EcoHealth survey of Herpetofauna in South Africa

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ABSTRACT

In this project, the samples were taken from reptiles' oral and cloacal cavities. The majority of samples originated from healthy-appearing captive reptiles therefore the bacteria detected in this study form part of the natural bacterial microbiome of various reptile species. Samples were taken from *Bitis arietans, Dendroaspis polylepis, Dispholidus typus, Hemachatus haemachatus, Naja annulifera, Naja mossambica, Pseudaspis cana, Python natalensis, Matobosaurus validus, Varanus albigularis, Stigmochelys pardalis,* and *Crocodylus niloticus*. Cloacal samples (n = 80) underwent growth on selective media for screening of *Salmonella*. The results showed an isolation success of 88.75 % of *Salmonella* across all samples. The use of Xylose Lysine Deoxycholate agar (isolation success of 81.25 %) had a highly significant (p = 0.017) influence on the isolation success when compared to the use of Brilliant Green agar (isolation success of 61.25 %). Multiple other factors were tested but none had a statistically significant influence of the isolation success of *Salmonella.* The oral samples (n = 53) underwent analyses of the bacterial 16S rRNA gene, obtained from Next-Generation sequencing, to determine the microbiome of the snakes sampled. A total of 706 unique taxa were detected in the oral cavity. The most abundant bacteria detected were identified as *Staphylococcus* (77.78 %) followed by *Enterobacter* (4.56 %), an unidentified genus within Weeksellaceae (3.21 %), *Alcaligenes* (2.16 %), and *Bacteroides* (1.74 %). Generally, a host species that were part of the Elapidae family had lower levels of bacterial diversity and richness when compared to species from other families. The classification of Elapidae did not have a significant influence on bacterial richness (p = 0.0506) but greatly influenced bacterial diversity (p = 0.0202) and the composition of the microbiome (p = 0.019). Host species had the greatest influence on the bacterial richness (p < 0.0001), diversity (p < 0.0001) and the composition of the microbiome (p = 0.001). Days in captivity had no significant influence on bacterial richness (p = 0.462) but did highly influence bacterial diversity (p = 0.0004) yet both values increased as days in captivity increased.

KEYWORDS

1. Bacteria
2. Biodiversity
3. Microbiome
4. Next-Generation Sequencing
5. Phylogeny
6. Reptile
7. Serpentes
8. Zoonosis

ACRONYMS

- BG – Brilliant Green
- BPW- Buffered Peptone Water
- HGT – Horizontal Gene Transfer
- HRC – Hoedspruit Reptile Centre
- LVS – Lowveld Venom Suppliers
- MDR – Multi-Drug Resistant/Resistance
- NGS – Next-Generation Sequencing
- PCR – Polymerase Chain Reaction
- XLD – Xylose Lysine Deoxycholate

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CHAPTER 1 INTRODUCTION

1.1 Problem Statement

Reptiles are known to harbour multiple types of pathogens including fungal, viral, protozoan, and bacterial pathogens (Mendoza-Roldan *et al.*, 2020). Historically there have been multiple outbreaks of infectious diseases across the globe and more than half of which can be linked to zoonotic pathogens (CDC, 2022; Mathur, 2022). The high rate of urbanisation has resulted in the encroachment of previously rural and/or remote regions, increasing the frequency of interactions with potentially dangerous animals. Within South Africa, this implies an increased frequency of interactions with reptiles. Reptiles are important members of the ecosystem but are considered threats to communities by humans.

The interactions with reptiles and their habitats can act as a pathway for the transmission of zoonotic pathogens. Reptile owners and workers who are commonly associated with reptiles may not be aware of the potential zoonotic risks their interactions with reptiles pose to their health and wellbeing. If the proper hygiene protocols are not followed in these circumstances, the risk of infection in humans is great. *Salmonella* is commonly shed in the faecal matter of reptiles and can cause serious infections in humans, most commonly, a self-limiting gastroenteritis. These infections can cause complications for immunocompromised individuals, young children and older people. Many of these infections associated with reptiles will go unreported due to their self-limiting nature, resulting in inaccurate numbers reported but reptile-associated Salmonellosis has been linked to outbreaks in the past (Cartwright *et al.*, 2016; Cota *et al.*, 2021; Ebani, 2017).

Globally, snake bites are considered one of the most neglected tropical diseases and the resulting envenomation accounts for an estimated 30 000 reported deaths per year within Africa with an even great number of debilitating injuries (Benjamin *et al.*, 2020). Globally there are between 1.2 and 1.5 million snakebites per year with 125 000 resulting in death or disability (Chuang *et al.*, 2022). Many of these snake bites do not involve the release of venom and are referred to as “dry bites”. While the risk of dry bites is much lower compared to bites where envenomation has occurred, both instances act as a pathway for pathogens to enter the human system and these bites can potentially cause a secondary infection in the victim (Chuang *et al.*, 2022).

Reptiles are known to house zoonotic pathogens that pose a great risk to humans and other animals (Jho *et al.*, 2011). The microbiota of reptiles can be used to determine these potential risks to humans and other animals. Bacterial infections are part of the ten most common causes of death in the world (Abayasekara *et al.*, 2017). A better understanding of the bacteria associated with reptiles may create a better understanding of the risks of human-reptile interactions.

1.2 Aims and Objectives

This study aimed to provide insight into the potential zoonotic risks different reptilian species pose to humans from human-reptile interactions. The potential risk is determined by the microbiota detected in the oral and cloacal cavities of the sampled reptilian species from captive facilities. Examples of these zoonotic risks included Salmonellosis (Bjelland *et al.*, 2020; Fagre *et al.*, 2020; Rush *et al.*, 2020), Mycobacteriosis, Chlamydiosis, and Leptospirosis (Ebani, 2017) from interactions with reptile faecal matter or surfaces contaminated with faecal matter. This study will focus on the early detection of *Salmonella* from the cloacal cavity of selected reptiles.

A secondary infection after a snakebite has occurred, poses additional risks to the snakebite victim. Some of these secondary infections have been linked to the following bacteria: *Bacillus* spp., *Enterococcus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Acinetobacter* spp., *Aeromonas* spp., *Citrobacter* spp., *Corynebacterium* spp., *Enterobacter spp., Escherichia coli, Klebsiella* spp., *Morganella* spp., *Proteus* spp., *Providencia* spp., *Pseudomonas* spp., *Salmonella* spp., *Serratia* spp., *Yersinia* spp., *Bacteroides* spp., *Clostridium* spp. (Baylock, 2001; Chuang *et al.*, 2022; Dehghani *et al.*, 2016; Garg *et al.*, 2009; Jho *et al.*, 2011; Smith *et al.*, 2021). The aim is to detect and catalogue bacteria from the oral cavities of selected snake species.

These aims were achieved through the following objectives:

Objective 1 – Determine the presence of intestinal *Salmonella* in captive reptiles from Hoedspruit Reptile Centre and Lowveld Venom Suppliers in Limpopo and Mpumalanga, South Africa. This was determined through culturing bacteria on selective media.

Objective 2 – Compare the isolation success of two types of media selective for *Salmonella*; Xylose Lysine Deoxycholate agar and Brilliant Green agar.

Objective 3 – Determine the statistical significance of various factors on the isolation success of *Salmonella*; host species, type of reptile, habitation status per enclosure, source of reptile, and the use of a dry cotton swab or Amies transport media.

Objective 4 – Determine the bacterial microbiome composition of the oral cavity of the various snake species. With this, determine the overall abundance of different bacterial phyla, the most abundant bacterial genus across the samples, and the indicator species of the oral microbiome.

Objective 5 – Determine the bacterial richness and diversity within the oral cavity of each host species. Determine whether the days in captivity and host phylogeny influenced bacterial diversity and richness.

Objective 6 – Determine the possible influence of host phylogeny on the oral microbiome composition, bacterial richness and bacterial diversity.

1.3 Expected Outcomes

Reptiles are known hosts of *Salmonella* spp. (Bjelland *et al.*, 2020; Ebani, 2017; Fagre *et al.*, 2020; Rush *et al.*, 2020) and our results will indicate a high isolation success in line with other studies. The two media tested will exhibit different efficacies for the selection of *Salmonella*. The variants used will have little to no influence on the isolation success of *Salmonella* due to its ubiquitous presence in reptiles.

The results from the Next-Generation sequencing of the 16S rRNA will allow the bacteria in the oral cavity to be identified to genus level and the respective abundance levels will be determined. The indicator species analysis will give an insight into expected bacteria for future studies with snakes under similar conditions. The diversity and richness levels of bacteria will be determined per host species. Clarity will be reached as to which of the tested variants has the greatest influence on the bacterial microbiome within the oral cavity of the snakes sampled. This research on the bacterial microbiome has not previously been conducted in this manner on South African reptile species and our data will therefore be a novel contribution to the description of the occurrence of bacteria in selected local reptiles. These results will guide future studies where in-depth microbiome work may be done on specific host species or geographical regions.

1.4 Chapter Layout

The above aims and objectives were used to develop a study plan for this project. Within chapter two, various literature was reviewed in alignment with this study. Reptiles were introduced and the species sampled in this study were presented. Some risks associated with snake bites and other human-reptile interactions were presented. This led to the review of some of the bacteria associated with reptiles with focus on the gut and oral microbiome of reptiles. The antimicrobial resistance of the expected bacteria was reviewed. The influence of diet on the microbiome was determined to aid the identification of possible influences on bacteria. The following section reviewed the culture-based and molecular methods of bacterial identification that were used in later chapters this allowed for a better understanding of the study. The review of these methods allowed the number of samples required to be determined based on the numbers used in other studies.

After the review of relevant literature, the knowledge obtained was implemented in chapters three and four. Chapter three focuses on intestinal *Salmonella* and its isolation success across the reptiles sampled. The efficacies of Xylose Lysine Deoxycholate and Brilliant Green agar media were tested to aid in future studies. The influence of various factors on the isolation success of *Salmonella* were determined using various statistical tests. The results of this could aid the understanding of the risks of human-reptile interactions.

The bacterial microbiome in the oral cavity of snakes was determined in Chapter four to increase the understanding of the risks associated with snake bites. Through the use of Next-Generation sequencing and various bioinformatic tools, the abundance of different bacterial phyla was determined per host sample and across all samples of individual species. Next, the genera of the bacteria present in the oral cavities were determined, their respective abundance was calculated and the indicator species were established. The identification of these genera allowed for further testing which determined the bacterial diversity and richness per host species. The influence of host species' phylogenetic placement was determined through PERMANOVA testing. These variations and other lesser variants were discussed to aid the understanding of their influence on the oral microbiome. The implications on human health of the bacteria found in the oral cavity were discussed to finish the chapter.

Chapter five acts as a summary of the previous chapters and creates a link between chapters three and four. The literature reviews in chapter 2 were incorporated into the

results of chapters three and four. The objectives mentioned in chapter one were revisited and the degree of completion was explained. Conclusions are drawn with consideration of all the results mentioned in previous chapters.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction To Reptiles

Placed under the Chordata phylum and Sauropsida clade, the class Reptilia contains three orders of reptiles relevant to this project; Testudines, Squamata and Crocodilia (Zug & Dowling, 2021). The majority of Reptilia are almost entirely covered by epidermal scales made from beta keratin (Zug & Dowling, 2021). Reptiles are also classified by their internal fertilization and amniotic development that occurs outside the body (in the majority of species) but lack specialized feeding glands as seen in mammals (Zug & Dowling, 2021). Reptiles are among the oldest tetrapods and vertebrate groups and remain diverse in their respective morphologies and ecological niches (van Hoek, 2014) but the list of extinct reptiles is far more diverse than their extant relatives (Zug & Dowling, 2021).

Reptiles are often viewed as unimportant, simplistic and expendable to society (de Miranda, 2017). Due to many of the world's developed countries being concentrated in the northern hemisphere, a lot of the research and data on reptiles is biased toward those found in these regions (de Miranda, 2017). Many aspects of reptiles in undeveloped or lower developed countries are understudied and many aspects of their cultural and ecological importance have not been researched as extensively as reptiles in developed countries (Cortes *et al.*, 2014; de Miranda, 2017). As advances in the technology used to study the history of animals have occurred, so has the understanding of reptiles' ecological importance (Cortes *et al.*, 2014; de Miranda, 2017). Reptiles play multiple roles in the ecological processes of their habitats and their abundance and larger body sizes contribute to their importance in these ecological processes (de Miranda, 2017).

Ecologically, reptiles are beneficial in many ways. Reptiles act as pollinators and aid seed dispersal thereby increasing the biodiversity of the surrounding ecosystem (Cortes *et al.*, 2014; de Miranda, 2017). Reptiles also help increase biodiversity through symbiotic relationships with other species by acting as sites for refuge or as reproduction sites (Cortes *et al.*, 2014; de Miranda, 2017). Reptiles are also important parts of the food web as predators of smaller animals and certain types of vegetation and by acting as prey for other animals in the ecosystem (Cortes *et al.*, 2014; de Miranda, 2017; Roll *et al.*, 2016). Some species favour meals such as small mammals (or pests) which can pose a great risk to human health if their populations are not kept under control. Some species are

also used as food sources in some human communities (Zug & Dowling, 2021). Reptiles are not excluded from agricultural land and although they may provide pest control in the fields they pose a risk to some livestock, especially due to snake bites (Rush *et al.*, 2020). Reptiles can act as a link between different types of ecosystems, such as marine and terrestrial, aiding the transfer of nutrients, seeds, pollen, parasites etc. between these ecosystems thereby supporting the ecosystems involved (Cortes *et al.*, 2014; de Miranda, 2017).

Culturally, reptiles play important roles in many different cultures, some as omens, myths and legends with some cultures using them for medicinal purposes and as a source of food (Nieman *et al.*, 2019; Roll *et al.*, 2016). Traditional healers in South Africa and other African cultures are held in high esteem in their communities and they believe welfare and health issues are connected by supernatural forces, social relationships and ancestral relationships (Nieman *et al.*, 2019). Nieman *et al.* (2019) conducted research in the Western Cape and Simelane and Kerley (1997) conducted research in KwaZulu-Natal. They determined which species were being used as traditional medicine in the sampled settlements. The reptiles used in traditional medicine include, but are not limited to *Bitis arietans*, *Python* spp.*, Naja nivea, Dendroaspis* spp., *Hemachatus haemachatus,* various unidentified snakes, *Varanus* spp., *Chersina angulata, Crocodylus niloticus, Agama atra, Acanthocercus atricollis,* various Cordylidae species, and some unspecified chameleons and chelonians (Nieman *et al.*, 2019; Simelane & Kerley, 1997). In traditional medicine, the uses of these reptiles appear to be species-specific and can be used for various reasons such as warding off curses, protection charms, treating mental or physical illnesses, treating epilepsy, improving physical attributes, as charms to aid aspects of life such as a winning a court case, retrieving a lost lover and many others (Nieman *et al.*, 2019; Simelane & Kerley, 1997). Other species that are not commonly used in traditional medicine are linked to witchcraft and are killed on site, such as geckos (Simelane & Kerley, 1997). Some other reptiles are seen as embodiments of their ancestors, such as *Boaedon capensis* and some chameleon species (Simelane & Kerley, 1997).

Despite their importance, they are still underrepresented in research and conservation efforts (de Miranda, 2017; Roll *et al.*, 2016) with the greatest interest focusing on large reptiles that are widely distributed or venomous and/or endangered reptiles (Roll *et al.*,

2016). According to the IUCN Red List[1], 21% of reptiles are under threat with 1847 species listed between vulnerable and extinct in the wild across the globe. According to the Reptile database, as of March 2022, there are currently 11 733 species of reptiles globally and 568 in South Africa (Uetz, 2022). Table 2-1 indicates the species that will be sampled for this study. Ten samples of each species, when available, were taken in accordance with reviewed literature to allow for variation among species and variation across species to be determined.

Table 2-1 Taxonomic classification of the species of interest

Order	Suborder	Family	Genus	Species	Common Name
Squamata	Serpentes	Colubridae	*Dispholidus*	*Dispholidus typus*	Boomslang
		Elapidae	*Naja*	*Naja annulifera*	Snouted Cobra
				Naja mossambica	Mozambique Spitting Cobra
			Hemachatus	*Hemachatus haemachatus*	Rinkhals
			Dendroaspis	*Dendroaspis polylepis*	Black Mamba
		Lamprophiidae	*Pseudaspis*	*Pseudaspis cana*	Mole Snake
		Pythonidae	*Python*	*Python natalensis*	Southern African Python
		Viperidae	*Bitis*	*Bitis arietans*	Puff Adder
	Cordyloidea[2]	Gerrhosauridae	*Matobosaurus*	*Matobosaurus validus*	Giant Plated Lizard
	Anguimor-pha	Varanidae	*Varanus*	*Varanus albigularis*	White-throated Monitor
Testudines	Cryptodira	Testudinidae	*Stigmochelys*	*Stigmochelys pardalis*	Leopard Tortoise
Crocodilia		Crocodylidae	*Crocodylus*	*Crocodylus niloticus*	Nile Crocodile

2.2 Risks Associated with Human-Reptile Interactions

2.2.1 Snake Bites

Within Africa, there are over half a million reported cases of envenomation from snakes every year that result in approximately thirty thousand deaths (Benjamin *et al.*, 2020). The remaining cases can result in injuries that vary in severity, with many resulting in life-altering disabilities or disfigurements (Benjamin *et al.*, 2020). These disfigurements and disabilities are seen as a disease of poverty as they are most often seen in communities that live below the poverty line or in remote communities with limited to no access to

[1] https://www.iucnredlist.org/
[2] Superfamily, suborder is not applicable

healthcare (Benjamin *et al.*, 2020). The majority (95 %) of snake bites in Africa occur within these impoverished communities and most are commonly experienced by manual labourers (Panagides *et al.*, 2017). The victims of these snake bites may lose their ability to work thereby forcing the family to remain in poverty (Benjamin *et al.*, 2020; Panagides *et al.*, 2017).

Antivenoms are used in the cases of envenomation of snakebites (Katali *et al.*, 2020). In many cases, victims of envenomation cannot correctly identify the snake that bit them and the administration of the wrong antivenom can lead to severe, adverse effects. In the majority of snake bite cases, the administration of antivenom is not required, even when envenomation has occurred as the victim can usually be treated by alternative methods according to the displayed symptoms (Chuang *et al.*, 2022; Wakasugi *et al.*, 2021). In South Africa, there are three types of antivenom available (SAVP, 2022). There are two types of monovalent antivenom: one that is effective against envenomation from *Dispholidus typus*, and the other is effective against envenomation from *Echis carinatus* and *Echis ocellatus* (SAVP, 2022). The last type of antivenom is a polyvalent antivenom that is effective against envenomation from *Bitis arietans, Bitis gabonica, Hemachatus haemachatus, Dendroaspis jamesoni, Dendroaspis polylepis, Dendroaspis angusticeps, Naja mossambica, Naja melanoleuca,* and *Naja nivea* (SAVP, 2022). South Africa has recently formed The National Snakebite Advisory Group[3] whose goal is to provide free support to medical personnel to aid with the treatment and management of snakebites.

The venom of any animal is seen as a defensive and predation mechanism (Avella *et al.*, 2021). The composition of venom experiences extreme selection pressure and relies on many aspects of the snakes' behaviours including preferred prey and habitat and the potential of this prey to escape after capture (Youngman *et al.*, 2019). The development of snake venom is thought to be linked to the immobilization of their prey as the main driver, as different varieties of venom have prey-specific effects (Youngman *et al.*, 2019). Snake venom is a complex mixture of proteins and peptides with salts and small organic molecules (Avella *et al.*, 2021). The venom of snakes is delivered to the prey in multiple ways but all originate from a venom gland (Avella *et al.*, 2021; Cleuren *et al.*, 2022). A snake can have a hollow or grooved fang and its venom is injected into its prey with the use of skeletal muscles (Avella *et al.*, 2021; Cleuren *et al.*, 2022). Other snakes can

[3] https://www.medicalacademic.co.za/post-summary/?post_refered=18468

spit/spray their venom through an anteriorly directed cavity in their fangs or along a channel in their fangs (Avella *et al.*, 2021; Cleuren *et al.*, 2022). This can be combined with the ability to remove the physical sheath around their fangs (Avella *et al.*, 2021).

The abundance of snake species allows for a variety of venom types with some species only containing one type of venom but the majority have developed a unique cocktail of venom types. Some of these venom types include hemotoxic, cytotoxic, neurotoxic and myotoxic (Benjamin *et al.*, 2020; Corbett *et al.*, 2020; Megale *et al.*, 2021; Youngman *et al.*, 2019). Hemotoxic venom affects blood cells causing coagulopathy which is a disruption in the blood's ability to form clots (Corbett *et al.*, 2020; Youngman *et al.*, 2019). Cytotoxic venom affects tissue and is the most common type of venom encountered in Africa (Benjamin *et al.*, 2020). Envenomation with cytotoxic venom can cause local tissue destruction resulting in blistering, bruising and necrosis (Benjamin *et al.*, 2020; Megale *et al.*, 2021). These wounds have a higher risk of infection as they allow a greater risk of secondary infection due to the types of wounds (Baylock, 2001; Benjamin *et al.*, 2020; Megale *et al.*, 2021). Neurotoxic venom affects the nervous system and can result in the paralysis of various muscles (Benjamin *et al.*, 2020; Erulu *et al.*, 2018). Myotoxic venom affects muscle tissue, specifically skeletal muscle tissue and is most commonly found in elapids and viperids (Oliveira *et al.*, 2022).

