PROFILES OF BACTERIAL COMMUNITIES MEDIATING PRODUCTION OF TSETSE ATTRACTIVE PHENOLS IN URINE OF SELECTED MAMMALS

HARRY ASENA MUSONYE (B.Sc.)
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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Harry Asena Musonye
I56/CTY/PT/32671/2015
Signature……Date 25/06/2019
Department of Biochemistry, Microbiology and Biotechnology

Approval by Supervisors

This thesis has been submitted for examination with our approval as the University supervisors.

Dr. James Nonoh
Signature…… Date 26/06/2019
Department of Biochemistry, Microbiology and Biotechnology
Kenyatta University

Dr. Ezekiel Mugendi Njeru
Signature…… Date 26/06/2019
Department of Biochemistry, Microbiology and Biotechnology
Kenyatta University

Prof. Ahmed Hassanali
Signature…… Date 26/06/2019
Department of Chemistry
Kenyatta University

DEDICATION

I dedicate this thesis to Eng. Musonye Fenwicks, my elder brother, who financially supported (personal contribution) this study. Your continued support and encouragement have enabled me reach this far and produce this thesis.

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ABBREVIATIONS AND ACRONYMS

AAT	Animal African Trypanosomiasis
AMDIS	Mass Spectral Deconvolution System
ANOVA	Analysis of the Variance
BLAST	Basic Local Arrangement Search Tool
CFU	Colony forming units
CLED	Cysteine-Lactose-Electrolyte Deficient
CTAB	Cetyl trimethylammonium bromide
DCM	Dichloromethane
DNA	Deoxyribonucleic Acid
DNABaser	Deoxyrebonucleic acid Baser
dNTP	deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic
Exo-SAP	Exonuclease-Shrimp Alkaline Phosphatase
GC-MS	Gas Chromatography-Mass Spectrometry
gDNA	Genomic DNA
HSD	Honestly Significant Difference
IAEA	Internationa Atomic Energy Agency
ICIPE	International Centre of Insect Physiology and Ecology
KWS	Kenya Wildlife Services
LLE	Liquid liquid extraction
LLME	liquid-liquid micro-extraction
LSD	Least Significant Differences
MEGA 7	Molecular Evolutionary Genetics Analysis 7
MR-VP	Methyl Red - Voges-Proskauer
NA	Nutrient agar
NCBI	National Centre for Biotechnological Information
NIST	National Institute of Standard Technology
PAST	Paleontological statistics software package
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
rRNA	ribosomal ribonucleic acid
SAS	Statistical Analysis System
Spp.	Species
SSU	Small sub unit
TAE	Tris-acetate- Ethylenediaminetetraacetic
Taq	Thermus aquaticus
TBE	Tris-borate- Ethylenediaminetetraacetic
TE buffer	Tris Ethylenediaminetetraacetic
TPC	Total phenolic compounds
TSI	Tripple sugar iron
UV	Ultra Violet

