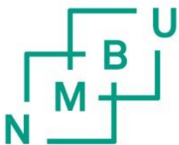

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Prosjektretning

Characterization of the Nasopharyngeal Microbiota and Occurrence of Respiratory Pathogens in Wild Artiodactyls in Zambia

Beskrivelse av nasofaryngeal mikrobiota og forekomst av luftveispatogener hos ville klovdyr i Zambia

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Summary

Title: Characterization of the Nasopharyngeal Microbiota and Occurrence of Respiratory Pathogens in Wild Artiodactyls in Zambia

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Knowledge on respiratory health, microbiota and respiratory pathogens in wild artiodactyls is limited. This thesis aims to investigate the respiratory health, nasopharyngeal microbiota and presence of selected respiratory pathogens in wild Zambian artiodactyls. Nasopharyngeal samples were collected from 14 animals within the species impala (*Aepyceros melampus*), sable antelope (*Hippotragus niger*), roan antelope (*Hippotragus equinus*), puku (*Kobus vardonii*) and kudu (*Tragelaphus stresiceros*) of the order Artiodactyla in Zambia. The sampled animals seemed generally of good respiratory health. Samples were analysed for selected respiratory pathogens by bacterial culture and MALDI-TOF. Amplicon 16S rRNA sequencing was used to describe the nasopharyngeal microbiota. Alpha- and beta-diversity of the microbiota was assessed using Shannon, Bray-Curtis and Weighted Unifrac analyses. Sable antelope had a less diverse nasopharyngeal microbial community compared to impala and roan antelope. Further, the microbiota clustered according to impala, sable and roan antelope, but the identified taxa were related. The three most abundant genera were *Moraxella*, *Ralstonia* and *Pantoea*. *Mannheimia haemolytica* was identified in one sable antelope, while the other selected pathogens *Pasteurella multocida* and *Histophilus somni*,

were not identified. There is a need for further research on these respiratory pathogens and the microbiota of wild artiodactyls to determine their impact on respiratory health.

Definitions & Abbreviations

BA	Blood agar
BAL	Bronchioalveolar lavage
BCS	Body condition score
bp	Base pair
BRD	Bovine respiratory disease
Clean Tags	Merged and pre-treated paired end raw reads
DNA	Deoxyribonucleic acid
DNS	Deep nasal swab
EC	*Escherichia coli* (*E. coli*)
EDTA	Ethylene diamine tetraacetic acid
EHS	Epizootic haemorrhagic septicaemia
ET	Effective tags
HR	Heart rate
HS	*Histophilus somni (H. somni)*
ID	Identity
IgG	Immunoglobulin G
Location A	Noah's Ark Conservatory in Mkushi
Location B	Kabwe
Location C	Protea Game Reserve Chisamba
LPS	Lipopolysaccharide
LRT	Lower respiratory tract
MALDI-TOF	Matrix-assisted laser desorption/ionization – Time of Flight

MH	*Mannheimia haemolytica (M. haemolytica)*
NMBU	Norwegian University of Life Sciences
NS	Nasal swab
OTU	Operational taxonomic unit
PCoA	Principal Coordinate Analysis
PCR	Polymerase chain reaction
PE	Paired end
PM	*Pasteurella multocida (P. multocida)*
QC	Quality control
qPCR	Quantitative polymerase chain reaction
Raw reads	All obtained sequences
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
RR	Respiratory rate
rRNA	Ribosomal ribonucleic acid
TTW	Trans tracheal wash
UNZA	University of Zambia
URT	Upper respiratory tract
ZAWA	Zambian Wildlife Authority

Introduction

Wildlife and wildlife health

Wildlife in Africa, with special focus on artiodactyls

Wildlife in Africa is well known for the enormous diversity, and it includes a variety of species, among them different ungulate species. Ungulate is a term referring to all mammals having hoofs and considers a vast majority of herbivores. The true ungulate refers to animals belonging to the order Artiodactyla and Perissodactyla (Figure 1), meaning even-toed and odd-toed animals respectively (Huffman, 2023c). Antelope is not a taxonomically defined group, but rather a term for artiodactyls belonging to the Bovidae family and not being cattle, sheep, or goats (Huffman, 2023a).

Artiodactyls are closely related to domesticated cattle (Figure 1), and research and surveillance programs are based on existing information in related species (Rhyan & Spraker, 2010), like cattle.

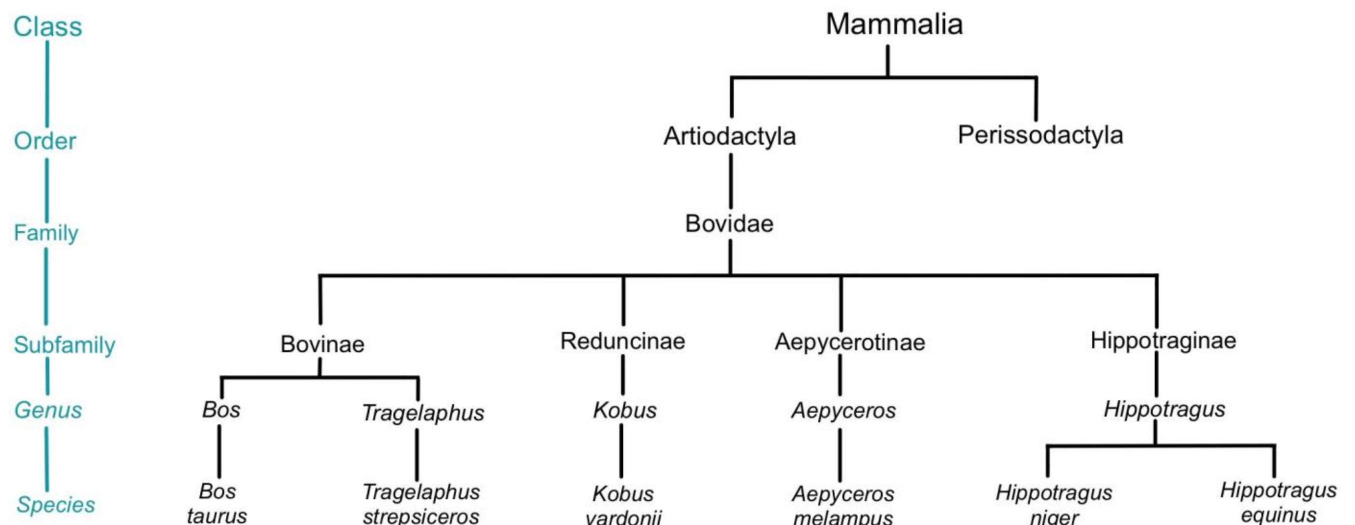


Figure 1: *Illustration showing the taxonomy of some African artiodactyls and how closely related they are to domesticated cattle. Length of the branch lines are not proportional to evolutionary distance. Figure based on information regarding taxonomic ranks from the website of Huffman (2023b). Bos taurus: domesticated cattle, Tragelaphus stresiceros: greater kudu, Kobus vardonii: puku, Aepyceros melampus: impala, Hippotragus niger: sable antelope, Hippotragus equinus: roan antelope.*

Wildlife in Zambia

Zambia has had a rapidly growing wildlife ranching industry over the last twenty years, and vast geographical areas are designated to wildlife conservation (Lindsey et al., 2013). Many wild animals are kept in national parks, and extensive or fenced wildlife ranches. Most of these privately owned ranches are registered with the Zambia Wildlife Authority (ZAWA) (Lindsey et al., 2013). The fenced ranches must apply for certification of ownership of wildlife, while the owners for unfenced ranches need to apply for hunting quotas and licenses. Some of the most popular species to keep in these ranches are wild ungulates like impala (*Aepyceros melampus*), kudu (*Tragelaphus strepsiceros*) and bushbuck (*Tragelaphus scriptus*) (Lindsey et al., 2013).

Wildlife ranching provides a good alternative for utilizing areas where agriculture is not viable, which is the case for several areas in Zambia (Lindsey et al., 2013). Many farmers choose to combine livestock farming with wildlife ranching to strengthen their financial case and to optimize the use of available land. Since the industry also contributes to food security, employment for locals and ecotourism, the wildlife industry is considered to be a positive addition to the country with both financial and social benefits (Lindsey et al., 2013).

There is a shortage of experts within the field of wildlife medicine and conservation, but as the industry has grown, so has the number of specialists; the number of wildlife veterinarians, ecologists, and other experts are increasing in southern Africa (Carruthers, 2008; Lindsey et al., 2013). This development is an important step towards improved wildlife conservation.

Wildlife as a reservoir and origin of emerging infectious diseases

Wildlife is a reservoir and a carrier of numerous pathogens that affect both humans and livestock (Rhyan & Spraker, 2010; Ryser-Degiorgis et al., 2015). Contact between humans, livestock and wildlife is increasing due to public interest in wildlife, mixed farms, and loss of wildlife habitats (As reviewed in Ryser-Degiorgis, 2013), forcing wildlife to move in closer contact with human activities. Shared water holes and grazing areas between livestock and wildlife creates interfaces where disease transmission in either direction can occur, dubbed so-called spillover events (Figure 2). Some evidence of such spillover events are well documented, for instance an event of transmission of bovine tuberculosis from wildlife to livestock in South Africa (Musoke et al., 2015) and brucellosis from elk to cattle in USA (Rhyan et al., 2013). However, more research is needed to better understand which diseases are circling in the wildlife population and the threats they can pose to the health of livestock populations (Caron et al., 2013; Rhyan & Spraker, 2010).

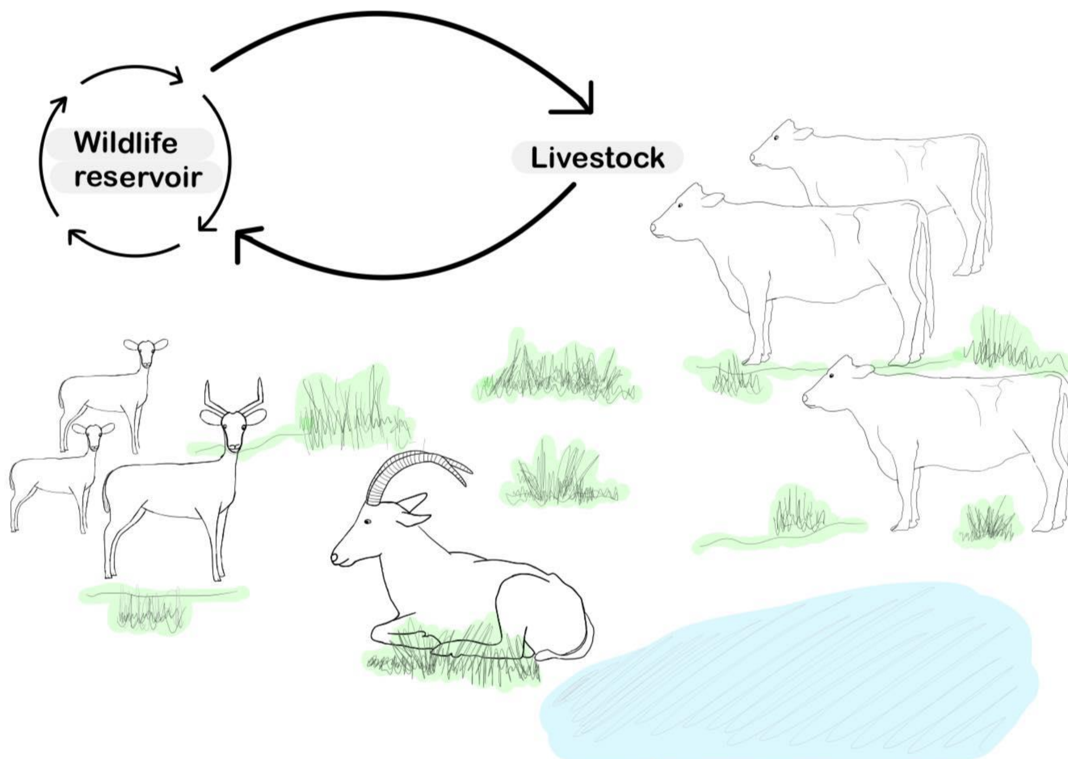


Figure 2: *Disease transmission at wildlife-livestock interface, spillover events. Transmission can occur both ways, and wildlife can serve as a reservoir.*

Respiratory health of artiodactyls

Little is known on the respiratory health of wild artiodactyls, but a few reports of respiratory diseases have been noted. These includes pasteurellosis (Nalubamba et al., 2012), tuberculosis (Schmitt et al., 1997), and infestation with lungworm (Pyziel et al., 2020; Pyziel et al., 2023). Of these, bovine tuberculosis has received considerable research attention in Zambia, as the disease is endemic to cattle and wildlife in certain areas of Zambia (Mwacalimba et al., 2013). Wild artiodactyls and especially the Kafue lechwe (*Kobus leche kafuensis*), a subspecies of lechwe antelopes (*Kobus leche*) found in the Kafue plains in Zambia, is an important reservoir of bovine tuberculosis for livestock (Malama et al., 2014; Muma et al., 2011).

In cattle, bovine respiratory disease (BRD) complex has a major impact on health, economy, and welfare of livestock. Nothing is currently known on the occurrence of BRD in wild artiodactyls, thus most of our knowledge on the epidemiology, aetiology, and pathophysiology of respiratory disease in Bovidae stems from cattle. BRD is multifactorial, and stressors contribute to the development of respiratory disease, for instance heat/cold, injury, chasing, dehydration, handling by humans, shipping, and adapting to a new environment with a mixing of different animal groups. Combined with viral and/or bacterial pathogens and the response from the host's immune system, BRD can develop (McGill & Sacco, 2020). The viruses commonly isolated from cattle with respiratory disease include bovine respiratory syncytial virus, bovine herpes virus 1, parainfluenza virus 3 and bovine coronavirus (Hodgins et al., 2002), while pathobionts commonly isolated includes *Pasteurella multocida (*PM), *Mannheimia haemolytica* (MH)*, Histophilus somni* (HS) and *Mycoplasma bovis* (Blakebrough-Hall et al., 2020; Callan & Garry, 2002; *Pasteurellosis*, 2023; Smith, 2015). Except for three impalas diagnosed with pneumonic pasteurellosis after necropsy (Nalubamba et al., 2012) and pathological findings of PM in impalas with sepsis in South

Africa (As reviewed in Chu et al., 2020), the role of these bacteria in respiratory disease in wild artiodactyls in Africa is unknown to us.

PM is however the cause of haemorrhagic septicaemia, an acute, fatal, septicaemic disease in ungulates, primarily in cattle and water buffaloes (*Bubalus bubalis*) caused by specific serotypes of *Pasteurella multocida* (PM). These serotypes vary with host species and geography (Mosier, 2021). A massive outbreak of haemorrhagic septicaemia occurred in wild artiodactyls in 2015, causing the death of more than 200.000 saiga antelopes in Central Kazakhstan caused by a latent strain of PM (Kock et al., 2018). In Zambia, an outbreak of haemorrhagic septicaemia in livestock occurred in 1977/78 in the Southern Province. The outbreak was caused by PM Type E (Francis et al., 1980), and more than 10 000 cattle died during six months, inflicting quarantine restrictions on the whole of Southern Province resulting in an acute meat shortage. The cattle herd in which the first deaths were reported had spent the entire dry season in the Kafue flood plains (Francis et al., 1980), and although the origin of the disease outbreak was not investigated, there is a possibility that wildlife in that area was involved. Indeed, PM has been isolated from wild animals (As reviewed in Chu et al., 2020)

Bacterial pathogens of bovine respiratory disease

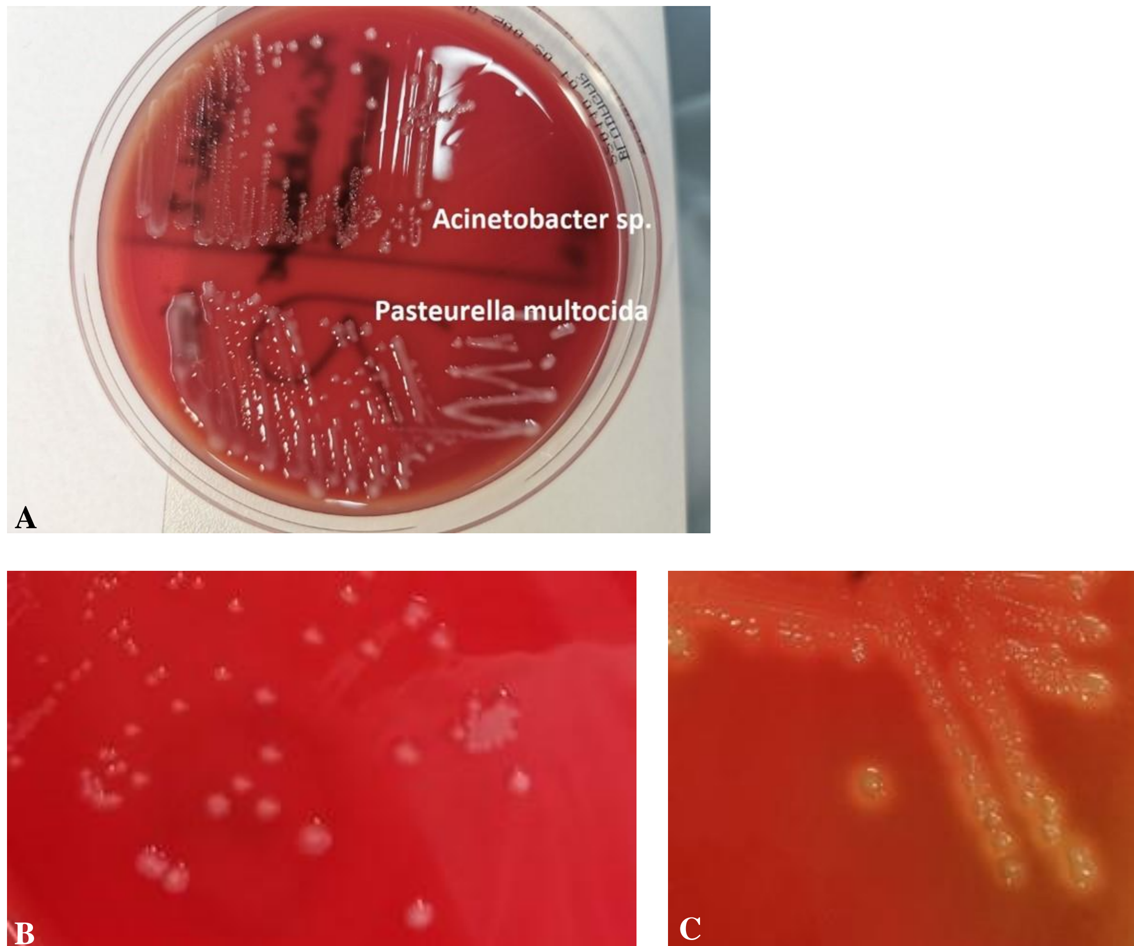
PM, MH, and HS are opportunistic pathogens of the family *Pasteurellaceae*, which have a wide host spectrum and can be found around the globe (Markey et al., 2013). The bacteria are mainly commensals found in the oral and respiratory tract of animals and humans; however, the carrier state varies greatly between species. PM, MH, and HS can cause disease under certain conditions, and animal to animal transmission enhance the virulence, and the bacteria often enter via the respiratory tract, resulting in pneumonic pasteurellosis if the conditions of disease is met (Markey et al., 2013).