Snakes have an innate immune system which remains of great interest to scientists and the drive to identify these antimicrobial peptides (AMPs) has increased over recent years (van Hoek, 2014). The properties of these immune system peptides are linked to the success of reptiles over many thousands of years (van Hoek, 2014). Due to the development and availability of genome sequencing, the genomes of many reptilian species have been sequenced allowing the identification of the genes responsible for the production of AMPs (van Hoek, 2014). The identification of these genes can provide a better understanding of the production, development and evolution of these AMPs (van Hoek, 2014). This provides an avenue to further understand the evolutionary success of reptiles in comparison to other clades of animals (van Hoek, 2014). AMPs possess rapid killing action and broad-spectrum antimicrobial activities (Tajbakhsh *et al.*, 2018). They range from 12 to 50 amino acid residues, with two or more positively charged residues with a large number of hydrophobic residues (Tajbakhsh *et al.*, 2018). The use of AMPs as a treatment for human infections is restricted due to the side effects of these peptides (Tajbakhsh *et al.*, 2018). Some of the side effects of using AMPs to treat infections include

haemolytic activity against erythrocytes and other cytotoxicity effects (Tajbakhsh *et al.*, 2018). van Hoek (2014) and Nair *et al.* (2007) specify multiple peptides that contain antimicrobial properties which fall under the groups of cathelicidin, waprin, proline-rich, β-defensin and crotamine. Many of these AMPs have been used in pharmaceuticals as therapeutic agents and their effect on human physiology is of great interest (Oliveira *et al.*, 2022). Many of these AMPs have valuable antibacterial and antifungal activity that may influence the natural microbiota of their host (Nair *et al.*, 2007; Oliveira *et al.*, 2022; van Hoek, 2014)

2.2.2 Bacteria Associated with Reptiles

The microbiome of a host animal can influence its development, evolution, nutrient absorption, physiology, immune system, development and susceptibility to disease (Filek *et al.*, 2021; Siddiqui *et al.*, 2022; Youngblut *et al.*, 2019). The bacterial genes of a host's microbiome are thought to contain 100 times more genes than that of the host (Filek *et al.*, 2021). It is thought that a host's genomic data and the genomic data of their microbial community can be considered a biologically distinct unit, therefore, their susceptibility to the evolutionary process should be looked at as a whole and not as separate entities evolving on their own (Filek *et al.*, 2021). Understanding how environmental changes influence the microbiome of animals is important to the management of the host population and aids in determining their adaptive potential to further environmental changes (Youngblut *et al.*, 2019). Reptiles' microbial communities are underrepresented and require further studies to allow for a better understanding of what influences them the most (Filek *et al.*, 2021; Siddiqui *et al.*, 2022).

Oral Microbiome

The oral microbiome of animals is linked to the microbiome of their prey and their environment (Zancolli *et al.*, 2015). While the mouths of reptiles are home to AMPs, treatment for reptile bites and secondary infections with these AMPs has not been put into practice. The risk of secondary infections from snakebites and other reptile bites can be lessened by the use of prophylactic antibiotics in some cases. The penicillin family of antibiotics appears to be the most commonly used during the treatment of snakebites in combination with a tetanus booster injection (Corbett *et al.*, 2020; Erulu *et al.*, 2018; Garg *et al.*, 2009; Lukač *et al.*, 2017; Wakasugi *et al.*, 2021). However, the efficacy of a single type of antibiotic is not sufficient to prevent the risk of a secondary infection. Because there is a drastic variation of the oral microbiome among snakes, regional knowledge of

the oral microbiota of snakes would be greatly beneficial to allow more appropriate treatment methods to be developed

There have been multiple studies since the 1950s identifying the bacteria associated with reptiles and their respective pathogenicity. An infection that is secondary to a snakebite has been linked to the oral bacterial flora of some snakes (Dehghani *et al.*, 2016; Zancolli *et al.*, 2015). Garg *et al.* (2009) conducted a study, in India, of the bacteria found within debrided tissue from bite sites and the majority of bacteria detected, were Gram-positive bacteria. Baylock (2001) conducted a study on the oral bacterial flora of snakes in southern Africa and was able to identify several species through culture-based methods from oral swabs. The snakes studied by Baylock (2001) were *Hemachatus haemachatus*, *Naja mossambica*, *Causus rhombeatus*, *Boaedon fuliginosis*, *Lamprophis guttatus, Lycodonomorphus inornatus*, *Bitis arietans*, *Bitis gabonica*, and *Python natalensis*. Chuang *et al.* (2022) studied wild-caught snakes (*Naja atra, Bungarus multicinctus, Protobothrops mucrosquamatus, Trimeresurus stejnegeri, Deinagkistrodon acutus,* and *Daboia siamensis*in) from southern Taiwan and identified the oral bacteria with culture-based methods and mass spectrometry of oral swabs. Dehghani *et al.* (2016) used culture-based methods from wild-caught snakes from regions of Iran that were kept in captivity for 6 months before sampling and bacteria was identified through culture-based methods. The snakes involved in the study by Dehghani *et al.* (2016) were *Coluber ravergieri, Spalerosophis diadema, Vipera lebetina, Spalerosophis microlepis, Coluber rhodorachis, Coluber ravergieri, and Coluber karelini.* Smith *et al.* (2021) used molecular methods to study the gut and oral microbiome of snakes (*Laticauda laticaudata*, *Trimeresurus flavomaculatus* and *Boiga dendrophila*) from the Babuyan Island Group of the Philippines.

From the above-mentioned literature, the Gram-Positive bacteria that have been associated with snakes include *Bacillus* spp*., Enterococcus* spp*., Staphylococcus* spp*.,* and *Streptococcus* spp*.* (Baylock, 2001; Chuang *et al.*, 2022; Dehghani *et al.*, 2016; Garg *et al.*, 2009; Smith *et al.*, 2021). The Gram-Negative bacteria identified include *Acinetobacter* spp*., Aeromonas* spp*., Citrobacter* spp*., Enterobacter spp., Escherichia coli, Klebsiella* spp*., Morganella* spp*., Proteus* spp*., Providencia* spp*., Pseudomonas* spp*., Salmonella* spp. and *Serratia* spp. (Baylock, 2001; Chuang *et al.*, 2022; Dehghani *et al.*, 2016; Garg *et al.*, 2009; Smith *et al.*, 2021). The anaerobic bacteria include

Shewanella putrefaciens, Yersinia enterocolitica, Bacteroides spp*., Clostridium* spp*.,* and *Peptostreptococcus* spp*. (Chuang et al., 2022).*

Chuang *et al.* (2022) tested antibiotic resistance against the oral bacterial flora of snakes and the majority of bacteria detected were susceptible to an array of antimicrobials, with some exceptions. Some samples of the anaerobic bacteria *Bacteroides* spp. and *Clostridium* spp. were resistant to penicillin (Chuang *et al.*, 2022). For the Gram-positive aerobic bacteria, *Staphylococcus sciuri* exhibited a decreased susceptibility to clindamycin, erythromycin, oxacillin and penicillin, while *Enterococcus casseliflavus* exhibited a low susceptibility to vancomycin (Chuang *et al.*, 2022). Of the Gram-negative bacteria tested by Chuang *et al.* (2022), *Proteus* spp., *Citrobacter* spp. and *Escherichia coli* exhibited low susceptibility to cefazolin with *Proteus vulgaris* being almost entirely resistant to cefazolin and cefuroxime. Garg *et al.* (2009) observed monomicrobial secondary infections in 77% of their cases of snakebites and the remainder of their cases had a mixed infection with two or more types of bacteria. With 52% of their samples being positive for *Staphylococcus aureus,* resistance to penicillin was determined in 53% of the isolates. In the literature studied, there is a consensus that clinical specimens should be taken and cultured before antibiotic treatment commences to ensure the best treatment is given. The knowledge of the expected microbiota in the region may aid the identification of the associated bacteria and their respective risks while allowing the best culturing technique to be used.

Gut Microbiome

Reptiles and other ectothermic animals are known reservoirs of *Salmonella* bacteria. The transfer of these bacteria to humans can occur through interactions with infected faecal matter or contaminated surfaces (Bjelland *et al.*, 2020; Fagre *et al.*, 2020; Rush *et al.*, 2020; Wikström *et al.*, 2014; Zajac *et al.*, 2021). *Salmonella* is a group of bacteria under the family Enterobacteriaceae and is Gram-negative (Bjelland *et al.*, 2020). Many species of *Salmonella* can withstand dry, cold or freezing environments and can survive in water for months (Bjelland *et al.*, 2020; Cartwright *et al.*, 2016; Harker *et al.*, 2011). *Salmonella enterica* can be divided into six subspecies with over 2 500 serovars and the *S. enterica* subspecies *enterica* is the most important to public health assessments (Pulford *et al.*, 2019). The serovars of *S. e. enterica* are grouped by typhoidal *Salmonella* and non-typhoidal *Salmonella*, with the former presenting as severe systemic infection and the latter as a self-limiting gastrointestinal disease in human hosts (Pulford *et al.*, 2019).

Human salmonellosis associated with snakes is a considerable threat in the United States (Rush *et al.*, 2020) and the United Kingdom (Harker *et al.*, 2011). Salmonellosis is contracted through the ingestion of contaminated food or water infected with *Salmonella* and through direct interaction with infected faeces (Awang *et al.*, 2021; Bauwens *et al.*, 2006; Ebani, 2017; Wabeto *et al.*, 2017; Wikström *et al.*, 2014). The resulting from Salmonellosis can be potentially fatal from a systemic infection (Awang *et al.*, 2021).

There has been an increase in the testing of antimicrobial resistance in *Salmonella* serovars from human hosts, predominantly from *S. enterica* strains (Cartwright *et al.*, 2016). The first antimicrobial resistance of *Salmonella* was seen against chloramphenicol in 1948; only two years after its introduction to treat enteric fever (Awang *et al.*, 2021). Other strains have developed resistance to ampicillin, sulphonamides, nalidixic-acid, ciprofloxacin, extended-spectrum cephalosporins, and trimethoprim (Awang *et al.*, 2021; Cota *et al.*, 2021). This antimicrobial resistance has been linked to horizontal gene transfer through mobile plasmids to other bacteria (Awang *et al.*, 2021).

There is a concern regarding studying faecal samples for *Salmonella* in snakes due to the intermittent shedding of *Salmonella* in the faeces. This intermittent shedding makes cloacal sampling less reliable than more invasive methods of sampling the gut microbiota however, cloacal sampling remains the most ethical sampling method (Bauwens *et al.*, 2006; Bjelland *et al.*, 2020; Cartwright *et al.*, 2016; Ebani, 2017; Ramos *et al.*, 2019; Rush *et al.*, 2020). Rush *et al.* (2020) detected *Salmonella* spp. in 35.6 % of their 45 wild Grenadian tree boa (*Corallus grenadensis*) samples. Upon further testing, 43 isolates were detected and after serological testing, there were all samples fell under 6 serovars of *Salmonella enterica* subsp *enterica*. Of the serovars detected by Rush *et al.* (2020), *S.* e. *enterica* ser. Rubislaw, *S.* e. *enterica* ser. Braenerup, and serovar IV:48:g.z51 (previously *S*. e. *enterica* ser. Marina) had previously been the source of illness in humans (Rush *et al.*, 2020). Rush *et al.* (2020) stated their prevalence rates were lower than other studies with other studies having prevalence levels closer to 60 %. Bjelland *et al.* (2020) studied the prevalence of *Salmonella* in captive reptiles at Norwegian Zoos and found a 62 % prevalence of *Salmonella* in snakes, 67 % in lizards and 3% in chelonians. Their results were dominated by *S. enterica* subsp*. enterica* (40%) and *S. enterica* subsp*. arizonae* (4 %). Other subspecies found included *S. enterica* subsp. *diarizonae, S. enterica* subsp. *salamae* and *S. enterica* subsp. *houtenae*. Cartwright *et al.* (2016) reviewed clinical samples of infected patients that had been sent in for testing in multiple

states in the USA. After a review of the patients, 60 % had reported owning a reptile and the others had reported recent interactions with reptiles at school, amusement parks or a friend's home. This outbreak had been linked to the contamination of frozen feeder rats that were used as food for these reptiles (Cartwright *et al.*, 2016). The detection methods of these studies are further explained in section 2.3.1.

2.3 Methods of Detection of Bacteria

2.3.1 Culture-Based Methods

According to Awang *et al.* (2021), the conventional guidelines for the identification of *Salmonella* follow four stages; non-selective pre-enrichment (buffered peptone water), selective enrichment (Rappaport Vassiliadis soy [RVS] broth), inoculation on selective isolation agar (such as Xylose Lysine Deoxycholate), and biochemical and serological tests. Buffered peptone water (BPW) is the most commonly used pre-enrichment broth for the recovery of *Salmonella* as it provides the appropriate conditions for the resuscitation of injured cells and the growth of cells before they undergo selective enrichment (Baylis *et al.*, 2000). It is valuable during pre-enrichment as it buffers the pH against the changes that result from the growth and metabolism of cells (Baylis *et al.*, 2000).

Brilliant Green (BG) agar is used for the differentiation and selection of *Salmonella* spp., excluding *S. e. enterica* ser. Typhi, from pathological matter, food and dairy products (Aryal, 2022b; Neogen, 2019). BG agar is highly selective therefore it is a preferred media for testing highly contaminated samples, such as faecal matter (Aryal, 2022b). The combination of tryptone, yeast extract and peptone creates a medium that is highly nutritious while supplying amino acids and long-chain peptides (Aryal, 2022b). The media contains lactose and sucrose that act as the source of fermentable carbohydrates for bacteria while the brilliant green inhibits the majority of gram-negative and gram-positive bacteria (Aryal, 2022b). The bacteria that ferment sucrose (*Salmonella* excl. *S. e. enterica* ser. Typhi) become red to white coloured colonies surrounded by a strong red media while lactose fermenting bacteria appear yellow to green with yellow media due to the indicator, brilliant green (Aryal, 2022b). It is recommended that BG agar is used in parallel with other selective media such as Xylose Lysine Deoxycholate agar, Hektoen enteric agar, Bismuth Sulphite Agar or Deoxycholate Citrate agar (Aryal, 2022b).

Xylose Lysine Deoxycholate (XLD) agar is used for differentiation and the isolation of enteric pathogens, especially *Salmonella* spp. from nonsterile origins (Aryal, 2022c; Neogen, 2021). *Salmonella* spp. can ferment the xylose resulting in an acid which changes the media yellow due to the presence of phenol red (Aryal, 2022c; Neogen, 2021). Once the xylose has been metabolized, *Salmonella* will decarboxylate lysine which shifts the pH back to neutral (Aryal, 2022c; Neogen, 2021). The neutral pH allows *Salmonella* to reduce the sodium thiosulphate and produce hydrogen sulphide which binds with the ferric ammonium citrate and produces black colonies (Aryal, 2022c; Neogen, 2021). The production of hydrogen sulphide is exclusive to the *Salmonella* genus, excluding a few species (Baylis *et al.*, 2000; Mitchell & Shane, 2001). Other organisms can decarboxylate lysine but the acid resulting from the fermentation of the lactose and sucrose results in a pH that is too low for hydrogen sulphide production (Aryal, 2022c; Neogen, 2021).

Bauwens *et al.* (2006) collected environmental samples and faecal samples (n = 100) of reptiles at a zoo in Antwerp and used BG and XLD agar after enrichment with BPW, RVS broth, and Mueller Kauffman Tetrathionate (MKTT) broth. The samples from Bauwens (2006) underwent biochemical tests for identification. Bjelland *et al.* (2020) assessed the prevalence of *Salmonella* in reptile cloacal swabs (n = 103) at various Norwegian zoos. Bjelland *et al.* (2020) pre-enriched swabs in BPW and selenite broth and directly plated samples into Bromthymolblue-Lactose-Agar then suspected *Salmonella* was streaked into urea agar and blood agar. Biochemical tests followed the streaking of the urea and blood agar. Cota *et al.* (2021) investigated the prevalence of *Salmonella* in pet reptiles (n = 78) in Portugal and tested the efficiency of chlorhexidine gluconate against the isolated bacteria. Cota *et al.* (2021) used BPW, MKTT, RVS, Hektoen agar, XLD agar and Triple Sugar Iron (TSI) agar and biochemical testing before testing the antimicrobial susceptibility of the isolates. Goupil *et al.* (2012) sampled various captive snakes (n = 12) in the United States over a period of 10 weeks. Faecal and cloacal samples were used to detect *Salmonella*. Goupil *et al.* (2012) made use of tetrathionate enrichment, BG, XLD and TSI agar followed by serotyping. Marin *et al.* (2021) sampled the skin, oral cavity and cloacal cavity of various captive reptiles (n = 123) from households and pet shops in Spain. The samples from Marin *et al.* (2021) were enriched with BPW, semi-solid modified RVS, XLD agar and chromogenic agar followed by biochemical and antimicrobial susceptibility testing. Merkeviciene *et al.* (2022) sampled faecal matter and took cloacal

swabs from various wild and captive reptiles (n = 97) from Lithuania. Merkeviciene *et al.* (2022) did not state which pre-enrichment was used but they specified the use of XLD and *Salmonella Shigella* (SS) agar followed by serotyping and antimicrobial susceptibility testing. Pulford *et al.* (2019) took faecal samples from various wild-caught venomous snakes (n = 106) from different countries in Africa. Pulford *et al.* (2019) determined the prevalence of *Salmonella* with the use of BPW, selenite broth, RVS, XLD agar and BG agar and underwent a polymerase chain reaction to identify the serovars. Rush *et al.* (2020) identified the *Salmonella* serovars in wild snakes (n = 45) from Grenada by testing cloacal and distal colon swabs. Rush *et al.* (2020) used tryptic soy broth and plated it on XLD agar and tryptic soy agar followed by serotyping. Wikström *et al.* (2014) sampled the terraria, bedding, and cloacal cavity or faecal matter of pet reptiles (n = 63) in Sweden. Samples were used to determine the prevalence of *Salmonella* with the use of BPW, RVS, XLD agar and BG agar or BPW and semi-solid modified RVS agar and reinoculated on XLD agar, and BG agar if negative results were seen. Any suspected *Salmonella* colonies were inoculated onto Bromocresol Purple Lactose agar and subsequently serotyped (Wikström *et al.*, 2014)

2.3.2 Molecular Methods

While culture-based methods remain the primary methods for bacterial identification, they can be time-consuming and require multiple additional tests for the certainty of identification (Clifford *et al.*, 2012). The automation of identification techniques has become more common as the equipment has increased in reliability and accuracy since their introduction (Clifford *et al.*, 2012), however, some facilities lack the required funding for the necessary equipment and/or reagents involved with molecular methods. When large amounts of DNA are sequenced it is referred to as high-throughput sequencing and allows for parallel sequencing (Brooker, 2012). Molecular techniques provide significant advantages over methods that rely on phenotypic variation as they have faster turnaround times, increased reliability, high sensitivity and scalability (Clifford *et al.*, 2012). When used with the appropriate bioinformatic tools and careful primer design, the issues previously mentioned can be alleviated (Clifford *et al.*, 2012).

Next-Generation sequencing (NGS) refers to specific methods with specific systems of sequencing such as the Pacific Biosciences SMRT (PacBio SMRT) (Brooker, 2012). With PacBio SMRT, the individual DNA molecular growth is monitored by fluorescent imaging and the DNA fragments and the polymerase are trapped within holes on a thin metal film

during sequencing (Brooker, 2012). NGS allows for the simultaneous sequencing of multiple DNA fragments, sometimes called parallel sequencing (Abayasekara *et al.*, 2017). This parallel sequencing negates the limitations of the older style of Sanger sequencings where a chain termination method was used and was limited to an amplified product of a single DNA molecule (Abayasekara *et al.*, 2017). Sanger sequencing would require clinical isolates that had been cultured *in vitro* which adds to its limitations and disadvantages (Abayasekara *et al.*, 2017). Since it becomes problematic to study the whole genome, genetic sequencing techniques make use of specific genes for different aspects of the study. For microbial communities, as with this study, a gene that is found within all possible species of a community is key to an efficient analysis.