ABSTRACT

Animal African Trypanosomiasis (AAT) is a major constraint to livestock health and production. The disease has for years continued to contribute towards economic and developmental setback in the sub-Saharan Africa. Tsestse flies belonging to *Glossina* species are the main vectors for transmission. They are attracted to livestock and wildlife hosts where they feed on blood meal contaminated by trypanosomes, the disease causing parasites. Host ageing urine has been shown to be the source of phenolic blend attractive to the tsetse. The identities and ability of bacteria to mediate production of these phenols has however, not been well investigated. This study aimed at profiling bacterial communities mediating production of tsetse attractive phenols in mammalian urine. Urine samples were collected from, disease free, African buffalo *(Syncerus caffer*), domestic cattle (*Bos taurus)* and eland (*Taurotragus oryx)* at Kongoni Game Valley Ranch in Nakuru County and Kenyatta University in Nairobi County. Urine samples collected for each animal species were aseptically pooled together. Each animal urine sample was then divided into two portions; 400 ml portions were used for fresh urine analysis while the other 400 ml portions were left open to age at ambient conditions. Bacteriological and phenols analysis was carried out on the ageing urine portions at 4 days ageing interval for 24 days. Morphological and biochemical analyses of bacterial isolates from fresh urine tentatively revealed presence of *Proteus* spp., *Enterobacter* spp *Serratia* spp., *Klebsiella* spp., *Stapylococcus* spp., *Streptococcus* spp. and *Enteroccoccus* spp. Bacterial counts were significantly different across ageing intervals in each of the urine samples of the study animals ($p \leq 0.0001$). Highest (9.40×10^7 CFU/ml) and lowest (4.36×10^5 CFU/ml) bacterial counts were observed in urine samples of cattle (day 20) and eland (fresh) respectively. Bacteriological analysis of ageing urine revealed ninteen morpholically different isolates. On subjecting the isolates to PAST software for morphological diversity, Shannon Weiner (*H*) showed highest diversity index at days twelve and sixteen, both at 2.639 and lowest diversity index in fresh urine, at 1.099. GC-Phenols analysis of ageing urine extracts showed a total of nine volatile phenols: P-cresol, o-cresol, m-cresol, phenol, 3-ethylphenol, 3-propylphenol, 2-methyloxyphenol, 4-ethylphenol and 4-propylphenol. There was important qualitative difference among ageing intervals for these phenols in all the urine samples. Phenols concentrations in urine extracts of the animals were statistically different, at $P \leq 0.05$, across ageing intervals. The concentration increased with ageing urine up to day 20 and depreciated at day 24. Comparison between animals, ageing intervals and their interaction revealed significant difference in amount of phenols emitted across days ($P \leq 0.0001$). Statistical comparison of specific phenols concentration among animals showed no significant difference at $P \leq 0.05$. When screened for potential to mediate phenols production, 8 isolates were found positive. Morphological, biochemical and 16S rRNA gene characterization of the isolates closely resembled *Enterococcus faecalis* KUB3006, *Psychrobacter alimentarius* PAMC 27887, *Streptococcus agalactiae* 2603V, *Morganella morganii* sub.sp. *morganii* KT, *Micrococcus luteus* NCTC2665, P*lanococcus massiliensis* strain ES2, *Ochrobactrum pituitosum* AA2 and *Enterococcus faecalis* OGIRF. This study established that some of the bacterial communities colonising mammalian urine are well characterized by certain phenols, which influence tsetse-host seeking behaviour. The study opens potential pathways of reducing attraction of tsetse to the host animals and enhancing "push-pull" models in vector control that combines the use of these bacteria.

CHAPTER ONE: INTRODUCTION

1.1 Background of the Study

Animal African Trypanosomiasis (AAT) has for years continued to suppress livestock health and productivity in the sub-Saharan Africa. It is an endemic parasitic disease of domestic livestock with negative impact on people's income on the African continent (Meyer *et al*., 2018). The direct and indirect cost caused by this disease is estimated at billions of dollars (Chitanga *et al.,* 2011). The disease covers about 11 million kilometre squares of sub-Saharan Africa occurring in 37 countries (Cecchi *et al*., 2009; Yaro *et al*., 2016). Elsewhere, studies have reported prevalence of AAT beyond its conventionally defined margins (Osorio *et al*., 2008). The disease has turned into an established risk to animal health in Asia and South America (Mekata *et al*., 2009). Infections by the disease causing parasites are rated as emerging disease in South America (Batista *et al.,* 2012). In the past, AAT has caused losses of more than US$ 160 million in Brazil and Bolivia where cattle ranching is the single most important economic activity (Da Silva *et al.,* 2011).

Cattle, sheep, camels, goats and pigs are the main economically important domestic species in which the infection is most commonly diagnosed (Merck, 2013). In spite of the age long interventions to control the challenge, an estimated 70 million cattle, as well as other small ruminants are still at threat from AAT (Simarro *et al.,* 2010; Yaro *et al*., 2016). Fatality cases in cattle due to the disease are estimated at three million cattle annually (Vreysen, 2006). This generates annual losses of approximately US $4.5 billion to the African continent agricultural industry (Yaro *et al*., 2016). As reviewed by Gitonga *et al.* (2017), other precious livestock such as camels and horses also suffer from AAT.

During the clinical disease, livestock experience an assortment of debilitating chronic symptoms for example oedema, fever, anaemia, hair loss, and paralysis (Bauer *et al.,* 2010). These conditions reduce fertility, weight gain, milk and meat production and make livestock too weak to be used for ploughing or transport. These in turn affect crop production.

Four biological factors have been described as variables that manipulate epidemiology of AAT in tsetse infected areas of Africa. These factors are parasite, vector, livestock and reservoir hosts (Van den Bossche *et al.,* 2010). *Trypanosoma* species are the causative parasites for AAT. These species include *T. vivax*, *T. brucei brucei* and *T. congolense* The parasites cause moderately mild infections in wild animals as compared to domestic animals where they cause cruel, often fatal disease (Lai *et al.,* 2008). Remarkably, many wild animals in Africa can host more than one trypanosome species hence can act as reservoirs for human and livestock infective trypanosomes (Auty *et al.,* 2012). Studies have also revealed ability of wild South American fauna to harbour *T. vivax* thus acting as reservoir of infection (Osorio *et al*., 2008). *T. congolense and T. vivax* are considered the most important cause of AAT in East and West Africa correspondingly (Lai *et al.,* 2008). Other AAT pathogens of economic importance are *Trypanasoma evansi* and *Trypanosoma equperdum*. The former is the main agent responsible for the disease in camels. It is transmitted mechanically by biting flies. The later causes dourine in horses and other equids and is transmitted through coitus (Desquesnes *et al.,* 2013).