As *Pasteurella, Mannheimia* and *Histophilus* belong to the same family of bacteria, they share several characteristics presented in Table 1 (Markey et al., 2013). *Pasteurellaceae* grow best on media containing blood or serum, so the routine medium for isolation of *Pasteurella*, *Mannheimia* and *Histophilus* is blood agar (BA) with ox or sheep blood. These plates are then incubated at 35°C for 24-48 hours in aerobic atmosphere (Markey et al., 2013). Pictures 1A, B, and C show examples of the colony morphology of PM, MH, and HS on BA.

Table 1*: Showing characteristics of PM, MH and HS.*

Species	Gram	Shape	Cat	Oxi	Haemolysis	Blood agar	MacConkey
PM	-	R/CB	+	+	No	Smooth, grey, big or small	No
MH	-	R/CB	+	+	B-haemolytic*	Mucoid or pudding-like, light grey to grey, small to medium	Pinpoint, pink
HS	-	R/CB	-	+	Variable	Yellow, mucoid, small or pinpoint	No

Species: Bacteria species. PM: *Pasteurella multocida.* MH*: Mannheimia haemolytica*. HS: *Histophilus somni.*
Gram: Results of Gram staining of bacteria. Positive (+), negative (-).
Shape: Shape of bacteria. R: rods, CB: coccobacillary.
Cat: Result of catalase test. Positive (+), negative (-).
Oxi: Result of oxidase test. Positive (+), negative (-).
Haemolysis: Bacterial haemolysis on blood agar? * Not always on sheep's blood agar.
Blood agar: Colony morphology on blood agar after 24 hours incubation.
MacConkey: Growth of bacteria on MacConkey agar, colony morphology.



Picture 1*: Example of colony morphology of* ***A****: Pasteurella multocida,* ***B****: Mannheimia haemolytica,* ***C****: Histophilus somni on sheep's blood. Photos by Lise Marie Ånestad*

Pasteurella multocida

PM is a bacterium within the *Pasteurellacea* family, genus *Pasteurella* and part of the *sensus stricto* together with *P. canis, P. stomatis* and *P. dagmatis* (Markey et al., 2013). PM can be both a primary and a secondary agent of respiratory diseases such as pasteurellosis and pleuropneumonia, and can also cause haemorrhagic septicaemia (Markey et al., 2013; Mosier, 2021). Transmission occurs through direct contact with infected animals, either with healthy carriers or diseased individuals, as well as through fomites. PM can also survive for longer periods in organic material, as well as over a year in water (Bredy & Botzler, 1989; Kehrenberg et al., 2001; Markey et al., 2013). Not all animals fall ill, and several animals are healthy carriers of the bacteria in the upper respiratory tract (URT) (Thomas et al., 2019).

PM can be differentiated into different serotypes (A, B, D, E and F), based on capsular and lipopolysaccharide (LPS) genotyping (Calderón Bernal et al., 2022; Markey et al., 2013). Different types of PM have different host species where they cause disease. This difference in host species is due to a variety of surface proteins on the bacteria and on host cells. However, some serotypes of PM are generalists, having multiple host species, and one host species can be susceptible to several serotypes of PM (Markey et al., 2013). PM serotype A has been isolated from cattle, sheep, pigs, rabbits, poultry, and many other domestic and wild animals. In cattle, PM serotype A is a part of enzootic pneumonia complex as well as shipping fever complex, and occasionally cause severe mastitis. Both serotype B and serotype E PM can cause epizootic haemorrhagic septicaemia (EHS) in cattle and water buffalo. Serotype E has only been found in Africa, while serotype B has been isolated in Southeast Asia and other countries. Serotype B has also caused EHS in bison yak and other ruminants, and has been isolated from healthy cattle (Abed et al., 2020; De Alwis & Australian Centre for International Agricultural, 1999; Elsayed et al., 2021; Markey et al., 2013). Type D has been seen in pigs, poultry, and less commonly other domestic animals, and causes atrophic rhinitis, pneumonia, or fowl cholera. Lastly type F has caused fowl cholera in poultry, and fibrinopurulent or diffuse haemorrhagic pneumonia in rabbits (Calderón Bernal et al., 2022; Markey et al., 2013).

Mannheimia haemolytica

Within the *Mannheimia* genus, MH is the main species of veterinary significance (Markey et al., 2013). MH is both a commensal of the oral and respiratory tract, and can infect cattle and sheep, causing diseases such as pneumonia, septicaemia, and mastitis, and is also a pathogen of the shipping fever complex. Most cases of pneumonia are caused by strains of MH carried

by the animal itself, but in a pneumonia outbreak molecular evidence indicates some degree of horizontal transmission between animals (Confer & Martino, 2022; Markey et al., 2013).

MH is divided into 12 serotypes based on indirect haemagglutination A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16 and A17 (Katsuda et al., 2008, as cited in Markey et al., 2013). *M. glucosida* is the new name of the previous serotype A11 of MH, and T3, T4, T10 and T15 is now under the species *Bibersteinia trehalosi* (BT) (Abed et al., 2020; Confer & Martino, 2022).

Histophilus **somni**

HS is a commensal of mucus membranes in both humans and animals. HS is commonly found in the upper respiratory or lower genital tracts of healthy cattle and sheep (Markey et al., 2013). HS can attach to several cell types and interaction with epithelial cells in the respiratory tract is important in the pathogenesis. Septicaemia can occur when HS crosses the pulmonary alveolar membrane (Inzana, 2016). HS has a high level of strain variability, some avirulent and others possessing a range of virulence factors which are constantly isolated from pathological specimens. These virulence factors are mainly associated with immune evasion (Inzana, 2016; Markey et al., 2013).

Microbiota in respiratory tract of Bovidae

To our knowledge, studies on the composition of the microbiota in the respiratory tract in wild artiodactyls are lacking. There has been some research on the gut and rumen microbiota in impalas and antelopes (Cersosimo et al., 2015; Shi et al., 2021). Regarding the respiratory microbiota, most of the existing knowledge is on domesticated species of Bovidae, especially in feed-lot cattle relative to the development of BRD (Centeno-Martinez et al., 2022; As reviewed in Timsit et al., 2020).

Microbiota on mucus membranes of the respiratory system provides an essential line of defence against pathogen colonization and subsequent respiratory infection (Zeineldin, M. M. et al., 2017). It is a diverse and complicated community of different types of bacteria. The respiratory tract has its own microbiota from nostrils to bronchioles, which both protects, contributes, and changes in response to respiratory disease. In bovines, Zeineldin, M. M. et al. (2017) states that "the (healthy) cattle respiratory tract is inhabited predominately by five phyla (Proteobacteria, Firmicutes, Tenericutes, Actinobacteria, and Bacteriodes)". Further, studies show that the more diverse the respiratory microbiota in cattle is, i.e., higher alpha diversity, the more likely the animal is to withstand development of respiratory disease (Walker, 1992 as cited in Pettigrew et al., 2012). These studies have also shown that cattle with bovine respiratory disease have a lower alpha diversity within the respiratory microbiota and that the phylogenetic diversity decreases in animals with respiratory disease compared to that of healthy animals (Centeno-Martinez et al., 2022). A more diverse microbiota provides a community of bacteria competing for adhesion receptors and efficiently utilizing the nutrients necessary to replicate, making it harder for opportunistic pathogens to colonize the respiratory tract (Abt & Pamer, 2014; As reviewed in Timsit et al., 2020). Microbiota can also provide protection by altering the environment and producing growth-inhibiting factors. These factors are e.g., lactic, or acetic acid, bacteriocins, and hydrogen peroxide (H_2O_2) (Amat et al., 2019; As reviewed in Timsit et al., 2020).

Microbiota in the URT is more diverse than in the lower respiratory tract (LRT). This may be due to the constant exposure of environmental and transmissible airborne microbiota. However, it is suggested that in cattle the URT is the source of bacteria that make up the microbiota in the LRT, and therefore are correlations when it comes to specific taxa between

these two bacterial communities (As reviewed in Timsit et al., 2020; Zeineldin, M. et al., 2017).

Some of the bacteria in the URT are opportunistic pathogens, meaning that alterations to the microbial environment can facilitate colonization of the LRT by bacteria that otherwise do not cause any harm (As reviewed in Caswell, 2014; Timsit et al., 2016). Examples of such opportunistic bacteria are PM, MH, and HS (Pratelli et al., 2021). Stress can weaken the host's immune system and viral infections cause dysbiosis of the nasopharyngeal microbiota, making the conditions favourable for opportunistic bacteria. As these pathogens proliferate, they gain access to the lungs through inhalation and can subsequently cause respiratory disease (Abt & Pamer, 2014; As reviewed in Caswell, 2014).

Wildlife translocation and disease surveillance

Disease surveillance in the wildlife population is necessary to understand the potential risks to human and animal health and welfare (As reviewed in Ryser-Degiorgis, 2013). A key part of wildlife health investigation and disease surveillance is the detection of pathogens and diseases, but there are many challenges (As reviewed in Ryser-Degiorgis, 2013). There is a general lack of knowledge on transmission of disease, carrier status, pathogen-host interactions, and disease susceptibility in wild animal species (Jia et al., 2020; Rhyan & Spraker, 2010). Thus, most surveillance programmes are based on existing knowledge of diseases in related species. Although this often is the best solution, it can lead to inaccurate conclusions when trying to relate this information to wild animals (Rhyan & Spraker, 2010). Another challenging part is the collection of field data and sampling. It is difficult to get a representative sample size when the pathogen/disease prevalence is unknown, leading to the need for a large sample size. This is both time-consuming and expensive (As reviewed in Ryser-Degiorgis, 2013).

There are different approaches to disease surveillance in wildlife. One method is passive surveillance, where the data collected is mostly based on community submissions. Examples include observation of clinically diseased or dead animals and subsequently sampling them, routine sampling of animals being hunted, or evaluation of roadkill (Rhyan & Spraker, 2010). This passive form of surveillance is highly depended on public interest and knowledge, and their willingness to report to the correct authorities. Some of the challenges include whether sick animals display noticeable clinical signs of disease and if there are suitable material to sample from. Scavengers can contaminate the carcass or reduce the availability of sufficient amounts of sample material (Rhyan & Spraker, 2010).

Another method of disease surveillance is targeted surveillance, also referred to as active surveillance. Sampling is done to detect specific pathogens or diseases, whether the animal is showing clinical signs of disease or not (World Organization for Animal Health, 2015). Animals chosen for the surveillance could belong to one or more species from the wildlife population. Decisions on which pathogens to include in the programme are made based on the importance of the pathogen, whether it directly or indirectly affects public health or livestock production and trade. (World Organization for Animal Health, 2015). Targeted surveillance is used to gain statistical data on the prevalence of diseases or pathogens, and the distribution of infection regarding age, sex, and geographical location (World Organization for Animal Health, no date).

Health assessments of wildlife is essential to give important empirical and physiological data necessary for understanding the impact stressors may have on individuals and populations. Screening of health status prior to translocation may improve the animals' chances of survival, as well as being essential for planning of conservation programmes (As reviewed in Kophamel et al., 2022).

Wildlife translocation

Wildlife translocation entails the movement of a wild animal from one location to another (Langridge et al., 2020). There are several reasons for translocation, conserving endangered species, population control, and trade between game ranches, among others (Langridge et al., 2020; Mengak, 2018; Dr. A. C. M. Sitima, 2023, personal communication, 24 June).

Risks of translocation

Translocation of animals comes with risks of pathogen transmissions and possible disease outbreaks. When an animal is being moved to a new area, all microbes, and parasites the animal carries move with it (Rhyan & Spraker, 2010). The translocated animals can introduce pathogens to naïve animal populations in the new environment. In addition, the translocated animals themselves can become infected by pathogens already present in the new environment due to lack of acquired immunity. A certain amount of stress is inherent when immobilizing animals, regardless of precautions and method of immobilization. Exposure to stress or stressors affect the immune system and may cause a flare up of latent infectious disease within the animals or make them more susceptible to infection (As reviewed in Kock et al., 2010). Another consequence of translocation is that a microbe which previously was avirulent can become virulent when introduced to a new host. In all regards, the results are emergence of disease (As reviewed in Kock et al., 2010; Rhyan & Spraker, 2010). There are well documented incidents where translocation has been the origin of disease outbreaks, like the introduction of ovine footrot to Norway in 2008 (Gilhuus et al., 2014).

Because of the risks previously stated, it is important to do a health assessment of the animals that are being translocated to ensure that only healthy animals are being moved. Health assessment can include an observation from a distance coupled with simple registrations on body condition, fur quality, look for any lesions and measure heart rate and body temperature

(Nelson et al., 2022). Many wildlife species being translocated are preys and exhibit qualities making identifying diseased animals difficult. They are experts at hiding weaknesses, and signs of disease may not always be evident (Rhyan & Spraker, 2010). In addition, it can be challenging to do a full health assessment when immobilizing and translocating animals, due to time restrictions among other factors. The result can be translocation of sick animals to a new area and therefore disease transmission.

Methods for immobilization

Immobilizing the animal is necessary for translocation. There are two main ways to immobilize: chemical and physical (Kaarakainen, 2019). In countries with warmer climate, such as Zambia, it is common to conduct the immobilization in the winter months and at the cool time of day, preferably in the morning or late afternoon to avoid the animal overheating, which is a common challenge when immobilizing animals (As reviewed in Laubscher et al., 2015).

Physical immobilization

Physical immobilization can be performed using several methods, including capture boma, capture nets, capture traps and capture baits. Several factors of both the animal, the area of capture and the goal of the capture must be taken into consideration when selecting capture method (As reviewed in Laubscher et al., 2015). Below the physical capture method used in this study will be described.

Capture bomas

Capture bomas are used for mass capture and is suitable for a large variety of species (As reviewed in Laubscher et al., 2015). When using a boma, animals are herded onto transportation units using funnel shaped fencing made of tarp. The tarp is strung between

trees using wires. As long as the tarp is opaque and free of holes, the animals treat it as a solid wall and will not run through it (As reviewed in Laubscher et al., 2015). Tarp curtains are hung at several points along the funnel shaped fencing. These tarp curtains are manually closed by a person hiding inside as the animals enter the boma (Dr. A. C. M. Sitima, 2023, personal communication, 24 June). Communication within the capture team is important for efficient capture, ensuring all target animals enter the boma before closing it off. The animals are then chased further into the boma as more curtains are closed, making the area smaller and smaller, forcing them onto the transport vehicle (As reviewed in Laubscher et al., 2015; Lekolool, 2012).

