate results. After that, it was stored at a cool environment to slow down bacterial growth and preserve sample integrity. Microscopic method will be used for microbial analysis.

1.6 Significant of Study

Effective wildlife health management in captive deer populations requires a comprehensive understanding of potential pathogens, antibiotic resistance, and the overall microbial diversity present. Such insight can be invaluable in crafting targeted disease prevention and control strategies, including vaccination campaigns, robust biosecurity protocols, and judicious antibiotic usage, all of which are essential for enhancing the overall health and welfare of captive deer. Recognizing that certain bacteria can pose significant threats to deer health, the ability to identify and monitor microbial communities becomes a critical tool in early illness detection, disease prevention, and outbreak management. This research endeavour in Gunung Reng, Jeli, Kelantan's captive deer facilities holds great promise in formulating proactive measures to mitigate disease transmission, bolster biosecurity practices, and safeguard the health of these captive populations.

Beyond its immediate applications, a study focused on identifying bacteria within Gunung Reng, Jeli, Kelantan's captive deer population carries broader significance. It contributes to our understanding of host-microbe interactions, microbial ecology, and the intricate world of wildlife microbiology. Moreover, this research serves as a platform for fostering collaborations among researchers and wildlife experts, offering a foundational benchmark for future comparative studies, and advancing the scientific knowledge base concerning captive deer populations and their complex microbial communities. In sum, such a study holds the potential to drive progress in wildlife health management and conservation efforts while shedding light on the intricate microbial dynamics at play within these captive ecosystems.

CHAPTER 2

LITERATURE REVIEW

2.1 Gut Microbiota in Deer Captive

The complex intestinal microorganism system that inhabits the mammalian gut consists of a large number of microorganisms. According to Nagy-Szakal and Kellermayer (2011), the animal gut is a complex ecosystem that is in a state of dynamic equilibrium based on the interaction of host variables, nutrition, and gut microbiome. Many investigations have shown how intricately the gut microbiota interacts with the host. For example, microecological balance and gut health can be influenced by environment, nutrition and host health (Waite et al., 2014 & Kers et al., 2018).

Recent investigations show that the gut microbiota is not only part of the host, but also has a significant influence on the health of the host, for example, improving immunity, digestion, metabolism, and enteroendocrine (Nicholson et al., 2012). Abundant herbivore foods require a specialized microbiome for disintegration and digestion (Dearing & Kohl., 2017, St-Pierre & Wright., 2013). At the same time, the complex and flexible gut microbiota micro-ecosystem can be influenced by various environments and host genotypes (Benson et al., 2010). For example, with changes in diet, the function, diversity, and relative abundance of certain microbes in the gut microbiota can change, and diet-induced loss of microbial function and diversity can increase the risk of diversity loss and extinction through amplification over generations (Zhang et al., 2009 & Ge et al., 2018).

However, according to the International Union for Conservation of Nature (IUCN) in 2008, both *R. unicolor and R. timorensis* (Figure 2.1) have been categorized as species that are vulnerable to extinction (Hedges et al., 2015).

Figure 2.1 *R. timorensis* kept in captivity for entertainment purposes at Gunung Reng, Jeli

2.2 Microbiome Dynamics in Captive Wildlife of Implications for Health and Welfare

Due to extensive human manipulation of the animals'-built environment, feeding, healthcare, and social interactions, wild animals kept in captivity are subjected to settings and lifestyles that they would not encounter in their native habitat (Carthey et al., 2020). Some species' longevity and welfare can be increased by these new surroundings (Mason, 2010), but other species experience severe health issues when kept in human environments and cared for by humans. Numerous health difficulties, such as challenges with metabolism and digestion (Amato et al., 2016), infections (Ushida et al., 2016) & Roth et al., 2019), stress (Yan et al., 2021), and reproduction (Tubbs et al., 2016), have been linked to captivity. For example, *R. timorensis* that live in the captivity at Gunung Reng (Figure 2.2) may have microbiota in gut that can negatively impact their health due to impractical lifestyle. The water they drink may have been contaminated with their defecate and at the same time, it can cause stress to *R. timorensis*.

Figure 2.2 *R. timorensis* drinking contaminated stagnant water in captive deer at Gunung Reng, Jeli

The microbiome has been considered as a mediator of host state in captivity, reflecting its expanding significance in other disciplines of organismal research (Bahrndorff et al., 2016).

Different animal reactions when kept in captivity may also be influenced by microbial plasticity or diverse microbial sensitivity. The gut microbiome is known to be influenced by diet and environment (Carmody et al., 2015 & Scepanovic et al., 2019), with subsequent impacts on the host's nervous, immunological, and metabolic systems in both human and animal hosts (Heijtz et al., 2011). However, not all hosts will experience physiological impacts as a result of the plasticity of the microbiome (Kohl et al., 2014 & Greene et al., 2019) and not all microbiomes will react similarly or to the same extent when exposed to new diets or living arrangements. There is no one type of domesticated gut microbiota, although domestication has been demonstrated to regularly affect it (Reese et al., 2021). Domestication-related microbial alterations can be attributed to diet, captive environments, and evolutionary change. For example, wild horses maintained in zoos have more gut microbial resemblance to domesticated horses than do wild animals in their natural habitat (Metcalf et al., 2017).

2.3 Factors Influencing Gut Microbiome Changes in Captivity

Although it is obvious that moving from the wild to captivity can result in changes to the gut microbiome, the ecological parameters altered in confinement that particularly result in microbiome shifts are frequently yet unknown. Natural diets and captive diets might differ significantly (Clayton et al., 2018), and it has been demonstrated that diet has a significant impact on the gut bacteria composition of caged animals (Bragg et al., 2020; van Leeuwen et al., 2020 & Martínez-Mota et al., 2019). As shown in captive sifakas, even small changes in diet can cause changes in the microbial composition (Greene et al., 2018). This suggests that environmental factors other than nutrition can potentially be important. Additionally, the gut microbiome of caged animals might differ between holding facilities due to microbial dissemination from abiotic environmental substrates such as water, soil and plants, as well as via contact with conspecifics or other species including humans (Narat et al., 2020). While human handling has been connected to the movement of microorganisms between mouse cages, Komodo dragons, for instance, have gut bacteria that are also present in their enclosures (Hyde et al., 2016). Animal social networks have a significant impact on the gut microbiome in the wild, probably changing the microbiome by physically transferring germs from one person to another (Perofsky et al., 2017 & Tung et al., 2015).

Interaction with people provides extra stressors that are specific to the captive setting and may have an impact on the microbiome. The most notable result of antibiotic use is the rapid removal of significant portions of the wild microbiota, which lowers the natural flora of the gut and may possibly increase the effects of captivity (Willing et al., 2011). By influencing the immune system, isolation from natural infections such helminths through anthelminthic medication or improved hygiene might cause alterations to the microbiome (Eleftheriou, 2021; Leung & Loke, 2013). Additionally, direct physical contact with people, such as that of keepers or guests, has the potential to both directly introduce bacteria associated with humans (Clayton et al., 2016 & Houtz et al., 2021) as well as to trigger stress, which in turn has the potential to change the microbial composition (Murakami et al., 2017).