Currently, there is little evidence of successful vaccine development for the trypanosomes as the parasite is said to have capacity to evade mammalian immune defences (Esterhuizen *et al*., 2011; Cnops *et al*., 2015; Scolari *et al.*, 2016; Wilkowsky 2018). This, according to Horn, (2014) is achieved by continuous change of the parasites' surface coat. Control of AAT as a measure to improve production capacities of rural based communities in regions infested by tsetse has been addressed by other methods (Hargrove *et al.*, 2012). The methods primarily include; use of insecticides, trypanocidal drugs, trapping vectors, pesticide treatments and sterile male release strategies (Hargrove *et al.*, 2012; Holmes, 2013; Shaw *et al.*, 2015). Nevertheless, the methods have their own weaknesses. For instance, use of trypanocidal drugs has been limited by efficacy factors due to multiple drug resistance (Moti *et al.*, 2012). The drugs have also been in continuous use for more than half a century and are limited in number. Another key concern is drug safety, in terms of residues in food-producing animals (Baker *et al.*, 2013).

Several reports indicate the widespread phenomenon of counterfeit and poor quality drugs of isometamidium based trypanocides in sub-Saharan Africa (Mungube *et al.*, 2012). In addition to drug resistance, other treatment failure could be as a result of new infections, under dosage, incorrect drug use or poor health state of the animal (Sutcliffe *et al.*, 2014; Moti *et al.*, 2015). Elsewhere, studies have shown that use of insecticides is costly, toxic and drives the advancement of resistance in target vectors (Toure *et al.*, 2015; Scolari *et al.*, 2016). As much as these control measures have not yielded optimum

solutions for AAT, there is optimism that control of the disease causing vector can offer solutions to the problem.

Tsetse flies belonging to *Glossina* species are the main vectors for trypanosomes. The species constitute an effective and steady risk to livestock over much of sub-Saharan Africa. The degree of spread of AAT at any given time depends on the circulation and the vectorial capacity of the involved species. Biting flies may act as mechanical vectors, but their impact in Africa is not well characterized (Desquesnes *et al.,* 2004; Krafsur, 2009).

Tsetse flies inhabit different environments. Their type of habitation is the basis used to classify them into different subgroups. The groups include morsitans, palpalis and fusca that inhabit savannah, riverine and forests in that order (Rayaisse *et al*., 2011). Of the three groups of *Glossina* spp., the savannah and riverine are of livestock and wildlife importance given that they inhabit areas suitable for grazing and watering. They live among livestock and wildlife where they feed on bloodstream meal from infected hosts and pick trypanosomes, the animals' disease causing parasites. The vectors ability to detect and locate suitable hosts from which to feed on is critical for their survival and reproduction (Lord *et al.,* 2017). They achieve this through short-range visual and long-range odour sensation (Omolo *et al*., 2009).

Different odour sources, case in point; urine, faeces, skin surface, feeds, animal breath and manure have been shown to enhance host location by tsetse. Among these, mammalian urine has been researched on. According to Hassanali *et al.* (1986) and Baldacchino *et al.* (2014), mammalian urine is composed of phenolic compounds which

are odorous and highly attractive to tsetse. Hassanali *et al.* (2015) mentioned that 3-n-propylphenol and 4-cresol phenols are critical tsetse attractant components of African buffalo, cattle and waterbuck urine.

Tsetse attractant phenols in mammalian host urine are believed to form slowly from the pro-attractants (Mihok and Lange, 2012). These pro-attractants have been identified as a combination of sulphates, glucuronates and other unabsorbed precursors in urine. Studies have suggested involvement of microbial communities in breakdown of these conjugates into tsetse attractive phenols (Omolo *et al*., 2009). The attraction of tsetse species, to livestock and wildlife is therefore attributable to phenolic compounds in urine. It was essential to therefore profile bacterial communities associated with production of tsetse attractive phenols in mammalian urine so as to help in generation of intervention tools in AAT control and eradication.