It is important that the entrance of the boma is both wide (100-120 meters) and well camouflaged with adequate space for the animals to enter when being chased (As reviewed in Laubscher et al., 2015). See Figure 3 for the layout of the boma used in this study. Different methods can be used for chasing, some possibilities are helicopters, ground vehicles or by foot. Human-animal contact is minimized when using helicopters for chasing, reducing the stress to the wild animal, making helicopters preferable when capturing highly stress susceptible species, such as kudu. Other suitable species for boma capture include impala, giraffes, zebra, buffalo and wild dogs (As reviewed in Laubscher et al., 2015; Lekolool, 2012).

This method enables capture of several animals, even entire herds, in a relatively short amount of time, although setting up the boma itself can be a time-consuming task (As reviewed in Laubscher et al., 2015). If the capture is unsuccessful a second chase could be attempted using the same boma. Chasing several times towards the same boma is rarely advantageous, and setting up the boma in a new area is advised (Dr. A. C. M. Sitima, 2023, personal communication, 24 June). Material for bomas can be expensive, and vehicles and

containers of high quality needed for the chasing and transportation can be costly. Boma captures is therefore only financially advantageous when capturing a large number of animals (As reviewed in Laubscher et al., 2015).

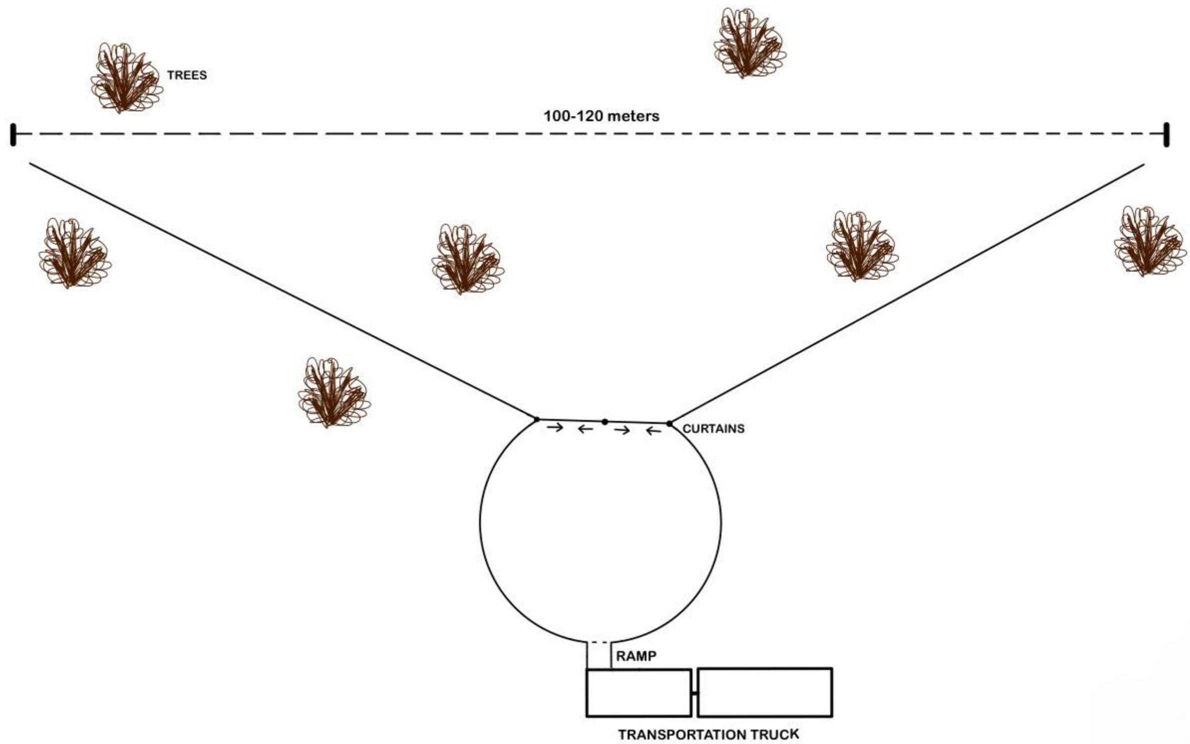


Figure 3*: Schematic illustration of capture boma used in Mkushi. The boma had a wide opening, curtains to manually close when the animals ran inside, and a ramp to ease the loading onto the transportation truck. It was set up in the woods where trees and bushes were used to hide the different structures of the boma.*

Chemical immobilization

Chemical immobilization includes use of drugs to sedate or anaesthetise the animal. These drugs are commonly administered by dart using a dart gun (Arnemo & Kreeger, 2018). Several considerations must be made when working with projectile devices and drugs. Different drug classes work through different mechanisms, and species may react to the same drugs differently. Experiences using different drugs and doses have been collected and made into different handbooks giving an overview of recommendations for different species, such as *Handbook of Wildlife Chemical Immobilization* (Arnemo & Kreeger, 2018). Drug protocols may vary between capture groups based on personal experience and purpose of immobilization (Dr. A. C. M. Sitima, 2023, personal communication, 24 June). In *Handbook of Wildlife Chemical Immobilization* Arnemo and Kreeger (2018) describes the different characteristics that should be taken into consideration when choosing immobilizing agents, including a high therapeutic index, available antagonist and low toxicity in humans.

Animals can be immobilized using different modes of injection e.g., from vehicles and helicopters (Dr. A. C. M. Sitima, 2023, personal communication, 24 June). It is important to monitor the animal after darting, during the immobilization and until recovery and release to prevent injuries and to treat any adverse side effects that may occur. Positioning of the animal is important, especially in ruminants, and breathing and other parameters should be monitored during immobilization (Arnemo & Kreeger, 2018).

When translocating the animal, it is transported from the immobilization site to the transportation vehicle while still immobilized (Dr. A. C. M. Sitima, 2023, personal communication, 24 June). Here it may be given a long-acting tranquiliser for reduction of anxiety and aggression to make the translocation process smoother for both the animal and personnel. If antagonizing the immobilizing agent is desirable, an antagonist can be

administered. Not all immobilizing agents can be reversed using an antagonist (Arnemo & Kreeger, 2018).

Sampling methods

Several different methods exist for sampling of the respiratory tract. The most frequently used antemortem methods are nasal swabs (NS), deep nasal swabs (DNS), transtracheal wash (TTW), and bronchioalveolar lavage (BAL) (Doyle et al., 2017; Godinho et al., 2007). Which method to choose is dependent on the field conditions and what the goal is, and each sampling method have advantages and disadvantages which should be considered when choosing a specific method. NS and DNS target the URT, and the samples are easy to collect, quick, and minimally invasive. TTW and BAL targets the LRT and is more invasive, time-consuming, and expensive than NS and DNS (Doyle et al., 2017; Pardon & Buczinski, 2020).

Principles of diagnosis of respiratory pathogens

There are several ways of detecting pathogens, from methods requiring special knowledge, expertise, and expensive equipment, to quite simple inexpensive methods. Here we describe methods used for bacterial detection in this thesis.

Bacterial diagnostics

Cultivation of bacteria allows the bacteria to multiply, increasing the material available for further examination and testing. Culturing methods include use of broth (liquid) and agar (solid) and can be used in a range of different atmospheres. Agar plates have varying qualities based on media type, some are suited for a wide variety of bacteria, while others are more specific (Steward, 2021). One of the commonly used agar plates is BA which grow most routine bacterial pathogens (Lappin, 2012)), and colony morphology on BA can also be used to identify the bacteria if the colonies are well isolated from each other.

Selective media can suppress or increase the growth of selected bacteria, sometimes including colour changes to identify the bacteria (Steward, 2021). CHROMagar™ Pasteurella (PR012, CHROMagar, Paris, France) is a selective culture media designed to detect bacteria belonging to the *Pasteurellaceae* family and inhibit growth of most environmental flora. The targeted bacteria will grow with mauve coloured colonies (CHROMagar, 2021).

Biochemical testing

Material from isolated colonies or pure culture can be subjected to biochemical tests to examine properties of the bacteria, such as their ability to ferment carbohydrates and production of different enzymes such as catalase (catalase test) and cytochrome c oxidase (oxidase test). Catalase test uses hydrogen peroxide (H_2O_2) to see if the bacteria produce the enzyme catalase (University of Wyoming, no date), while a test reagent tetra-methyl-p-phenylenediamine dihydrochloride assesses the bacteria’s ability to produce cytochrome c oxidase (Michigan State University, no date).

MALDI-TOF

MALDI is short for matrix assisted laser desorption ionization while TOF is short for time of flight. MALDI-TOF only requires a low sample volume for analysing, and the analysis only takes minutes (*MALDI-TOF and TOF/TOF MS*, 2023).

MALDI ionizes particles which are then separated based on their mass-to-charge ratio. It also determines the time ions take to travel to a detector. This analytical technique results in a spectrum with values describing mass-to-charge on the x-axis and the y-axis representing the intensity. The sample spectrum is compared to a database of spectrums for known organisms to determine the sample organism. MALDI-TOF is more reliable compared to traditional

methods of microorganism identification, except for species not included in the database and species like one another (Rychert, 2019).

The sample must be pure culture, but since the required sample volume is so small the analyses can oftentimes be performed on primary cultures, as long as the selected colony is pure and well isolated (Rychert, 2019).

Molecular diagnostics of pathogens and definition of the microbiota

Molecular diagnostics is a branch of medical and clinical laboratory science that uses molecular biology techniques to diagnose and monitor diseases, infections, and genetic disorders at the molecular level. It involves the detection and analysis of specific DNA, RNA, or protein markers that are associated with, in our case, the presence of different bacteria (YaleMedicine, no date). Below are the key steps taken to detect genetic material from specific bacteria:

DNA extraction

DNA extraction is a method for isolating DNA from the cell, and to separate it from other cellular components and debris. The extracted and purified DNA can then be used to detect pathogens or to characterize the entire genomic content of a selected matrix (metagenomics). DNA can be extracted from various biological samples such as body fluids, swabs, plant, and animal tissue among others (Shetty, 2020).

The DNA extraction process consist of three basic steps: lysis, precipitation, and purification. The lysis step is performed to disrupt and break open cytoplasmic and nuclear membranes to release the DNA (Shetty, 2020). Subsequently, the cell debris must be removed by centrifugation and wash stages. If RNA is not of interest for the following genomic analysis,

RNAse treatment is performed to break down and remove the unwanted RNA molecules (Gupta, 2019; Shetty, 2020).

After lysis, the DNA is mixed with cellular debris and contaminants like proteins, detergents, and reagents, which could inhibit downstream analysis such as PCRs (Benito, 2022). Precipitation is done to separate the DNA from other components of cell lysate (Shetty, 2020). Then, centrifugation is done to discard excess substances. Once all the unwanted material is removed, and the DNA is completely purified, the DNA precipitate can be redissolved in buffer solutions or double-distilled water (Gupta, 2019; Shetty, 2020).

DNA quantification

Nucleic acid quantitation can be done using photometry. DNA and RNA has absorptive properties that is utilised in photometric measurements of nucleic acids. The samples do not have to be prepared or dyed, and no standards are required. Photometry can also provide measurements of purity rations (A260/280 and A260/230). Photometry is not selective and uses algorithms to distinguish DNA and RNA (Thermo Fisher Scientific, no date).

Nucleic acid quantitation can also be done using fluorescence, as in Qubit™ Fluorometer (ThermoFisher, Eugene, OR, USA). Qubit™ Assays have fluorescent dyes highly specific to target molecules in the sample. The dyes emit fluorescence only when bound to their target molecules. The fluorescence emitted by the dyes are detected by Qubit™ Fluorometer. Qubit™ dsDNA (Broad Range) Assay (Q32850, ThermoFisher, Eugene, OR, USA) can be used to detect dsDNA concentration.

16S rRNA amplicon sequencing and shotgun metagenomics

All DNA-based life forms, including bacteria, have the 16S ribosomal RNA (rRNA) gene, which contain conserved and variable regions. The conserved regions make universal primers

possible, while the variable regions make the gene good for differentiating microorganisms (LC Sciences, no date). There are nine variable regions within the 16S rRNA gene which can be used to discern different specific bacteria from each other (Chakravorty et al., 2007).

The amplicon sequencing technique is based on PCR amplification of fragments of the 16S rRNA gene's (one or two hypervariable regions) followed by sequencing the amplicon products (Galan et al., 2016). After sequencing, the sequences of the resulting 16S rRNA is analysed using bioinformatic methods to describe and compare taxonomy of bacteria present in a matrix (*16S/18S/ITS Amplicon Metagenomic Sequencing*, 2023).

Aim

The aim of this project is to investigate the respiratory health of wild Zambian artiodactyls, with focus on nasopharyngeal microbiota and presence of selected respiratory pathogens.

The paper has the following three objectives:

1. Investigate the occurrence of PM, MH, and HS from deep nasal swabs in captured artiodactyls from the Central province of Zambia and correlate these findings with a short evaluation of respiratory health.
2. Characterize the microbiota of the nasopharyngeal mucosa of captured artiodactyls in Zambia.
3. Investigate the effect of species on the composition of the nasopharyngeal microbiota.

Material & methods

Ethical approval

Ethical clearance for final year students from University of Zambia health and research ethical committee IRBno.00011000, IORGno: 0009227 and FWA no 00026270.

Study design

Study area and selection of animals

In this cross-sectional study the study population consisted of five species of wild artiodactyls from the central province of Zambia: impala (*Aepyceros melampus*), puku (*Kobus vardonii*), kudu (*Tragelaphus strepsiceros*), roan antelope (*Hippotragus equinus*) and sable antelope (*Hippotragus niger*). The species and the number of animals of each were chosen based on available immobilization assignments for translocation from 20th June until 31st July 2023.

The immobilization and sampling of animals were conducted in three privately owned game ranches in the Central Province of Zambia. Location A: Noah's Ark Conservancy in Mkushi on the 25th of June, location B: Kabwe on the 7th of July, location C: Protea Game Reserve Chisamba on the 13th of July. See Figure 4 and 5 for location of sampling sites. The study selection consisted of a total of five impala, one puku, five roan antelopes, three sable antelopes and one kudu.

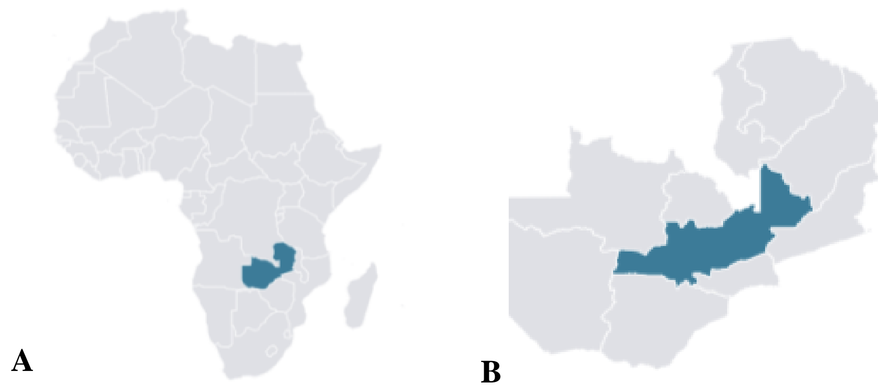


Figure 4*: **A**: Map over Africa, Zambia is marked. **B**: Map over Zambia, the Central Province is marked. Figures made using www.visme.co*

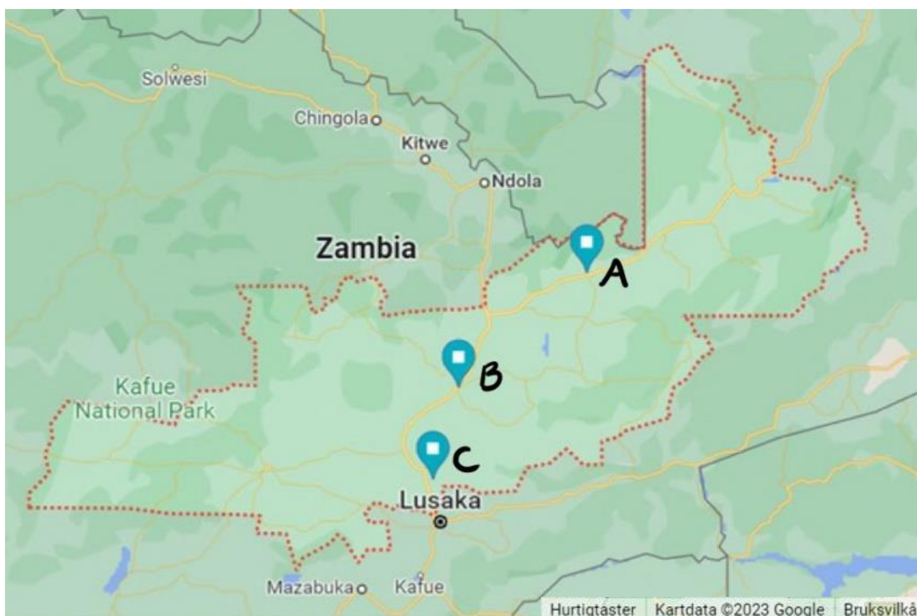


Figure 5*: Map showing the different capture sites in the Central Province of Zambia, also showing Lusaka the capitol of Zambia. **A**: Noah's Ark Concervancy, Mkushi. **B**: Kabwe. **C**: Protea Game Reserve, Chisamba.*

Selection and immobilization of artiodactyls

Animals were either physically or chemically immobilized with the primary goal being translocation. A professional capture team conducted the immobilizations. The three immobilization assignments were done by two different capture teams. Each team was led by one veterinarian. The capture in locations A and B was conducted by the same team, while a different team conducted the capture in location C.