2.4 Captivity Induced Changes in Gut Microbial Composition Impact Microbiome Function and Host Health

The composition and diversity of the gut microbiome can vary in captivity, however characterising these two metrics is insufficient to establish if the gut microbiome contributes to the health of captive animals because they do not reveal whether bacterial functions alter (Reese & Dunn, 2018). There are currently few studies that examine the composition and functional ability of the microbiome in the wild and in captivity. A few of them have performed functional metagenomic sequencing, and the majority of these have anticipated functionality using reference genomes connected to taxa represented in 16S rRNA gene sequencing data (Cheng et al., 2015 & Borbón- García et al., 2017). However, (Sun et al., 2019) for a situation in which no functional change was expected. Most of these studies demonstrate that predicted function differs between captive and wild microbiomes, and they argue that the altered functional capability of the bacterial community may have an actual impact biology. Alterations in functional capacity, however, may not always signify alterations in bacterial activity or host physiology (Reese & Kearney, 2019).

However, there have been certain occasions where the microbial composition and health outcomes in caged animals have been more closely linked. Numerous studies have linked the nature of the gut microbiome to particular disease phenotypes in animals kept in captivity, such as chronic wasting disease in deer (Minich et al., 2021), digestive disease in wolves (Bragg et al., 2020), and iron overload condition in rhinoceroses (Roth et al., 2019). That research offers evidence in favour of a functional link between microbial composition and health effects in at least some situations. faecal microbiota transplantation (FMT) of a wild microbiota community has been used in mammals to successfully reduce inflammation and improve survival against infectious diseases (Rosshart et al., 2017) and expand dietary niche (Blyton et al., 2019), supporting the idea that the wild microbiome in particular can be beneficial. The generalizability of these results, however, is still unknown because it has not always been specifically studied whether microbial alterations could result in positive health consequences. Although the inconsistent results of FMTs and nutritional interventions in agricultural contexts underline the possibility that an altered microbiota would not cure all diseases, microbial manipulations in captive animals have also been employed to try to address health concerns (Clemmons et al., 2018).

CHAPTER 3

METHODOLOGY

3.1 Study Area

This study was executed at Gunung Reng, Jeli, Kelantan (5.7158° N, 101.7444° E). The location is close to Kampung Batu Melintang which is on the East-West Highway (Gerik- Jeli), at a distance of about 15 km from Jeli (Figure 3.1). Gunung Reng is a leisure area located near the Pergau River, next to An-Nur Mosque. This location has distinct historical significance and has become famous among locals and visitors from outside the region.

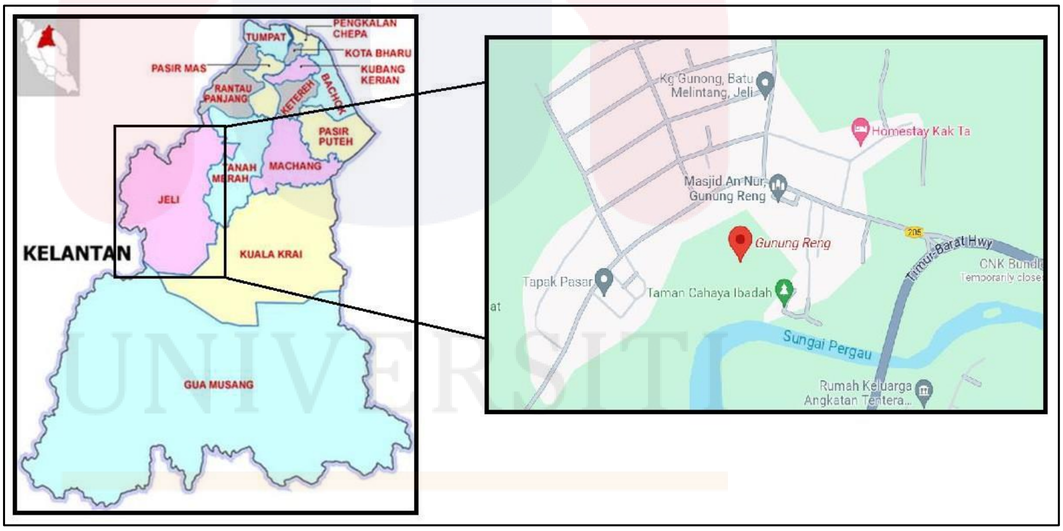

Figure 3.1 Sampling area to collect faecal samples *R. timorensis* which is located at Gunung Reng, Jeli District, Kelantan (Kamaruzzaman et al., 2016)

There are many interesting activities which is play at a dip in a shallow and clean river water, hiking the mountain or caving the caves like Gua Harimau and Gua Gajah. Moreover, inside the confines of the Gunung Reng cave, a large number of over 1000 bats may be seen coming from the cave during dusk. Additionally, several of *R. timorensis* are kept inside cages surrounding the Gunung Reng area, for entertainment purposes.

3.2 Sample Collection

Firstly, Javan *R. timorensis* (Javan Rusa) captive was observed at Gunung Reng, Jeli (Figure 3.1). In this research, faecal samples were gathered using disposable gloves and zip lock bag was used to store the sample. The zip lock bag need to be in clean condition to avoid any contaminant from outside. During the transportation to the laboratory, the sample has ensured remain well-preserved by using cold storage and insulated containers with ice pack. The faecal sample need to keep cool, ideally 4°C, to slow microbial growth and maintain sample integrity.

3.3 Serial Dilution of *R. timorensis* faecal

The sample of *R. timorensis* faecal was brought to the Microbiology and Biochemistry Laboratory, the Faculty of Earth Science (FSB) at Universiti Malaysia Kelantan, Jeli Campus. Through successive re-suspension of an initial solution (solution 0) into fixed volumes of a liquid diluent (blanks). These blanks usually consist of 0.45% saline, although the composition can be varied (Sanders, 2012). Any volume for each diluent can be choose, but it is most often a multiple of 10, facilitating logarithmic reduction of the sample. Solution 0 contains a total of stock dilution cells (faecal) was suspended in 10 ml of distilled water. Solution 0 of 1 ml was removed and was added to 9 ml of saline (diluent 1), the new solution (solution 1) was contained 1/10th of the initial concentration of stock dilution. The new solution (solution1) contained 10 stock dilution cells. After that, another 1 ml of solution 1 was removed and added into another 9 ml of saline (diluent 2) will yield solution 2, containing only a single stock dilution. After that, the process was repeated until 3- fold serial dilution since each new solution (9 ml of diluent + 1 ml of solution) includes a total of 10 ml. (Solution 1, Solution 2 and Solution 3 (Figure 3.2).