1.2 Statement of the problem

Animal African trypanosomiasis (AAT) is a devastating livestock disease that poses serious challenges to livestock health and productivity in sub-Saharan Africa. The disease is responsible for total annual losses estimated in the billions of dollars (US$) in the affected regions. The direct impact on livestock productivity include, increase in abortion and calf mortality, reduced meat and milk off take, restriction from international livestock trade, reduction in calving rate and increase in cost of livestock management (Malele *et al.*, 2016). Indirect impacts of AAT include decreased traction power of oxen,

degenerated human health, decreased crop production and reduction in work efficiency of both man and animals (Swallow, 2014; Alsan, 2015).

According to Bizuayehu *et al*. (2012), AAT has negatively impacted on the economic development and settlement of a major part of the African continent. The disease and its vector stop the integration of crop agricultural and livestock keeping. Integration is significant factor of growth of sustainable agricultural systems (Feldmann *et al*., 2005). AAT is therefore a serious challenge for socio-economic development, agricultural activity, animal health and production in sub-Saharan Africa. Furthermore, the situation is getting worse as the prevention and control of the disease is facing a challenge due to limitation of vector control activities and the development of multiple drug resistance by the parasite and drug safety in terms of residues in food-producing animals (Baker *et al.*, 2013).

The epidemiology of AAT is influenced by parasite, vector, reservoir hosts and livestock (Van *et al.*, 2010). Interaction of tsetse and the host is enhanced by different odour sources for example mammalian urine. Mammalian urine is composed of phenolic compounds which are odorous and highly attractive to tsetse. According to Mihok and Lange, (2012), the mammalian urine phenolic compounds are formed from pro-attractants already existing in urine. Also, Omolo *et al.* (2009) in their study have reported involvement of microbial communities in the formation of the tsetse attractive phenols in mammalian urine. Formation and production of phenols in mammalian urine is therefore, associated with attraction of tsetse species to livestock and wildlife.

In view of these, there have been concerted efforts, by different scientist, to come up with knowledge and solutions appertaining different variables of the spread of AAT. However, despite considerable investments towards its control and eradication, AAT has consistently remained a fatal livestock disease and a livelihood limiting factor among livestock-based rural dwellers (Shaw, 2009). Therefore, there is need for bacterial analysis of phenols formation in mammalian urine as this will help in generation of intervention tools in AAT control.

1.3 Justification of the Study

Agriculture is critical to the economy of countries in sub-Saharan Africa especially in rural areas (World Bank, 2011). Livestock farmers depend on their animals for food, draught power, home consumption, transport and monitory value from the sale of animals and animal products. Ensuring food security in rural parts of sub-Saharan Africa is an imperative challenge, and sustainable growth of agriculture is recognised as a key strategy for poverty reduction (Christiaensen *et al*., 2011). However, livestock farming is constrained by AAT in sub-Saharan Africa. There is need to carry out investigation whose results can help contribute to methods of reducing the transmission and spread of AAT. There are many problems related to the spread and transmission of AAT diseases in the populations that live in tropical climates in Africa. These problems range from issues related to livestock health, political, environmental, social and economic variables.

Loss of livestock due to the affliction of the diseases related to AAT has affected economic productivity in the society (Shaw, 2004). Livestock keepers incur extra cost on

detection and treatment of infected animals, purchasing trypanocidal drugs, equipment and veterinary services. Preventive operations in use for instance, vector control measure, and development of trypanotolerant livestock are costly to farmers too (Holt *et al.,* 2016). New insights into vector control, as an intergral part of the solution for AAT, can be useful in reduction of government spending on livestock health. The deficit in animal production compels countries where trypanosomiasis is widespread to resort to imports of meat and dairy products, a practice harmful to their balance of trade. It is also worth noting that considerable resources by affected governments have been allocated to areas affected by the disease for research on animal trypanosomiasis control. This puts pressure of the government budgetary allocation (Selby *et al.,* 2013).

Tsetse control is important in controlling land use in the affected areas. AAT affects livestock health and productivity to the degree that it influences where people settle, as well as the diversity and intensity of crop and livestock industries (Ilemobade, 2009). As mentioned by Scholari *et al.* (2017), trypanosomiasis causes livestock to be concerted in restricted grazing quarters. This results in land overuse and deterioration (Simarro *et al.,* 2011). According to Reid *et al.* (2000b), populations often respond to the problem of tsetse infestations. In response to the infestation, affected populations have had to move to other lands (Mweempwa *et al.,* 2015). This migration leads to clearing of forests in search of fresh and safe habitats or it leads to exertion of population density pressure on lands already inhabited by other people (Cecchi *et al.,* 2008).