The 16S rRNA gene is a highly conserved gene among bacterial genera and is used for identification and basic phylogenetic studies (Abayasekara *et al.*, 2017; Clarridge, 2004). The 16S rRNA gene contains highly conserved regions that are interspersed with hypervariable regions that facilitate the identification through metagenomics (Abayasekara *et al.*, 2017). Metagenomics is a relatively new field of study that involves the study of microbial diversity with a sample based on the available genetic material (Abayasekara *et al.*, 2017). Due to the widespread use of the 16S rRNA gene for identification, its GenBank database is large with multiple strains of bacteria (Clarridge, 2004) which allows for easy identification in a project of any size. Its widespread use is because the 16S rRNA encodes for critical components that are required for cell function and are highly conserved over time (Clarridge, 2004). Mutations within the 16S rRNA gene are slow overall but the 16S rRNA gene does contain regions that mutate at higher rates depending on the host cell (Clarridge, 2004). This gene is also the target for some antimicrobial agents and the mutations in these regions can impact their susceptibility to these agents (Clarridge, 2004).

Some issues associated with 16S gene analysis are the hypervariable regions that can change within a short period, sometimes within the same cell. Another issue is that some bacterial species can have identical sequences (Galloway-Peña & Hanson, 2020). These issues are amplified when the gene has undergone polymerase chain reaction (PCR) as multiple instances of these errors are detected (Galloway-Peña & Hanson, 2020). To negate these errors, the incorporation of operational taxonomic units (OTUs) has been included in the analysis of these sequences (Galloway-Peña & Hanson, 2020). OTUs group sequences that fall within a threshold of divergence of 97 % or 99 % to allow the

control of biological and technical discrepancies (Galloway-Peña & Hanson, 2020). An OTU is seen as a distinct group and can be used for the analysis of diversity and richness. There are many ways to interpret and manipulate the 16S sequences and, in this study, we will make use of alpha and beta diversity tools.

Alpha diversity is used to quantify diversity and richness within samples and can be used for comparisons across groups (Galloway-Peña & Hanson, 2020; Smith *et al.*, 2021), such as host species. Beta diversity is used to compare sample diversity often by comparing feature dissimilarity to result in a distance matrix between all samples (Galloway-Peña & Hanson, 2020). A form of multivariate analysis that will be used in this study is a permutational multivariant analysis of variance (PERMANOVA) that is based on the beta diversity distance matrices to form clusters (Anderson, 2017; Galloway-Peña & Hanson, 2020). P-values of these variants were obtained and any value below 0.05 was counted as statistically significant as it ensures the probability of the finding being true is above 95 % (Andrade, 2019).

The following literature identified bacterial microbiome and stated the phylum present. Esmaeilishirazifard *et al.* (2022) used NGS of the 16S gene to construct the bacterial microbiome present in the venom extracted from snakes (n = 20) and spiders (n = 8). The venoms used originated from *Bothrops atrox, Naja nigricollis, Crotalus atrox, Oxyuranus scutellatus* and *Bitis arietans*. These venom samples were collected by third parties and the results were used to determine the influence of venom on microbial communities (Esmaeilishirazifard *et al.*, 2022). Esmaeilishirazifard et al. (2022) indicated the bacteria detected in their samples were predominately of Gammaproteobacteria, Cyanobacteria, Alphaproteobacteria, Flavobacteria, Bacilli, Actinobacteria and Bacteroidia. Filek *et al.* (2021) sampled the oral and cloacal cavities of wild and rehabilitated loggerhead turtles, *Caretta caretta* (n = 12), using molecular methods. Filek et al. (2021) was able to determine the composition of the microbiome of their samples which consisted predominately of Proteobacteria, Bacteroidetes and Firmicutes. Krishnankutty *et al.* (2018) determined the oral bacterial microbiome of wild snakes (n = 12) from Kerala state, India. Krishnankutty *et al.* (2018) sampled *Naja naja*, *Ophiophagus hannah* and *Python molurus*. Krishnankutty et al. (2018) revealed Proteobacteria and Actinobacteria were the most common within their samples. Smith *et al.* (2021) studied the oral and cloacal microbiome of wild venomous snakes (n = 22) from the Babuyan Island Group. The snakes sampled were *Laticauda laticaudata*, *Boiga dendrophila* and *Trimeresurus*

flavomaculatus. Smith et al. (2021) identified Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Epsilonbacteraeota, Acidobacteria, Fusobacteria, Chlamydiae, Chloroflexi, Tenericutes and Verrucomicrobia.

The literature referenced next identified the bacterial microbiome to family, genus and species. McNally *et al.* (2021) conducted research on Kemp's ridley turtles, *Lepidochelys kempii* (n = 35), by swabbing oral and cloacal cavities of turtles obtained for rehabilitation for hypothermia at New England Aquarium, Massachusetts, USA. The turtles were swabbed at intake, periodically during their stay and before their release and their bacterial microbiomes were established. McNally et al. (2021) identified Porticoccaceae, Arenicellaceae, Flavobacteriaceae, Rhodobacteraceae, Vibrionaceae, Stappiaceae, Shewanellaceae, Burkholderiaceae, Fusobacteriaceae, Ruminococcaceae and Arcobacteraceae as part of the microbiome of their samples. Panda *et al.* (2018) studied the oral microbiome of wild-caught *Naja naja* (n = 6) from India. Panda et al. (2018) identified *Salmonella* spp., *Pseudomonas* spp., *Proteus* spp., *Escherichia coli*, *Morganella* spp., *Citrobacter* spp., *Aeromonas* spp., *Enterobacter* spp., *Acinetobacter* spp., *Neisseria* spp., *Serratia* spp., *Bacillus* spp., *Enterococcus* spp., *Staphylococcus* spp., *Alcaligenes* spp., *Chryseobacterium* spp. and *Micrococcus* spp. within their samples. Hill *et al.* (2018) sampled wild snakes (n = 12) from Tennessee and Arkansas, USA. They used the results of their chosen molecular techniques and challenge assays to determine how the cutaneous bacterial microbiome resists the development of snake fungal disease from *Ophidiomyces ophiodiicola*. The snakes sampled were *Crotalus horridus* and *Coluber constrictor*. Hill et al. (2018) identified 15 strains of bacteria that exhibited antifungal properties, these included strains within the genera *Aeromonas, Erwinia, Morganella, Myroides* and *Stenotrophomonas*. Mao *et al.* (2021) compared the oral bacterial microbiome of wild *Naja atra* from central Taiwan to samples that had been taken from the wounds of snakebite victims that were suspected to be infected in the nearby hospital. Mao *et al.* (2021) identified the microbiome of their samples consisted of *Enterococcus* spp., *Morganella morganii, Proteus mirabilis, Proteus penneri, Proteus vulgaris, Providentia rettgeri, Pseudomonas aeruginosa, Serratia marcescens,* and *Shewanella* spp. with *Morganella morganii, Proteus vulgaris,* and *Proteus mirabilis* being identified in the bite wounds of snakebite victims.

2.4 Final Remarks

Ecologically, reptiles play many important roles while simultaneously being part of societal and cultural practices. However, even with this importance they are still seen as expendable to society and have little importance in research conservation efforts. There are over half a million reported cases of snakebites every year and countless unreported cases. Reptiles, especially snakes, pose a significant threat to humans through snakebites, primary or secondary infections, and/or through life-changing injuries that may result from the wounds of a snake bite, envenomation and subsequent infections.

Multiple genera of bacteria have been detected in the microbiome of snakes, most prominently *Salmonella, Staphylococcus, Proteus, Providencia,* and *Morganella*. The microbiome of reptiles contains many beneficial AMPS which are of great significance to their value to research and conservation based on their physiological and pharmacological uses. The AMPs found in reptiles have been linked to their success over thousands of years. The influence of these AMPs on the microbiota of reptiles is still relatively unresearched.

The high levels of zoonotic bacteria found in reptiles pose significant risks to human health, for example, the multiple instances of secondary infections after a snakebite are the result of zoonotic bacteria. In previous studies, some of the bacteria in the oral cavity have exhibited resistance to one or more antimicrobial agents. Many instances of infections with *Salmonella* have been researched and there is a high number of infections linked to recent interactions with reptiles and their environments.

The microbiomes of reptiles have been evaluated through the use of culture-based methods and molecular methods or a combination of both. The study of the gut microbiome is generally focused on *Salmonella* and a combination of culture-based and molecular methods is preferred for identification at the serovar level. The study of the oral microbiome has been tested with culture-based methods previously, however, there is a greater number of studies that use molecular-based methods for more accurate results. A few studies have been conducted on South African reptiles resulting in a large deficit in the knowledge of the resident bacteria.

Overall, the threat reptiles pose to humans has been established and is known, however, there are many differences observed in the bacteria found. This indicates multiple factors influence the microbiomes of reptiles and region-specific studies are beneficial to better

understand the risks they pose to humans. Understanding the microbiomes of reptiles may also provide insight into the health and well-being of the reptile. The better the understanding of a group of animals, the greater chance these animals will be included in further research and conservation efforts.

CHAPTER 3 SCREENING FOR *SALMONELLA* IN THE CLOACAL CAVITY OF REPTILES

3.1 Introduction

Salmonella is a flagellated Gram-negative bacterium with many serovars that are pathogenic to humans (Awang *et al.*, 2021; Bjelland *et al.*, 2020). *Salmonella* is listed as one of the main causes of diarrheal disease by the World Health Organisation (WHO) with the majority of cases resulting from contaminated animal products (Awang *et al.*, 2021; Bjelland *et al.*, 2020). The serovars of *Salmonella enterica* subspecies *enterica* are classified as Typhoidal (caused by *S. e. enterica* ser. Typhi and *S. e. enterica* ser. Paratyphi which can only infect humans) and non-Typhoidal (caused by *S. e. enterica* ser. Typhimurium and *S. e. enterica* ser. Enteritidis which can infect animals and humans) (Awang *et al.*, 2021; Bjelland *et al.*, 2020). Nontyphoidal *Salmonella* accounts for over 400 deaths per year with 26 000 hospitalisations each year (Bjelland *et al.*, 2020). The total number of infections with typhoid and nontyphoidal *Salmonella* was estimated at 14.3 million with 136 000 deaths in 2017 (Awang *et al.*, 2021).

Reptiles and other ectothermic animals are often associated with the spread of *Salmonella* (Goupil *et al.*, 2012; Pulford *et al.*, 2019; Rush *et al.*, 2020). *Salmonella* spp. are part of the natural microbiome found within the gastrointestinal tract of 90% of reptiles and are known to be asymptomatic to their reptilian hosts (Bjelland *et al.*, 2020; Cartwright *et al.*, 2016). While *Salmonella* is a natural part of reptiles' healthy gut microbiota an increased level of *Salmonella* may indicate the snakes are experiencing stressors that are affecting their natural microbiota (Bjelland *et al.*, 2020; Cartwright *et al.*, 2016). Human-reptile interactions pose a risk of transmission of *Salmonella* and other pathogens and human infections with *Salmonella* can be life-threatening (Bauwens *et al.*, 2006; Fagre *et al.*, 2020; Pulford *et al.*, 2019). Early detection of infection with *Salmonella* in humans could be a lifesaver by allowing necessary treatment early on (Awang *et al.*, 2021). The understanding of *Salmonella* in commonly handled reptiles can lessen the risk of infections as important safety precautions can be implemented. The shedding of *Salmonella* in reptiles is intermittent (Bauwens *et al.*, 2006; Bjelland *et al.*, 2020; Cartwright *et al.*, 2016; Ebani, 2017; Ramos *et al.*, 2019) therefore the monitoring of *Salmonella* shedding in faeces may give an indication if the reptiles are experiencing stressors (Verbrugghe *et al.*, 2016).

The reptile service sections of a zoo had the highest level of *Salmonella* when compared to other departments in a study by Bauwens *et al.* (2006). Studies have detected over 80 % of reptiles in pet shops are carriers of *S. enterica* (Pulford *et al.*, 2019). The presence of *S. enterica* in non-venomous reptiles is responsible for 6 % of human Salmonellosis infections per year (Pulford *et al.*, 2019). As high as 91 % of venomous snakes in a study by Pulford *et al.* (2019) were positive for *S. enterica*. Interactions with animals that are known reservoirs for *Salmonella* are thought to account for 11 % of all human infections with *Salmonella* (Bjelland *et al.*, 2020; Cartwright *et al.*, 2016). The infection with *Salmonella* can cause vomiting, diarrhoea, fever, and potentially lethal septicaemia in mammals (Awang *et al.*, 2021; Bjelland *et al.*, 2020). While *Salmonella* is predominately an enteric pathogen, it can become a systemic infection (Chaudhuri *et al.*, 2018; Verbrugghe *et al.*, 2016).

The objective of this chapter is to determine the presence of intestinal *Salmonella* in captive reptiles from Hoedspruit Reptile Centre and Lowveld Venom Supply in Limpopo and Mpumalanga, South Africa, through the culturing of bacteria on selective media. The difference in isolation success of *Salmonella* on Xylose Lysine Deoxycholate agar and Brilliant Green agar will be determined. The statistical significance of various factors (host species, type of reptile, habitation status, source of reptile, and whether the reptile was captive or wild caught) on the isolation success of *Salmonella* will be determined.

3.2 Methods

3.2.1 Permits and Ethical Clearance

Category 3 Ethical approval was obtained from NWU-AnimCareREC at North-West University, Potchefstroom Campus, for Courtney Siobhan Ransom with ethics number: NWU-00411-21-A5. The wild-caught snakes at Lowveld Venom Supply were collected under permit number MPB/V/2003[4]. The samples obtained from Hoedspruit Reptile Centre were collected under the supervision of an on-site veterinarian. Transport of samples was covered under permit 20210329ORK - UMPI[5]. A Section 20 permit[6] was obtained from the Department of Forestry and Fisheries, according to requirements

[4] Annexure A.
[5] Annexure B.
[6] Annexure C

stipulated in the *Animal Diseases Act (Act 35 of 1984)* for the study of animal diseases. Re: 12/11/1/3 (1711AC)[7].

3.2.2 Sampling

Cloacal samples were taken from resident and wild-caught snakes that were being kept at Lowveld Venom Supply (LVS) and Hoedspruit Reptile Centre (HRC). All the animals were handled by the staff at the respective locations to reduce the risks to the animals as well as the researchers. Venomous snakes were held by appropriate methods that considered the handler's and researcher's safety, depending on species and ease of handling. Some species were necked and held during sampling and others were held with the head of the snake in tubes to reduce fatigue for the handlers. Spitting snakes were kept in tubes and eye protection was used when sampling. For the longer snakes, such as *Dendroaspis polylepis* and *Python natalensis*, multiple people aided in supporting the body of the snake during sampling. Handlers and researchers used F10sc Veterinary Disinfectant[8] to sanitize gloves and the work area between animals to prevent any contamination between animals. A veterinarian, a veterinarian technician and experienced animal handlers were on site for the sedation and handling of *Crocodylus niloticus* specimens. The species sampled and their respective numbers and origins are listed in table 3-1.

An individual sterile cotton swab stick was used to swab the cloacal cavity of most reptiles. The swab was carefully inserted in an anterior direction and lightly swirled between the thumb and index finger. Cotton swabs were stored in their respective tubes or Amies transport media. The cloacal samples of the *Stigmochelys pardalis, Varanus albigularis,* and *Matobosaurus validus* were stored in Amies agar gel transport media tubes[9]. These transport media tubes contain salt and buffers to preserve the cells without allowing for the growth of cells (Aryal, 2022a; Condalab, 2020; Rijal, 2021). The transport media contains agar, magnesium chloride anhydrous, calcium chloride, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium hydrogen phosphate, and sodium thioglycolate (Condalab, 2020). All samples were transported in a cold box and returned to 4°C in the lab until tests were conducted. Aseptic techniques were followed during all the steps to prevent any contamination or spread to the researcher.

[7] Annexure E.
[8] http://healthandhygiene.co.za/f10-sc-veterinary-disinfectant-product-information/
[9] Condalab: Amies Transport Medium without Charcoal

Table 3-1 Species sampled, the number of each species sampled, source and origins.

Family	Genus	Species	Number	Source	Origin	Sample type	Habitation status
Colubridae	*Dispholidus*	*Dispholidus typus*	10	LVS	Captive	Dry swab	Singular
Elapidae	*Naja*	*Naja annulifera*	10	LVS	Captive	Dry swab	Singular
		Naja mossambica	10	9 – LVS	Captive	Dry swab	Singular
				1 – HRC		Dry swab	Singular
	Hemachatus	*Hemachatus haemachatus*	3	HRC	Captive	Dry swab	Singular
	Dendroaspis	*Dendroaspis polylepis*	10	LVS	Captive	Dry swab	Shared
Pseudaspididae	*Pseudaspis*	*Pseudaspis cana*	2	HRC	Captive	Dry swab	Shared
Pythonidae	*Python*	*Python natalensis*	4	HRC	1 – Captive	Dry swab	Singular
					3 – Wild Caught (in Quarantine)	Dry swab	Singular
Viperidae	*Bitis*	*Bitis arietans*	10	LVS	Captive	Dry swab	Singular
Gerrhosauridae	*Matobosaurus*	*Matobosaurus validus*	5	HRC	Captive	Amies transport media	Shared
Varanidae	*Varanus*	*Varanus albigularis*	3	HRC	Captive	Amies transport media	Shared
Testudinidae	*Stigmochelys*	*Stigmochelys pardalis*	10	HRC	Captive	Amies transport media	Shared
Crocodylidae	*Crocodylus*	*Crocodylus niloticus*	3	HRC	Captive	Dry swab	1-Singular 2-Shared

3.2.3 Culture-Based Methods

The culture-based methods used for this section of the study were adapted from Pulford *et al.* (2019) and the procedure was adapted according to available media components and equipment. Some steps were omitted due to the inability to source relevant broths in time. The omission of these steps proved inconsequential to the isolation success of *Salmonella* in this study.

Pre-enrichment of Samples

Buffered Peptone Water[10] (BPW) was used as pre-enrichment for samples to allow for the resuscitation of viable bacteria that were lost during transport and storage. The BPW was prepared by adding 8.05 g of powdered BPW to 500 ml of double-distilled H_2O (ddH_2O). The mixture was heated for 15 minutes on a hot plate with a stirrer until the powder was completely dissolved. The BPW was then autoclaved at 121°C for 15 minutes and stored at room temperature until needed. The BPW was decanted into smaller aliquots for each sample set of 10 to 20 samples to minimise the risk of contamination. BPW is able to support *Aspergillus brasiliensis, Candida albicans, Bacillus subtilis, Escherichia coli, Salmonella typhimurium,* and *Staphylococcus aureus* (Condalab, 2021). All cloacal samples taken with dry sterile cotton swabs underwent pre-enrichment with BPW. The samples that were stored in transport media did not undergo pre-enrichment. For pre-enrichment, 3ml of buffered peptone water was added directly to the sterile cotton swab tubes to prevent the loss of any bacteria. These were incubated for 24 hours at 37°C.

Culturing of Bacteria

Xylose Lysine Deoxycholate Agar

The Xylose Lysine Deoxycholate[11] (XLD) agar was prepared 500 ml at a time to prevent the need for a water bath as XLD media can be damaged if overheated. For the XLD plates, 27.5 g of powdered XLD agar was added to 500 ml ddH_2O and the mixture was heated on a hot plate with a stirrer until boiling as the media cannot be autoclaved. The XLD agar was then left to cool until the glass was able to be handled. A total of 500 ml of XLD agar equated to 25 - 30 90 mm X 15 mm Petri dishes. The plates were poured in a Class II Biosafety Cabinet and underwent UV-C decontamination for 20 minutes while cooling. The XLD media plates were sealed with Parafilm and stored at 4°C until use. Characteristics of growth pattern and media colour were used to identify bacterial species on the XLD agar (Table 3-2).

[10] Condalab: Buffered Peptone Water EP/USP
[11] Neogen: Xylose Lysine Deoxycholate (XLD) Agar (NCM0027)

Table 3-2 Expected growth pattern for XLD agar, adapted from Neogen (2021).

Microorganism	Growth	Identification
Enterococcus faecalis	Complete inhibition	N/A
Escherichia coli	Partial to complete inhibition	Yellow to yellow-red colonies
Salmonella enterica subsp. *enterica* serovar Abony	Full growth	Red colonies w/ black centres
Salmonella enterica subsp. *enterica* serovar Enteritidis	Full growth	Red colonies w/ black centres
Salmonella enterica subsp. *enterica* serovar Typhimurium	Full growth	Red colonies w/ black centres
Shigella sonnei	Full growth	Red colonies

Brilliant Green Agar

Brilliant Green[12] (BG) agar is used for the differentiation and selection of *Salmonella* spp., excluding *S.* Typhi, (Neogen, 2019). A total of 26 g of powdered BG agar was added to 500 ml ddH_2O and left to soak for 10 minutes before heating to a boil on a hot plate with a stirrer. The same pouring, decontamination, sealing and storage methods were used as explained for XLD agar. *Salmonella* spp. were identified according to the guidelines of the manufacturer (Table 3-3).

Table 3-3 Expected growth pattern for BG agar, adapted from Neogen (2019).