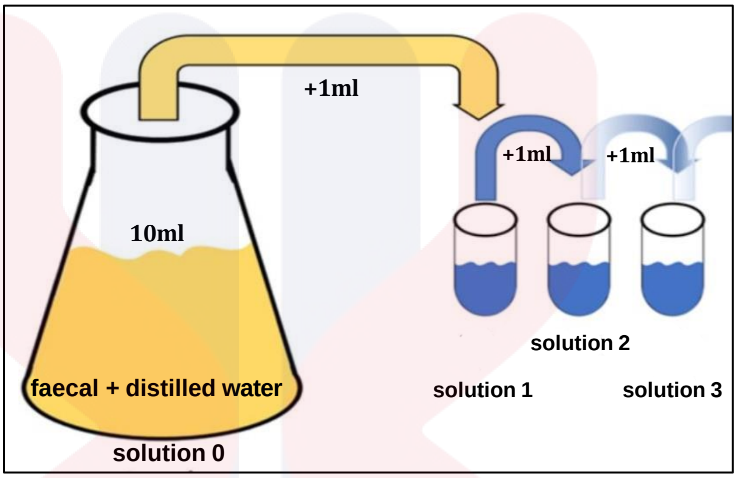

Figure 3.2 serial dilution of stock solution to prepare (solution 1, solution 2 and solution 3)

3.4 Preparation of Different Agar Media for the *R. timorensis* Gut Microbial Analysis

Six different agar media was used in this study, which is *Lactobacillus* MRS agar, Malzextrakt Agar, Nutrient Agar, Potato Dextrose Agar, Simmon Citrate Agar and Sulfate API Agar.

MRS Agar was prepared using 70g of *lactobacillus* MRS Agar (HiMedia, India) and distilled water was added with one litter. *Lactobacillus* MRS Agar was dissolved and sterilize in an autoclave (TerasLab Saintifik, Malaysia) at 121°C for 15 minutes. After the media is autoclaved, it will be allowed to cool to 45–50°C. The mixture then be poured into sterilized Petri plates, and it was settled to create a firm medium. The liquid media or prepared plates was kept refrigerated (De MAN et al.,

1960) in the Microbiology and Biochemistry Laboratory, Faculty of Earth Science (FSB), Universiti Malaysia Kelantan (UMK), Jeli Campus, at 2°C to 8°C. The method was repeated for the other agar.

3.5 Culturing of faecal Microbiota from *R. timorensis* on Different Agar Media

For this section, media that was prepared in section 3.4 was used to inoculate the samples diluted in section 3.3 sterilization. Using micropipette, 200µl of Solution 1 was transferred onto MRS agar and was spread using a L-shaped spreader. Before spread, L-shaped glass spreader was dipped in alcohol. Then, the glass spreader was flamed over a Bunsen burner. After that, the sample was spread evenly over the surface of MRS agar using the sterile glass spreader (Aryal, 2022). Then, the spreader was moved back and forth across the plate as the turntable is spinning (Figure 3.2). Lastly, before inverting the plate for incubation, the sample was allowed at least five minutes to absorb thoroughly (Sanders, 2012). Following this preparation, all the prepared agar plates was incubated 24 hours day at 37°C in an incubator (Memmert, Germany).

All these steps were done in a clean laminar flow hood in Microbiology and Biochemistry Laboratory. The similar procedure was repeated for all the solutions (Solution 2, Solution 3) and also for all the media prepared (*Lactobacillus* MRS agar, Malzextrakt Agar, Nutrient Agar, Potato Dextrose Agar, Simmon Citrate Agar and Sulfate API Agar). In total, for each media, three different diluted solutions was cultured and duplicates were prepared and labeled accordingly (Solution 1a and Solution 1b).

Table 3.1 Culture and duplicates for each media of three different diluted solutions

Agar	Data label	Dilution
***Lactobacillus* MRS**	Solution 1A (MRS Agar)	
	Solution 1B (MRS Agar)	10^{-1},10^{-2},10^{-3}
Malzextrakt	Solution 2A (MA)	
	Solution 2B (MA)	10^{-1},10^{-2},10^{-3}
Nutrient	Solution 3A (NA)	
	Solution 3B (NA)	10^{-1},10^{-2},10^{-3}
Potato Dextrose	Solution 4A (PDA)	
	Solution 4B (PDA)	10^{-1},10^{-2},10^{-3}
Simmon Citrate	Solution 5A (SCA)	
	Solution 5B (SCA)	10^{-1},10^{-2},10^{-3}
Sulfate API	Solution 6A (API Agar)	
	Solution 6B (API Agar)	10^{-1},10^{-2},10^{-3}

Table 3.2 The abbreviations and Full Names of Common Agar Media Used in Microbiology

Indicator	
MA	**Malzextrakt Agar**
NA	Nutrient Agar
PDA	Potato Dextrose Agar
SCA	Simmon Citrate Agar

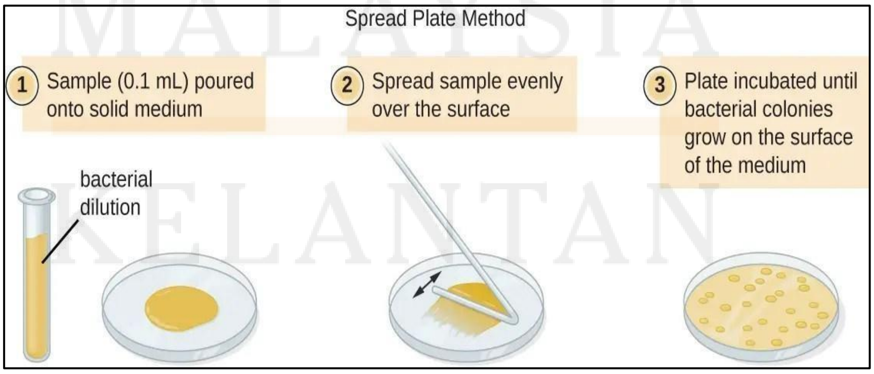

Figure 3.3 Spreading method to analyze microbial activity (Rijal and Rijal, 2022)

3.6 Morphology Identification through Microscopy Technique and Colony-Forming Units (CFU) Counting

Microscopes was used for the identification of microorganisms based on their morphology. Initially, a clean, dry glass slide was covered with a smeared layer of the pure culture microorganisms grown on each of the six different media. A Bunsen burner was used to heat the smear. After applying crystal violet, the slide was left to stand for one minute. After that, distilled water was used to rinse the slide. After applying Gram's Iodine to the slide, it was left for a minute. After that, distilled water was used once again to rinse the slide. Subsequently, the slide was cleaned with distilled water and 1-2 drops of acetone applied. After applying safranin and letting it left for a minute, the slide was cleaned with distilled water. Finally, the slides was examined under a light microscope after being allowed to air dry (Ku Hasan, 2017).

Colonies were counted to find the colony-forming units (CFU) on the agar plate when the incubation period was over. By multiplying the colony count by the appropriate dilution factor, the CFU value of the sample may be computed (Figure 3.3), providing information on the concentration of viable bacteria in the original sample (Aryal, 2022).

$$\text{CFU / ml} = \frac{\text{Total number of colonies obtained} \times \text{dilution factor}}{\text{Volume of specimen used (aliquot)}}$$

Figure 3.4 Colony Forming Units (CFU) formula (Aryal, 2022)

CHAPTER 4

RESULT AND DISCUSSION

4.1 Microbial Diversity Assessment in Male and Female Deer Fecal Samples Using Spread and Streak Plate Techniques

In this study, the spread plate and streak plate technique were used. Each of the six types of agars represented three dilutions of 10^{-1}, 10^{-2}, and 10^{-3}. Initially, 1 gram of deer faecal was dissolved in distilled water using a vortex mixer. Subsequently, 1ml of the solution was transferred into 9 ml of saline in the first test tube. Then, 1ml of the solution from the first test tube was transferred into the second test tube, and finally, 1ml of the second solution was transferred into the third test tube.