In Mkushi (location A), ground vehicles and personnel on foot chased the animals into a capture boma. Using a boma was justified as the capture assignment was mass capture of 100 impalas, the only selection criterium was being an impala. At the end of the funnel shaped boma, a big enclosure was placed for the animals to settle overnight before being herded onto containers the following morning for transportation, as illustrated in Figure 3.

In Kabwe (location B) and Protea Game Reserve Chisamba (location C), the animals were chemically immobilized. A dart rifle (Model 389, Pneu-Dart Inc., Williamsport, Pennsylvania, USA) loaded with etorphine and ketamine was used to immobilize the animals (Picture 2A and B). Animals were chemically immobilized, and when the animal was hit and restrained, a soft field stretcher was put underneath the animal before carrying it to one of the vehicles (Picture 3A and 3B).

***Picture 2: A**: Eira Moen Attaei Kachouie is holding the dart rifle used for chemical immobilization in Kabwe.* ***B**: Previously used dart, like the ones used in Kabwe. Photos by Elise Lismoen.*

***Picture 3A**: a roan antelope was carried using a soft field stretcher onto the field truck after chemical immobilization using a dart rifle. **B**: lifting the same roan antelope from the field truck into the transportation truck. Pictures from capture assignment in Kabwe. Photo by Eira Moen Attaei Kachouie and Elise Lismoen.*

Data collection in the field

After locating the herd of interest, initial observations were made before proceeding with the immobilization of animals. These observations were made based on the team's experience of choosing suitable animals.

Clinical examination and parameters

Descriptives of species, sex, estimation of age (two categories, "young" and "adult") and weight (kg) were collected after capture. The category "young" described animals up to two years of age, and the "adult" category were animals over the age of two. Estimations on age and weight were based on the knowledge of the capture team.

A scale from 1-3 to assess body condition score was made. A score was given based on a visual inspection considering the body composition of the animal. Score 1 equalled an

emaciated animal, score 2 described a normal-looking animal, and score 3 described an overweight or obese animal. The assessment of body score index was done by members of the immobilization team, using their knowledge of what a normal or healthy artiodactyl should look like.

To evaluate the respiratory health status, an assessment panel consisting of the following clinical parameters were developed, respiratory rate and pattern, auscultation of the lungs, assessment of nasal discharge, and measurement of rectal temperature.

These protocols were used regardless of the immobilization method. When the animal was immobilized, the first parameters to be assessed were respiratory rate and pattern, and whether the animal had a spontaneous cough. This was done by observing the animal from approximately one meters distance while the capture team restrained it. Then the animal was approached, and auscultation of the heart and lungs was performed. Only one side of the thorax was auscultated depending on the positioning of the animal. The heart rate was registered, and auscultation of the lungs was performed by listening to three different spots (Figure 6). Lung sounds were qualified as normal (N), wheezing (W) or crackling (C). The body temperature was measured using a rectal thermometer.

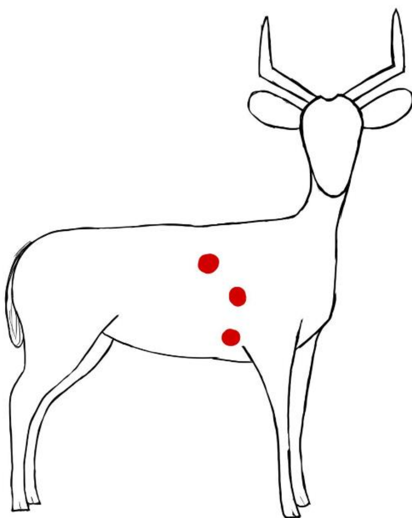


Figure 6*: The three auscultation points used for evaluation of lung sounds; caudodorsal, craniodorsal and cranioventral.*

The quantity and quality of the nasal discharge were visually evaluated before the nostrils were wiped prior to the sampling of DNS. A scoring system was made for both the quantity and quality of the nasal discharge, and this data plus registrations of spontaneous cough were classified by the severity of affection to the animal's respiratory health. The categories are no affection, mild affection, moderate affection, and severe affection, see Table 2. Parameters like respiration rate, heart rate, and body temperature were not included as they were registered after the animals were chased and/or chemically sedated.

Table 2*: Classification of affection of respiratory health in the study population and the data each category includes. Traumatic epistaxis is not included in evaluation of respiratory health, and therefore not included in the table.*

Respiratory sign	No affection	Mild	Moderate	Severe	Very severe
Quantity of nasal discharge	0	1	2	3	4
Quality of nasal discharge	N	S	M	P	H
Cough	0	1	1	1	1

Quantity of nasal discharge*: 0: No discharge. 1: Minimal discharge from one nostril. 2: Minimal discharge from both nostrils. 3: Moderate amount of discharge from both nostrils. 4: Excessive amount of discharge from both nostrils.*
Quality of nasal discharge*: N: No discharge. S: Serous discharge. M: Mucoid discharge. P: Purulent discharge. H: Haemorrhagic discharge.*
Cough*: Presence of spontaneous cough. 1: yes, 0: no.*

Sampling

For physical capture, the animals had been chased into the boma, rested in the enclosure overnight, and then chased into the truck the following day. They trapped one animal at a time and lifted it out of the truck and restrained it on the ground for sampling. Then the animal was lifted back onto the truck.

For chemical capture, the point of sampling was done either in the field where it had been darted, on the field vehicle, or by the transportation truck before the animal was lifted onto the truck and given the reversal drug.

A venous blood sample, saliva sample, DNS, hair, and faecal sample were collected from each animal. Only the DNS will be discussed further in this thesis as these were used to

examine the nasopharyngeal microbiota and screened for respiratory bacteria associated with BRD.

Deep nasal swabs

Samples from the nasopharynx were collected through bilateral nasal access using one double guarded Laryngeal Swab (MW128, MWE medical wire, England & Wales) for each nasopharynx. A clean compress saturated with NaCl, was used to clean the nostril. Insertion length of the swab was determined by measuring the distance between the nostril and medial canthus of the eye, before the covered swab was inserted medioventrally in the nasal cavity. After the covered swab was retracted 3-4 cm and the swab was pushed 3-4 cm out of the protective cover, the swab was firmly rotated for approximately 10-30 seconds against the nasopharyngeal mucosa, depending on how calm the animal was. The swab was then retracted back into the plastic protection before the protected swab was pulled out of the animal's nasal cavity. Using side-cutting pliers the swab was cut off and transferred to a transport medium which was then marked with the ID number of the tested animal. Between each animal, the pliers were cleaned by removing visible debris before disinfecting with alcohol.

Every swab was stored in liquid Amies transport medium in a Copan eSwab® vial (480C, COPAN Diagnostics Inc., Murrieta, CA, USA). The transport medium was activated by swirling the accompanying nasal swab in the media for a few seconds, before transferring the DNS to the vial with activated Amies. The vial was kept in a cooling bag with cooling elements after the samples were taken and stored until arrival at the laboratory where they were seeded. Swabs were subjected to bacterial cultivation within 55 hours (range 3,92-54,5 hours, median 6,12 hours), before both swabs were frozen at -80 °C.

Figure 7 shows the workflow of sample collection and sample processing.

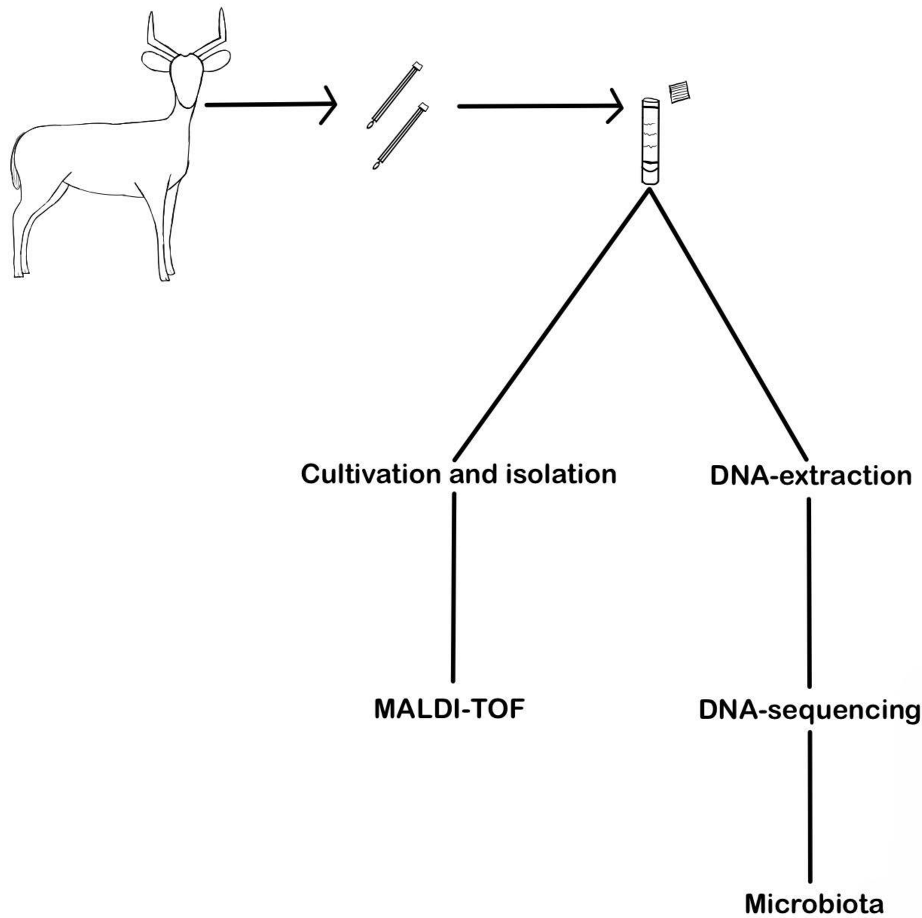


***Figure 7**: Flowchart showing collection of two deep nasal swabs from one animal and pooling of the samples in the same vial containing liquid Amies. These samples were then used for cultivation and isolation of respiratory pathogens Pasteurella multocida, Mannheimia haemolytica and Histophilus somni, and the findings were verified using MALDI-TOF. The same samples were also used for DNA extraction and sequencing for information about the nasopharyngeal microbiota.*

Bacterial cultivation for respiratory bacteria.

Each DNS was seeded onto two different agar plates: a sheep's BA (CM0271B, Oxoid Limited, Basingstoke, UK) and a selective agar CHROMagar™ Pasteurella (PR012, CHROMagar, Paris, France). There were two DNSs collected from each animal, and these were kept in the same vial and seeded on each their half of the same agar plate.

Deep nasal swab on blood agar

The DNS were seeded onto BA using three (A) or two (B) dilutions as illustrated in Figure 8, depending on number of available swabs from each animal. The plates were then incubated for 24-48 hours at 37°C in aerobic atmosphere.

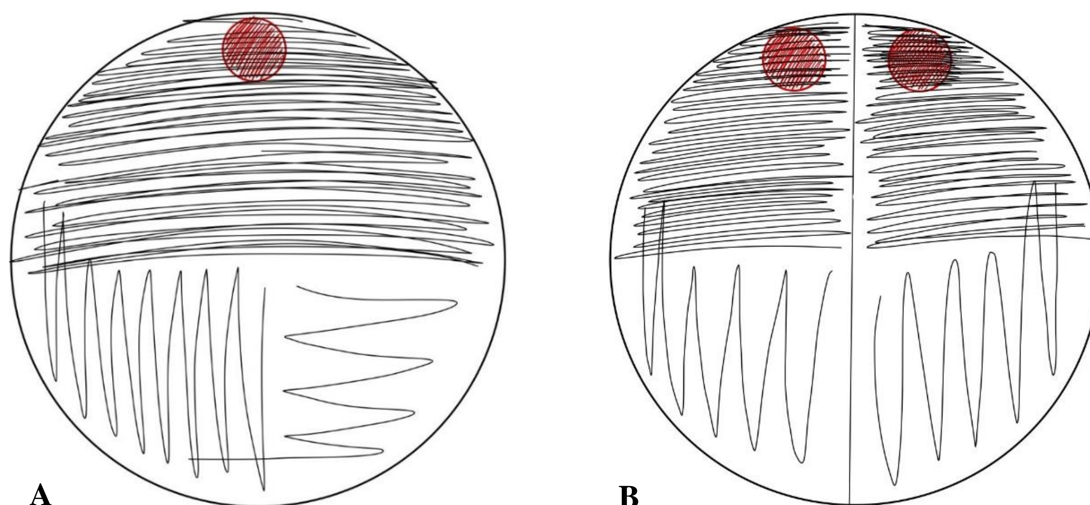


Figure 8: A*: Seeding one DNS on blood agar, three dilutions.* ***B****: Seeding two DNSs on blood agar, two dilutions. Red area: Direct smear of DNS.*

Deep nasal swab on CHROMagar™ Pasteurella

One plate was used per animal. The DNS was directly smeared onto the plate, ensuring all sides of the swab had been in contact with the media. Vials containing one swab was seeded as illustrated in Figure 9A, while those containing two swabs were seeded as illustrated in Figure 9B. A sterile tweezer was used to pick up the swab from the transport medium.

The CHROMagar™ was incubated for 24-48 hours at 37°C in an aerobic atmosphere.

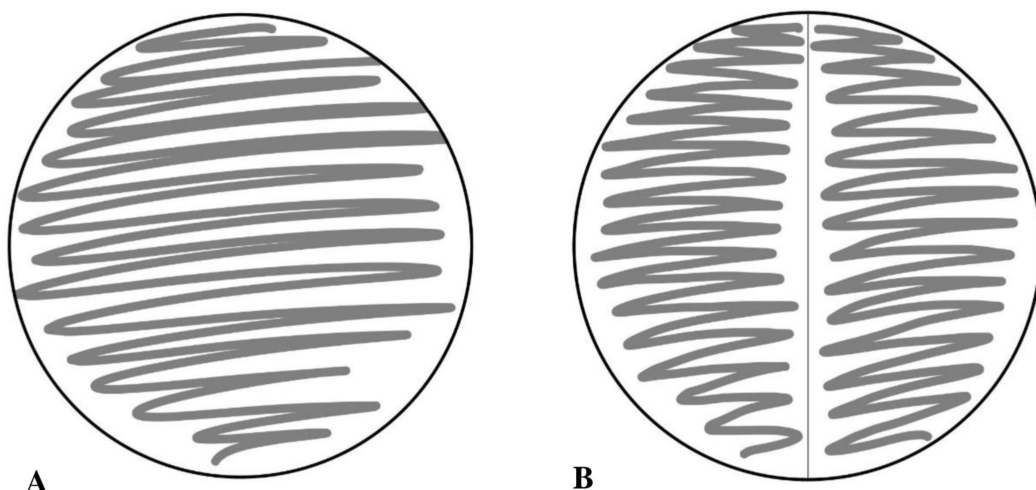


***Figure 9: A**: Streaking of one DNS on CHROMagar™. **B:** Streaking of two DNSs on CHROMagar™*

When the swabs had been applied to both BA and CHROMagar™, the DNSs were returned to the same vial they came in. Except for three vials (001, 002, 003) kept at -20°C for 10 days before being moved to -80°C freezer, all vials and transport media were stored at -80°C in a resealable plastic bag awaiting shipment to Norway.

All colonies showing growth on CHROMagar™ after 24 hours were seeded onto BA to obtain pure cultures. If there were any new colonies at 48 hours (compared to 24 hours), these were seeded onto BA as well.

Choosing presumptive target colonies

Bacteria of interest were PM, MH, and HS. Colonies conforming with the expected colony morphology of PM (BA: non-haemolytic, smooth, grey, varying size colonies with a characteristic sweet or indole-like odour, Picture 1A), MH (BA: small or medium-sized grey colonies, haemolytic, and pudding/butter-like, Picture 1B) or HS (BA: slow-growing, yellow mucoid unclear haemolytic colonies, small/pinpoint, Picture 1C) were subcultured for presumptive confirmation using simple biochemical tests.

From blood agar

Presumptive colonies were described and secondary seeded onto a new BA to achieve pure culture, with four (Figure 10A) to eight (Figure 10B) colonies on each plate. The BA was then incubated for 24-48 hours at 37°C, except for two plates incubated for 115[1] hours.