Microorganism	Growth	Identification
Enterococcus faecalis	Complete inhibition	N/A
Escherichia coli	Suppressed to inhibited	Yellow colonies
Proteus mirabilis	Suppressed	Translucent pinpoint colonies
Salmonella enterica subsp. *enterica* serovar Enteritidis	± 50%	Pink colonies
Salmonella enterica subsp. *enterica* serovar Typhimurium	± 50%	Pink colonies

After pre-enrichment, the cultures from the buffered peptone water were used to inoculate XLD and BG agar plates. All plates were incubated at 37°C for 48 hours and checked periodically during this time.

[12] Neogen: Brilliant Green Agar (Modified) (NCM0058)

Long-Term Storage of Samples

After the samples had been used to inoculate the relative plates, 500 µl of the buffered peptone water culture was added to 500 µl of the glycerol stock into 2ml Eppendorf's, labelled and stored at -80°C. The 50 % Glycerol Stock was made by adding 300 ml double distilled water and 300 ml molecular-grade glycerol together which was subsequently autoclaved for 15 minutes at 121°C. For the samples that had not been used to inoculate the plates, the samples underwent incubation in the BPW for 24 hours at 37°C before being added to glycerol stock and stored at -80°C.

Statistical testing

The influence of different variants on the isolation success of *Salmonella* was determined using various statistical tests using GraphPad Prism 9.4.1 (GraphPad Software, 2022). A one sample t-test was run to calculate the values of significance of host species. A one-way ANOVA was conducted to determine the significance of the use of XLD agar or BG agar on the isolation success rate. The influence of host species was tested with a one-sample t-test. A simple t-test was run to determine the influence of host phylogeny, habitation status, source (LVS or HRC) and if a dry swab or Amies transport media was used during transport. All tests were conducted using the isolation success rate per species.

3.3 Results

The isolation success of *Salmonella* on the attempted isolates was determined by the percentage of plates that showed positive isolation of *Salmonella* on the more successful media after 48 hours of incubation at 37°C i.e., if XLD agar had a greater number of positive plates than BG agar, the isolation success was determined by the number of plates that successfully isolated *Salmonella* on XLD agar divided by the total number of samples for that species. For the XLD agar, the detection of *Salmonella* was confirmed by red media with red colonies that had a black centre. The detection of the black centre was needed to ensure *Shigella* was not interpreted as *Salmonella*. For BG agar, the detection of *Salmonella* was confirmed with the growth of pink/red colonies on red/pink media with the colonies having a slightly opaque and liquid appearance. At 24 hours, the start of black colonies on the XLD agar could be seen but the media remained yellow (neutral pH). At 48 hours of incubation ensured the development of the black colonies and the change of the media back to red (acidic pH) to ensure the detection of *Salmonella*

was achieved according to the technical data sheet provided with XLD agar. The BG agar was the most consistent over the 48 hours and only minor changes were observed.

Table 3-4 Isolation success of *Salmonella* in sampled tortoises, lizards and crocodiles.

Species	n	Time (hours)	XLD	BG	Time (hours)	XLD	BG	Isolation success
S. pardalis	10	24	1	7	48	1	7	0.7
V. albigularis	3	24	3	2	48	3	2	1.0
M. validus	5	24	4	1	48	4	1	0.8
C. niloticus	3	24	3	2	48	3	2	1.0
Total	**21**		**11**	**12**		**11**	**12**	**0.81**

In total, 17 of 21 (80.95 %) of the samples indicated positive isolation of *Salmonella*. There was a 100% isolation success from *V. albigularis* and *C. niloticus.* A high level of isolation success from *S. pardalis* and *M. validus* was seen. All samples, except one sample of *C. niloticus*, originated from animals that shared enclosures with members of the same species. Every sample in table 3-4 originated from captive reptiles that were sourced from HRC.

The isolation success of our samples originating from snakes (table 3-5) depicts many of the host species exhibiting high levels of *Salmonella* isolation. The majority of snakes were sourced from LVS, with the exceptions *P. cana, P. natalensis* and *H. Haemachatus* sourced from HRC. The one sample of *N. mossambica* from HRC did not yield any growth. The majority of snakes were in singular enclosures except for *D. polylepis* which were all housed in an enclosure with other members of their species.

Table 3-5 Isolation success of *Salmonella* within snake samples for two different culture methods.

Species	n	Time (hours)	XLD	BG	Time (hours)	XLD	BG	Isolation Success
B. arietans	10	24	10	7	48	10	7	1.0
D. polylepis	10	24	9	9	48	9	9	0.9
D. typus	10	24	7	7	48	9	7	0.9
H. haemachatus	3	24	3	2	48	3	2	1.0
N. annulifera	10	24	10	4	48	10	6	1.0
N. mossambica	10	24	9	5	48	8	4	0.8
P. cana	2	24	0	0	48	1	1	0.5
P. natalensis	4	24	4	2	48	4	1	1.0
Total	**59**		**52**	**36**		**54**	**37**	**0.92**

An overall isolation success of 91.53 % (54 out of 59) from samples originating from snakes was exhibited. There was a 100 % isolation success of *Salmonella* in *B. arietans, N. annulifera,* and *P. natalensis* and the lowest isolation success can be seen in *P. cana* (figure 3-1). *Salmonella* was isolated in 71 out of 80 (88.75 %) of all samples with the mean isolation success for these reptiles being 88.33 %. Overall, XLD agar (81.25 %) had 20.00 % greater isolation success than BG agar (61.25 %).

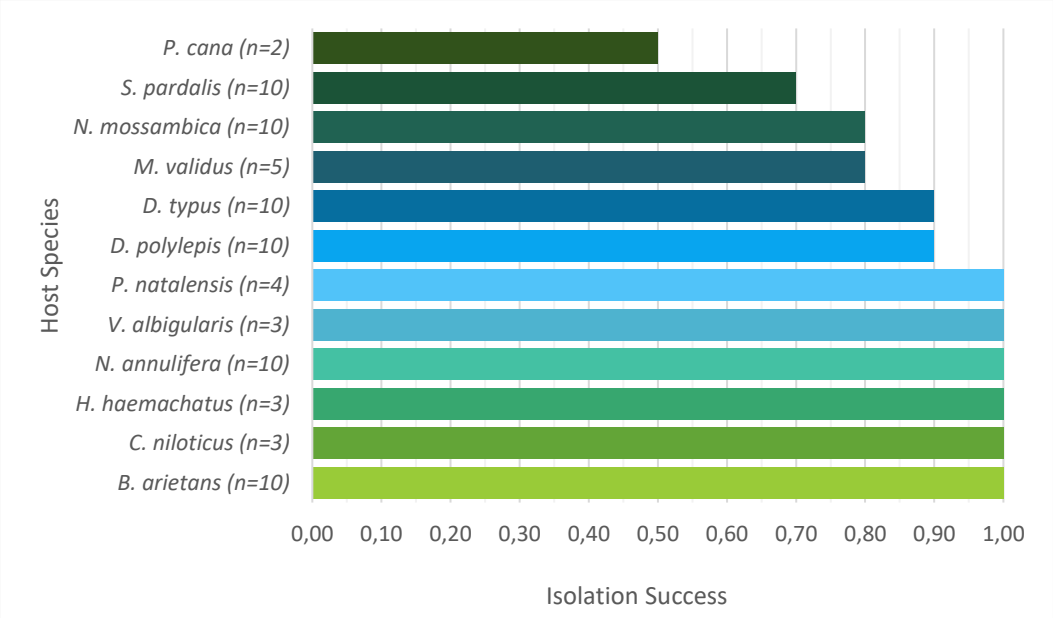

Figure 3-1 Isolation success of *Salmonella* after 48 hours of incubation.

Statistical Influence of Variables

The results of various statistical tests (table 3-6) depict the significance of different variants on the isolation success of *Salmonella*. Host species ($p < 0.001$) had a very significant influence on the isolation success of *Salmonella*. If the host species was considered a snake or lizard, tortoise or crocodile ($p = 0.905$) there was no significant influence on the isolation success of *Salmonella*. In all samples, we can see a significant difference in the isolation success of XLD agar ($n = 65$) compared to BG agar ($n = 49$). The use of XLD agar or BG agar ($p = 0.017$) had a high statistical significance influence on the isolation success, with XLD agar exhibiting higher isolation success. Whether the host was housed in their own enclosure or cohabitated with other members of their species ($p = 0.318$), whether the host was from Lowveld Venom Supply or Hoedspruit Reptile Centre ($p = 0.962$), and whether a dry swab or Amies transport tube was used ($p = 0.638$) had no significant influence on the isolation success of *Salmonella*.

Table 3-6 P-values of different variants on the isolation success of *Salmonella.*

Variant	P value	Test
Host Species	<0.001	One Sample t-test
Snakes or lizards, tortoises and crocodiles	0.905	Unpaired t-test
XLD vs BG	0.017	One-way ANOVA
Singular habitation or cohabitation	0.953	Unpaired t-test
Source (LVS or HRC)	0.962	Unpaired t-test
Dry swab or Amies transport media	0.638	Unpaired t-test

3.4 Discussion and Conclusion

3.4.1 Factors That May Influence Isolation Success.

Salmonella was detected in all the host species and high levels of isolation success were seen across the sample groups. The mean isolation success of 88.33 % and *Salmonella* was successfully isolated in 88.75 % of all reptiles sampled. Multiple factors may influence these high levels of *Salmonella.*

Stress is a necessary part of life yet constant stressors can have negative implications (Verbrugghe *et al.*, 2012). In animals and humans, long periods of stress can alter hormone levels, neurotransmitters, peptides, and cytokines, and can suppress the immune system and increase vulnerability to infection (Bjelland *et al.*, 2020; Verbrugghe

et al., 2012; Verbrugghe *et al.*, 2016). The stress response is governed by epinephrine, norepinephrine, cortisol and corticosterone and these stress-related hormones are known to affect the immune system (Verbrugghe *et al.*, 2012; Verbrugghe *et al.*, 2016). The presence of norepinephrine has been proven to increase the growth and motility of *S. enterica*, this indicates there is a link to increase shedding during stressful periods which increases the risk of transmission (Verbrugghe *et al.*, 2012; Verbrugghe *et al.*, 2016).

The amount of stress experienced by the snakes should be monitored as prolonged exposure to stressors can lead to an infection from the natural microbiota (Verbrugghe *et al.*, 2012). Because *Salmonella* is shed periodically through snakes' faeces, (Bauwens *et al.*, 2006; Bjelland *et al.*, 2020; Cartwright *et al.*, 2016; Ebani, 2017; Ramos *et al.*, 2019) the monitoring of *Salmonella* levels in captive snakes may help reduce the risk to their handlers while allowing the snakes' health and stress levels to be monitored as well. The intermittent shedding of *Salmonella* through faeces may influence the levels detected in cloacal swabs (Bauwens *et al.*, 2006; Bjelland *et al.*, 2020; Cartwright *et al.*, 2016; Ebani, 2017; Ramos *et al.*, 2019; Rush *et al.*, 2020). All reptiles sampled were kept within facilities focused on the care of reptiles. All host species experienced similar levels of husbandry regimens thereby reducing the chances of uneven captivity-induced stress that could have caused an increased shedding of *Salmonella,* however there may be species-specific responses to captivity that cannot be determined in this study. Because this study was only used as a screening for *Salmonella*, the exact levels of *Salmonella* were not tested; only the presence of *Salmonella* was studied. While *Salmonella* levels do fluctuate, *Salmonella* will remain present in the cloacal cavity.

Host species did have a highly significant influence ($p < 0.001$) on the isolation success of *Salmonella*. The was no statistically significant difference in the isolation success of snakes or lizards, tortoises and crocodiles ($p = 0.905$). The isolation success from samples from snakes (91.53 %) was 10.58 % higher than that seen in lizards, tortoises and crocodiles (80.95 %). This discrepancy may be linked to the lower sample pool used for lizards, tortoises and crocodiles due to the limited individuals at HRC. This may indicate the phylogenetic placement of the host species does not influence the presence of *Salmonella*, only that *Salmonella* is a commensal part of the reptilian gut microbiome.

Marin *et al.* (2021) indicated reptiles kept by private owners had lower levels of *Salmonella* shedding when compared to reptiles kept in enclosures with other individuals. This higher level is thought to be a result of stress and less strict cleaning regimens. Some

(*D. polylepis, H. haemachatus, P. cana, S. pardalis, V. albigularis, M. validus,* and *C. niloticus*) of the reptiles were kept in enclosures with other members of their species while the other (*B. arietans, D. typus, N. annulifera, N. mossambica,* and *P. natalensis)* were housed in individual enclosures. The results in this study did not correlate with the findings of Marin *et al.* (2021) which indicates cohabiting with other members of the same species may not have influenced these results ($p = 0.318$). The source of the reptiles from either HRC or LVS had no influence ($p = 0.962$) on the isolation success either.

In reviewed literature, both dry cotton swabs and cotton swabs placed in Amies transport media have been used for cloacal sampling of reptiles. There was no observable difference in the isolation success within the samples in this project. Statistically, the use of dry cotton swabs and the use of amies transport media did not influence isolation success ($p = 0.638$).

3.4.2 Comparison of Isolation Success of Two Types of Selective Media

While XLD and BG agar are both listed as being used for the selection/isolation and differentiation of *Salmonella*, XLD is intended to be used with non-sterile products and BG is for food and feed-stuffs and inhibits *Shigella* and *S. e. enterica* serovar Typhi. According to Neogen (2021), the selectivity of XLD agar to *Salmonella* is achieved by the presence of sodium deoxycholate and phenol red as an indicator. Both types of agars were used to help in identification as the *Shigella* colonies can be confused for *Salmonella* at early stages on the XLD agar. The XLD media showed a 20% greater isolation success than the BG. This difference is thought to be the BG agars ability to only support 50% of the *Salmonella* and due to the ambiguous appearance of the *Salmonella* colonies on the BG agar that can be interpreted as other bacteria whereas *Salmonella* is the only bacteria that can form the hydrogen sulphide complex and. (Neogen, 2021).

The isolation success of *Salmonella* was very significantly ($p=0.017$) influenced by the different types of media. The only instance of XLD limiting the growth was experienced with *S. pardalis* samples. This may be a result of *S. pardalis* harbouring a different serovar than the other reptiles which BG agar is more selective for than XLD agar. Further research into this result would be beneficial to understanding this discrepancy as this study was only screening for *Salmonella* and not species-specific and serovars. This discrepancy indicates the benefits of using multiple media types to ensure different species and serovars can be isolated. With this project, the XLD was preferred as it gave easier-to-interpret results due to the formation of black colonies but the joint use of the

agars did allow for added certainty when screening for *Salmonella*. The use of both BG and XLD agar is encouraged for the detection of *Salmonella* spp. to aid the accuracy and validity of results (Aryal, 2022b) and removes the risk of losing results if there is contamination or failure of one of the agar plates.

3.4.3 Implications of *Salmonella* on Snake and Human Health

The gut microbiome of reptiles is said to consist of mostly Proteobacteria, Firmicutes and Bacteroidetes (Siddiqui *et al.*, 2022). *Salmonella* is found in the Proteobacteria phylum. The gut microbiome is thought to contribute to the success of the host's immune system responses, with between 70 % - 80 % of immune cells being found within the gut (Siddiqui *et al.*, 2022). The immune system of reptiles exhibits similar components to those found in mammals (Siddiqui *et al.*, 2022). Understanding the gut microbiome of any animal can allow for a better understanding of the immune system of said animal. The high detection of *Salmonella* in reptiles that appear healthy provides evidence it forms part of their natural gut microbiome. Multiple researchers have identified *Salmonella* as commensal bacteria in the gut of reptiles but there is still a risk of disease from *Salmonella* in the host species due to overgrowth of *Salmonella*. Extended periods of stress in the host increase the virulence of *Salmonella* and increase the risk of infection in the host (Verbrugghe *et al.*, 2012; Verbrugghe *et al.*, 2016). The understanding of the presence of *Salmonella* in the gut of reptiles provides insight into the commensal bacteria in the gut of reptiles. The gut microbiome of reptiles is greatly understudied (Siddiqui *et al.*, 2022) yet the prevalence of *Salmonella* in reptiles is of great interest. This bias towards the study of *Salmonella* levels appears to be linked to its ability to infect humans. The risk of infection with *Salmonella* from the handling of reptiles is high and has resulted in many countries implementing legislation covering the owning of pet reptiles due to multiple outbreaks associated with pet reptiles (Bjelland *et al.*, 2020). There is also a risk of the colonisation of *Salmonella* in the food given to pet reptiles, such as frozen mice and rats (Cartwright *et al.*, 2016).

Salmonella can withstand dry, cold and freezing environments and can survive in water for several months (Bjelland *et al.*, 2020; Cartwright *et al.*, 2016; Harker *et al.*, 2011). Some infections with *Salmonella* can present as severe systemic infections and others as a self-limiting gastrointestinal disease in human hosts (Pulford *et al.*, 2019). The infection with *Salmonella* in humans is known as Salmonellosis which is contracted through the ingestion of contaminated food or water that has come into contact with

animal faeces or those of an infected human or through direct contact with the faeces containing *Salmonella* (Awang *et al.*, 2021; Ebani, 2017; Fagre *et al.*, 2020; Rush *et al.*, 2020; Wabeto *et al.*, 2017; Wikström *et al.*, 2014). There has been an increase in the testing of antimicrobial resistance in *Salmonella* serovars from human hosts, predominantly from *Salmonella enterica* strains (Cartwright *et al.*, 2016). The first antimicrobial resistance of *Salmonella* was seen against chloramphenicol in 1948; only two years after its introduction of treating enteric fever (Awang *et al.*, 2021). Other strains have developed resistance to ampicillin, penicillin, sulphonamides, nalidixic-acid, ciprofloxacin, extended-spectrum cephalosporins, and trimethoprim (Awang *et al.*, 2021; Cota *et al.*, 2021).

Salmonella also can exhibit biofilm production on different surfaces (Cota *et al.*, 2021). The development of a biofilm reduces the bacteria's susceptibility to antimicrobial agents (Cota *et al.*, 2021). A biofilm is an aggregation of microorganisms that are embedded with a substance or extracellular polymeric matrix that is self-produced (Assefa & Amare, 2022; Oluwole, 2022). Biofilms have altered phenotypic characteristics regarding their gene transcription and growth rates (Oluwole, 2022). Biofilms can be attached to living or non-living surfaces which allows the microorganisms to survive under certain environmental conditions (Assefa & Amare, 2022; Oluwole, 2022). Biofilm production increases virulence and resistance to multiple methods of disinfection of surfaces (Assefa & Amare, 2022; Oluwole, 2022). This increase in virulence and growth rate increases the risk of transmission to humans and animals which come into contact with the biofilm (Assefa & Amare, 2022; Oluwole, 2022).

3.5 Conclusion

Two different types of selective media used for this study had different levels of isolation success. XLD agar had an isolation success rate of 81.25 % and Brilliant Green agar had an isolation success rate of 61.25 %. XLD agar had a 20 % higher isolation success than BG agar. The use of XLD agar ($p = 0.017$) had a highly significant influence on the isolation success of *Salmonella*. The use of both types of media was beneficial as it allowed all growth of *Salmonella* to be detected if one type of media had a lower isolation success than the other type of media.

Multiple factors were thought to influence the isolation success rate but only the host species ($p < 0.001$) and the use of XLD or BG agar ($p = 0.017$) had a statistically relevant

influence on the isolation success of *Salmonella*. There was no statistically significant influence on isolation success if the samples originated from snakes or other reptiles ($p = 0.905$), if the host species were housed individually or with other members of its species ($p = 0.318$), if the samples originated from LVS or HRC ($p = 0.962$) and if a dry swab or Amies transport media was used ($p = 0.638$). The cumulation of these results indicate *Salmonella* is commensal bacterial within the gut of reptiles and not from an external source. The isolation success levels in this study were high than those detected in the reviewed literature (Bauwens *et al.*, 2006; Bjelland *et al.*, 2020; Briones *et al.*, 2004; Cota *et al.*, 2021; Fagre *et al.*, 2020; Goupil *et al.*, 2012; Jang *et al.*, 2008; Marin *et al.*, 2021; Ramos *et al.*, 2019; Rush *et al.*, 2020; Wikström *et al.*, 2014). The isolation success rate was similar to the isolation success of Zajac *et al.* (2021).

Salmonella was detected in all of the host species sampled in this study with a mean isolation success of 88.33 %. *Salmonella* was successfully isolated in 88.75 % of the samples from various reptiles. There was an isolation success rate of 91.53 % in samples originating from snakes and 80.95 % in samples originating from lizards, crocodiles and tortoises. With 88.75 % of our samples showing positive growth for *Salmonella*, there is a real risk for exposure to zoonotic pathogens as a consequence of handling these reptiles.

Salmonella is known to be part of the natural intestinal flora of healthy snakes, which could result in complacency towards pathogen hygiene protocols on the part of the handler. The interaction with the sample set of snakes and with their habitats shows a high risk of contracting *Salmonella* and possibly other zoonotic infections for the handlers. There is the potential for snake-to-snake transmission within the facility across different individuals. All reptile enclosures need to undergo decontamination with appropriate disinfectants to counteract biofilm production and good personal hygiene needs to take place during any interaction with reptiles. All of the snakes and reptiles handled appeared healthy and underwent rigorous husbandry practises that included strict hygiene protocols; thus, the risk of transmission was decreased within our sample set.