After the three test tubes been shaken, 1ml of the solution was transferred onto each agar plate representing the three dilutions using a micropipette calibrated to 1000µL. Subsequently, the solution on each agar plate was spread using an L-shaped spreader to flatten the surface. Once all solutions were transferred on the agar plates and been spread, each agar plate was placed in incubation to encourage bacterial growth. After 24 hours, numerous colonies of various shapes and sizes were observed on the agar plates from male faecal samples (Figure 4.1) and the characteristics of colony is provided in Tables 1. While Figure 4.2 are numerous colonies of female faecal sample that grow on agar plate and the colony attributes at Table 2.

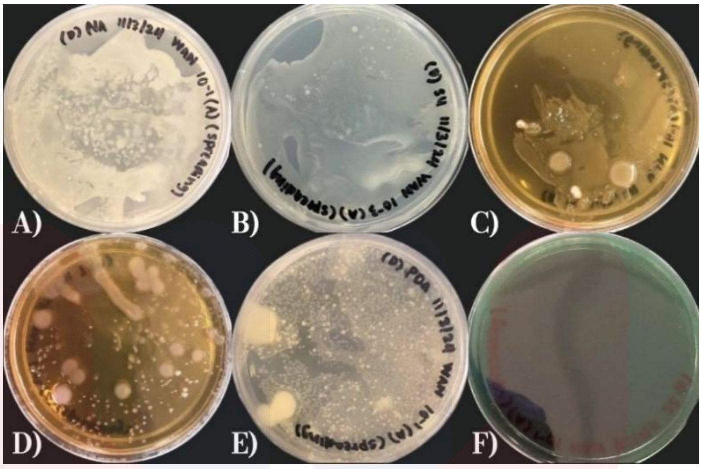

Figure 4.1 The colonies of bacteria that grow on six types of agars from male faecal

Table 4.1 Colony attributes of different agar types at various dilutions from male deer faecal

	Types of agars	Colony attributes
A)	Nutrient 10^{-1} (A)	Margin: Undulate Elevation: Raised Shape: Circular Colour: White Smell: Strong smell
B)	Sulphate API 10^{-3} (A)	Margin: - Elevation: Flat Shape: Circular Colour: White Smell: Strong smell
C)	Malzextrakt 10^{-1} (A)	Margin: - Elevation: Raised Shape: Circular Colour: White Smell: Strong smell
D)	Lactobacillus MRS 10^{-2} (A)	Margin: - Elevation: Convex/ Raised Shape: Circular Colour: White Smell: Strong smell

E)	Potato Dextrose 10^{-1} (A)	Margin: - Elevation: Convex Shape: Circular Colour: White Smell: Strong smell
F)	Simmon Citrate 10^{-1} (A)	Margin: - Elevation: - Shape: - Colour: - Smell: Strong smell

Figures 4.1 and Figures 4.2 show the growth of colonies on each agar according to elevation, shape, colour and margin. However, colony growth cannot be considered at the end of its growth because it is determined by where the colony should grow (Wimpenny, 1988). The characteristics of the colonies in Figures 4.1 and 4.2 can be found in Table 4.1 and Table 4.2, which respectively describe the shape of each colony growing on each type of agar. Other tests, such as metagenomics, are needed to gain a deeper understanding of the colony's taxonomy. However, in this study, only gram staining and microscopic techniques were used to identify the type of bacterial form.

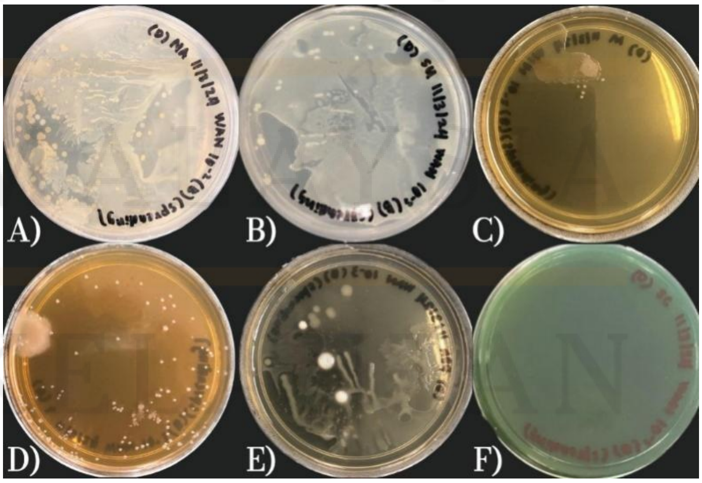

Figure 4.2 The colonies of bacteria that grow on six types of agars from female faecal

Table 4.2 Colony attributes of different agar types at various dilutions from female deer faecal

	Types of agars	Colony attributes
A)	Nutrient 10^{-2} (B)	Margin: Filiform/ Lobate Elevation: Raised/ Convex Shape: Circular/ Irregular Colour: Yellow Smell: Strong smell
B)	Sulphate API 10^{-3} (B)	Margin: - Elevation: Raised Shape: Circular Colour: Yellow Smell: Strong smell
C)	Malzextrakt 10^{-2} (B)	Margin: - Elevation: Convex/ Raised Shape: Circular Colour: White Smell: Strong smell
D)	Lactobacillus MRS 10^{-3} (B)	Margin: - Elevation: Convex Shape: Circular Colour: White Smell: Strong smell
E)	Potato Dextrose 10^{-3} (B)	Margin: - Elevation: Convex/ Raised Shape: Irregular/ Circular Colour: White Smell: Strong smell
F)	Simmon Citrate 10^{-3} (B)	Margin: - Elevation: - Shape: - Colour: - Smell: Strong smell

4.2 Comparative Analysis of Bacterial Colony Morphology on Nutrient and Sulfate API Agar from Male and Female faecal Samples

i. Nutrient agar

When bacteria grow on a solid media such as nutrient agar, they show a variety of physical attributes that are typically referred to as the morphology of the colonies. To distinguish and identify distinct species of bacteria, these characteristics are essential. Based on Figure 4.1(A) and Figure 4.2(A), respectively from male faecal and female faecal, there is a significant difference with the margin, elevation, shape and colour that appear on both of colonies growth.