The control of tsetse and trypanosomiasis is necessary in order to alleviate poverty, hunger and food insecurity in Africa continent**.** All these can be addressed through generation of enough information that can be used to model interventions for control of the AAT vector. In the past, there have been suggestions, of involvement of microbial communities in the formation of phenols that influence tsetse-host seeking behaviour (Okech and Hassanali, 1990). Profiling bacterial communities that mediate production of tsetse attractive phenols in mammalian urine is therefore, essential as it will help in development of intervention tools in AAT control. It is thus based on these factors that this study was carried out to come up with objective information about formation of the tsetse attracting phenols in mammalian urine. The results and information generated from this study are useful for scientists and policy makers in the African continent.

1.4 Hypotheses

i. Urine from different mammalian animals harbour diverse bacterial communities
ii. Generation of urine based phenols differ according to the mammalian species and the stage of fermentation.
iii. Bacterial communities in mammalian urine mediate hydrolysis of phenol conjugates to tsetse phenolic cues.

1.5 Objectives

1.5.1 General objective

To profile bacterial communities mediating production of tsetse attractive phenols in mammalian urine

1.5.2 Specific objectives

i. To isolate and characterize bacterial communities in fresh and ageing urine of cattle, buffalo and eland

ii. To determine generation of phenols in cattle, buffalo and eland urine at different stages of fermentation

iii. To analyze the potential of production of tsetse phenolic cues by urine based bacterial communities in cattle, buffalo and eland urine

1.6 Significance of the study

In this study, there were three outputs. In the first output, isolates of bacterial communities in fresh mammalian urine were characterised. The second output was the characterization of tsetse attractive phenols resulting from activities of bacterial communities in ageing mammalian urine. In the last output, bacterial communities that mediate production of tsetse attractive phenols in mammalian urine were identified and documented. Results of this study are essential in modelling AAT epidemiology as well as rationalizing present tsetse control methods. Knowledge generated from this study is helpful in developing effective and sustainable tools and strategies to control AAT and break the cycle of disease transmission. An understanding of bacterial communities may lead to improved development of vector-control strategies that aim to disrupt vector host-seeking behaviour. In addition, observations from this study might help design an important biotechnological model for controlled release of phenols in the field. All these can be adapted and integrated into other disease management efforts.

CHAPTER TWO: LITERATURE REVIEW

2.1 The Epidemiology of African Animal Trypanosomiasis

Vector-borne diseases have complex epidemiology because of the differences in the ecology of variables involved. These variables include hosts, parasites and vectors. The AAT is a complex protozoal disease whose mediating factors have been suggested to be tsetse flies, trypanosomes, livestock and reservoir hosts (Bourn *et al*., 2001). The disease is characterised by some pathologies ranging from acute, chronic to fatal effects.

2.1.1 The Vector and their role in AAT transmission

Tsetse flies are insects of order *dipterian* belonging to genus *Glossina.* Thirty-one species and subspecies of these insects have been identified, and a majority of them are potential vectors of AAT. *Glossina* is the genus with most trypanosomes transmitting tsetse flies. The members of this genus are highly specialized in parasite transmission (Wamwiru *et al.,* 2016). *Glossina* are small in size. Apart from being vectors for AAT parasite, tsetse flies also serve as hosts to trypanosomes (Sharma *et al.,* 2009). Tsetse feeds exclusively on blood. They are holometabolous insects, and their female give birth to full-grown larvae, which rapidly pupate in the soil. Their longevity, mobility and frequent feeding make these flies' highly efficient vectors. Tsetse flies can fly at speeds of up to 25 km per hour, but they usually fly more slowly and only for short periods, for example, up to 50 minutes, and usually can rest for more than 23 hours per day in trees to avoid desiccation. This short duration of flight per day requires that they feed on more blood to sustain the long period of resting.

Tsetse flies' role in AAT transmission involves complex steps. First step is location of the host. Mechanisms used for host location are vision and odour detection. Location of hosts from greater distances is achieved by odour sensing while location from shorter distance of about 15 m, by the vision mechanism (Rayaisse *et al.,* 2010). The second step in AAT transmission is feeding on the host blood. Unlike other members of order *diptera*, for instance mosquitoes, both male and female adult tsetse flies feed on vertebrate blood (Geiger *et al.,* 2015). However, in terms of infection, the female tsetse is less infectious compared to male tsetse (Krinsky, 2019). The tsetse use stylet-like proboscis for feeding on their host. By feeding on blood meal from a trypanosomes infected vertebrate, adult tsetse also become infected and the infection remains permanent (Simo *et al.*, 2008).