CHAPTER 4 ASSESSMENT OF THE BACTERIAL MICROBIOME IN THE ORAL CAVITY OF SNAKES

4.1 Introduction

A bite from any animal poses a risk to human health through the introduction of bacteria, fungi, protozoans, and viruses (Mendoza-Roldan *et al.*, 2020). While all humans are colonized by bacteria that are part of our microbiomes, the introduction of additional microorganisms can threaten this delicate balance of resident bacteria. A bite from an animal introduces the microbiome of the animal directly into the human system allowing a direct pathway for infection. The microbiome of the animals contains their naturally occurring symbiotic or commensal microorganisms but also can contain organisms that have been encountered elsewhere in the animal's habitat (Galloway-Peña & Hanson, 2020; Willey *et al.*, 2014). Microorganisms are naturally found in/on soils, water, flora, and prey of these wild animals (Willey *et al.*, 2014).

While snake bites pose the obvious risk of envenomation and the complications from this pose a great enough risk, there is an additional risk of secondary infection from the bacteria and other microorganisms from their microbiomes that can threaten the health of the victim. In many cases of snake bites, little to no venom has been administered and it can be considered a dry bite (Benjamin *et al.*, 2020). These "dry bites" can be overlooked and no medical attention is received or sought after by the victim. Whether envenomation has occurred or not, a bite from a snake introduces many unknown microorganisms into an open wound.

Different bacteria and microorganisms require different treatments and how they interact with the human immune system needs to be considered. In situations where antibiotics are being considered during post-exposure treatment, the appropriate antibiotic needs to be administered (Vardanega *et al.*, 2022). Whilst some of the bacteria pose no immediate threat, a potential long-term risk exists if not treated. For the correct and informed choice to be made, the microbiome of the oral cavity of the snake needs to be understood and incorporated into the treatment plan. As with the treatment with antivenom, the snake should preferably be identified before treatment, the same could be said regarding antibiotic treatment as different snakes could house different bacterial strains. While identification is not always possible, understanding the general microbiome provides an advantage to the possible treatment of snakebite wounds.

The objectives for this section are to determine the composition of the bacterial microbiome of the oral cavity of various snake species from Hoedspruit Reptile Centre (HRC) and Lowveld Venom Suppliers (LVS) in Limpopo and Mpumalanga, South Africa. The resulting sequences from Next-Generation sequencing of the 16S rRNA gene were used to determine the overall abundance of different bacterial phyla, the most abundant bacteria across all samples, and the indicator species of the oral microbiome of snakes. The bacterial diversity (Shannon index) and richness were determined per host species. Through various statistical means, the influence of various factors (host phylogeny and days in captivity) on the composition of the oral microbiome was determined.

4.2 Methods

4.2.1 Sampling

Oral samples were taken from resident and wild-caught snakes that were being kept at LVS and HRC. The previously mentioned permits (specified in chapter 3) covered the collection and transport of samples from the aforementioned facilities. All the reptiles were handled by the staff at the respective locations to reduce the risk of injuries to the animals as well as to the researchers. Venomous snakes were held by appropriate methods that considered the handler's and researcher's safety, depending on species and ease of handling. All snakes were restrained in a transparent tube to allow safe and easy access to their heads from the anterior terminal end of the tube. The snakes (table 4-1) were sampled by the insertion of a sterile cotton swab into the oral cavity and swabbing as much of the oral cavity as possible by swirling the swab between the thumb and index finger and moving it around the oral cavity. In some cases, the swab was held with a long pair of forceps to extend the reach into the tube. Handlers and researchers used F10sc Veterinary Disinfectant[13] to sanitize gloves and the work area between samples to prevent cross-contamination. The samples were transported in a cold box and returned to 4°C in the lab until tests could be conducted. Aseptic techniques were followed during all steps to prevent any contamination of the samples.

[13] http://healthandhygiene.co.za/f10-sc-veterinary-disinfectant-product-information/

Table 4-1 Species samples, their numbers, sources and origins

Order	Suborder	Family	Genus	Species	Number	Source	Origin
Squamata	Serpentes	Colubridae	*Dispholidus*	*Dispholidus typus*	10	LVS	Captive
		Elapidae	*Naja*	*Naja annulifera*	10	LVS	Captive
				Naja mossambica	10[14]	9 – LVS 1 – HRC	Captive
			Dendroaspis	*Dendroaspis polylepis*	10[15]	LVS	8 - Captive 2 – Born in captivity
		Pseudaspididae	*Pseudaspis*	*Pseudaspis cana*	2	HRC	Captive
		Pythonidae	*Python*	*Python natalensis*	4	HRC	1 – Captive 3 – Wild caught (in quarantine)
		Viperidae	*Bitis*	*Bitis arietans*	10	LVS	Captive

4.2.2 Next-Generation Sequencing and Bioinformatics

The DNA extraction and Next-Generation sequencing (NGS) were conducted by Inqaba Biotec™. The ZymoBIOMICS DNA Miniprep kit[16] was used for DNA isolation and the whole 16S rRNA gene PCR was performed using the universal primers (27-F: AGRGTTYGATYMTGGCTCAG; 1492-R: RGYTACCTTGTTACGACTT) with Taq NEB Q5 high Fidelity 2x MM[17]. Sequencing was done on Pacific Biosciences (PacBio) Sequel IIe system and analysis was done using PacBio SMRTLink software 10.1.

There were 56 samples underwent DNA extraction, however, only 53 samples were viable for NGS. The resulting FASTQ files from 53 samples were loaded into R version 4.2.1 (R Core Team, 2022) interfaced with R Studio (4.2.1) (RStudio Team, 2020). The libraries used in R were; Bioconductor v3.16 (Callahan *et al.*, 2022a): *DADA2* (Callahan *et al.*, 2022b), *phyloseq* (McMurdie & Holmes, 2013) *biostrings* (Pagès et al., 2022) and *BioGenerics* (Callahan *et al.*, 2022a), with *ggplot2* v3.4.0 (Wickham *et al.*, 2022b), *dplyr* v1.0.10 (Wickham *et al.*, 2022a), *vegan* v2.5.7 (Oksanen *et al.*, 2022), *labdsv* v2.0-1 (Roberts, 2019), *microbiome* (Lahti & Shetty, 2019) and *lme4* v1.1-31 (Bates *et al.*, 2022).

[14] 10 individuals were sampled but one sample from HRC was not able to be amplified resulting in 9 viable samples from LVS
[15] 10 individuals were sampled but 2 captive samples were not able to be amplified
[16] https://www.zymoresearch.com/products/zymobiomics-dna-miniprep-kit
[17] https://international.neb.com/products/m0492-q5-high-fidelity-2x-master-mix#Product%20Information

The package *dada2* (Callahan *et al.*, 2022b) was used to denoise raw sequencing reads and identify Amplicon Sequence Variants (ASVs) using a new protocol specifically designed for PacBio data. The raw sequences were filtered to only those between 1380bp and 1520bp, the expected full-length 16S rRNA gene amplicon size. Samples with low reads (100 sequences or less) were removed from the data pool. Eight samples in total were lost; one sample of *D. polylepis,* 1 sample of *P. cana,* 2 samples of *B. arietans* and 4 samples of *N. annulifera.* The removal of the low reads resulted in 45 samples being viable for analysis. The ASVs were assigned using *phyloseq* (McMurdie & Holmes, 2013) and *vegan* (Oksanen et al., 2022) libraries. Taxonomy was assigned to ASVs using the *phyloseq* (McMurdie & Holmes, 2013) and SILVA[18] 138 v1.2.11 database (Quast *et al.*, 2013) with a 97 % threshold for divergence from other sequences, resulting in the allocation of 706 taxa by 7 taxonomic ranks among the 45 appropriate samples. Where SILVA (Quast *et al.*, 2013) and *phyloseq* (McMurdie & Holmes, 2013) were not able to classify ASVs to genus or species level, the BLAST[19] rRNA/ITS online database (Altschul *et al.*, 1990) was used to attempt to identify finer-resolution taxonomy. Post-quality-control reads were linked to the relevant metadata containing information about each sample's host phylogeny and days in captivity using the R package *phyloseq* (McMurdie & Holmes, 2013). Host phylogeny was listed as whether the host species was classified as Elapidae or not. This variable was chosen to allow for the separation of our samples based on phylogeny type while ensuring the subset included multiple species.

Stacked bar plots were created using the *microbiome* (Lahti & Shetty, 2019) library in R studio (RStudio Team, 2020). These plots were used for the abundance of different phyla as per other studies. Genus level was then used for abundance levels across all samples and the abundance per genus was calculated based on the number of repeated unique sequences per genus that were assigned with *phyloseq* (McMurdie & Holmes, 2013), SILVA (Quast *et al.*, 2013) and BLAST (Altschul *et al.*, 1990). The indicator analysis was determined in R Studio (RStudio Team, 2020) using the *labdsv* library (Roberts, 2019).

For alpha diversity calculations, set.seed(1234) was used to initialize repeatable random subsampling. The bacterial richness and Shannon index (diversity) of each species were determined. The richness and Shannon index were then calculated in relation to the days

[18] https://www.arb-silva.de/
[19] https://blast.ncbi.nlm.nih.gov/Blast.cgi

in captivity of the host. This analysis could only be run for *B. arietans*, *D. typus* and *N. annulifera* as the data was not available for the other sampled species.

For beta diversity analysis, permutational multivariant analysis of variance (PERMANOVA) analysis was run in the *vegan* (Oksanen et al., 2022) R package (R Core Team, 2022) using the *adonis2* function with Euclidean distance and 999 permutations. The predictors tested were host species and if the host species was classified under the Elapidae family or others. A Principal Components Analysis (PCA) was run with Euclidean distance using *ggplot2* (Wickham et al., 2022b), to visualise the variation among species using the ASV distance matrix.

4.3 Results

4.3.1 Bacterial Abundance by Host Species

The abundance of the four different bacterial phyla (Firmicutes, Bacteroidota, Actinobacteria and Proteobacteria) identified in the oral cavity of our samples are shown in figure 4-1. Most of these bacterial phyla were not unique to any sample or host species except the “other” group was only detected in *N. mossambica*. The group of other bacteria was identified as Tenericutes and Fusobacteria. The abundance of different bacterial phyla varied across the snake species. As seen in figure 4-1, *D. typus* has the highest abundance of Firmicutes at 95 % with 2 % of Proteobacteria, 2 % Actinobacterota and only 1 % Bacteroidota. The second highest abundance of Firmicutes with 78 % was found in *B. arietans* with 15 % Proteobacteria, 4 % Actinobacteriota and 3 % Bacteroidota. The third greatest abundance of Firmicutes at 72 % was found in *N. annulifera* with 20 % Proteobacteria, 7 % Bacteroidota and 1 % Actinobacteriota. The *D. polylepis* group had the fourth-highest abundance of Firmicutes at 69 % with 19 % Bacteroidota, 8 % Actinobacteria and 4 % Proteobacteria. The *N. mossambica* group had 43 % Proteobacteria, 35 % Firmicutes, 19 % Bacteroidota, 2 % other and 1 % Actinobacteria. The *P. natalensis* group had 40 % Proteobacteria, 35 % Firmicutes, 24 % Bacteroidota and 1 % Actinobacteria. The average abundance of different phyla across the samples are Firmicutes 78.56 %, Proteobacteria 10.77 %, Bacteroidota 7.68 %, Actinobacteria 2.1 % and Other 0.75 %. The abundance levels were calculated by the number of bacterial sequences detected in each phylum. The remaining 3.14 % was comprised of bacteria groups that accounted for less than 0.01 % each of the whole microbiome.

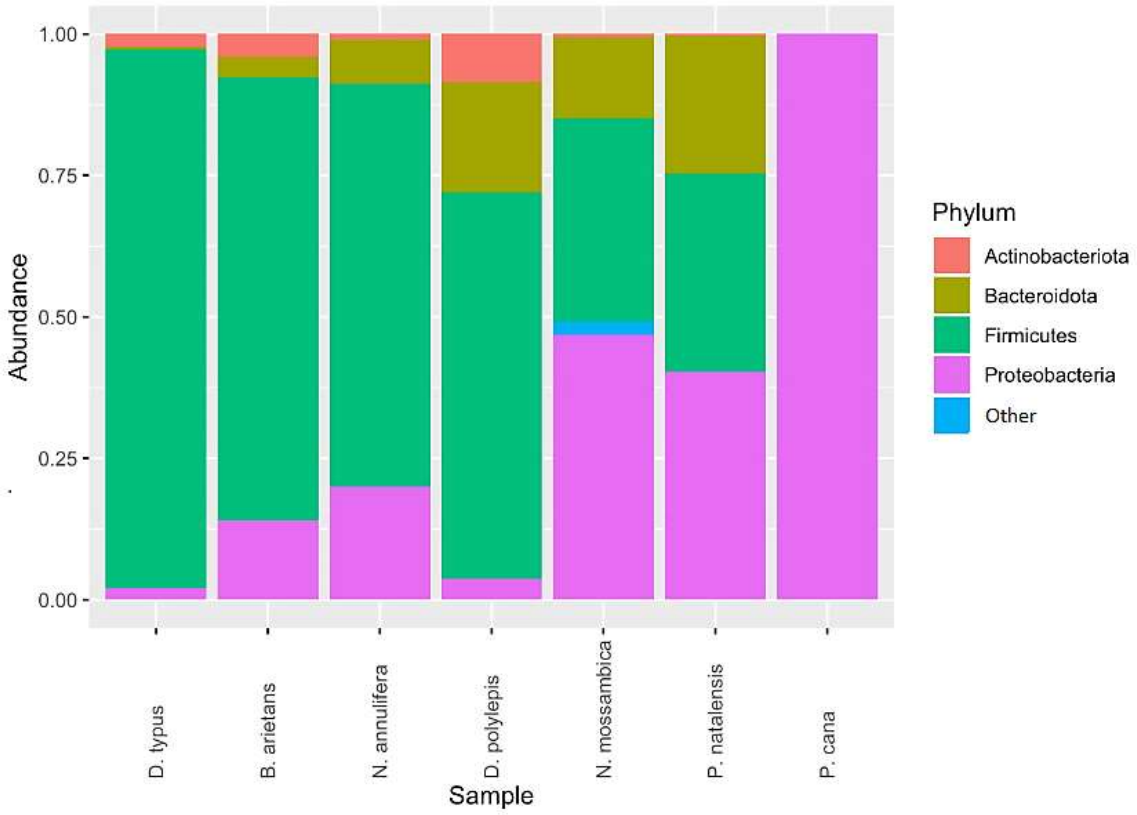

Figure 4-1 The average abundance of bacterial phylum in the oral cavity of various snake species based on the results of Next-Gen sequencing of the bacterial 16S rRNA gene.

Across our samples, Firmicutes was the most dominant phyla (figure 4-2), with some samples only containing Firmicutes. The majority of samples were captive snakes that were wild-caught, except two *D. polylepis* specimens (HHL015 and HHL 017) that were born in captivity from a gravid wild-caught female. Three *P. natalensis* specimens (HHL104, HHL105 HHL106) were formally wild-caught problem snakes that were bought to HRC and undergoing temporary quarantine with the intention of subsequent release back to the wild. The majority of our samples came from snakes that appeared to be healthy with the exceptions of HHL007, HHL013, HHL023, HHL038, and HHL106. The two specimens of *N. annulifera*, HHL007 and HHL013, had a localised infection in their tail tissue and unspecified dermatitis localised to their neck respectively.

These samples had elevated levels of Proteobacteria but HHL005 also showed higher levels of Proteobacteria indicating the elevated levels were unlikely linked to their infections. HHL004 was the only sample of *N. annulifera* that showed high levels of Bacteroidota but the snake appeared healthy upon inspection and was kept under the same conditions as the other members of the species. The unhealthy-appearing *D. polylepis* (HHL023) had unspecified dermatitis with flaking scales across the length of its body but the oral sample exhibited no unique pattern of abundance. HHL038 had visible lesions in its mouth and a higher abundance of Bacteroidota was detected than within other *B. arietans* samples indicating the infection may be linked to certain bacteria in that phylum.

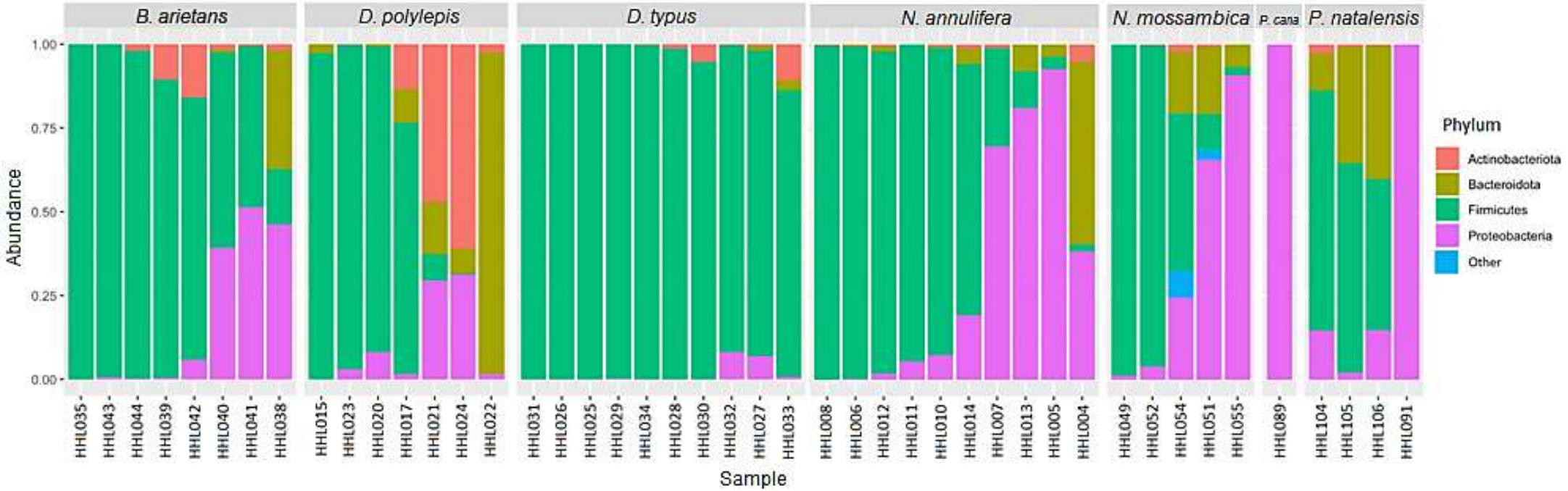

Figure 4-2 The abundance of bacterial phylum in the oral cavity of various snake species per sample based on the results of Next-Gen sequencing of the bacterial 16S rRNA gene.

4.3.1.1 Bacterial Abundance by Genus

The following section depicts the identification of the unique sequences that resulted from the NGS of the snake oral swabs. It was possible to identify all sequences to genus level (table 4-2), except for members of the Weeksellaceae family. Across all samples, there were 706 unique taxa identified with a total of 249 286 sequences found. Only 0.69 % of the total sequences were not able to be aligned via the SILVA (Quast *et al.*, 2013) database or BLAST (Altschul *et al.*, 1990). *Staphylococcus* accounted for 77.76% of all unique bacterial sequences detected followed by *Enterobacter* at 4.56 %, *Alcaligenes* at 2.16 %, and *Bacteroides* at 1.74 % (table 4-2 and 4.3). An unidentifiable genus under Weeksellaceae accounted for 3.21 % of the total sequences.

Table 4-2 Taxonomic classification of identified bacteria detected at levels higher than 0.01% with the number of repeating sequences and percentage of the microbiome

Phylum	Class	Order	Family	Genus	Repeats	Per cent (%)
Actinobacter-iota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Corynebacterium*	1686	0,68
			Dietziaceae	*Dietzia*	1490	0,60
		Micrococcales	Brevibacteriaceae	*Brevibacterium*	1722	0,61
			Micrococcaceae	*Kocuria*	726	0,21
Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	4355	1,75
			Dysgonomonadaceae	*Petrimonas*	1839	0,74
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Myroides*	2282	0,92
			Weeksellaceae	Not Found	7995	3,21
	Sphingobacteriia	Sphingobacteriales	Filobacteriaceae	*Filobacterium*	2636	1,06
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	1638	0,66
			Staphylococcaceae	*Staphylococcus*	193835	77,76
		Lactobacillales	Enterococcaceae	*Enterococcus*	338	0,14
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Alcaligenes*	5395	2,16
				Bordetella	3688	1,48
	Gammaproteo-bacteria	Enterobacterales	Enterobacteriaceae	*Citrobacter*	435	0,17
				Enterobacter	11368	4,56
				Salmonella	2147	0,86
			Morganellaceae	*Morganella*	455	0,18
				Providencia	479	0,19
			Yersiniaceae	*Serratia*	448	0,18
		Pasteurellales	Pasteurellaceae	*Seminibacterium*	390	0,16
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	674	0,27
			Pseudomonadaceae	*Pseudomonas*	480	0,19
		Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	917	0,37
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	*Mycoplasma*	1160	0,47
Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	*Fusobacterium*	708	0,28

While many variants of the above genera were seen, the majority of these variants aligned with the same species and/or strain (table 4-3). The variants were determined by having multiple instances that had been identified to the same species, possibly due to the hypervariable regions found with the 16S rRNA gene. The most common strain and species are depicted in table 4-3. The variants of *Staphylococcus* were the highest (n = 187) but the majority aligned with *Staphylococcus saprophyticus* or *Staphylococcus sciuri.* All the variants of *Enterobacter* (n = 44) were aligned to the cloacae complex species group and the majority were aligned to *Enterobacter hormaechei* subspecies *xiangfangensis*. The variants of the Weeksellaceae (n = 13) family were not able to be aligned to any genus*.* The variants of *Alcaligenes* (n = 14) were all aligned to *Alcaligenes faecalis* but no further classification was available. The variants of *Bacteroides* (n = 22) were all aligned to the same *Bacteroides nordii* strain.