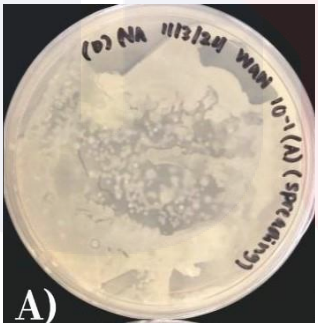

Figure 4.1(A)

Figure 4.1(A) Colony growth on Nutrient agar from male faecal

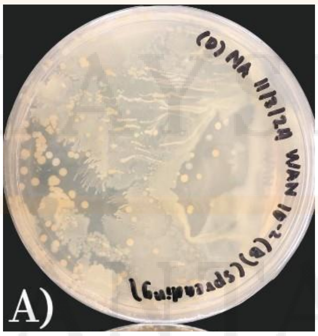

Figure 4.2(A)

Figure 4.2(A) Colony growth on Nutrient agar from female faecal

As can see above, the characteristic between both of the Figure 4.1(A) and Figure 4.2(B) were different where the colonies from female faecal (Figure 4.2)(A) were way more than colonies from male faecal (Figure 4.1)(B). From male faecal, the shape that were found is circular, which is it is round and symmetrical, undulate margin and the elevation is raised. While from female faecal, it shows circular, irregular, rhizoid and filamentous shape. Other than that, the margin are filiform and lobate and the elevation are raised and convex. The size of both colonies on agar are large which can be describe the significant portion covered the whole agar plate.

The higher number of microbial colonies observed in female faecal samples on Figure 4.2(A) on nutrient agar compared to male samples (Figure 4.1)(A) can be explained by several factors which is diet. Males and females often exhibit different dietary habits that in turn affect the composition of their gut microbiota. According to Mueller et al. (2006), dietary differences are important factors influencing gut bacteria, and women generally consume more fibrous foods that support a diverse microbial environment.

In wild deer, females exhibit greater microbiota diversity than males. Gender-related changes in microbial communities can be associated with hormones (Fraser et al., 2003). For instance, female growth hormone levels during pregnancy can influence the faecal microbiota. Additionally, reproductive hormones have been connected to sex-related and gut microbial variations in wild animals (Sun et al., 2023). Increasing evidence indicates a link between sex steroid hormone levels and human gut microbiota (Dominianni et al., 2015), (Flores et al., 2012). Edwards et al. noted that estrogen and progesterone impact intestinal function. Haro et al. (2016) highlighted that the higher bacterial diversity in female significantly contributes to the observed differences in colony counts.

ii. Sulfate API agar

Sulphate API agar is specifically designed for the cultivation of sulfate reducing bacteria (SRB). Sulfate-reducing bacteria (SRB) are of great ecological importance in the mineralization of organic matter in anaerobic environments (Vester & Ingvorsen, 1998). For example, in marine sediments up to 50% of organic matter can be oxidized through sulfate reduction (Jørgensen, 1982). In some low-sulfate environments, in addition to being found in freshwater lakes and salt marshes, it can also be found in the human body, especially in the gut microbiota (Heggendorn et al., 2014). Based on Figure 4.1(B) and Figure 4.2(B), respectively from male and female deer faecal, as can see below, there are colonies growth that appear on the agar plate.

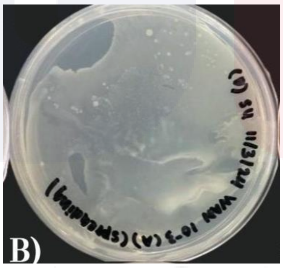

Figure 4.1 (B)

Figure 4.1(B) Colony growth on Sulfate API agar from male faecal

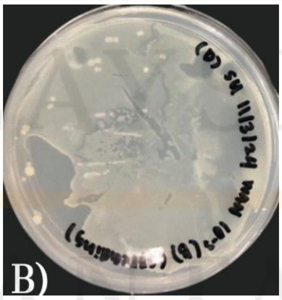

Figure 4.2 (B)

Figure 4.2(B) Colony growth on Sulfate API agar from female faecal

The characteristics that can be seen between both of the Figure 4.1(B) and Figure 4.2(B) have a same attribute where the size of colony are big and it closely to fill the agar plates. From Figure 4.1(B), the shapes that appear on the agar plates are circular and have a flat elevation. Figure 4.2(B) the shape that grow are also in circular but have a raised elevation. Sulfate-reducing bacteria (SRB) are strictly anaerobes that thrive in environments with ideal temperatures of 25–44°C and pH ranges of 5.5–9.0. At present, there exist more than 20 prominent taxa, including but not limited to *Desulfovibrio*, *Desulfomonas*, *Desulfotomaculum*, *Desulfolobus*, *Desulfobacter*, *Desulfococus*, and *Desulfosarcina* (Heggendorn et al., 2014). Beside that, the species of *Desulfovibrio desulfuricans* are often detected in intestinal microbiota (Bozo-Hurtado et al., 2013).

4.3 Morphology of Bacteria

Bacterial morphology is the study of the shape, size, arrangement, and structural characteristics of bacteria. These aspects of microbiology are critical to identifying and classifying bacteria, as well as understanding their ecological role and pathogenic mechanisms such as *Salmonellae*, *E Coli*, and *Vibriones* (Prabakar et al., 2010). Bacteria come in a variety of shapes, including spheres (cocci), rods (bacilli), and spirals (spirilla) (Mohamad et al., 2014), each adapted to a particular environment and function. As can see below, there are characteristics of bacteria that can be seen using microscopic techniques.

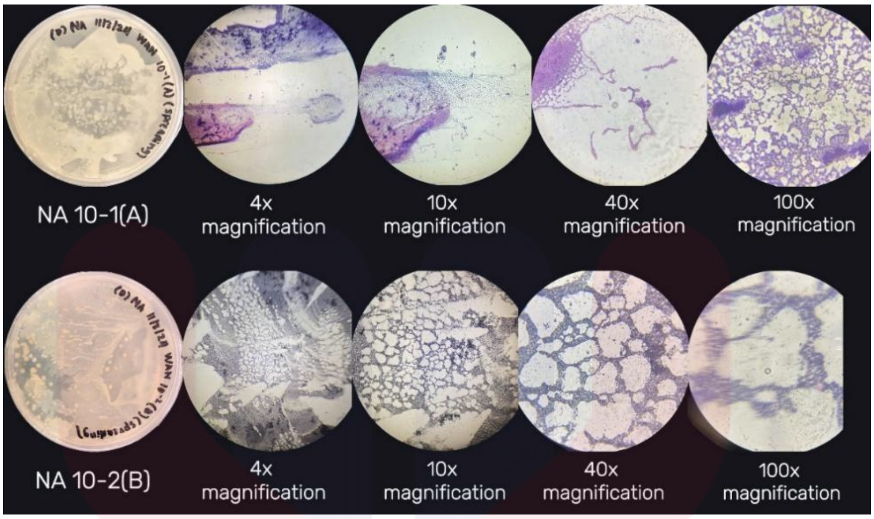

Figure 4.3 Structure of bacteria under microscope on Nutrient agar (NA) 10^{-1}(A) from male faecal and on Nutrient agar (NA) 10^{-2}(B) from female faecal

Based on Figure 4.3 on Nutrient agar (NA) 10^{-1}(A) which are from male faecal, the characteristics that can be seen under oil immersion 100x magnification are gram positive cocci (spherical) which is coccus, diplococci, tetrad and streptococci. While for NA 10^{-2}(B) which are from female faecal, under 100x magnificent, there are positive cocci (spherical) which is coccus and staphylococci. The similar form of bacteria can also be seen on Figure 4.4 below.