When pure culture was achieved the plates were inspected for colony morphology. Colonies that did not fit the description of PM, MH, and HS were excluded from further testing. Each colony fulfilling the morphologic criteria of PM, MH, and/or HS was tested for Gram stain, production of catalase, and oxidase. Results from microbiological testing and staining were listed in a table along with a description of colony morphology. Cultures of bacteria with correct colony morphology on BA, that were oxidase and catalase positive and Gram-negative rods were classified as presumptive PMs. Cultures were classified as presumptive MH if they had correct colony morphology, showed rods-stained Gram-negative, and had positive results for catalase and oxidase tests. Cultures that had correct BA colony morphology and had variable results when using catalase and oxidase test were classified as presumptive HSs. These presumptive PMs, MHs and HSs were prepared for deep freeze.

From CHROMagar™

All colonies that grew on CHROMagar™ were secondary seeded, inspected and tested as described for colonies from BA. Some colonies had promising morphology but limited available material. Due to a lack of BA, those colonies were transferred to cryotubes without microbiological testing to ensure as much material for freezing as possible. When there was limited material, all available material was transferred to the cryotubes.

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[1] Due to lack of laboratory access during weekend and national holidays.

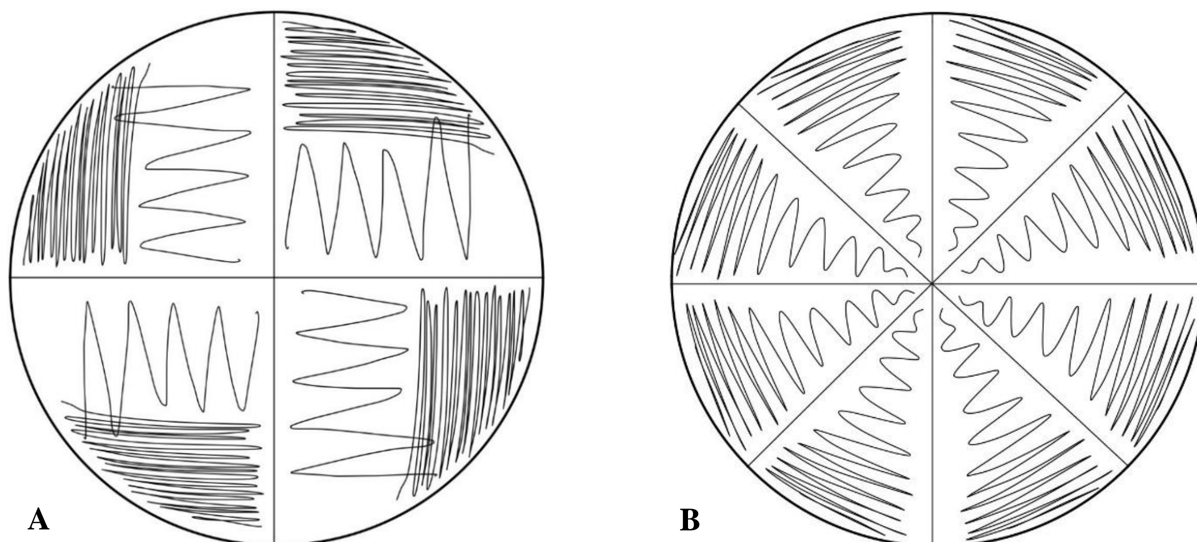

***Figure 10A**: Standard secondary seeding of colonies. **B:** Secondary seeding of colonies if large number of colonies on one primary plate.*

Biochemical testing

Biochemical testing was performed using oxidase test, catalase test, and Gram staining. Oxidase test was performed using Millipore® Oxidase Strips (40560, MerckKGaA, Darmstadt, Germany). The paper part of the oxidase strip was either smeared directly onto the colonies if enough material (and monoculture) or a plastic inoculation loop was used to transfer bacteria onto the paper part of the strip. A positive oxidase test was evident by the colour change to violet/dark blue within 3 minutes.

For the catalase test, one colony was transferred to a glass objective using a sterile inoculation loop. One drop of catalase reagent (H_2O_2) was added. Positive bacteria produced O_2 from H_2O_2, seen as bubbles. Gram staining was performed using basic fuchsin and examined using a 100X objective. Shape and stain of the bacteria was recorded. If colony morphology was indicative of any of the bacteria of interest, but the biochemical tests were not according to the expected results of the bacteria, the colony was still chosen for freezing in cryotubes.

Species confirmation of selected colonies

Preparation for MALDI-TOF

From each presumptive PM, MH and HS, colony material was transferred to a Microbank® cryotube (PL.170C/R, Pro-Lab Diagnostics, Richmond Hill, ON, Canada) using a sterile plastic inoculation loop. The cryotube was turned 4-5 times and stored at -80 °C in a resealable plastic bag. Cryotube 1, 2, 3, 4, and 5 were mistakenly stored at -20 °C for nine (n=1), seven (n=1) and one (n=3) days before being moved to the -80 °C freezer.

MALDI-TOF

Cryotubes were removed from the freezer and 10 µL of colony material was seeded to BA plates containing bovine blood. The plates were incubated for 24 hours at 37°C in aerobic atmosphere before secondary seeding to new BA plates that were incubated for another 24 hours at 37°C in an aerobic atmosphere to ensure monoculture. Material from these new plates was used for MALDI-TOF after overnight incubation, using NMBU protocol. The reference strain (*Escherichia coli* ATCC 8739) was incubated using BA for 12-24 hours at 37 °C aerobic atmosphere. Sterile 1 µL inoculation loops were used to transfer bacteria from a colony to one spot on the MS-DS slide where it was smeared using circular movements. One µL of MS CHCA matrix was applied to the slide spot right after inoculation with bacteria. For every 16 test slots one calibration spot were inoculated with *E. coli* reference strain. After all test and calibration spots were dry the MS-DS slide was analysed using MALDI-TOF. MALDI-TOF was performed using VITEK ® MS (bioMérieux) at NMBU, Ås. All isolates were investigated in two technical replicates. VITEK MS (BioMérieux) use VITEK ® MS Expanded V3.2 and SARAMIS ® Database as databases.

Defining the microbiota of nasopharynx

DNA extraction

DNA was extracted from the samples using QIAamp® DNA Mini Kit (51306, QIAGEN, Hilden, Germany) following the Buccal Swabs Spin Protocol described in QIAamp® DNA Mini and Blood Mini Handbook with alterations as described in the following:

Swabs were thawed and 400 µL of liquid Amies from the transport vials was added to a microcentrifuge tube (1,5 mL). Twenty µL of proteinase K and 600 µL Buffer AL was added and mixed by vortexing for 15 seconds on a Heidolph vortexer. After incubation at 56 °C for 10 min., the microcentrifuge tubes were centrifuged briefly to remove drops from the lid of the microcentrifuge tube. Four hundred µL of ethanol (96%) was added to the sample before vortexing again to mix the sample. Again, the sample was briefly centrifuged to remove any drops from inside the lid.

Seven hundred µL of contents from the microcentrifuge tube was transferred to the QIAamp® Mini spin column placed in a 2 mL collection tube. The collection tube with the QIAamp® Mini spin column was centrifuged at 8000 rpm for 1 minute, before the QIAamp® Mini spin column was transferred to a new collection tube (2 mL). The remaining 700 µL of sample in the microcentrifuge tube was then added to the QIAamp® Mini spin column in the new collection tube before centrifuging at 8000 rpm for another minute.

The QIAamp® Mini spin column was transferred to a new 2 mL collection tube and the used collection tube discarded, before 500 µL of Buffer AW1 was added to the spin column. The collection tube with spin column and Buffer AW1 was centrifuged at 8000 rpm before the QIAamp® Mini column was transferred to a new collection tube of 2 mL, and the collection tube with filtrate was discarded.

Five hundred µL of Buffer AW2 was added to the spin column before centrifugation at full speed (14000 rpm) for 3 minutes. The spin column was then transferred to a new collection tube (2 mL) and centrifuged for 1 minute at 14000 rpm. The collection tube was discarded as the QIAamp® Mini spin column was transferred to a clean 1,5 mL microcentrifuge tube. The QIAamp® Mini spin column was then carefully opened before adding 50 µL of Buffer EB[2] (19086, QIAGEN, Hilden, Germany) and incubated at room temperature for 1 minute and centrifuged for 1 minute at 8000 rpm.

Assessing DNA purity and quantity using Qubit and mySPEC.

The DNA concentration was measured using the fluorometric method by Qubit™ (Thermo Fischer Scientific inc., Waltham, MA, USA) with the Qubit™ dsDNA BR (Broad Range) Assay Kit (Q32850, Thermo Fischer Scientific Inc, Waltham, MA, USA). DNA purity was measured using the spectrophotometric method using mySPEC (732-2534, LS Scientific ltd., London, GB) and assessed purity based on 260/280 relation. A 260/280 of ≥1,8 is in general accepted as pure regarding DNA.

DNA Sequencing and library preparation

Amplicon sequencing was performed by Novogene Co., Ltd. (Cambridge, UK) using amplification of V3/V4 regions of the 16S rRNA gene, aiming for 30 000 paired end (PE) reads per sample. The primers 5'-CCTAYGGGRBGCASCAG-3' and 5'-GGACTACNNGGGTATCTAAT-3' with barcodes were generated in a Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The amplicons were quantified and qualified running a 2% agarose electrophoresis using equal volumes of PCR products and

[2] Novogene recommends EB, TE or TB buffer for sequencing samples (Novogene, 2023).

loading buffer for size detection. PCR products were purified, end-repaired and A-tailed before they were ligated with Illumina adapters. The generated library was checked using Qubit and qPCR for quantification. Size distribution detection was done using a bioanalyzer. Paired-end sequencing was done on the Illumina platform generating 250 bp PE raw reads.

Quality control of raw reads

All bioinformatics analyses were performed by Novogene UK. In short, raw PE reads were demultiplexed using a custom Python script (V3.6.13) (Van Rossum & Drake, 2009) and barcodes and primers were removed using cutadapt (V3.3) (Martin, 2011). PE reads were merged using FLASH (V1.2.11) (Magoč & Salzberg, 2011), creating splicing sequences hereafter referred to as raw tags. Raw tags were filtered for quality using fastp (Version 0.23.1)(Chen, 2023) to obtain Clean Tags (Bokulich et al., 2013). Chimeras were identified using the UCHIME Algorithm (Edgar et al., 2011) and the reference database (SILVA database (16S/18S)) (Quast et al., 2013), then removed using vsearch (V2.16.0) package (Rognes et al., 2016), creating Effective Tags (ET) used in all downstream analyses.

Clustering the Effective Tags in operational taxonomic units

Sequence analysis was performed by UPARSE software (UPARSE v7.0.1001) (Edgar, 2013). Sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs). A representative sequence for each OTU was taxonomically annotated against the SILVA Database (Quast et al., 2013) using the Mothur algorithm (Schloss, 2009). For sequences that could not be annotated, a dmp file provided by NCBI would be used to supplement the taxonomic information.

Microbial community analysis and visualization

Top 10 taxa of each sample at phylum and genus level were selected to plot the distribution histogram of relative abundance in Perl through SVG. Relative abundance of top 10 taxa calculated based on ETs classified excluding “Others” (less common) is presented here.

Venn diagrams visually display the common and unique information between different samples or groups. Venn diagrams were produced in R (R Core Team, 2022) with VennDiagram function and in perl with SVG.

OTU abundance information was normalized using a sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on the normalized data over the variable “species”, namely impala, roan antelope, and sable antelope. Alpha diversity analyses the complexity of species for a sample, and here we applied the Shannon index, to evaluate both the richness and the evenness of the microbial community in question. Beta diversity analysis was used to evaluate differences of samples in species complexity, and Weighted Unifrac and Bray-Curtis indexes were calculated by QIIME software (Version 1.9.1) (Caporaso et al., 2010). Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualised from complex, multidimensional data. A distance matrix based on Bray-Curtis diversity index among samples were transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by first principal coordinate, and the second maximum one by the second principal coordinate, and so on. PCoA analysis was displayed by ade4 package (Bougeard & Dray, 2018; Chessel et al., 2004; Dray & Dufour, 2007; Dray et al., 2007; Thioulouse et al., 2018) and ggplot2 package (Wickham, 2016) in R software (Version 4.0.3)(R Core Team, 2022).

Results

Locations and study selection

Five impala, one puku, one kudu, four roan antelopes, and three sable antelopes were sampled across three different locations in the Central Province of Zambia from June 24th to July 13th, 2023. The study selection included five males and 10 females. Three of the animals were physically immobilized, while 12 animals were chemically immobilized. See Table 3 for the general data collected in the field during immobilization.

Location A – Mkushi, Noah's Ark Conservancy

A total of 12-15 animals were captured using a boma. Two impalas, one male and one female, and one female kudu were sampled the following day. As the other impalas captured in the boma were pregnant and there was a risk of death if exposed to further stress, no other animals were samples from this capture site.

Location B – Kabwe

Five roan antelopes and one sable antelope were chemically immobilized and translocated. A male roan antelope was too awake to allow sampling, therefore only five animals were sampled from this location.

Location C – Protea Game Reserve Chisamba

One kudu, two sable antelopes, and three impalas were chemically immobilized and sampled.

Clinical observations

All animals were categorized as a 2 on BCS, meaning all immobilized animals were considered of normal body composition by the teams conducting the immobilizations.

No observation of spontaneous cough was made in any of the animals. Most of the animals (n=12) had no visible nasal discharge. One animal (002) was bleeding from both nostrils and mouth pre sampling, presumed to be due to trauma during capture/loading, while three animals (004, 005 and 012) had epistaxis post sampling. See Table 3 for registrations regarding respiratory health and DNS collection.

Respiratory rate (RR) was registered for nine animals. Physically immobilized animals (n=2) ranged from 48-76 breaths per minute (median 62), while chemically immobilized (n=7) ranged from 28-64 breaths per minute (median 32). Body temperature was measured in 10 animals. The body temperature in both physically immobilized animals were 39,4°C. Chemically immobilized animals (n=8) ranged from 37,0°C-41,7°C (median 38,6°C). Animals had been chased, handled, and/or sedated for a varying amount of time at the point of temperature measurement.

Due to unexpected time restrictions during the immobilization, a more rapid assessment of respiratory health was developed. This included assessment of the nasal discharge and the presence of spontaneous cough. We classified 12 of the 14 sampled animals as showing no signs of respiratory disease. Animal 001, the deceased impala, was excluded from this evaluation because of the lack of clinical data. Animal 002 is not included due to traumatic epistaxis and difficulty assessing nasal discharge in this individual.

Table 3: *Data collected in the field regarding the immobilized animals.*

Animal ID	Location	Method	Species	Sex	Cough	Quality	Quantity	RR	Temp	Samples
001	A	P	Impala	F						DNS
002	A	P	Impala	M	0	T	*	76	39,4	DNS
003	A	P	Puku	F	0	N	0	48	39,4	DNS
004	B	C	Roan antelope	F	0	N	0	36		DNS
005	B	C	Roan antelope	F	0	N	0	28	38,2	DNS
006	B	C	Roan antelope	F	0	N	0			DNS
007	B	C	Roan antelope	F	0	N	0	28	38,7	DNS
008	B	C	Roan antelope	M				32		No samples
009	B	C	Sable antelope	M	0	N	0	48		DNS
010	C	C	Impala	M	0	N	0	64	38,5	DNS
011	C	C	Sable antelope	F	0	N	0		37,0	DNS
012	C	C	Sable antelope	M	0	N (T)	0		37,9	DNS
013	C	C	Impala	F	0	N	0		41,7	DNS
014	C	C	Impala	F	0	N	0		40,5	DNS
015	C	C	Kudu	F	0	N	0	28	38,6	DNS

Location: *Location of immobilization. A: Noah's Ark Concervancy, Mkushi. B: Kabwe. C: Protea Game Reserve Chisamba.*
Method*: Immobilization method. P: Physical. C: Chemical.*
Sex*: Sex of immobilized animal. F: Female. M: Male.*
Cough*: Spontaneous cough observed? 1: Yes. 0: No.*
Quality*: Quality of nasal discharge. N: No discharge. S: Serous discharge. M: Mucoid discharge. P: Purulent discharge. H: Haemorrhagic discharge. T: Traumatic epistaxis.*
Quantity*: Quantity of nasal discharge. 0: No discharge. 1: Minimal discharge from one nostril. 2: Minimal discharge from both nostrils. 3: Moderate amount of discharge from both nostrils. 4: Excessive amount of discharge from both nostrils.*
** not able to evaluate due to traumatic epistaxis.*
RR*: Respiratory rate, breaths per minute.*
Temp*: Rectal temperature in °C.*
Samples*: Collected samples. DNS: Deep nasal swab.*

A full clinical evaluation was performed on two animals physically captured in Mkushi, see Table 4.