Table 4-3 Species and strains (when available) of the Top 5 bacteria detected in the oral cavity of snakes and the number of variants detected per genera

Family	**Genus**	**Species**	**Strain**	**Variants within genus**
Staphylococcaceae	*Staphylococcus*	*Staphylococcus saprophyticus* *Staphylococcus sciuri*	ATCC 15305 DSM 20345	187
Enterobacteriaceae	*Enterobacter*	*Enterobacter hormaechei*	*xiangfangensis* 10-17	44
Weeksellaceae	Not available	Not available	Not available	13
Alcaligenaceae	*Alcaligenes*	*Alcaligenes faecalis*	Not available	14
Bacteroidaceae	*Bacteroides*	*Bacteroides nordii*	WAL 11050 = JCM 12987	22

4.3.1.2 Indicator Species Analysis

Indicator species and other ecological indicators are commonly used as a method to monitor and assess the state of current environmental conditions (Niemi & McDonald, 2004; Siddig *et al.*, 2016). While indicator analyses are usually done at higher population levels, bacterial indicator species of microbiomes can be used to determine the general health of their hosts (Siddig *et al.*, 2016). Our samples mostly came from captive snakes and can be used as a survey of the bacteria that would be present in the oral cavity of a healthy snake with similar husbandry. It could be used as a method to monitor the health of snakes over longer periods. The indicator species for our samples and their significance values are depicted in table 4-4.

Table 4-4 List of bacterial species and their respective weights for conducting an indicator analysis for the health of the host species

Strain	Indicator Value	P-value
Staphylococcus saprophyticus_1	0.5367	0.003
Weeksellaceae	0.7500	0.002
Staphylococcus sciuri	0.6525	0.002
Staphylococcus saprophyticus_2	0.6293	0.004
Enterobacter hormaechei_1	0.7500	0.002
Enterobacter hormaechei_2	0.5000	0.038

The indicator bacteria of the oral microbiomes of various snake species include *S. saprophyticus,* Weeksellaceae, *S. sciuri*, and *E. hormaechei* (table 4-3). The indicator values indicate the relative abundance of a bacterial strain detected after multiple random subsampling and the P-values indicate the probability that a specific bacterial strain will be detected in a random subset of the host species. For *S. saprophyticus*_1 the indicator value indicates this strain was detected in 53.67 % of the samples and has a probability of 99.7 % of being detected in a random subset of the samples taken. Weeksellaceae and *E. hormaechei_1* had the highest relative abundance with 75.00 % followed by *S. sciuri* 65.25 %, *S. saprophyticus_2* 62.93 %, *S. saprophyticus_1* 53.67 % and *E. hormaechei_2*. The p-values indicate all of these bacteria have more than a 96.2 % of being detected in random subsampling.

4.3.2 Alpha Diversity

Alpha diversity quantifies the diversity within a sample and is used to quantify and visualise bacterial diversity and richness (Galloway-Peña & Hanson, 2020; Smith *et al.*, 2021). Figure 4-3 depicts the richness of the bacteria found within the oral cavities of each species. The host species have been separated by whether the host species is classified as part of the Elapidae family or other families. The snake species that are classified as Elapidae generally have lower bacterial species richness than the species that are not elapids. Of these species that are classified as Elapidae, *D. polylepis* (15) had the lowest bacterial species richness followed by *N. annulifera* (17) and *N. mossambica* (19). The *P. natalensis* (29) species had the greatest bacterial species richness and *B. arietans* (28) had the second highest richness followed by *D. typus* (25). According to the p-values calculated, host species ($p < 0.0001$) has a highly significant

influence on the richness of the bacteria in the oral cavity and if the sample is classified as Elapidae (p = 0.0506) there is no statistically significant influence on the richness of bacteria in the oral cavity, however, the host species that classified as Elapidae do have an observable lower bacterial species richness.

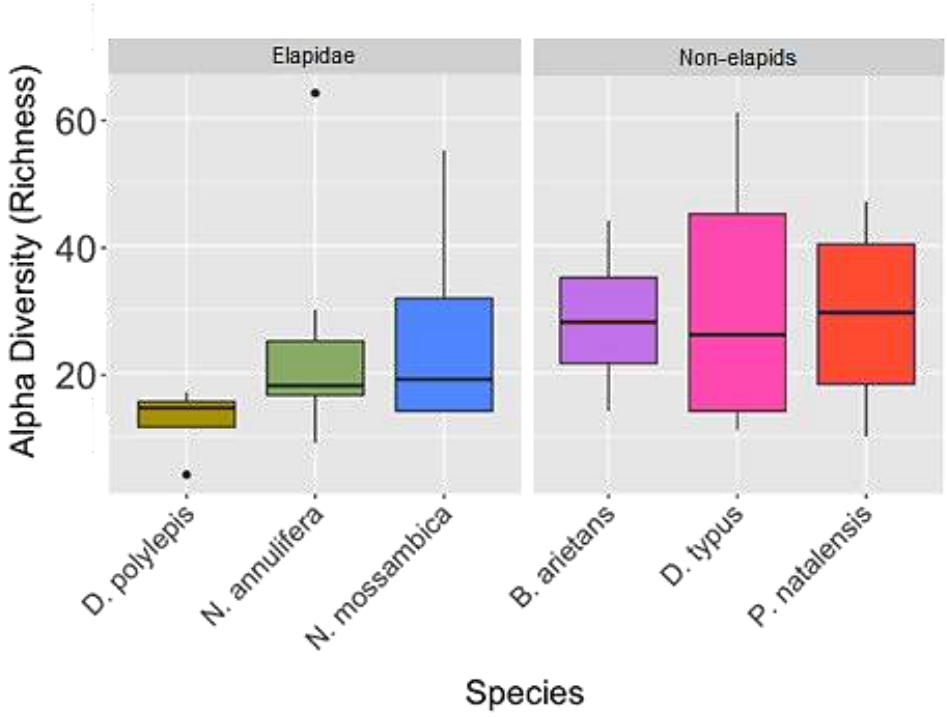

Figure 4-3 Bacterial richness based on Alpha Diversity split by whether the host species is classified under the Elapidae family (Y) or not (N). Based on results from Next-Gen sequencing of the bacterial 16S rRNA gene.

To determine the variety of bacteria that are found within the host samples the Shannon index was calculated for the samples. The results in figure 4-4 were also split by host species and the classification of Elapidae. The species which had the classification of Elapidae had lower diversity than those that did not. The p-values for these predictors and their influence on the Shannon index were: host species ($p < 0.0001$) and Elapidae Y/N ($p = 0.0202$). The *B. arietans* (2.60) species had the highest diversity of bacteria followed by *D. typus* (2.25) and *P. natalensis* (2.20). For the species that are classified as Elapidae, *N. annulifera* (2.00) had the highest bacterial diversity followed by *N. mossambica* (1.90) and *D. polylepis* (1.55). The host species *N. annulifera* had the highest diversity of the elapids and had the greatest outliers, for richness and diversity, within the sample set. The host species ($p < 0.0001$) predictor indicates the diversity of bacteria was highly influenced by the host species. The classification of Elapidae (p =

0.0202) does significantly influence the bacterial diversity in the oral cavity of various snake species.

From the analyses mentioned above, host species had the greatest influence on the richness and diversity of the bacterial oral microbiome. The classification of Elapidae does significantly influence the bacterial species diversity and it was observed the classification of Elapidae does decrease the bacterial richness and diversity in the oral cavity.

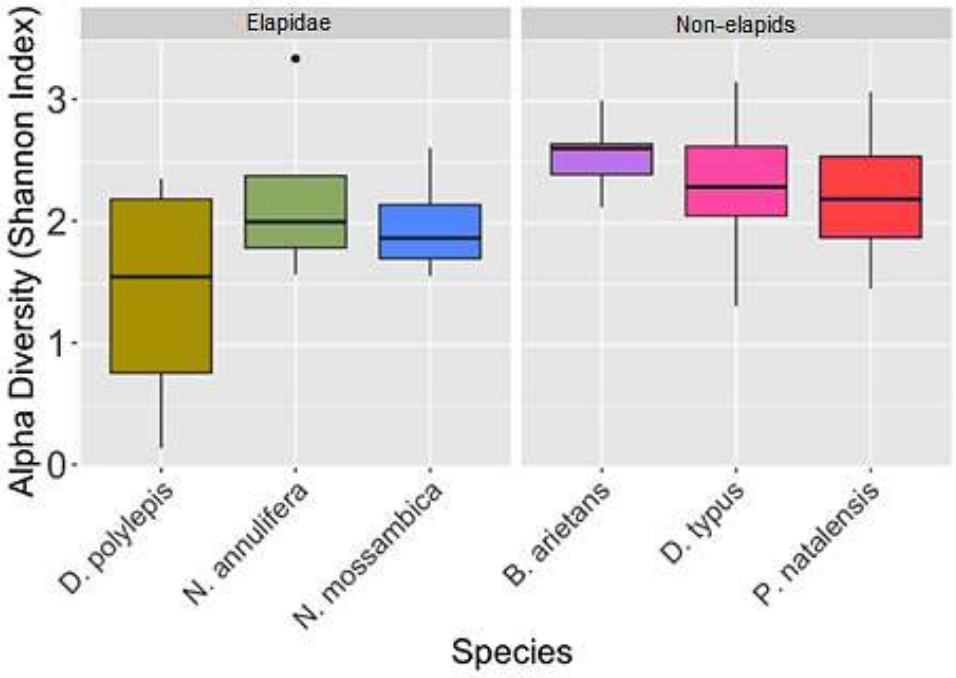

Figure 4-4 Shannon index of diversity among sampled species separated by whether the host species is classified under the Elapidae family (Y) or not (N). Based on results from Next-Gen sequencing of the bacterial 16S rRNA gene.

The variant of days in captivity was tested to determine if there was any influence on bacterial diversity and richness. The host species *B. arietans, D. typus* and *N. annulifera* were used for these calculations as this data was not available for the other host species. The days in captivity did influence the diversity ($p = 0.0004$) (figure 4-5) and there is an observable increase in bacterial diversity when compared to days in captivity. The *B. arietans* group showed the least difference in diversity when compared to days in captivity while *D.* typus and N*. annulifera* had the greatest difference in bacterial diversity in relation to days in captivity. Both *D. typus* and *N. annulifera* had outliers that could have influenced the trend line.

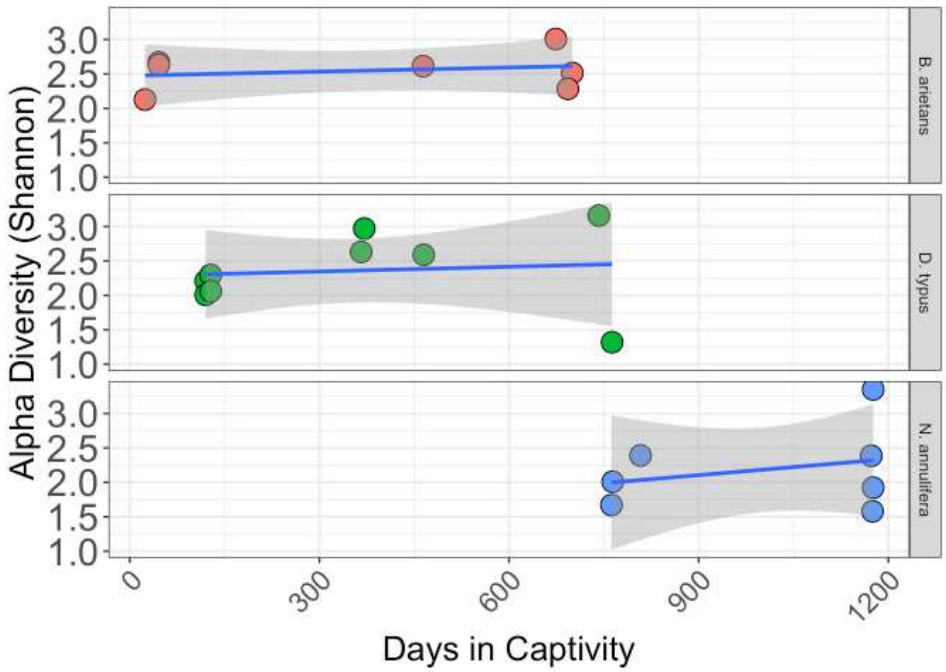

Figure 4-5 Shannon Index of bacterial diversity of the oral cavity, compared to the number of days spent in captivity of various snake species. Each point represents one sample and the number of days spent in captivity of that host species. Based on results from Next-Gen sequencing of the bacterial 16S rRNA gene.

The days in captivity did not have a significant influence on the richness of bacteria ($p = 0.462$) but there is an increasing trend in richness compared to days in captivity (figure 4-6). There is an increase in bacterial richness compared to days in captivity, however, this does not rely on the number of days in captivity and may have other factors increasing the richness. In the group of *B. arietans,* a clear increase can be seen even with one outlier point indicating a lower diversity. The *D. typus* group indicates the greatest increase in richness as the days in captivity increase with the outlier point occurring in the middle of the trend. For the *N. annulifera*, the cluster of samples that had spent the most days in captivity was lower in richness than the samples that had spent fewer days in captivity. The outlier of *N. annulifera* that had spent the most days in captivity had a much greater richness than the other samples and caused the trend to increase, as seen with diversity.

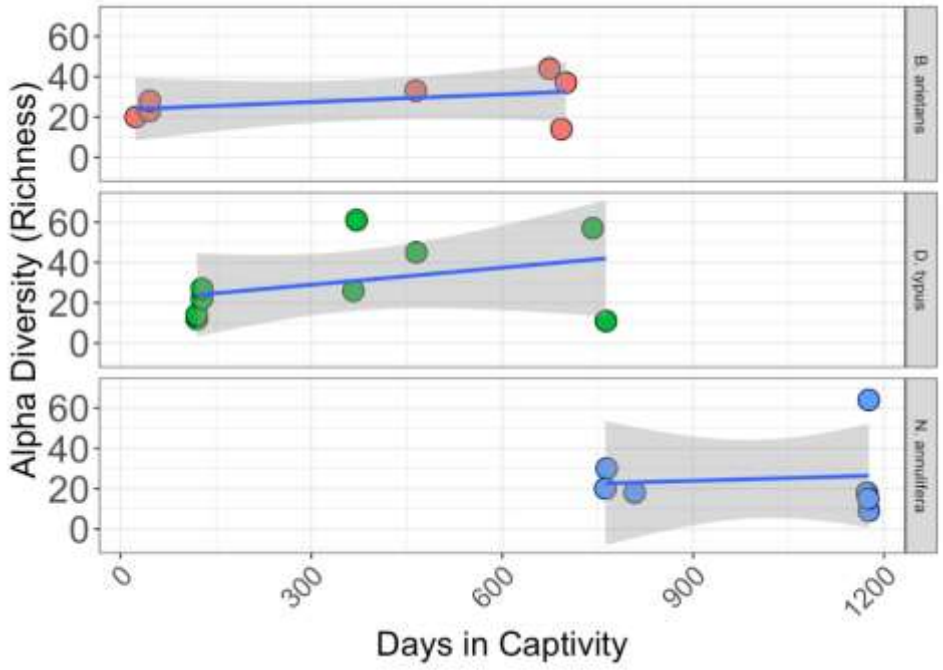

Figure 4-6 Bacterial richness of the oral cavity, compared to the number of days in captivity of various snake species. Each point represents one sample and the number of days spent in captivity of that host species. Based on results from Next-Gen sequencing of the bacterial 16S rRNA gene.

4.3.3 Beta Diversity

Beta diversity studies the diversity between individual samples and is calculated by comparing dissimilarity between samples (Galloway-Peña & Hanson, 2020). These results focus on the comparisons of the oral microbiomes as a whole and compare the microbiomes to each other. The PERMANOVA analysis was run with the predictors of host species and the if the host species is classified as Elapidae or not. There was a significant difference in Beta diversity between host species ($p = 0.001$) indicating that bacterial community compositions were distinctly dissimilar for the snake species tested. If the host species was classified as Elapidae or not ($p = 0.019$) indicated a significant influence on the microbiome composition of the snake species that are classified as Elapidae and the species that are classified as other families. These values show host species highly influences the composition of the microbiome and the classification of Elapidae also influences the composition of the microbiome, indicating a link to phylogeny. The Principal Components Analysis (PCA) (figure 4-7), is a visual representation of the dissimilarities of the microbiomes.

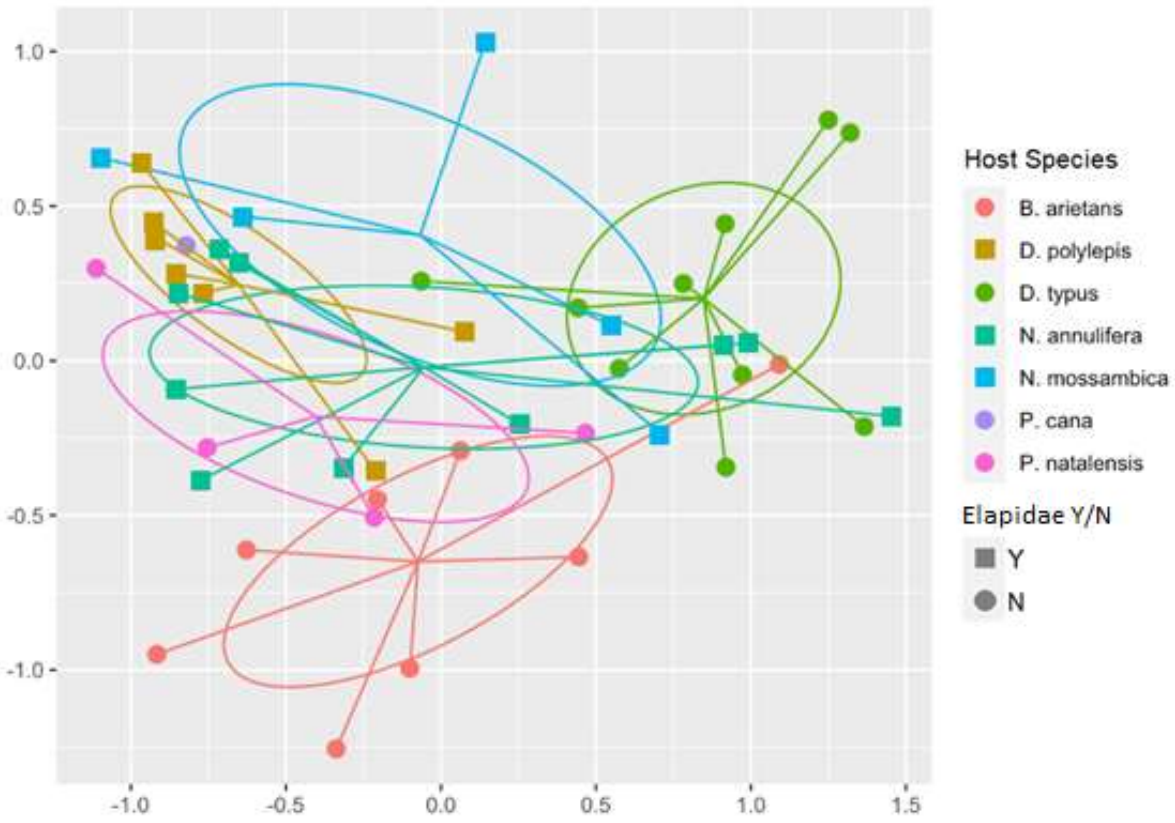

Figure 4-7 Principal Components Analysis (PCA) plot of variation among host species with an indication of whether the host species is classified under the Elapidae (☐) or not (○). Each (○ or ☐) point represents the average Amplicon Sequence Variants (ASVs) (used in place of operational taxonomic units) of each sample's microbiome. The exact coordinates of each point are irrelevant; only the distance between points is of value. The distance between two points represents the variance of the ASVs between the two microbiomes; the further apart the two points the more dissimilar the microbiomes. Constructed using the 16S rRNA sequences of the bacterial oral microbiome of various snake species from Next-Gen sequencing. This plot acts as a visualisation tool to allow the variance between samples to be seen on a two-dimensional plane. The circles surrounding the central points indicate the statistically expected range of the microbiomes of samples from the same group (host species). The central point (centroid) of each group indicates the average ASV makeup of the group. Where these circles overlap is where the similarity in the microbiomes of the different species can be expected.