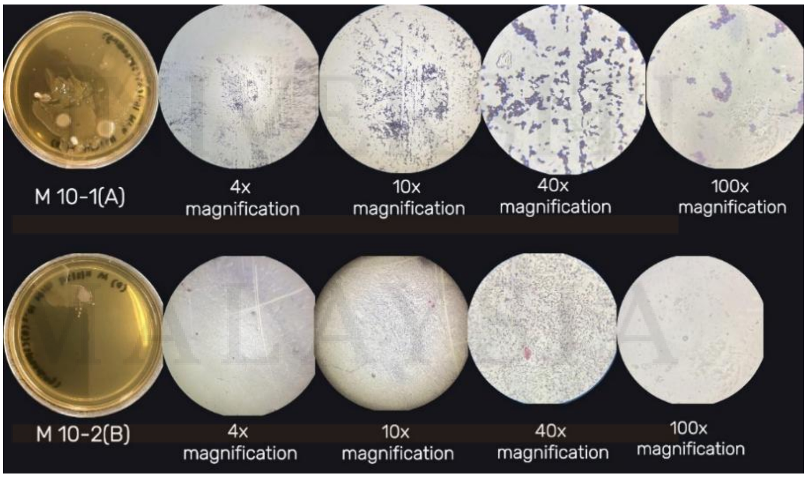

Figure 4.4 Structure of bacteria under microscope on Malzextrakt agar (M) 10-1(A) from male faecal and on Malzextrakt agar (M) 10-2(B) from female faecal

Under microscope in oil immersion 100x of magnification Figure 4.4 on Malzextrakt agar (M) 10^{-1}(A),which is from male faecal, it can be seen the gram is positive cocci (Spherical) which is coccus, diplococci, tetrad and streptococci. But, on Malzextrakt agar (M) 10^{-2}(B), which from female faecal, the shape of form that can be found in oil immersion 100x of magnification are negative cocci (spherical), which is coccus and tetrad.

Basically, to describe more about shape of cocci bacteria that have been mentioned above, cocci bacteria can appear as single cells, in pairs, in groups of four, in chains, in clusters, or in cubes of eight cells (Mohamad et al., 2014). These types of structures include monococci, which are single spherical cells, and diplococci, which are pairs of spherical cells that are frequently associated with specific bacterial infections. Streptococci, form chains of spherical cells and are commonly associated with streptococcal infections. Staphylococci have a distinct morphology characterized by clusters of spherical cells resembling grape bunches, as seen in Staphylococcus infections. Tetrads are also made up of groups of four spherical cells, whereas sarcinae are made up of cubes of eight spherical cells, which is less common but important in certain microbial contexts. Other than that, the other types of bacteria form can be viewed under Sulphate API agar (SU) (Figure 4.5), Lactobacilli MRS agar (L) (Figure 4.6) and Potato agar dextrose (PDA) (Figure 4.6).

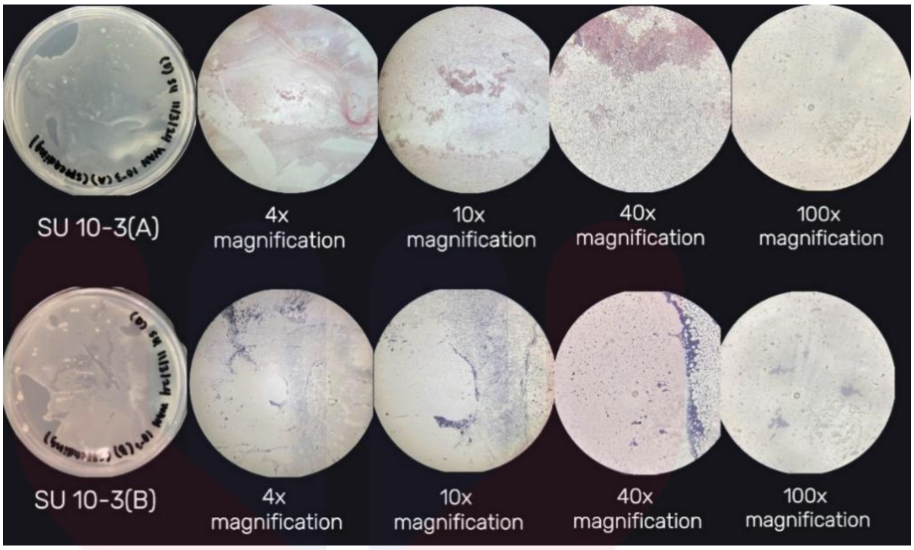

Figure 4.5 Structure of bacteria under microscope on Sulphate API agar (SU) 10-3(A) from male faecal and on Sulphate API agar (SU) 10-3(B) from female faecal

Regarding to Figure 4.5 on Sulphate API agar (SU) 10-3(A), from male faecal, under microscope in immersion oil at 100x of magnification are negative bacilli (rods), which are coccobacillus, bacillus, diplobacilli and streptobacilli. While for Sulphate API agar (SU) 10-3(B), from female faecal, it can be seen at 100x of magnification the gram positive of bacilli (rods) which are also contain *coccobacillus*, bacillus, streptobacilli and palisades structure. For *Lactobacillus* MRS agar, it have the same shape of structure of bacilli (rods) (Figure 4.6) below.

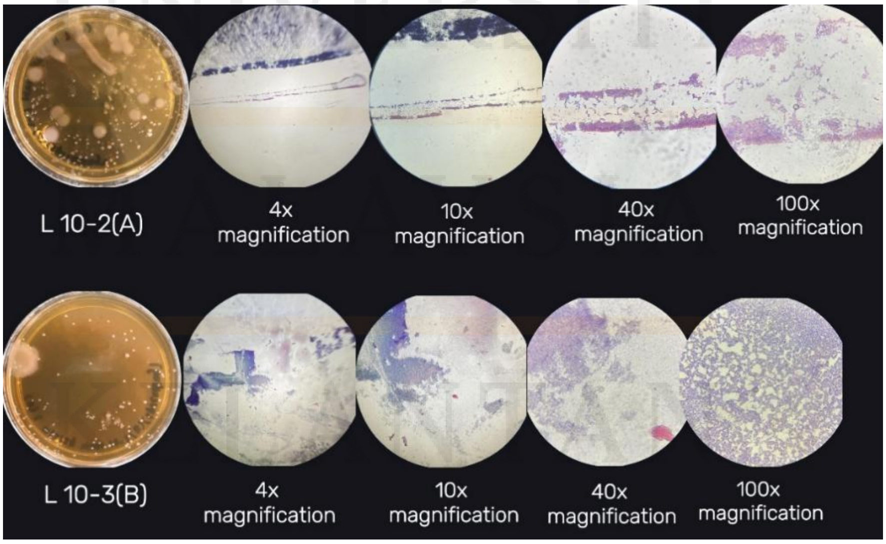

Figure 4.6 Structure of bacteria under microscope on Lactobacillus MRS agar (L) 10-2(A) from male faecal and on Lactobacillus MRS agar (L) 10-3(B) from female faecal