***Table 4**: Data from two individuals with full clinical evaluation.*

ID	Species	Age	Weight	BCS	Temp	HR	RR	Ausc	Cough	Qlt	Qty
002	Impala	Adult	45-50	2	39,4	68	76	N	0	T	*
003	Puku	Young	25	2	39,4	96	48	N	0	N	0

***ID**: Animal ID.*
***Age**: Estimated age. Young: ≤2 years of age. Adult: >2 years of age.*
***Weight**: Estimated weight.*
***BCS**: Body condition score. 1: Emaciated. 2: Normal. 3: Overweight.*
***Temp**: Rectal temperature in °C.*
***HR**: Heart rate in beats per minute.*
***RR**: Respiratory rate in breaths per minute.*
***Ausc**: Auscultation of lungs. N: No abnormal lung sounds. C: Crackles. W: Wheezes.*
***Cough**: Observed spontaneous cough? 1: Yes. 0: No.*
***Qlt**: Quality of nasal discharge. N: No discharge. S: Serous discharge. M: Mucoid discharge. P: Purulent discharge. H: Haemorrhagic discharge. T: Traumatic epistaxis.*
***Qty**: Quantity of nasal discharge. 0: No discharge. 1: Minimal discharge from one nostril. 2: Minimal discharge from both nostrils. 3: Moderate amount of discharge from both nostrils. 4: Excessive amount of discharge from both nostrils.*
** not able to evaluate due to traumatic epistaxis.*

Sampling

DNS was obtained from 14 of 15 immobilized animals. See Table 3 for an overview of animals sampled using DNS. Swabs were seeded on BA and CHROMagar, either two DNSs (n=12) or one DNS (n=2) per animal.

Animal 001 died in the boma before being loaded onto the transportation vehicle. This pregnant female impala was sampled upon arrival at the boma and before sampling the other animals. After sampling the impala was transported to a field slaughterhouse for necropsy. Necropsy showed abundant ectoparasites and no macroscopically visible endoparasites. Some lung lobes were nodular by palpation and had a red-grey discoloration. The impala was pregnant, estimated to be in the second trimester. The preliminary diagnosis made by the examining veterinarian was pneumonia. Lungs were not swabbed due to faecal contamination during necropsy.

Microbiology

Seven of 14 sampled animals gave rise to 21 colonies with candidate PM, MH, or HS. These 21 colonies were subjected to biochemical testing and storage in cryotubes for diagnosis with MALDI-TOF. See Appendix 1 for results of biochemical testing, gram staining and colony morphology of colonies selected for cryotubes. Stored bacteria were transported to Norway and subjected to species determination using MALDI-TOF. Bacteria from cryotube 10 containing colony VII from animal 009 had no growth after seeding on BA after 24- and 48-hour incubation and was therefore excluded from further analyses.

MALDI-TOF was conducted on the 20 remaining colonies. Isolates from tube number 5, 6, and 20 could not be identified. Cryotube 9 had two possible matches with 49.4% and 50.6% confidence and was therefore not species identified, while the rest of the samples were identified with 99.9 % confidence. Results from MALDI-TOF are depicted in Table 5. In Picture 4 the colony morphology of MH found in animal 012 is shown.

Picture 4*: Secondary seeding of DNS from animal 012 after 24 hours of incubation at UNZA. Colony VII was later identified as Mannheimia haemolytica with MALDI-TOF at NMBU. Photo by Mali Sofie Aadal.*

***Table 5**: Overview of each cryotube, their corresponding animal ID, location of capture, colony number, species, sex, and identified colonies, including confidence of identification. Cryotubes belonging to the same individual have identical colour (same as Appendix 1).*

C no.	Animal ID - Location	Colony	Species	Sex	Results (confidence, %)
1	003-A	I	Puku	F	*Acinetobacter lwoffii* (99,9)
2	003-A	VI	Puku	F	*Acinetobacter lwoffii* (99,9)
3	001-A	VI	Impala	F	*Acinetobacter lwoffii* (99,9)
5	001-A	V	Impala	F	No ID
4	002-A	IV	Impala	M	*Acinetobacter lwoffii* (99,9)
6	006-B	I	Roan antelope	F	No ID
7	006-B	II	Roan antelope	F	*Escherichia coli* (99,9)
8	006-B	V	Roan antelope	F	*Escherichia coli* (99,9)
9	009-B	II	Sable antelope	M	*Staphylococcus haemolyticus* (49,4) / *Acinetobacter ursingii* (50,6)
10	009-B	VII	Sable antelope	M	No growth, not tested
11	009-B	X	Sable antelope	M	*Bibersteinia trehalosi* (99,9)
12	009-B	XI	Sable antelope	M	*Neisseria flava/perflava/subflava* (99,9)
13	012-C	XVII	Sable antelope	M	*Mannheimia haemolytica* (99,9)
14	012-C	XV	Sable antelope	M	*Mannheimia haemolytica* (99,9)
15	012-C	XVI	Sable antelope	M	*Mannheimia haemolytica* (99,9)
16	012-C	XXI	Sable antelope	M	*Mannheimia haemolytica* (99,9)
17	012-C	VII	Sable antelope	M	*Mannheimia haemolytica* (99,9)
18	012-C	XIII	Sable antelope	M	*Mannheimia haemolytica* (99,9)
19	012-C	XXIII	Sable antelope	M	*Neisseria flava/perflava/subflava* (99,9)
20	011-C	X	Sable antelope	F	No ID
21	011-C	VIII	Sable antelope	F	*Neisseria mucosa/sicca* (99,9)

***C. no.:** Cryotube number.*
***Animal ID – Location**: Animal ID number – Place of location. A: Noah's Ark Concervancy, Mkushi. B: Kabwe. C: Protea Game Reserve Chisamba.*
***Colony**: Colony number on blood agar using Roman numerals.*
***Species**: Species of sampled animal.*
***Sex**: Sex of sampled animal. F: Female. M: Male.*
***Results (confidence, %):** Results of MALDI-TOF identification. Bacteria and confidence of identification in %.*

DNA and sequencing

DNA extraction, concentration, and purity

DNA extraction and Qubit analysis resulted in DNA concentration ranging from 13,5 ng/µL to 107,0 ng/µL (median 33,4 ng/µL). According to mySPEC analysis, the 260/280 ratio were in the range 1.861 – 2.111 (median 1.961) indicating sufficient DNA yield and quality. See Table 6 for Qubit and mySPEC results.

Table 6*: Results from Qubit and mySPEC analysis.*

Animal ID	DNA concentration (ng/µL)	260/280
001	61.0	1.861
002	25.0	2.025
003	43.8	1.878
004	27.4	1.997
005	15.1	1.981
006	10.6	1.930
007	15.7	2.002
009	18.1	2.007
010	59.4	1.907
011	36.8	1.938
012	30.0	2.062
013	13.5	2.111
014	28.4	1.940
015	107	1.908

DNA Concentration: *Concentration of DNA in ng/µL. Measured using Qubit.*
260/280*: Ratio between absorption at 260 and 280 nm, used to assess DNA purity. Measured using mySPEC.*

16S rRNA sequencing

Quality Control

All samples sent for sequencing passed quality control (QC) by Novogene and was prepared for library and sequencing. A median of 65721.5 (range 62280-71211) raw PE reads was achieved, of which 82.65% (range 51.39-91.39%) were resultant ET after QC. Therefore, a median of 54323 ET (range 33075 to 59263) was used for downstream analysis. The median length of ET was 404.97bp (range 366.91-422.73). See Table 7 for more details on the sequence effort.

Table 7: *Results from 16S rRNA sequencing showing raw paired end reads, average length of effective tags and percentage of effective tags.*

Sample	RawPE	Avglen(nt)	Effective%
001	64360	392.94	51.39%
002	64845	422.73	91.39%
003	66764	383.04	63.35%
004	66290	390.20	71.73%
005	71211	390.79	76.77%
006	66393	421.00	81.30%
007	62280	410.95	84.00%
009	63766	411.35	89.40%
010	65658	421.65	88.07%
011	66506	404.87	84.14%
012	64424	422.49	87.12%
013	64717	399.69	90.25%
014	65785	405.60	73.72%
015	66500	366.91	80.56%

Sample: *ID number of animal.*
RawPE: *Original paired end reads after sequencing.*
Avglen(nt): *Average length of effective tags.*
Effective%: *Percentage of non-chimera sequences in raw paired end reads.*

OTU analysis and taxonomic composition according to samples and species

Most ETs were classified (93,8% of ET, Figure 11), and these ETs yielded an average of 951 OTUs per sample (Figure 11). On average, 3234 tags were unique to each sample (Figure 11). The taxonomic annotation of the classified reads will be presented in the following.

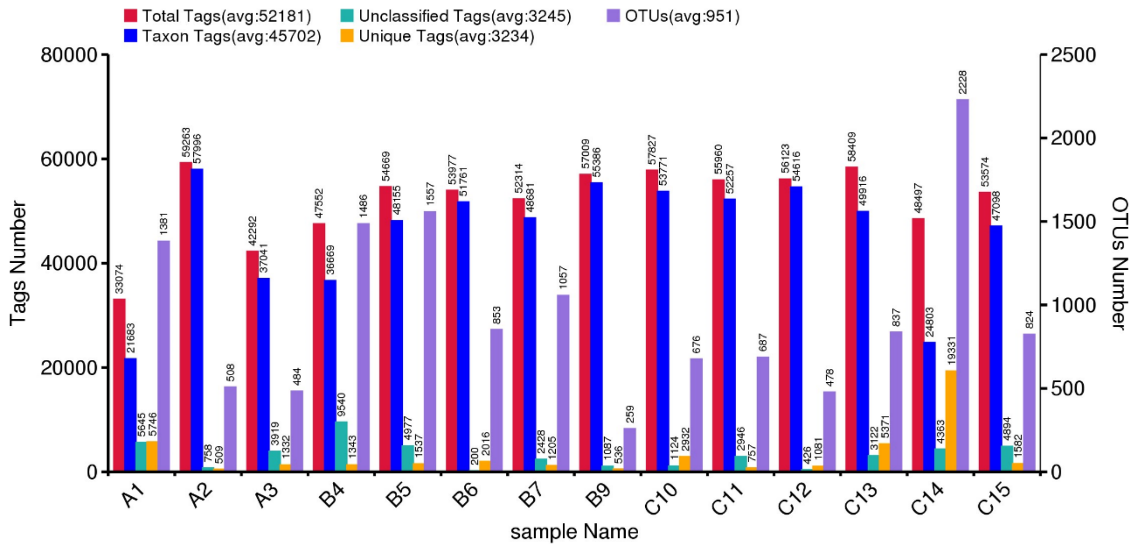

Figure 11: *Bar chart showing number of classified sequences for each sample.*

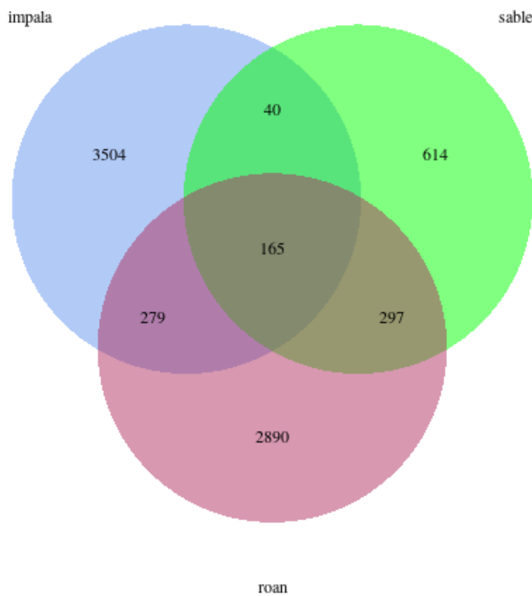

Figure 12: *Venn diagram presenting number of OTUs for the species impala, sable antelope (sable) and roan antelope (roan). The diagram shows numbers of shared and unique OTUs.*

The absolute number of OTUs for impala, sable antelope and roan antelope and the number of shared and unique OTUs in each species is presented in Figure 12. There are 165 core OTUs shared between all three species, and a varying number of unique OTUs. Impalas have the largest number of unique OTUs (3504), and shares 40 OTUs with sable antelope, and 279 OTUs with roan antelope. Roan antelopes have 2890 unique OTUs, and 297 shared OTUs with sable antelopes. Sable antelopes have the lowest amount of unique OTUs of the three compared species at 614. The relative abundance of the top 10 genera in each artiodactyl species and in each sample is depicted in Figure 13 and 14, respectively. The top 10 genera across all species were *Corynebacterium, Acinetobacter, Suttonella, Fervidobacterium, Anaplasma, Streptococcus, Ralstonia, Macrococcus, Pantoea* and *Moraxella*. Other genera are gathered and classified as “Others”, depicted as the yellow bar in Figure 13 and 14. Figure 15 depicts the taxonomic cluster heatmap of the top 35 genera of all samples.

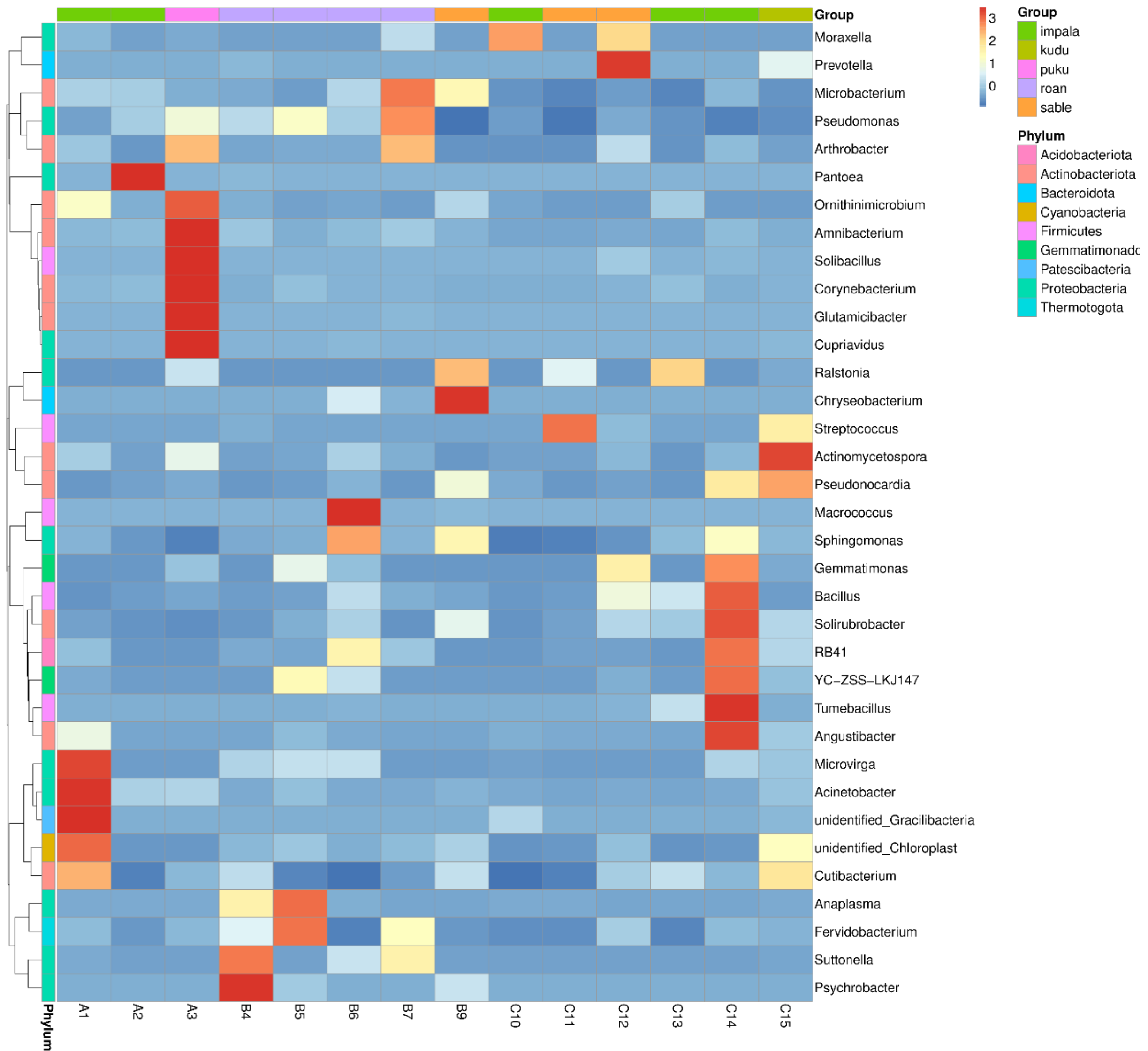

Figure 15: *The taxonomic abundance cluster heatmap according to the top 35 genera of all samples. Top bar represents species (see legend for details), the phylum is represented as bars on the leaves of the phylogenetic tree.*

The heatmap in Figure 15 indicates some deviant samples with high occurrence of some bacteria of which the remaining samples lack or have a very low abundance of. Samples collected at the same time and with the same method (marked as A, B and C) were not similar to each other (see Figure 15, 16 and 17).