The final step in transmission is the pricking and penetration of a healthy animal. To achieve the transmission of trypanosomes, infected flies prick and penetrate the animal skin getting access to the blood circulation system where they deposit the AAT causing parasite (Farikou *et al.,* 2010). Transmission period between animals should be short enough to ensure survival of parasites in the insect mouthparts, as shown in experimental infections in goats (Moloo *et al.,* 2000; Thuita *et al.,* 2008).

Even though tsetse is the chief biological vector of AAT causing parasites in Africa, it is not a guarantee that when they feed on the trypanosomes infected hosts, they will transmit AAT. Several other complimenting factors for the transmission of AAT are intrinsic factors, ecological factors and host factors (Despommier, 2000; Peacock *et al.,*

2012). Mechanisms for these factors, however, involve multi-stage complex processes which are beyond the scope of this study.

2.1.2 Tsetse's life cycle

The life cycle of tsetse has been described by literature in several publications and books, for example, in Peacock *et al.* (2012), Leak, (1999), Chapman and Chapman (1998). When the tsetse imago hatches, its genital organs are already formed and mating can take place shortly within the hours that follow. Four days after the first meal of the female, ovulation then takes place. After ovulation, the egg then moves to the uterus. In the uterus, the egg is then fertilised by the sperm from the spermatheca. After about three days the egg hatches and gives rise to a first stage larva (L_1). The females have a pocket similar to the uterus of the mammal, in which it preserves its larva until maturity. During its intra-uterine life, the larva is fed by secretions of lactiferous glands annexed to the uterus. At 25°C the first instar lasts for about one day, the second instar (L_2) for about two days and the third instar (L_3) for about three days. The larvae are deposited by the mother in a favourable shaded place.

The L_3 larva once deposited, moves actively and buries it-self a few centimetres under the ground. Then, it immobilises and metamorphoses to pupae. The entire process of metamorphosis occurs within the puparium, leading to the perfect adult tsetse. When metamorphoses are complete, the young adult fly leaves its puparium by breaking a circular slit in the anterior end using its putilinum. The subsequent larvi-positions generally occur with intervals of 8 to 12 days. However, the occurrence is majorly

determined by the ambient temperature and the species, for example, *G.morsitans*, at 30°C and 18°C for 8 and 25 days respectively. On the other hand, sex (shorter for females) and ambient temperature (on average 30 to 35 days, but pupal periods from 17 up to 88 days have been observed) are critical factors in determining the length of pupal period (Figure 2.1).