Based on Figure 4.6 on Lactobacillus MRS agar (L) 10^{-2}(A), from male faecal, in immersion oil at 100x of magnification, there are a positive bacillus (rods) such as coccobacillus, bacillus, diplobacilli and palisades. While from female faecal, (L) 10^{-3}(B), the shape that can seen is also positive bacilli (rods), which are at 100x of magnification, under microscopic, it can be seen coccobacillus, bacillus and streptobacilli. At Potato Dextrose agar (PDA) 10^{-3}(B) (Figure 4.7), from female faecal are positive bacilli (rods) also found at the 100x of magnification in oil immersion, which are contain coccobacillus, bacillus, streptobacilli and diplobacilli. While, at PDA 10^{-1}(A), from male faecal, the other shape of bacteria appear as positive spirochetes (spiral) such as vibrios and spirochete in oil immersion at 100x of magnification. However, . However, it is possible for bacteria to grow on PDA because the media has a low pH value. The cultures had an optimal pH of 3.5–4.0 for growth (Spllttstoesser et al., 1994).

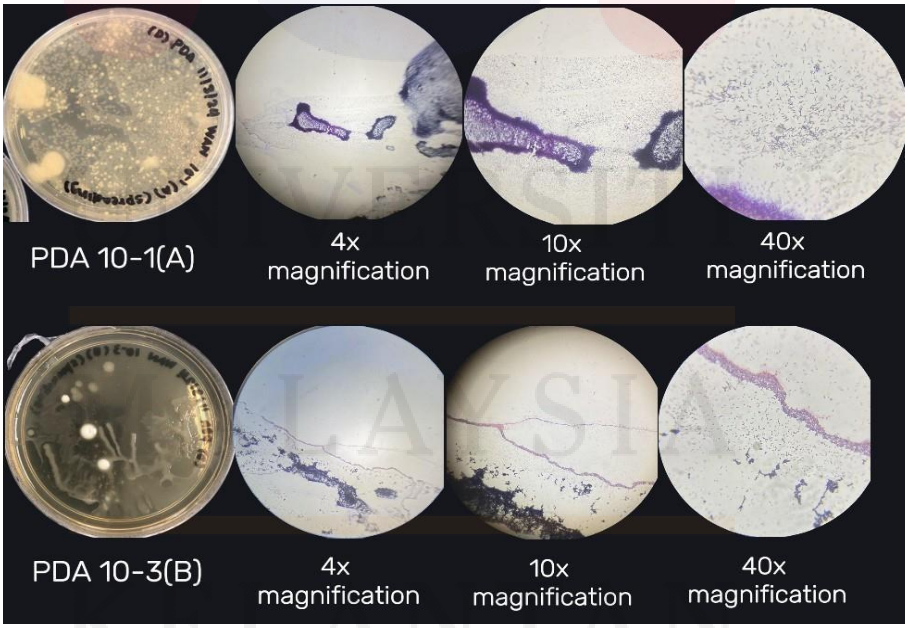

Figure 4.7 Structure of bacteria under microscope using 4x, 10x and 40x of magnification on Potato Dextrose agar (PDA) 10-1(A) from male faecal and on Potato Dextrose agar (PDA) 10^{-3}(B) from female faecal

Bacilli display a diverse morphological arrangement, usually showing a less complex pattern than cocci. Most often, it occurs as a single cell or in pairs. (Tankeshwar & Tankeshwar, 2023). This arrangement consists of a single bacillus in which individual rod-shaped cells exist independently, as seen in many bacterial species, including *Escherichia coli*. Certain bacteria, such as *Moraxella*, are characterized by *diplobacilli*, which are made up of two rod-shaped cells that remain attached after division. *Streptobacilli* are a chain of rod-shaped cells formed through successive divisions in one plane. Palisades are made up of rod-shaped cells aligned in a parallel or V-shaped pattern, which are often the result of fractured divisions. Coccobacilli are short, oval-shaped rods that fall somewhere between cocci and bacilli. Finally, filamentous bacilli form chains of rod-shaped cells that extend like threads to create an intricate network.

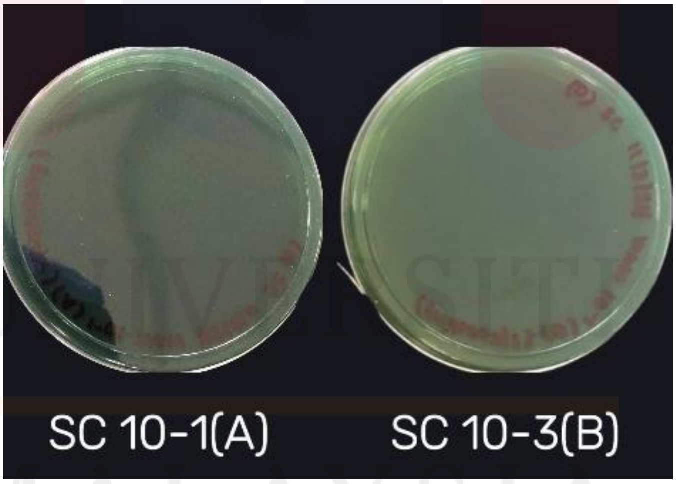

Figure 4.8 No change of colour occur on both of the Simmon Citrate agar (SC) 10^{-1}(A) from male faecal and 10^{-3}(B) on female faecal

Simmons Citrate Agar contains ammonium dihydrogen phosphate, the sole source of nitrogen, and sodium citrate, the only source of carbon. Organisms that can use ammonium dihydrogen phosphate and citrate will grow freely on this medium. Bromothymol blue acts as a pH indicator, causing the medium to change from green (neutral) to blue (alkaline) as the pH increases. Citrate utilization creates an alkaline carbonate, causing a deep blue colour to change in the agar. The medium will remain green if organisms are unable to metabolize sodium citrate (MacWilliams, 2009).

4.4 Role and Impact of Gastrointestinal Microflora in Animal Nutrition

Animals are classified according to their own digestive physiology and the microorganisms in the gastrointestinal tract can be examined according to the location of the intestine, which is through the foregut (before the gastric stomach), midgut (small intestine) and hindgut (large intestine) (Stevens & Hume, 1998). Most microorganisms live in communities in the gastrointestinal tract. This gastrointestinal microflora is known to play a nutritional role in ruminants, pseudoruminants, and monogastric mammals, with only moderate or no foregut fermentation but extensive hindgut fermentation in the blind cecal pouch (Savage, 1986).

Microflora in adult animals hydrolyze both exogenous (dietary) and endogenous polymers, supplying all or most of the adult's carbon, energy, vitamins, and macromolecular building blocks. Flora also conserves nitrogen, which would otherwise be released as urea. In exchange, flora competes directly with host tissues for nutrients consumed through diet, and also indirectly by slightly suppressing animal tissues' absorptive capacity (Savage, 1986). When the synergy is balanced, animal tissues and microflora work together to promote the host's overall health and nutritional well-being. However, this system may be disrupted by antibacterial drugs that destroy microflora and diseases in animal tissues that destroy the controls that regulate where indigenous communities are located in the tract, their microbial composition, and their biochemical activity. At such times, animal tissue nutrition can be severely compromised.