We can see every host species does have exclusive aspects of its microbiome as there are parts of the circles that do not overlap with other species. There is a higher level of

clustering for the species classified as Elapidae while those that are not classified as Elapidae are more dispersed. The species group of *N. annulifera* has the highest variation within a species as this group has the greatest distance between two points. The species group of *N. annulifera* has the highest level of overlap with other host species. The *D. polylepis* group has the least variation within a host species and has the greatest clustering of points and second greatest overlap with other species. The host species *B. arietans* have the least overlap indicating the most exclusive expected microbiome.

4.4 Discussion and Conclusion

4.4.1 Implications of Bacteria Detected on Human and Animal Health

The microbiome of humans is made up of Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria phyla (Forster *et al.*, 2022); the same phyla are found within these samples from snakes. Baylock (2001) indicated there is a strong correlation between the bacteria located within the oral cavity of snakes and the bacteria located in the wounds of snakebite victims.

Firmicutes are Gram-positive bacteria that are solitary and form endospores (Willey et al., 2014). *Staphylococcus* is a Firmicute which accounted for 77. 76 % of the microbiome of the sampled snakes. Firmicutes bacteria are responsible for many diseases in humans including endocarditis, mastitis, bacteraemia, cellulitis, and pneumonia (Khazandi *et al.*, 2018; Willey *et al.*, 2014). There are multiple instances of multidrug-resistant (MDR) strains of *Staphylococcus*, especially methicillin resistance (Khazandi *et al.*, 2018; Rey Perez *et al.*, 2021). This implies there is a risk with the strains that were discovered in the oral cavity of snakes, as these could be one of the MDR strains or they could mutate into an MDR strain. Some *Staphylococcus* spp. can generate biofilms which can impair the defence system of the host and reduce the efficiency of antibiotic actions (Silva et al., 2020). A biofilm is an aggregation of microorganisms that are embedded with a substance or extracellular polymeric matrix which is self-produced (Assefa & Amare, 2022; Oluwole, 2022). Biofilms have altered phenotypic characteristics and often have increased gene transcription and growth rates (Oluwole, 2022). Biofilms can become attached to surfaces which allows the bacteria to survive under harsher environmental conditions (Assefa & Amare, 2022; Oluwole, 2022). Biofilms in hospitals are responsible for 65 % of nosocomial infections and 60 % of all human infections (Assefa & Amare, 2022). The result of biofilms is an increase in virulence and resistance to multiple methods of

disinfecting the surfaces to which the biofilms are attached (Assefa & Amare, 2022; Oluwole, 2022).

Staphylococcus saprophyticus is a common coagulase-negative bacterium that is most commonly found on the skin of humans but can be an opportunistic pathogen that causes uncomplicated urinary tract infection (Silva et al., 2020; Stephens et al., 2020). Coagulase is an enzyme found in some species of *Staphylococcus* that can trigger the clotting of blood plasma, which aids the bacteria's virulence (Willey et al., 2014). The strain that was found within the oral microbiome of snakes was *S. saprophyticus* ATCC 15305. The virulence of *S. saprophyticus* in animals can be enhanced by a polysaccharide capsule that this species can sometimes possess but this capsule does not enhance the virulence in humans (Silva et al., 2020). When compared to other coagulase-negative bacteria, *S. saprophyticus* does not have multiple virulence factors including the adhesion proteins that other species have resulting in it being of lower clinical value (Silva et al., 2020). While this strain can threaten humans, it can threaten agricultural farming as well. MDR *Staphylococcus* species have been documented in infections in swine (Bonvegna et al., 2021) and can cause bovine mastitis, and more serious infections, as determined by Dabele et al. (2021), and Khazandi et al. (2018). *Staphylococcus. sciuri* is an opportunistic human pathogen and can be found in the environments and on the skin of many animals (including humans) (Khazandi et al., 2018). The *S. sciuri* species has been shown to have MDR strains in the available literature and has exhibited a decreased susceptibility to clindamycin, erythromycin, oxacillin and penicillin (Chuang *et al.*, 2022). Both these strains of *Staphylococcus* pose a threat to human health based on their ability to be opportunistic pathogens and possible MDR.

The Proteobacteria phylum contains the family of Enterobacteriales containing gam-negative enteric bacteria and contains many pathogens known to affect the human gut; *Enterobacter*, *Salmonella*, and *Escherichia* (Willey et al., 2014). *Enterobacter hormaeche*i, found at 4.56 % in the snake oral microbiome, is widespread in the environment (Chen et al., 2022; Shi et al., 2022). As part of the *Enterobacter* cloacae complex, it is predominately classified as a human enteric pathogen that causes nosocomial infections (Chen et al., 2022; Shi et al., 2022). In animals, *E. hormaeche*i has been found as the cause of diarrhoea, respiratory disease, and uterine infections (Shi *et al.*, 2022; Wang *et al.*, 2020; Wang *et al.*, 2021). Members of the Enterobacteriaceae family that can produce carbapenemases, therefore, are resistant to carbapenem and

have been added to the list of medically import MDR bacteria (Chen et al., 2022). These strains are considered a public health crisis as treatment with carbapenem is considered a last resort treatment due to high costs and severe clinical outcomes (Chen et al., 2022).

Another Proteobacteria that was found at 2.16 % of the snake microbiome was *Alcaligenes faecalis,* an opportunistic pathogen (Hasan *et al.*, 2019; Huang, 2020). The *A. faecalis* species is a common part of the human gut microbiome but can also be found in soil, water and some hospital environments (Huang, 2020; Tena *et al.*, 2015). Infections with *A. faecalis* are not common but there have been sporadic cases of nosocomial infections from contaminated hospital equipment and which can cause endocarditis, meningitis, bacteraemia, abscesses and others (Hasan *et al.*, 2019; Huang, 2020; Tena *et al.*, 2015). Infections with *A. faecalis* can be difficult to treat due to MDR (Hasan *et al.*, 2019; Huang, 2020; Tena *et al.*, 2015).

For Bacteroidota, the most dominant bacteria were unidentified bacteria from the Weeksellaceae family. These were the only sequences that were unable to be identified to the genus level as the BLAST results were equally shared between *Chryseobacterium* and *Riemerella anatipestifer*. The species of *R. anatipestifer* is most commonly found as a pathogen in ducklings and other young birds, with a high morbidity rate (Gong *et al.*, 2020; Nielsen *et al.*, 2021). It has demonstrated MDR to many bacteria (Nielsen *et al.*, 2021). It can cause acute sepsis in young birds and chronic issues in older birds (Gong *et al.*, 2020). The hemolytic action *R. anatispester* displays is similar to that seen in *Mycobacterium tuberculosis* and *Bordetella pertussis* and this hemolytic action has been linked to the virulence of these bacteria (Gong *et al.*, 2020). *Chryseobacterium gleum, Chryseobacterium meningosepticum* and *Chryseobacterium hominis* are bacteria that are pathogenic to humans that are often found in environmental sources such as water and soils (Yadav *et al.*, 2021). Some species are listed as major pathogens to fish (Michel *et al.*, 2005). These can be the source of nosocomial infections as *Chryseobacterium* can cultivate in taps, basins, and on multiple medical devices (Michel *et al.*, 2005; Yadav *et al.*, 2021). They have been described as pathogens that have resulted in pneumonia, menigitis, endocarditis, ocular infections, bacteremia, epidermal infections and other soft tissue infections (Lee & Mauger, 2021; Yadav *et al.*, 2021). Those most at risk are immunocompromised individuals and newborns (Yadav *et al.*, 2021). Among isolates of *Chryseobacterium,* MDR against rifampicin has been detected within 75% of the isolates and 33% across a

broad spectrum of antimicrobials (Michel *et al.*, 2005; Yadav *et al.*, 2021). Due to the inability to identify to genus level of the Weeksellaceae bacteria, the risk to humans cannot be determined.

The sensitivity of these bacteria to antibiotics can result from the acquisition of antibiotic-resistant genes within this microbiome (Forster *et al.*, 2022). Any bacteria can undergo horizontal gene transfer (HGT) allowing for the restructuring of the genetic makeup of the bacteria present (Lerner *et al.*, 2017; Zhu *et al.*, 2022). This constant exchange of genetic material allows for adaptations and mutations to be shared across communities allowing for constant evolution of the resistance to external damage of the bacterial populations (Lerner *et al.*, 2017). The overuse and incorrect use of antibiotics is the main driver for the development of MDR strains of bacteria and is facilitated by the HGT of antibiotic genes among bacteria (Lerner *et al.*, 2017). Most of the transfer of genetic material occurs between bacteria of the same genera within the same microbiome and at higher levels within pathogenic bacteria (Forster *et al.*, 2022).

The interaction with any bacteria different from the host's natural microbiome poses the risk of infections through various methods. An inflow of bacteria, even if part of the natural microbiome, has can disrupt the natural levels the human immune system can handle effectively (CDC, 2016). The speed at which bacteria mutate and the risk of horizontal gene transfer between the different populations is high at all times (Forster *et al.*, 2022). This transfer may result in the influx of genetic material that provides the bacteria with resistance to immune responses or antibiotics and other drugs (Forster *et al.*, 2022).

Of the literature reviewed, there are multiple instances of secondary infections following snakebites but these infections resulted from bacteria detected in low numbers in this study's samples. Some of the bacteria that caused secondary infections and were found in this study include *Morganella morganii, Serratia* spp*., Bacillus* spp*., Enterococcus* spp*., Acinetobacter* spp., *Citrobacter spp.,* and *Salmonella* spp. but none of the aforementioned bacteria were found at levels higher than 1% per genus with an average abundance of 0.35 % per genus. Indicating the snakes sampled in this study pose an apparent low risk of secondary infections if a bite to a human occurs. However even if the risk is low, appropriate prophylactic treatment is recommended in the event a bite occurs.

4.4.2 Implications of Bacteria Detected on Snake Health

The bacteria detected in this study act as a survey of the natural microbiome of snakes kept in captivity. The majority of the bacteria detected appear to have no negative influence on the health of the host species of snakes. The samples tested in this study had higher levels of Firmicutes than those seen in other literature. When comparing the results of this study to those found in other literature (Abba *et al.*, 2017; Dehghani *et al.*, 2016; Esmaeilishirazifard *et al.*, 2022; Krishnankutty *et al.*, 2018; Panda *et al.*, 2018; Resiere *et al.*, 2018; Smith *et al.*, 2021), the levels of Firmicutes (*Staphylococcus*) are higher than those of other studies on wild and captive snakes.

The majority of samples were taken from health-appearing snakes and the indicator species analysis provides insight into which bacteria form part of the natural microbiome of the snakes sampled that could be used in future research. There was no observable difference between the samples that originated from healthy-appearing snakes and the snakes that were ill, apart from one sample having elevated levels of Bacteroidota. From this we can determine, *S. saprophyticus,* Weeksellaceae, *S. sciuri*, and *E. hormaechei* are the most prominent bacteria in the oral cavity of the sampled snakes. These bacterial relative abundance levels can be used in future screenings to determine whether the appropriate levels of bacteria are present in the oral cavity.

Comparison of Bacteria Found Within the Oral Cavity to Other Literature

The bacteria that were found in this study and reviewed literature were *Bacillus, Enterococcus, Staphylococcus*, *Acinetobacter, Citrobacter, Enterobacter, Morganella, Providencia, Pseudomonas, Salmonella, Serratia,* and *Bacteroides.* The bacteria that were detected in the reviewed literature but not in this study were *Streptococcus, Aeromonas, Escherichia coli, Klebsiella, Proteus, Shewanella, Yersinia, Clostridium,* and *Peptostreptococcus*. The bacteria that were found in this study but not in the reviewed literature were *Corynebacterium, Dietzia, Brevibacterium, Kocuria, Petrimonas,* Weeksellaceae*, Filobacterium, Alcaligenes, Bordetella, Seminibacterium, Stenotrophomonas,* and *Mycoplasma.*

4.4.3 Factors Which Influence the Oral Microbiota

The host species had the most significant influence on multiple factors of the microbiome. It influences the richness, diversity and composition of the microbiome. The different host species had exclusive aspects of their microbiome which indicates the importance of

identification of a snake when considering treatment for snakebites. While all host species had high levels of *Staphylococcus* in the oral cavity, the presence of other bacteria cannot be overlooked when considering the risk of a secondary infection. The combination of the statistical significance of both host species and if the host species is classified as Elapidae or other families indicates the composition and diversity of the bacterial oral microbiome are influenced by host phylogeny. Snakes that are classified as Elapidae have lower bacterial diversity and their microbiomes are similar to each other, sharing closely related bacteria within their microbiome, based on the PCA. The group of *N. mossambica* was the only group to have *Mycoplasma* and *Fusobacteria,* seen as outliers in the various graphs, yet still had the second lowest bacterial diversity.

The PCA indicates the samples from *D. polylepis* contain bacteria that are similar based on their ASVs. All individuals of *D. polylepis* were kept in shared outdoor enclosures while the other host species were kept in singular enclosures. The similar compositions of microbiomes and low diversity could be a result of their shared outdoor enclosure. These results may indicate the bacterial microbiome can be influenced by the environment and habitation status. The exact influence of these variants could not be determined due to limited sample numbers.

The influence of host species could be linked to the presence of antimicrobial peptides (AMPs) that differ based on the phylogeny of the host species (Ageitos *et al.*, 2017; van Hoek, 2014). The AMP cathelicidin has been identified in members of the elapid family and the cathelicidin in the members of elapids may explain the lowered diversity and richness. Further research regarding the presence of cathelicidin in the oral cavity and its influence on the oral microbiota is required. There is a multitude of other AMPs that are present in snakes and other reptiles but their influence on the oral microbiome has not been established. There is a focus on the influence these AMPs have on the gut microbiota but their link to oral microbiota has not been established. The unique AMP profile of each snake may be the reason for the differences that are seen in their microbiomes. The unique AMP profile of each snake may be the reason why the bacterial diversity and richness differ between snake species as different AMPs profiles would have different efficacies against different bacteria.

Influence of Diet

The variant of diet is kept consistent across the board as all snakes from Lowveld Venom Suppliers (LVS) were fed from an in-house rat breeding facility and the snakes from

Hoedspruit Reptile Centre (HRC) were frozen rats or other appropriately sized small mammals. This allows the influence of diet to be consistent across the samples and there is a connection between the food source of our samples and the oral microbiome

Dehghani *et al.* (2016) showed that feeding can introduce *Staphylococcus* into the oral cavity. The majority of the samples analysed by Dehghani *et al.* (2016) did not have *Staphylococcus* present before feeding but the majority had *Staphylococcus* present in the oral cavity after feeding. This indicates the high levels of *Staphylococcus* in the results of this study are possibly due to their food. The consistent feeding from the in-house facility would explain the proportionate increase of bacterial diversity and richness as days in captivity increase. While days in captivity did not have a statically relevant influence on bacterial richness, an increase in richness can still be seen as days in captivity increase. The rats from the in-house breeding facility will share similar microbiomes to other rats in the facility which allows the influence of the rats as a food source to be consistent across the snake samples. All of the snakes would be introduced to similar bacteria through their food which reduces variation in the microbiomes of the snakes sampled.

4.4.4 Conclusion

The results from this study allow for an expansion of the understanding of the oral microbiome of various South African snake species. The species studied today are a small part of the many species found in South Africa but this research acts as a baseline study for future research into other species, variants and aspects that influence the microbiome. The most abundant bacterial phylum across the samples was Firmicutes with *Staphylococcus* being the most abundant, accounting for 77.76 % of all sequences detected. This was followed by *Enterobacter* at 4.56 %, *Alcaligenes* at 2.16 %, and *Bacteroides* at 1.74 %. The Weeksellaceae family accounted for 3.21 % and was the only bacteria that were not identified to the genus level. Of these bacteria *Staphylococcus, Enterobacter* and *Bacteroides* were found in the reviewed literature and Weeksellaceae and *Alcaligenes* were not mentioned in the reviewed literature. The bacteria that have caused secondary infections after snakebites that were detected in these samples were *Morganella morganii, Serratia* spp*., Bacillus* spp*., Enterococcus* spp*., Acinetobacter* spp., *Citrobacter spp.,* and *Salmonella* spp.

The influence of diet on the microbiome was negated by the majority of the host species being fed from an in-house breeding facility and the other host species that were fed from another source were fed similar small mammals from a singular source. The use of small

mammals as food may explain the high levels of *Staphylococcus* because it is commonly found on the skin of mammals.

The strains identified in the indicator species analysis can act as indicators for the healthy oral microbiome of the sampled snakes and their relative abundance can be used to estimate the expected abundance in new samples when the host species has been kept under similar conditions. These can be used during the monitoring of these snakes as any major differences in the microbiome may be an indicator of stressors. There is a low likelihood that these indicator species could be used for other populations of snakes as there are many aspects that could alter the natural microbiome of a population. These species could only be used as an estimation of a bacterial population that has similar husbandry but wild-caught snakes are likely to have a different oral microbiome.

The host species with the highest diversity was *B. arietans* and the host species with the highest bacterial richness was *P. natalensis*. From our results, *D. polylepis* had the lowest bacterial richness and diversity while having the second most similar microbiome composition to other members of the species based on the PCA. Which could be due to their cohabitation. The days in captivity did have a significant influence on the bacterial diversity ($p = 0.0004$) but did not influence the bacterial richness ($p = 0.462$) of bacteria. The host species had the greatest influence on the bacterial richness ($p < 0.0001$) and diversity ($p < 0.0001$) and the classification of Elapidae did not significantly influence the richness ($p = 0.0506$) but did significantly influence the diversity ($p = 0.0202$) of the bacteria in the oral cavity. The composition of the oral bacterial microbiome was highly influenced by the host species ($p = 0.001$) and the classification of Elapidae ($p = 0.019$). The combination of these values indicates the microbiome may be influenced by host phylogeny. There are multiple ways the hosts' phylogeny might influence their oral microbiomes that are avenues for future research. The results found today could act as a baseline survey for future research into the bacterial oral microbiome of South African snakes.

CHAPTER 5 REVIEW OF THE BACTERIAL MICROBIOME OF REPTILES

5.1 Link To Literature Review

Reptiles are an important part of many ecosystems but are underrepresented in conservation efforts and research. The microbiome of reptiles is not commonly the focus of research and the composition of their microbiomes is not as easily available as many other animals, especially mammals. Studying the microbiome of animals allows for an insight into their adaptive success as the microbiome of any animal forms part of their innate immune system and influences their physiology, nutrient absorption, and development.

The presence of *Salmonella* in reptiles has been established and they are known reservoirs of many *Salmonella* species and serovars. This study only screened for *Salmonella* in the cloacal cavity of various reptiles and exact species and serovars were not determined. The screening for *Salmonella* in the sampled reptiles was achieved through simplified culture-based methods. The presence of *Salmonella* in the sampled reptiles was higher than the prevalence seen in other literature at 88.75 % of reptiles sampled were positive for *Salmonella* in the study. There have been multiple outbreaks of Salmonellosis that have been linked to interactions with reptiles and the results from this study indicate a high risk of contracting *Salmonella* from the reptiles sampled.

Snakebites are a great threat to human health and the bacteria in the oral cavity of snakes have been linked to secondary infections. There are multiple bacteria in the oral cavity of snakes that have caused secondary infections including *Morganella morganii, Serratia* spp*., Bacillus* spp*., Enterococcus* spp*., Acinetobacter* spp., *Citrobacter spp.,* and *Salmonella* spp. which were all detected in the samples examined for this study. Providing an insight into the bacteria that are present in the oral cavity of snakes allows for a better treatment plan to be developed in the event of a snakebite. An appropriate treatment plan lowers the risk of a secondary infection. If these bacteria present are known to exhibit multidrug resistance (MDR) then understanding the susceptibility to antimicrobials of these bacteria allows for a more appropriate antimicrobial treatment to be used.

The bacteria that were found in this study and reviewed literature were *Bacillus, Enterococcus, Staphylococcus*, *Acinetobacter, Citrobacter, Enterobacter, Morganella, Providencia, Pseudomonas, Salmonella, Serratia,* and *Bacteroides* (Baylock, 2001;

Chuang *et al.*, 2022; Dehghani *et al.*, 2016; Garg *et al.*, 2009; Smith *et al.*, 2021) The bacteria that were found in this study but not in the reviewed literature were *Corynebacterium, Dietzia, Brevibacterium, Kocuria, Petrimonas,* Weeksellaceae*, Filobacterium, Alcaligenes, Bordetella, Seminibacterium, Stenotrophomonas,* and *Mycoplasma* (Baylock, 2001; Chuang *et al.*, 2022; Dehghani *et al.*, 2016; Garg *et al.*, 2009; Smith *et al.*, 2021)*.*

5.2 Completion Of Objectives

Objective 1 - Determine the presence of intestinal *Salmonella* in captive reptiles

Salmonella was isolated from 88.75 % of the samples from various reptile species. A 91.52 % isolation success was seen from snakes and 82.55 % isolation success from lizards, tortoises and crocodiles. *Salmonella* was detected in every species that were sampled, with a mean isolation success of 88.33 % per host species.