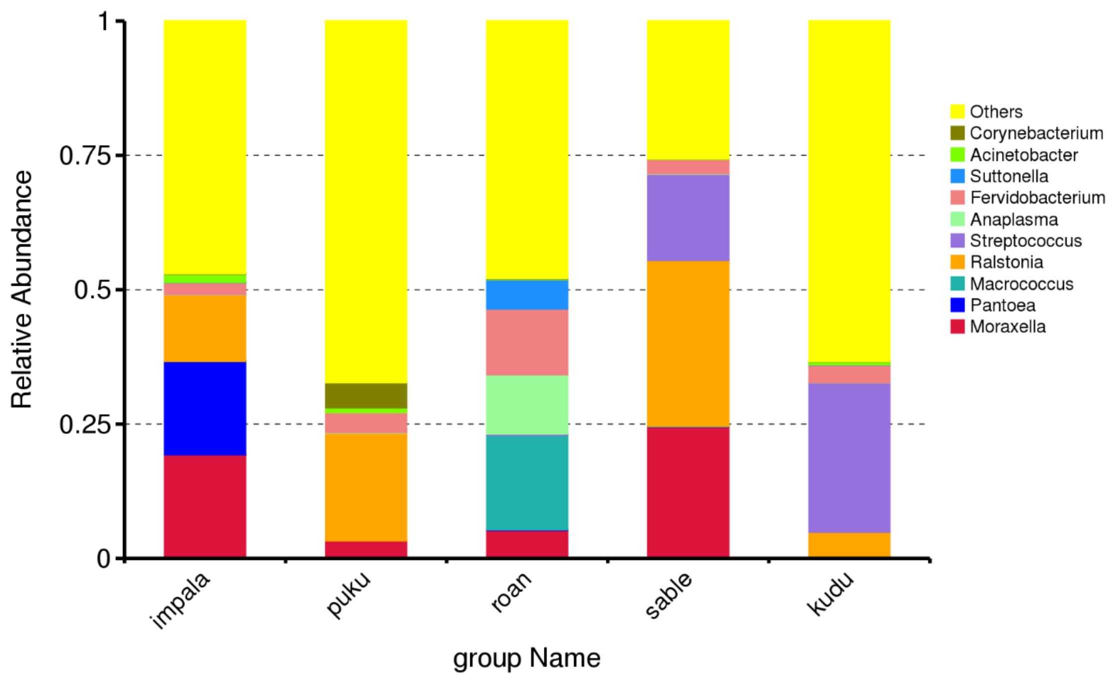

Figure 13: *Bar plot depicting relative abundance of bacterial genera within each of the captured species.*

The most common genera in all samples overall were *Moraxella*, *Ralstonia* and *Pantoea*, with relative abundance of 21.7%, 19.9% and 10.7% respectively. *Moraxella* was found in all species, and in most samples (not A2 and B9) with a relative abundance of 32.0%, 28.8% and 8.25% in impala, sable antelope, and roan antelope, respectively. *Ralstonia*, the second most common genus, was found in all species except roan antelope. *Ralstonia* was present in samples A2, A3, B9, C11, C13 and C15, and in relative abundances of 38.3%, 37.0% and 20.6% in puku, sable antelope and impala. *Pantoea* was the third most common genus and was found in all species except puku. Impalas were the species with the largest relative abundance of *Pantoea* at 32.1%. Roan antelope and sable antelope had notably lower relative abundance of *Pantoea* at 0.16% and 0.07%. The genus was not found in puku.

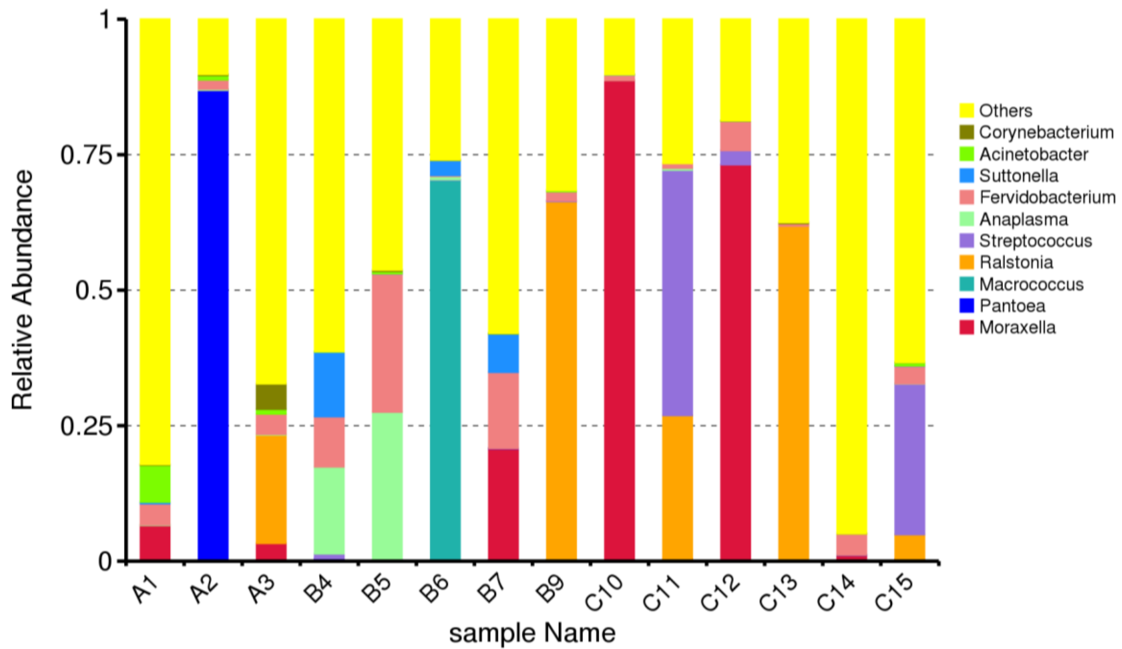

Figure 14: *Bar plot depicting relative abundance of bacterial genera within each sample.*

The fourth most common genus was *Streptococcus*. Genus *Streptococcus* had an average relative abundance of 8.66% across all samples, with kudu and sable antelopes being the artiodactyl species with the highest relative abundance at 52.9% and 18.9%. Impala and roan antelope had a much lower relative abundance of *Streptococcus* with 0.01% and 0.37% respectively. *Streptococcus* was not found in puku, and not in the samples A1, A3, C10 and C13.

Fervidobacterium was the fifth most common genus at 7.67% relative abundance on average and was found in all species and all samples. *Fervidobacterium* was most common in roan antelopes with relative abundance of 9.23 %. Impala and sable antelope had relative abundance of 2.51% and 3.14% respectively. *Macrococcus* was present in a relative abundance of 7.65% across all samples, but was not present in all, only in B6, B9 and C14. *Macrococcus* was common in roan antelope (28,0%), followed by sable antelope (0.07%), and was not detected in impala (< 0,004%), puku or kudu.

The seventh most common genus was *Anaplasma* with an average relative abundance of 4.74% across all samples. *Anaplasma* was found in all species, but not in all samples: B7, B9, C12, C13 and C14 did not get any tags annotated to *Anaplasma*. Again, roan antelope had the largest relative abundance of this genus at 17.0%. *Anaplasma* made up 0.14% and 0.10% of relative abundance in roan antelope and impala. *Suttonella* had an average relative abundance of 2.24% across all samples and 8.10% and 0.06 % in roan antelope and impala. The genus was detected in the samples A1, B4, B5, B6, B7, C10 and C15, and was not found in puku or sable antelope sample.

Lastly, we have *Acinetobacter* and *Corynebacterium* with on average 0.72% and 0.49% of relative abundance. *Acinetobacter* was most common in puku at 1.69%. It was found in all samples except C11 and C13 and was therefore present in all species. Impala, roan antelope, and sable antelope had relative abundance of 1.49%, 0.22% and 0.12% respectively. *Corynebacterium* was found in at least one sample from each species as well, but not in the samples B4, B9, C10 and C11. Impala, roan antelope, and sable antelope had relative abundance of 0.15%, 0.12% and 0.01%. Puku had the largest relative abundance of *Corynebacterium* with 8.96%.

Samples A1 and C14 had a higher proportion of tags (82.1% and 94.9%) annotated to “less common” designated as “Others”.

Alpha diversity

Alpha diversity measure Shannon was calculated for the different species with more than one individual, see Figure 18. Samples from impala had the highest variance in Shannon diversity index (median 4.625 ± 3.488), while limited variance was seen for samples from sable antelope (median 3.042 ± 0.406). The alpha diversity did not differ significantly between

impala and sable nor roan antelope, but the alpha diversity of the microbiota in the DNS from roan antelopes was eminently higher than in the microbiota collected from the sable antelopes. The difference was however not significant due to low sample size.

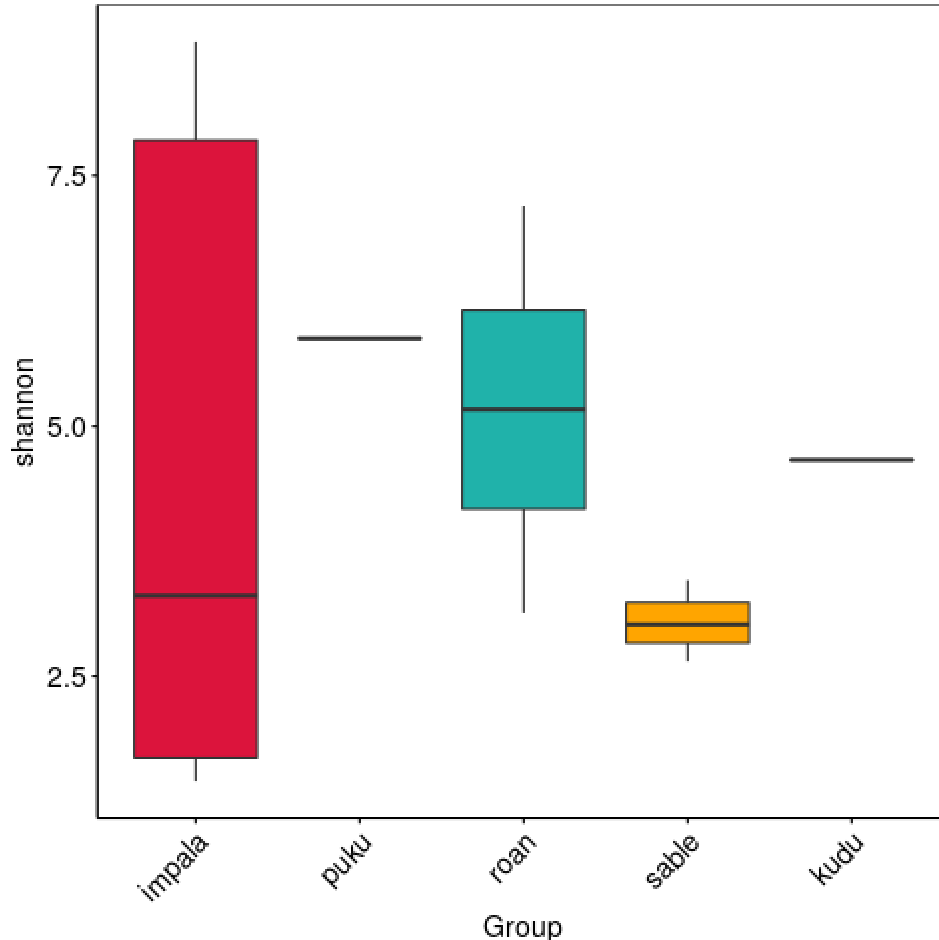

Figure 18: *Box plot for alpha diversity in all species.*

Beta diversity

The difference in microbiota composition between the five artiodactyl species included was investigated using Bray Curtis and Weighted Unifrac diversity index and visualized in a PCoA plot (Figure 16 and 17). Here, some evidence for clustering according to species can be seen for impala and roan, while the microbiota of the sable antelopes clustered with puku and kudu using Bray Curtis (Figure 16). When taking the phylogenetic relationship of the investigated taxa in consideration using the Weighted Unifrac beta diversity index instead, the observed diversification between species disappeared (Figure 17).

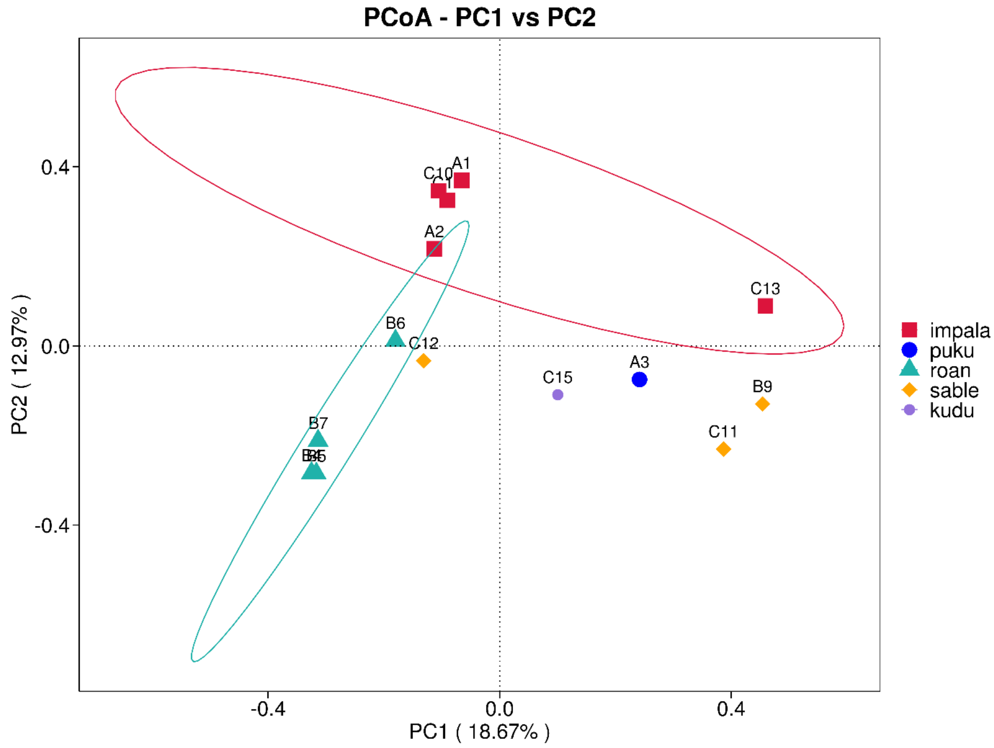

Figure 16: *PCoA plot based on Bray Curtis. Notes: each point represents a sample, plotted by principal component on the x-axis and another principal component on the y-axis.*

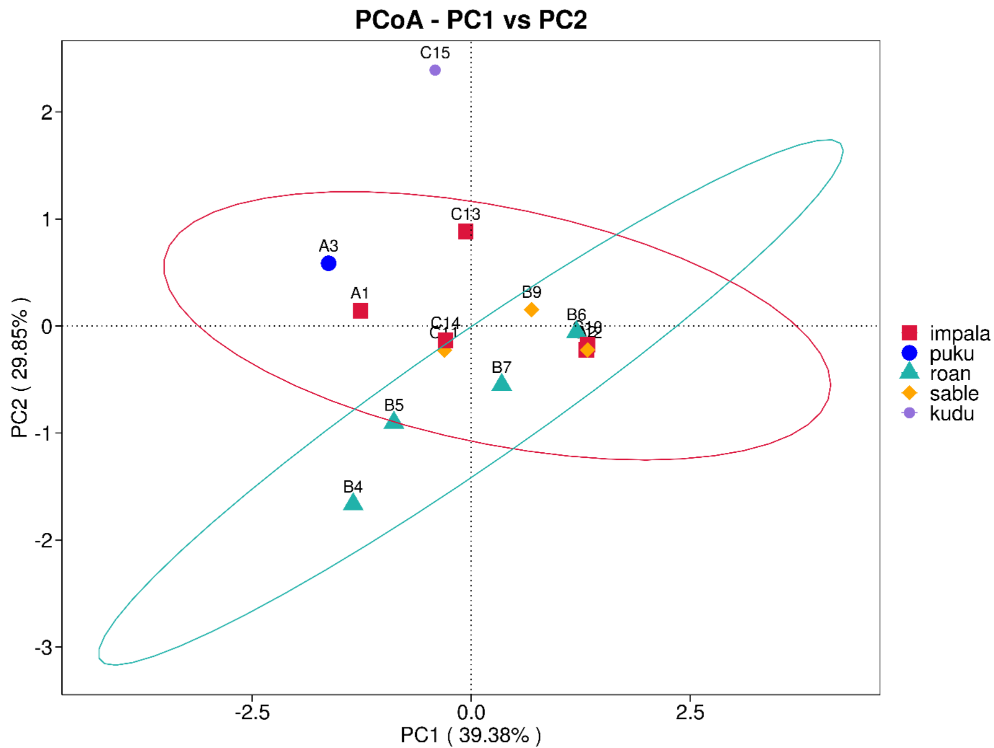

Figure 17: *PCoA plot based on Weighted Unifrac. Notes: each point represents a sample, plotted by principal component on the x-axis and another principal component on the y-axis.*

Discussion

This study aimed to investigate the respiratory health, with focus on nasopharyngeal microbiota and the presence of selected airway pathogens in wild Zambian artiodactyls. We found that one of fourteen tested animals was an asymptomatic carrier of a common bacterial opportunistic pathogen (MH) associated with BRD. PM or HS was not detected in any of the sampled animals. Our study also shows that the diversity of the microbiota of sable antelopes were lower than that of roan antelopes and impala, and that sable antelopes, roan antelopes, and impalas have a species-specific microbiota on their mucus membranes in the nasopharynx. This is, to our knowledge, the first study of the bacterial community of the nasopharynx of wild African artiodactyls, and as such provides important knowledge to a wider research community.