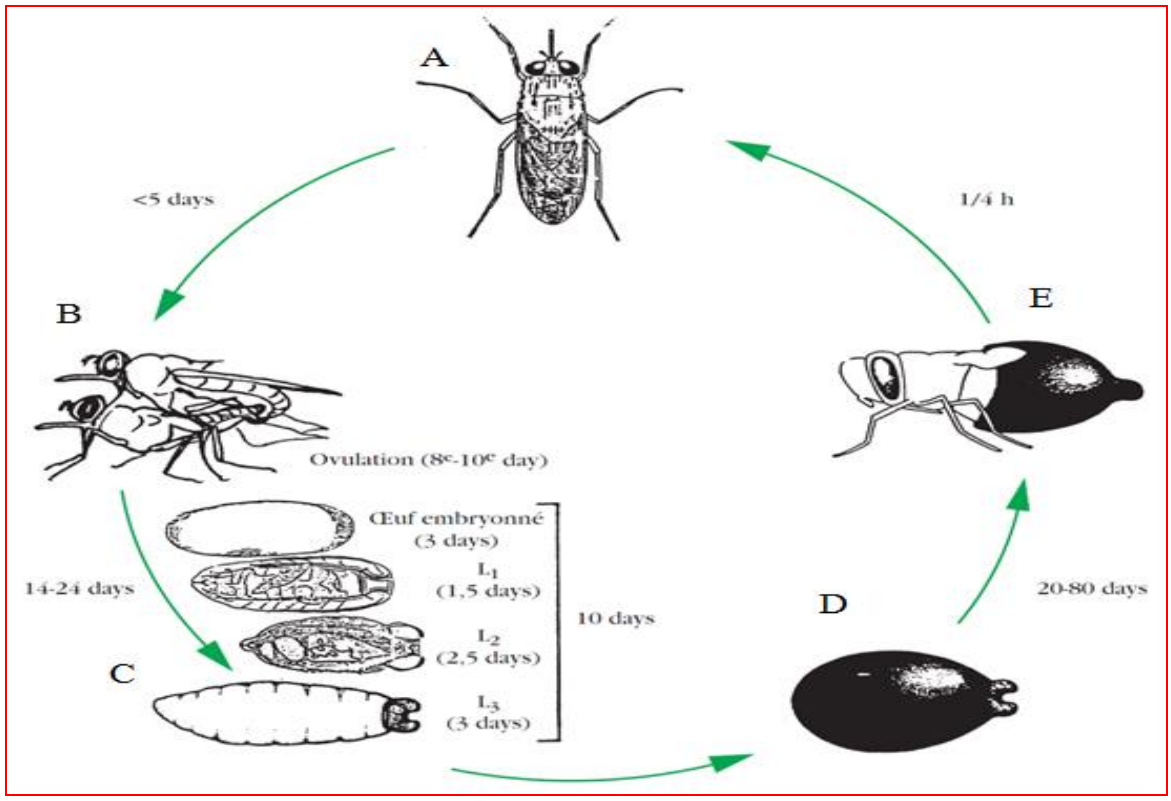

Figure 2.1: Reproductive cycle of a tsetse fly: **A**=Blood engorged female tsetse fly, **B**=Ovulation and larvipositing, **C**= larva stages (L_1, L_2 and L_3), **D**=puparium containing the pupa, **E**=fly emerging from the puparium (Adapted from Cuisance, 1989 with slight modification)

2.1.3 Tsetse fly hosts

Tsetse hosts range from livestock to wildlife. In wildlife, animals like buffalo, monitor lizard, bush pig, warthog, duiker and bush buck, are the natural hosts. These animals acquire prolonged trypanosome infections with no symptoms. On the other hand,

livestock show an assortment of vulnerability to infection. These vary from debilitating symptoms to fatality (Bauer *et al.,* 2010). The ability of some wildlife in Africa to tolerate trypanosomiasis infection helps in creation of reservoirs for trypanosomes that infect livestock and humans (Lai *et al.,* 2008). Studies have also indicated the ability of trypanosomes to infect other wildlife, for example, guinea pigs, mice, monkeys, rats, and rabbits (Krasfur, 2009; Desquesnes *et al*., 2004). Livestock vulnerability to trypanosomiasis is determined by age, breed, previous exposure to the parasite, behaviour and health status of the animal (Lumbala *et al.,* 2009).

Tsetse flies are triggered by urine and breath components of the host to fly upwind. With visual detection, tsetse responds quickly to stationary hosts as opposed to moving ones. Host colour also plays a critical role in tsetse attraction to host. Blue colour has been found to be more attractive. Apart from the blue colour, previous research works have shown that fawn and brown coloured host, for example some cattle, are more likely to be infected than those of other colours (Carty, 2002).

2.1.4 The Parasite

Animal African Trypanosomiasis is parasitic disease caused by an extracellular flagellate. Trypanosomes are unicellular protozoan belonging to phylum Sarcomastigophora, order Kinetoplastida, family Trypanosomatidae, and genus *Trypanosoma*. *Trypanosoma* species are the causative parasites for AAT. These species include *T. vivax, T.congolense and T. brucei brucei (*Lai *et al.,* 2008). *T.congolense*, which is the smallest of the pathogenic trypanosomes, is further subdivided into several types. These types are

distinguished by isoenzymatic differences and molecular techniques. They include *T. congolense* savannah type, *T. congolense* Tsavo type, *T. congolense* forest type and *T. congolense* Kilifi type (Auty *et al.,* 2015). Savannah group is considered the most virulent and of clinical importance in livestock. Nonetheless among this specific group substantial difference in virulence exists (Ahmed *et al.,* 2015).

Different species of trypanosome, once transmitted in livestock host body, undergo complex development processes involving a series of host defence and parasite counter-defence mechanisms that lead to trypanosomiasis (Thuita *et al.,* 2008). As reviewed by Federica *et al*. (2016), as opposed to other trypanosomes, *T. vivax* does not multiply in the tsetse mid-gut, but remains confined to the insect proboscis, where it completes its short life cycle. This is the reason why this species can also be transmitted mechanically by other hematophagous flies, for instance *Tabanus* spp. (horseflies) (Galiza *et al.,* 2011)