Objective 2 – Compare the isolation success of two types of media selective for *Salmonella*; Xylose Lysine Deoxycholate (XLD) agar and Brilliant Green (BG) agar.

XLD agar had an isolation success of 81.25 % and BG agar had an isolation success of 61.25 %. The use of XLD agar significantly ($p = 0.017$) influenced the isolation success of *Salmonella*. The BG agar can only support 50 % of the viable *Salmonella* which was apparent when reviewing the growth of all agar plates. XLD agar was the preferred agar as the identification of *Salmonella* on the XLD agar was simpler than that of the BG agar. The use of both types of agars is still preferred as it allowed all *Salmonella* to be detected across all samples.

Objective 3 – Determine the statistical significance various factors have on the isolation success of *Salmonella*

The host species ($p < 0.001$) and the use of XLD or BG agar ($p = 0.017$) had a very significant influence on the isolation success of *Salmonella.* The type of reptile ($p = 0.905$), habitation status per enclosure ($p = 0.318$), source of reptile ($p = 0.962$), and the use of a dry cotton swab or Amies transport swab ($p = 0.638$) and no statistically significant influence on the isolation success of *Salmonella*.

Objective 4 - Determine the bacterial microbiome composition of the oral cavity of the various snake species. Determine the overall abundance of different bacterial

phyla, the most abundant bacteria across the samples, and the indicator species of the oral microbiome of sampled snakes.

The average abundance of different phyla were Firmicutes at 78.56 %, Proteobacteria at 10.77 %, Bacteroidota at 7.68 %, Actinobacteria at 2.1 % and Other at 0.75 %. The remaining 3.14 % was comprised of various bacterial groups that accounted for less than 0.01% each. There were 706 taxa identified across all samples. *Staphylococcus* (77.76 % with 187 variants) was the most prevalent taxa in the oral cavities followed by *Enterobacter* (4.56 % with 44 variants), Weeksellaceae (3.21 % with 13 variants), *Alcaligenes* (2.16 % with 14 variants), and *Bacteroides* (1.74 % with 22 variants). The indicator species for the samples were *Staphylococcus saprophyticus,* an unknown Weeksellaceae species, *Staphylococcus sciuri*, and *Enterobacter hormaechei.*

Objective 5 – Determine the bacterial richness and diversity within the oral cavity of each host species. Determine whether the days in captivity and host phylogeny influenced bacterial diversity and richness.

For the species classified as Elapidae, *D. polylepis* had the lowest bacterial species richness followed by *N. annulifera* and *N. mossambica.* The *P. natalensis* species had the highest bacterial species richness and *B. arietans* had the second highest richness followed by *D. typus.* The *B. arietans* species had the highest diversity of bacteria followed by *D. typus* and *P. natalensis*. For the species that are classified as Elapidae, *N. annulifera* had the highest bacterial diversity followed by *N. mossambica* and *D. polylepis*.

Days in captivity had no statistically relevant influence on the bacterial richness ($p = 0.462$) but did have a highly significant influence on the bacterial diversity ($p = 0.0004$) within the oral cavity. Both richness and diversity increased as the days in captivity increased, however, only diversity correlated to the days in captivity.

Host species ($p < 0.0001$) had the greatest influence on the richness of bacteria and the classification of Elapidae ($p = 0.0506$) did not have a significant influence on the richness of bacteria. Species that are classified as Elapidae had lower bacterial richness than those that are not classified as Elapidae. Host species ($p < 0.0001$) had the greatest influence on bacterial diversity (Shannon index) and the classification of Elapidae ($p = 0.0202$) had a significant influence on bacterial diversity. The species that are under the classification of Elapidae had an overall lower bacterial diversity than the species that did

not. The combination of these results indicate host phylogeny may influence the bacterial diversity in the oral cavity.

Objective 6 – Determine the possible influence of host phylogeny on the oral microbiome composition.

Host species ($p = 0.001$) had the most influence on the composition of the bacterial oral microbiome and the classification of Elapidae or not ($p = 0.019$) had a significant influence on the composition of the microbiome. The combination of these results indicates phylogeny does influence the composition of the bacterial oral microbiome.

5.3 Conclusions

The results of this study act as a baseline study for further research to expand upon. These results form part of a survey of the natural microbiome of reptiles kept in captivity in the Limpopo and Mpumalanga provinces of South Africa. The results of this study have enforced the risks involved with human-reptile interactions. The screening for Salmonella in the cloacal cavity was successful and *Salmonella* was detected in every species sampled. The importance of proper hygiene practices has been emphasized by the presence of possibly zoonotic bacteria detected in the cloacal and oral cavities of various reptiles. The high availability of *Salmonella* in the cloacal cavity of reptiles indicates a high zoonotic risk if basic hygiene practices are not possible. The risks involved with an infection of *Salmonella* were discussed. *Salmonella* exhibits a high zoonotic potential and has exhibited MDR in previous studies. It was established that *Salmonella* forms part of the natural intestinal microbiome as it was detected in all healthy-appearing reptiles. *Salmonella* can be infectious to reptiles if normal levels are not maintained. A change in the levels of *Salmonella* in the gut of reptiles can result from extended periods of stress, another infection and periods of lowered immune system function. The monitoring of *Salmonella* in the gut of reptiles should form part of general health screenings to allow for early detection of any possible infection with *Salmonella*.

The natural oral microbiome of the sampled snakes was determined through the use of molecular methods. Many of the bacteria detected are opportunistic pathogens but still form part of the natural microbiome of humans and other animals. For the snakes sampled, *Staphylococcus* was the most abundant bacteria accounting for 77.76 % of the oral microbiome and was found within the oral cavity of all snakes sampled. The two most abundant species of *Staphylococcus* (*Staphylococcus saprophyticus* and

Staphylococcus) are commonly found as natural parts of the human and other mammals' microbiomes. These bacteria still pose a risk as they are considered opportunistic pathogens which have previously exhibited MDR in the past.

The microbiome of reptiles is greatly influenced by their environment and their diet. The presence of *Staphylococcus* in all indicates the presence of this bacteria is possibly linked to their diet as all host species were fed similar food of adult rats, rat pups or other small mammals depending on their size. The lack of variance in the diet of the sampled reptiles allowed for a lower variety of bacteria to be detected if compared to other studies of wild snakes (Smith *et al.*, 2021). The prevalence of *Staphylococcus* was higher than seen in other studies of the oral microbiome of reptiles. The reptiles sampled had low abundance levels of the bacteria which have caused secondary infections from snakebites in the reviewed literature. Further research is required before the risk of infection from these low levels of the bacteria mentioned can be determined.

Many factors influenced the bacterial oral microbiome of various snake species. The host species and whether the host species was classified as Elapidae or not significantly impacted the bacterial richness and diversity within the oral cavity. The snakes classified as elapids had lowered bacterial richness and diversity than the snakes that were classified as other families. The days in captivity did significantly influence the bacterial diversity but did not influence the bacterial richness. There was a trend observed as both bacterial diversity and richness increased when compared to days in captivity. The composition of the oral microbiome was significantly influenced by host species and if the host species was classified as Elapidae or not. These results indicate host phylogeny could influence the composition of the microbiome.

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ANNEXURES

A. Collection Permit of Lowveld Venom Suppliers

Mpumalanga

TOURISM AGENCY

PERMITS

Permit No. MPB/V/ 2003

PERMIT

TO ESTABLISH AND OPERATE A VENOM EXTRACTION FACILITY

(Issued in terms of the provisions of the Nature Conservation Act 10 of 1998)

Name of permit holder: *Christopher Hobkirk*
Residential address: *3 Akwameryn Street*
West Acres
Nelspruit 1200

In terms of and subject to the provisions of the Nature Conservation Act, 1998 (Act No. 10 of 1998) and the regulations framed thereunder, the above-mentioned person is hereby authorised, subject to the conditions and requirements appearing on this permit to establish or carry on the institution referred to hereunder during the period of validity of this permit.

PARTICULARS OF INSTITUTION

Name of institution: *Lowveld Venom Suppliers*

Place where business is carried out: *Cnr Sabie & Main Road - HAZYVIEW*

ANIMAL SPECIES WHICH MAY BE KEPT AND EXHIBITED IN TERMS OF THIS PERMIT

Number	Species	Scientific name
As per attached addendum		

- **Gaboon Adder should Not be sourced from Mpumalanga Province**
- **New Condition: TOPS regulations from DEAT will apply from 1st February 2008(*Gov. Gazette No. 29657 of 23 February 2007*)**

Period of validity of permit: From date of issue to: *30 June 2022*

For Chief Executive Officer

Signature of permit holder

As per permit MPB/V/2003 dd. 26/07/2019

10	Berg Adder	*Bitis atropos*
10	Many Horned Adder	*Bitis cornuta*
10	Horned Adder	*Bitis caudalis*
10	Peringuey's Adder	*Bitis peringueyi*
10	Common Night Adder	*Causus rhombeatus*
10	Snouted Night Adder	*Causus defilippii*
100	Cape Cobra	*Naja nivea*
99	Forest Cobra	*Naja melanoleuca*
99	Rinkhals	*Hemachatus haemachatus*
10	Coral Snake	*Aspidelaps lubricus*
10	Shield Nose Snake	*Aspidelaps scutatus*
10	Zambezi Garter Snake	*Elapsoidea boulengeri*
9	Twig Snake	*Thelotornis capensis*
10	Bibron's Stiletto Snake	*Atractaspis bibronii*
9	Natal Black Snake	*Mecrlaps microlepidotis*
10	Rufous Beaked Snake	*Rhamphiophis rostratus*
10	Olive Grass Snake	*Psammophis mossambicus*
10	Short Snouted Grass Snake	*Psammophis brevirostris*
10	Spotted Skaapsteker	*Psammophylax rhombeatus*
10	Striped Skaapsteker	*Psammophylax tritaeniatus*
10	Eastern Bark Snake	*Hemirhagerrhis nototaenia*
10	Common Tiger Snake	*Telescopus semiannulatus*
9	Herald Snake	*Crotaphopeltis hotamboeia*
10	Natal Purple-Glossed Snake	*Amblyodipsas concolor*
10	Common Purple-Glossed Snake	*Amblyodipsas polylepis*
30	Southern African Python	*Python natalensis*
10	Brown House Snake	*Lamrophis capensis*
10	Olive House Snake	*Lamraphis inornatus*
9	Aurora House Snake	*Lamrophis aurora*
10	Spotted Bush Snake	*Philothamnus semivariegatus*
10	Green Water Snake	*Philothamnus hoplogaster*
10	Natal Green Snake	*Philothamnus natalensis*
10	Cape Wolf Snake	*Lycophidion capense*
10	Cape File Snake	*Mehelya capensis*
10	Black File Snake	*Mehelya nyassae*
10	Rhombic Egg Eater	*Dasypeltis scabra*
100	Black mamba	*Dendroaspis polylepis*
100	Mozambique spitting cobra	*Naja mossambica*
100	Snouted Cobra	*Naja annulifera*
100	Boomslang	*Dispholidus typus*
97	Green mamba	*Dendroaspis angusticeps*
97	Puff adder	*Bitis arietans*
100	Gaboon adder Eastern	*Bitis gabonica*
50	Gaboon adder Western	*Bitis gabonica rhinoscerous*
50	Jamesons mamba	*Dendroaspis jamesoni*
19	Rock monitor	*Varanus albigularis*
19	Water monitor	*Varanus niloticus*
47	Leopard tortoise	*Geochelone pardalis*
20	Spekes hinged tortoise	*kinixys spekii*

Private Bag X11338 Mbombela 1200. N4 National Road, Halls Gateway, Matsulin, MBOMBELA - MPUMALANGA. www.mpumalanga.com
Tel: +27 (0) 13 759 5300/1 Fax: +27 (0) 13 759 5490

Conditions

1. The permit shall not be transferable
2. Only a person authorised thereto by the C E O may make an alteration on the permit
3. The permit shall be subjected to the provisions of any law in force during the period of validity of the permit in the area to which the permit applies
4. No wild animals may be brought into or removed from the property unless authorised by a permit.
5. All animals received from the public must be kept in separate facilities and must be handed to the MTPA
6. Register must be kept on all animals received
7. The animals may not be handled by the public and the public may not be allowed into the cages.
8. The necessary precautions must be taken to ensure the safety of the public.
9. The permit holder of this permit may not sell, donate, import, export or convey any wild or exotic animal/s referred to on the permit or any progeny of such wild animal/s, unless he is the holder of a permit.

General Information

(a) Keeping of live wild animals or exotic animals in certain conditions(section 30)
(b) Prohibited acts with exotic animals(section 34)
(c) This permit may be cancelled or amended at any time.(Section 86.)
(d) The holder of a permit who contravenes or a fails to comply with any one of the conditions or requirements to which the permit is subject, shall be guilty of an offence.(Section 86.)
(e) The holder of a permit shall, at the request of a person authorised in terms of the provisions of the Act so to demand, forthwith produce such permit to such person.(Section 103.)

B. Transport Permit

Permit in	Permit out
	/

DEPARTMENT OF AGRICULTURE

MPUMALANGA

Permit number: 20210329OFE - VRUPI

DEPT. LANDBOU

UMNYANGO WETEKULIMA

VETERINARY PERMIT TO MOVE ANIMALS / ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngeligunya leMtsetfo weTifo teTilwane No. 35 wa 1984:

Name: / Naam: / Libito:	CHÉ WELDON	ID No.:	7502075129088
Address: / Adres: / Likheli:	NORTH WEST UNIVERSITY, POTCHEFSTROOM, 2531		
to move with: / om te beweeg met: / unyakatise yekuhambisa:	THREE HUNDRED THIRTY SEVEN	VARIOUS REPTILE SPECIES	LIVE / PRODUCT
identified as follows: / geïdentifiseer as volg: / letikhonjiswe nga:	337 REPTILE SAMPLES (115 BLOODSMEARS, 112 FTA CARDS, 107 SKIN SAMPLES + 3 PARASITES)		
from the farm/diptank area/place: / vanaf die plaas/dipbakgebied/plek: / kusuka epulazini/edibhini/endzaweni:	HANS HOHEISEN WRS	STOCK CARD NUMBER	in the district of / in die distrik / esigodzini sase: BOHLABELA
to the farm/diptank area/abattoir/place: / na die plaas/dipbakgebied/abattoir/plek: / kuya epulazini/edibhini/emadzeleni/endzaweni:	POTCHEFSTROOM		in the district of / in die distrik / esigodzini sase: DR KENNETH KAUNDA

On the following conditions:
1. This permit:
(a) is valid for 10 days from date of issue and for one consignment only
(b) must accompany the animals / products mentioned above;
(c) must be produced for inspection on demand by any land or animal owner or police or veterinary official;
(d) must be kept at a destination until collected by a veterinary official.
2. For game / game products a nature conservation permit is also needed.
3. Animals and products must be visibly free from external parasites.
4. Animals are to be transported in accordance with animal welfare guidelines.
5. Other conditions:

Op die volgende voorwaardes:
1. Hierdie permit:
(a) is geldig vir 10 dae vanaf datum van uitreiking en slegs vir een besending;
(b) moet die diere / produkte hierbo vermeld vergesel;
(c) moet vir inspeksie getoon word op aanvraag van enige grond of dier-eienaar of polisie- of veeartsenybeampte;
(d) moet by bestemming gehou word totdat dit deur 'n veeartsenybeampte geneem word.
2. Vir wild / wildprodukte word 'n natuurbewaringspermit ook benodig.
3. Diere en produkte moet sigbaar vry van uitwendige parasiete wees.
4. Diere moet volgens dierewelsynriglyne vervoer word.
5. Ander voorwaardes:

[illegible]

* SAMPLE LIST TO REMAIN ACCOMPANY PERMIT *

6. Retention for / Quarantine for / Direct slaughter within: ... days after arrival at destination

Seal nos.	/	/	Place / Plek / Indzawo	HANS HOHEISEN WRS
Vehicle reg. nos.	KKM860 NW	/	Date / Datum / Lusuku	2021-03-29

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

DR HEIN CAMPER — Issuing Veterinary Official

Signature — State Veterinarian / Staatsveearts / Dokotela wetifuyo

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with this permit or any condition thereof is guilty of an offence.
Permithouer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
[illegible]

DISTRIBUTION: VETERINARY OFFICIAL AT DESTINATION

REPUBLIC OF SOUTH AFRICA
DEPARTMENT OF AGRICULTURE
OFFICIAL VETERINARIAN
2021-03-29
DR H.K.M. RAM...
REG. NO D18/159...
MPUMALANGA PROVINCE

C. Section 20 Permit of Animal Diseases Act

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development. Private Bag X138, Pretoria 0001
Enquiries: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@dalrrd.gov.za
Reference: 12/11/1/3 (1711AC)

Prof Ché Weldon
Unit for Environmental Sciences and Management
North-West University
Tel: 018 299 2375
Email: Che.Weldon@nwu.ac.za

Dear Prof Weldon,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application received on 21 October 2020 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The research project is approved as per the application form received 21 October 2020 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to HenryG@dalrrd.gov.za;
3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
4. Ethical approval for all components of the study must be obtained from the relevant authority before the study may start;
5. Only reptile (snake, monitor, tortoise and terrapin) samples may be collected as part of this study. Samples that may be collected include skin moulds, swab samples, scale biopsies, faeces, blood and organ samples. Samples must be fixed in 10% buffered formalin or PrimeStore MTM as indicated;

6. Sampling of reptiles may only be conducted in the specified areas for which a state veterinary letter of no objection has been received:
   a. NWP – Dr Kenneth Kaunda District
   b. MP – Mbombela (Nelspruit), Hazyview areas
   c. LP – Hoedspruit, Soutpansberg areas

   This includes the Hoedspruit Reptile Centre and Lowveld Venom Suppliers (Hazyview);
7. Only reptile samples may be obtained from the Hans Hoheisen Wildlife Research Station (HHWRS) Biobank for which a letter of permission has been received;
8. For transport of any reptile samples out of the Foot and Mouth Disease Controlled Areas and the African Swine Fever Controlled Areas, relevant movement permits from the responsible state veterinarian must be obtained;
9. All samples must be packaged and transported in accordance with the National Road Traffic Act, 1996 (Act No. 93 of 1996);
10. Processing, testing and analyses of the reptile samples must be performed in the Herp Health Laboratory (E6, G03) of the North-West University, Potchefstroom campus. These facilities may be subject to an on-site inspection by the Directorate Animal Heath. If any critical non-conformances are identified, the facility (including all Section 20 approved studies connected there to) will be suspended until these non-conformances are rectified;
11. Sampes may be tested for the following pathogens using the relevant test procedures, which may inlcude histopathology, Transmission Electron Microscopy and molecular diagnostics. No propagation of these agents is allowed:
   a. Mycoplasma agassizii
   b. Ophidiomyces ophiodiicola (Snake Fungal Disease)
   c. Plasmodium
   d. Ranavirus
   e. Salmonella
12. The detection of any controlled or notifiable animals diseases (including Ranavirus) must be reported to the responsible state veterinarian and the Director Animal Health;
13. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study using the specified waste contractor.
14. Records must be kept for five years for auditing purposes;
15. Extracted DNA from reptile samples may be stored under access control at -80°C in Laboratory G03, building E6, North-West University Potchefstroom campus;
16. Stored samples may not be outsourced or used for further research without prior written approval from the Director: Animal Health;
17. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@dalrrd.gov.za.

Title of research/study: *"Initiate and conduct disease surveillance in the North West province, Mpumalanga and Limpopo to primarily assess the prevalence and characterize infectious diseases in reptiles, including zoonosis"*

Researcher: Prof Ché Weldon

Institution: Unit for Environmental Sciences and Management, North-West University

Our ref Number: 12/11/1/3 (1711AC)

Your ref: NWU-00063-19-S5

Expiry date: December 2022

Kind regards,

DR. MPHO MAJA

DIRECTOR: ANIMAL HEALTH

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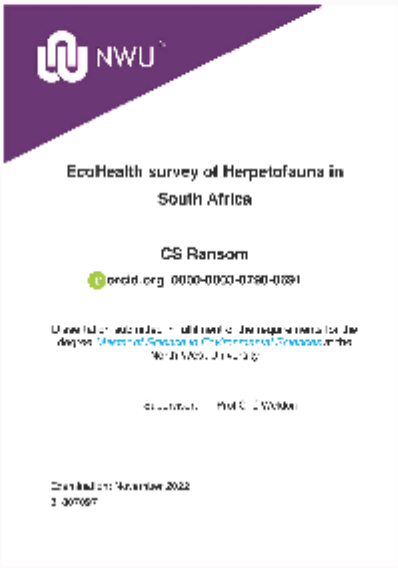
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