Respiratory health

The result of our assessment of respiratory health shows that our study population had no signs of respiratory disease, except the dead impala where the veterinarian made the diagnosis of pneumonia after performing a necropsy. We cannot, however, make any conclusions about the respiratory health of the study population based on our clinical data. Our limited study selection consists only of 14 animals being of five different species from three different locations. Thus, we can only conclude that the overall impression of the respiratory health of the sampled animals were good.

Sampling animals already being immobilized for reasons other than research saves animals from being subjected to the stress of immobilization only for the sake of sampling. In our experience, since the main aim was to translocate the animals, a short amount of time was set aside for sampling and clinical examination. In addition, the animals were only slightly

sedated during chemical immobilization or completely awake during physical immobilization, thus several members of the capture team were needed to restrain the animals. This minimised the available space around the animal and posed a challenge as several people were needed for data collection. Limited time, space and sedation depth are essential factors contributing to a less detailed clinical examination than originally planned.

Parameters like heart rate, respiratory rate and body temperature are usually important when determining the overall health of animals. However, both stress and drugs used for immobilization cause changes in the respiratory and circulatory system, in addition to affecting body temperature, thus, leaving the data unsuited for health evaluation. Since there is little information on what the normal parameters are, it is difficult to determine whether or not our findings are within the expected range for the different animals.

This project has shown that communication in the field is challenging due to time restrictions and the number of people present. Therefore, when animals are captured for dual purposes, clear communication between the capture and research team is necessary before entering the field. There must be clarification of expectations in advance about what is going to happen and how the sampling should take place to limit the number of unexpected events. Both parties' interests should be taken into consideration and a protocol conducted to satisfy both purposes in a good manner.

As we used existing knowledge about cattle as a basis for our study, a question is to which extent this knowledge is transferrable to wild African artiodactyls. Extrapolation from other species is often the best we can do when information regarding targeted species is lacking. Nevertheless, this can lead to incorrect conclusions and may cause surprises along the way (Rhyan & Spraker, 2010; As reviewed in Ryser-Degiorgis, 2013). An example of this is the protocol for sampling of DNS. We experienced that the measurement of how far to insert the

DNS could not be performed the same way with e.g. roan antelope, and epistaxis post sampling may be due to the method of measuring being wrong.

We found MH in one of the sampled animals upon cultivation. *Pasteurellaceae* have been isolated from wild artiodactyls in Europe, mainly from wild goat species with close contact with domesticated livestock (González-Candela et al., 2006). PM have been identified as the causative agent in a massive outbreak of haemorrhagic septicaemia in saiga antelopes in Kazakhstan, and it is suggested that latent strains of PM was endemic in that antelope population (Kock et al., 2018). This indicates that certain bacteria within *Pasteurellaecea* can circulate in wild artiodactyls, and PM have been detected in impalas in South-Africa (As reviewed in Chu et al., 2020). We speculate whether only finding one BRD-associated bacterium is due to good respiratory health in sampled animals. Nevertheless, these bacteria are considered commensals in cattle, and could therefore have been present in the sampled animals regardless of health status. Considering our small sample size, the finding of MH in one animal is relevant and could be of clinical importance. Further research is needed to draw conclusion on the presence and prevalence of *Pasteureallacea* in wild artiodactyls.

Sampling both nasopharynxes and pooling the swabs increase the sampled area, constituting a more representative sample of the nasopharyngeal microbiota. *Pasteurellaceae* are highly sensitive to the environment and therefore have a limited survival period outside of the host. Due to this sensitivity, samples are best transported with transportation media such as Amies (Fox et al., 2007).

Using the selective medium CHROMagar Pasteurella in addition to BA did not always aid the isolation of target bacteria as much as we hoped. It is said to be a selective media where only *Pasteurellacea* and EC will grow, but when we Gram-stained several of the colonies from CHROMagar, we also found Gram-positive bacteria. This indicates that bacteria other than

EC and species within *Pasteurellaceae* can grow on CHROMagar, although in general other bacteria seem to be inhibited.

Despite having little previous experience, we were able to isolate one target bacteria, MH. Several of the other isolated bacteria, albeit not target bacteria, have similar colony morphology. One of them was *Bibersteinia trehalosi*, which is also a part of *Pasteurellacea* family. Some of the bacteria identified with sequencing were also found using traditional culture. Four of the 21 selected colonies were identified as *Acinetobacter lwoffii*, originating from three different animals. We did not aim to find *Acinetobacter,* but on BA *Acinetobacter* species can be mistaken for PM (Alsan & Klompas, 2010). Considering that *Acinetobacter* was one of the top 10 most abundant genera found across all samples, the fact that we isolated four bacteria of this genus might not be that surprising, and points to the validity of 16s rRNA method to characterize the microbiota.

Nasopharyngeal microbiota

There have been several studies on the nasopharyngeal microbiota in both healthy cattle and cattle with BRD on feedlot farms. The most dominant genera found in the nasopharynx of healthy cattle include *Moraxella*, *Corynebacterium*, *Streptococcus* and *Acinetobacter* (Lima et al., 2016; McMullen et al., 2020; Zeineldin, M. M. et al., 2017). *Moraxella* has been recognized as the most abundant genera in the URT of cattle in several studies (McMullen et al., 2019; McMullen et al., 2020; Zeineldin, M. et al., 2017). There is little documentation regarding the nasopharyngeal microbiota in wild artiodactyls, but the same four genera have been isolated from wild goats in Europe using nasal swabs (González-Candela et al., 2006). Many of these bacteria were also found in our study; *Moraxella*, which was identified in 12 of 14 samples, *Acinetobacter,* found in 12 of 14 samples, *Corynebacterium,* found in 10 of 14 samples, and *Streptococcus* in 10 of 14 samples. We also found genera that to our knowledge

have not been found in animals before, namely *Ralstonia* and *Pantoea*. These genera are commonly found in water and soil (Ruan et al., 2022; Gilligan et al., 2003 as cited in Waugh et al., 2010), and it is possible that the bacteria either comes from ingested feed or that the samples were contaminated.

The most peculiar finding was *Fervidobacterium*, which is a thermophile and obligate anaerobe found in hyperthermal vents (Kanoksilapatham et al., 2016). To our knowledge, this genus has not been found previously in the respiratory tract of Bovidae. However, other obligate anaerobe genera have been isolated from the nasopharynx of cattle, for example *Fusobacterium* and *Bacteroides*. It has been suggested that anaerobic bacteria may make up a portion of the nasopharyngeal microbiota in cattle due to regurgitation from the anaerobic rumen and the formation of aerosols (Holman et al., 2017; Klima et al., 2019; Lima et al., 2016). Chasing of the sampled animals in this study could increase the risk of rumen content being regurgitated. One study conducted on the rumen microbiota in impalas did not mention *Fervidobacterium* (Cersosimo et al., 2015), and to our knowledge, this is the first finding of this genus in wild African artiodactyls. Alternative reasons are contamination of the transport media or extraction kit, as this genus was found in all samples. The laboratory analyses did not include negative controls, therefore, we cannot exclude whether procedural contamination was the potential source to these findings.

Several of our samples also included *Anaplasma*. *Anaplasma* lives within erythrocytes or granulocytes and use ticks as a vector of transmission. Species within *Anaplasma* are known to cause diseases such as bovine anaplasmosis, tick-borne fever and human granulocytic anaplasmosis (Kocan et al., 2015). Animals surviving an infection typically become persistent carriers of infection for life (Hurst, 2018). *Anaplasma* has been found in several wild artiodactyl species (Kuttler, 1984). It is possible that some of the sampled animals had

ongoing infections or were carriers of the bacteria, as we observed several animals with high tick infestation. From animals 004 and 005, both roan antelopes, we observed considerable amount of epistaxis after sampling, and that could be the reason why this genus was found in abundance in these samples. Another possibility could be that *Anaplasma* was introduced from the BA plates if the sheep from which the blood was collected had anaplasmosis. However, several samples were cultivated using BA from the same batch, and *Anaplasma* was not identified in all these samples.

When it comes to sampling methods, TTW and BAL have larger sampling areas and will give a better representation of the microbiota in the LRT compared to the use of swabs (Pardon & Buczinski, 2020), alas this was not feasible in our study. In addition, the goal should always be to use the least invasive diagnostic techniques whether it is in a clinical setting or in experimental studies (Godinho et al., 2007). NS have limited value for infectious diagnostics as the cultures are often overgrown by contaminants (Doyle et al., 2017; Pardon & Buczinski, 2020), therefore we used double guarded laryngeal swabs. It is suggested that there is a mutualistic interrelationship between the bacterial communities in the nasopharynx and the LRT, thus characterization of the nasopharyngeal microbiota could be used to predict the microbiota in the LRT (Centeno-Martinez et al., 2022; Pardon & Buczinski, 2020; Zeineldin, M. et al., 2017).

Differences in composition of microbiota

We found that the sable antelopes had a lower number of OTUs compared with impala and roan antelopes. This could indicate that the sable antelopes have a less diverse nasopharyngeal microbiota compared to that of impala and roan antelope. A less diverse microbiota could affect the animal's resilience to infections (Walker, 1992 as cited in Pettigrew et al., 2012),

and we speculate whether sable antelopes are more prone to respiratory disease. Indeed, the animal that carried MH was a sable antelope.

The most abundant genera were *Pantoea* 32,2%, *Moraxella* 32%, *Ralstonia* 20,6% in impalas, *Macrococcus* 27,9%, *Fervidobacterium* 19,2%, *Anaplasma* 17% in roan antelopes, and *Ralstonia* 36,9%, *Moraxella* 28,8%, *Streptococcus* 18,8% in sable antelopes. We experienced that the roan antelopes got nosebleeds post-sampling more easily than the other sampled species, and that could explain why *Anaplasma* was one of the most abundant genera in this species. In this study, comparing the top 10 most abundant genera overall, impala and sable antelope were the most similar both having *Ralstonia* and *Moraxella* within their top three most abundant genera.

The effect of species and individual seem to be stronger predictors of microbiota than collection and DNA extraction method, as samples collected at the same time were not similar to each other. When looking at the beta diversity of the five sampled species, it appears that animals within the same species clustered together, like the impalas and the roan antelopes, irrespective of sampling sites. This indicates that the microbiota of animals belonging to the same species is more similar than the microbiota of different species. However, when taking into consideration the phylogenetic relationship of the bacterial taxa, the clustering tendency disappeared. There may have been different bacterial taxa found in the different species, but the taxa themselves are closely related to each other, thus making the beta diversity lower than first assumed. As all samples were taken from the nasopharynx, and from phylogenetically related animals living in similar environments, it makes sense to find related bacterial taxa in their nasopharynx. Related bacteria tend to have the same qualities and thrive in the same type of environment (As reviewed in Martiny et al., 2015), e.g. the nasopharynx of artiodactyls.

General limitations

A limitation of this study is the small sample size, and inclusion of many different species of artiodactyls. The original goal was to collect samples from nearly 100 animals of two different species: impala and sable antelope. Several factors influenced the study selection. Selection of animals were done by the capture team and was dependent on capture assignments, available species and animals. This led to alterations in target species on short notice and a smaller and more diverse collection of samples than originally planned. These factors combined does not offer the necessary foundation for drawing conclusions to the artiodactyl population of the Central Province of Zambia, let alone for the entire country of Zambia. This study is therefore descriptive, aiming to describe the nasopharyngeal microbiota of sampled animals, as well as looking for selected respiratory bacteria.

Conclusion

To our knowledge this was the first study to describe the microbiota and investigate the occurrence of BRD associated bacteria in the nasopharynx of wild African artiodactyls. We found both unique and similar bacterial taxa compared to nasopharyngeal microbiota in cattle and wild artiodactyls. We found MH in one of the sampled animals, thus proving that it is present in the wild artiodactyl population in Zambia. The brief clinical examination showed that the sampled animals were in good respiratory health, thus suggesting that MH could be a commensal of the nasopharynx in the animal it was isolated from. Nevertheless, there is a need for further research regarding the respiratory microbiota, the prevalence of BRD associated bacteria and the impact they have on respiratory health of wild artiodactyls.

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Sammendrag

Tittel: Beskrivelse av nasofaryngeal mikrobiota og forekomst av luftveispatogener hos ville klovdyr i Zambia

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Det er begrenset kunnskap om mikrobiota og luftveispatogener hos ville klovdyr. Denne oppgaven har som mål å beskrive respiratorisk helse, den nasofaryngeale mikrobiotaen og forekomsten av utvalgte luftveispatogener hos ville klovdyr i Zambia. Nasofarynks ble prøvetatt hos 14 dyr innen artene impala (*Aepyceros melampus*), sabelantilope (*Hippotragus niger*), roanantilope (*Hippotragus equinus*), puku (*Kobus vardonii*) og kudu (*Tragelaphus strepsiceros*) innen orden Artiodactyla i Zambia. De dyrene som ble prøvetatt virket å ha god respiratorisk helse. Prøvene ble undersøkt for tilstedeværelse av utvalgte luftveispatogener ved hjelp av dyrkning og MALDI-TOF. Amplicon 16S rRNA-sekvensering ble brukt til å beskrive nasofaryngeal mikrobiota. Alfa- og beta-diversitet ble analysert ved hjelp av Shannon, Bray-Curtis og Weighted Unifrac. Sabelantilope hadde mindre divers mikrobiota i nasofarynks sammenlignet med impala og roanantilope. Videre clustret mikrobiotaen basert på impala, sabelantilope og roanantilope, men identifiserte taksa var i slekt. De tre mest tallrike genera var *Moraxella*, *Ralstonia* og *Pantoea*. *Mannheimia haemolytica* ble identifisert hos én sabelantilope. De andre utvalgte patogenene, *Pasteurella multocida* og *Histophilus somni,* ble ikke identifisert. For å fastslå innvirkningen av luftveispatogener og mikrobiota på den respiratoriske helsen hos ville klovdyr, er det behov for videre forskning.

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Appendix

Appendix 1: Results of biochemical tests and colony morphology

Results of biochemical tests, gram staining and colony morphology of bacteria chosen for MALDI identification. Cryotubes with cultures collected from the same animal have the same colour in the table

C. no.	Cat	Oxi	Gram	Colony morphology
1	+	-	-	White-grey, transparent edge, 3-4 mm, smooth, even
2	+	-	-	Pin-point colonies, white-grey
3				One colony, 2-3 mm, white-grey, mucoid, round, smooth, even
5	+	+	-	White-grey, one large single colony, 4 mm, mucoid, pudding-like, no haemolysis
4	-	+		1 mm, round, white grey, no haemolysis, mucoid
6	+	+	-	2-3 mm, white-grey, mucoid, round, smooth, even, opaque, non-haemolytic
7	+	-	-	1-2 mm, grey, no haemolysis, round, smooth, even
8	+	-	-	3 mm, grey, whiter centrally, mucoid, round, smooth, even, opaque
9	+	-	-	1-2 mm, grey, mucoid, no haemolysis, even, smooth, round
10	-	+	-	2-3 mm, grey, whiter centrally, no haemolysis, mucoid, even, smooth, round
11	-	+	-	Grey-white, 2-3 mm, whiter central, round, smooth, even, no haemolysis, mucoid
12	+	+	-	White-grey, 2-3 mm, whiter centrally, no haemolysis, round, smooth, even, opaque
13				Grey, whiter centrally, 2-3 mm, faint haemolysis, smooth, even, round
14	+	+	-	Grey, 2-3 mm, non-haemolytic, smooth, even, round
15	+	+	-	Pinpoint, grey, non-haemolytic, mucoid
16	+	+	-	Grey-white, 2-3 mm, shiny, non-haemolytic, whiter centrally
17	+	+	-	Grey, 2-3 mm, round, smooth, even, mucoid, non-haemolytic
18	+	+		Grey-white, 2 mm, non-haemolytic, somewhat mucoid
19	+	+	-	White-grey, discoloration, no single colonies
20	+	+	-	1-2 mm, grey, non-haemolytic, smooth, even, round, sharp smell (ammonia?)
21	+	+	-	Grey, 1-2 mm, round, smooth, non-haemolytic

C. no.: *Cryotube number.*
Cat*: Result of catalase test. Positive (+) or negative (-).*
Oxi*: Result of oxidase test. Positive (+) or negative (-).*
Gram*: Result of Gram stain. Positive (+) or negative (-).*
Colony morphology*: Colony morphology on blood agar.*

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