4.5 The Role of Gram-Positive and Gram-Negative Bacteria in Gut Health and Disease

Gram-positive bacteria, such as *Lactobacillus, Clostridium*, *Enterococcus,* and *Ruminococcus,* play an important role in the gut microbiome (Rajilić-Stojanović & de Vos, 2014). One of the most common diseases caused by Gram-positive bacteria is Clostridium difficile infection (CDI). This condition occurs when the normal gut flora is disrupted, which is usually caused by antibiotic use. This disruption allows C. difficile to proliferate and produce toxins, resulting in colitis and severe diarrhea (Leffler & Lamont, 2015). CDI symptoms include watery diarrhea, fever, loss of appetite, nausea, and abdominal pain. Treatment entails discontinuing the antibiotic that caused the problem, administering specific antibiotics such as vancomycin or fidaxomicin, and, in severe cases, faecal microbiota transplantation (FMT) (McDonald et al., 2018).

SIBO, or Small Intestinal Bacterial Overgrowth, is another condition associated with Gram-positive bacteria. This happens when there is an overgrowth of bacteria in the small intestine, including Gram-positive types such as *Lactobacillus* and *Enterococcus.* This can impair nutrient absorption and result in symptoms such as bloating, diarrhea, abdominal pain, and malnutrition. Antibiotics such as rifaximin, dietary changes, and probiotics are commonly used to help restore a healthy bacterial balance in the gut (Pimentel et al., 2020; Ghoshal et al., 2017).

In animals, the gut microbiome plays an important role in health and disease. For example, in deer, Firmicutes and Bacteroidetes dominate the gut microbiota, which aid in the digestion of complex plant materials such as cellulose (Li et al., 2015). These microbes are required for the fermentation process, which enables deer to extract nutrients from their fibrous diet. Disruptions in deer gut microbiome can cause malnutrition and other health problems, similar to those seen in humans.

Gram-negative bacteria play an important role in the gut microbiome. This group includes bacteria from the Bacteroidetes phylum (such as *Bacteroides* and *Prevotella*) and the Proteobacteria phylum (such as *Escherichia*, *Klebsiella*, and *Helicobacter*) (Ley et al., 2006; Shin et al., 2015). Inflammatory Bowel Disease (IBD) is characterized by an imbalance in the gut microbiota, which includes an increase in Gram-negative bacteria such as Escherichia coli (specifically adherent-invasive E. coli, AIEC) and a decrease in beneficial bacteria (Frank et al., 2007). Crohn's disease and ulcerative colitis are chronic inflammatory conditions that affect the gastrointestinal tract. IBD symptoms include abdominal pain, diarrhea, rectal bleeding, weight loss, and fatigue. Anti-inflammatory drugs, immunosuppressants, biologics, and dietary changes are among the treatments for inflammatory bowel disease (IBD).

Irritable Bowel Syndrome (IBS) is another condition caused by a gut microbiota imbalance, with elevated levels of specific Gram-negative bacteria (Crouzet et al., 2013). IBS is a functional gastrointestinal disorder that causes chronic abdominal pain and irregular bowel habits with no known cause. Dietary changes (such as a low FODMAP diet), probiotics, antibiotics such as rifaximin for bacterial overgrowth, and symptom-management medications are all used to treat IBS (Wu et al., 2024)

Colorectal cancer (CRC) has also been linked to dysbiosis, which involves high levels of Gram-negative bacteria such as *Fusobacterium nucleatum*. This bacterial imbalance could contribute to the development of cancer (Castellarin et al., 2012). Colorectal cancer symptoms include altered bowel habits, blood in the feces, weight loss, and abdominal discomfort. Surgery, chemotherapy, radiation therapy, and possibly microbiome-modulating therapies in the future are all common treatments (Brennan & Garrett, 2016).

The gut microbiome of other animals, such as ruminants like cows and sheep, demonstrates the importance of microbial balance. These animals rely heavily on gut bacteria to digest cellulose and other complex carbohydrates in their plant-based diets. Disruptions in their gut microbiota can cause ruminal acidosis, which can have serious health consequences (Hook et al., 2011).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

This study investigates the gut microbiota of captive deer (*Rusa timorensis*) at Gunung Reng, Jeli, Kelantan, with a focus on identifying and comparing the microbial communities present in captive and wild deer populations. *Rusa timorensis*, a species native to the Indonesian archipelago, has been introduced to various regions, including Malaysia, where it is valued for its adaptability and resistance to disease. The research aims to classify gut microorganisms using simple staining techniques and compare microbial classes via secondary data. Captive conditions, including diet and habitat, significantly influence gut microbiota, impacting health and adaptability. This study collected faecal samples from deer at Gunung Reng and conducted microbial analysis using various agar media and microscopy techniques. Results indicated distinct differences in microbial composition between male and female deer, with notable implications for health management and conservation efforts. Understanding gut microbiota dynamics in captivity can inform strategies to enhance animal welfare and ensure the sustainable management of captive populations.

5.2 RECOMMENDATION

Based on the study, several recommendations are suggested to improve the Gut Microbiota of Captive Deer (*Rusa timorensis*) at Gunung Reng, Jeli, Kelantan. In order to identify knowledge gaps and update the most recent findings, it is essential to first conduct a thorough literature review. A more thorough understanding of the microbiota can be obtained by increasing the sample size, gathering samples at various times, and using a variety of sample sites. The utilization of advanced analytical methods like metagenomics, metatranscriptomics and next-generation sequencing (NGS) is necessary to identify a larger variety of microorganisms and investigate their possible roles. Advanced bioinformatics tools like Mothur and QIIME can guarantee precise data analysis. Furthermore, it's essential to consider nutritional and environmental factors, analyzing deer diets in detail and keeping an eye on the environment to understand how these affect gut microbiota. Studies involving comparisons between species and between wild and captive deer populations may provide information about particular microbiota characteristics. Research can be expanded and innovative technologies can be accessed through collaboration with ecologists, veterinarians, microbiologists, bioinformaticians and universities and research facilities.

Additionally, through studying the correlation between the microbiota's composition and the health status of deer through routine health monitoring and the potential advantages of probiotics or supplements, research can be further enhanced. Contributions to the scientific community and data accessibility can be ensured by collecting sequencing data in public repositories like the NCBI's Sequence Read Archive (SRA) and publishing findings in peer-reviewed journals. Raising awareness and supporting research can be achieved through involving local stakeholders and doing educational outreach. The success of this research also depends on getting funding from governmental and non-governmental organizations and making sure that enough money has been set aside for fieldwork, laboratory supplies, and employee training. By putting these suggestions into practice, we may be able to gain a deeper comprehension of the gut microbiota of captive deer in Gunung Reng, Jeli, Kelantan, which will benefit their welfare and conservation.

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