Causes of AAT in Africa differ, depending on the regions. For example, the AAT in East Africa is majorly caused by *T. congolense* and the one in West Africa is majorly caused by T. *vivax* (Lai *et al.,* 2008). However, mixed infections in livestock, involving two or three species, are frequent in areas of medium to high tsetse challenge (Biryomumaisho *et al*., 2013; Takeet *et al.,* 2013; Moti *et al.,* 2015). Trypanosomes have ability to infect diverse livestock and more than 30 wildlife species (Auty *et al.,* 2015). Transmission through mechanical vectors has propelled the spread of *T. vivax* and *T. b. brucei* beyond the tsetse fly belt. The parasite *T. vivax* for example, is now established as a risk in Mauritius and in 13 South American countries. Although T. *vivax* remains enzootic in

South America primarily due to mechanical transmission, other potential modes of transmission include perinatal and iatrogenic routes or via alternative, but, as yet unidentified vectors (Osorio *et al.,* 2008) (Figure 2.2).

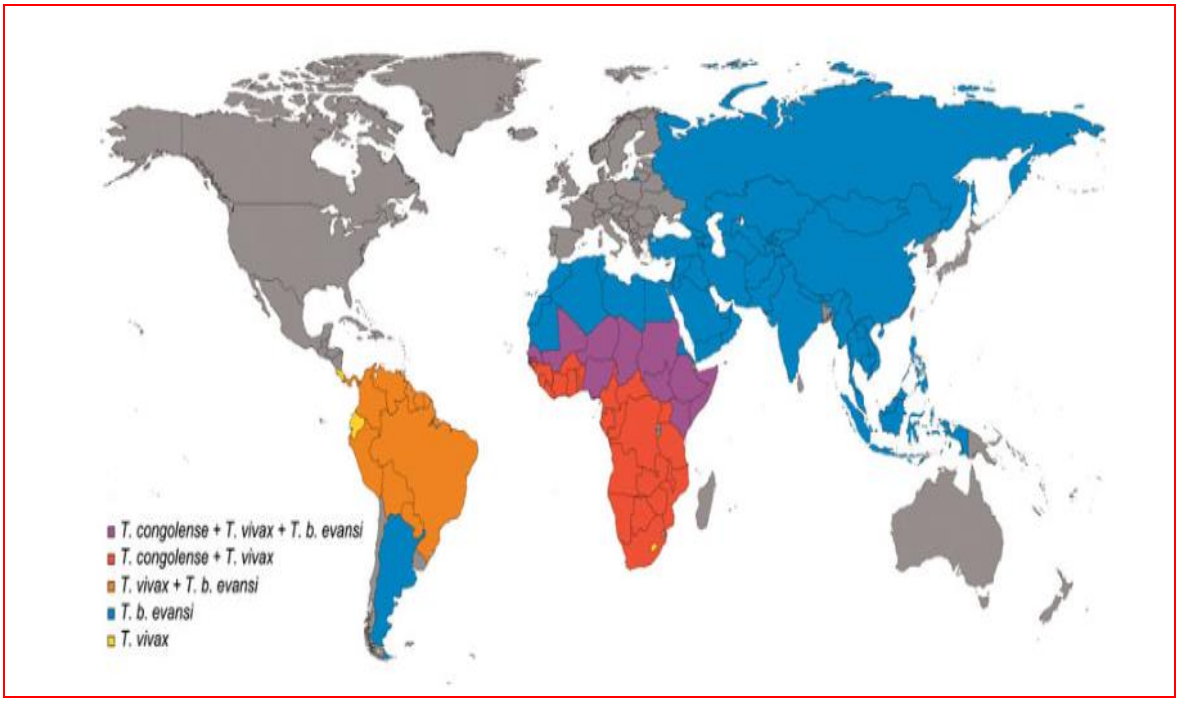

Figure 2.2: Geographical distribution of trypanosomes across the globe. (Courtesy of Federica *et al*. 2016)

Pathogenesis of the parasite in affected animals differs (Imhof *et al*., 2014). In cattle, for instance, pathogenesis is dominated by three features: anaemia, tissue lesions and immune-suppression. However, mechanisms that lead to anaemia are complex. Even though, trypanosomes release haemolysins, intravascular haemolysis is not a prominent feature that leads to anaemia. The condition is rather attributed to erythrophagocytosis by cells of the mononuclear phagocytic system in the spleen, bone marrow, lungs and lymph nodes. Stimulation of cells of these organs is achieved through formation of complexes

between immunoglobulin specific for antigen and trypanosomes or complement attached to red cells (Taylor and Authié, 2004).

Pathogenesis in tissues is associated with the ability of the parasites to invade extra-vascular spaces and organs such as lymph node, cerebrospinal fluid and eyes. Whereas *T. b. brucei* is distributed in both the circulation system and in the other tissues, *T. congolense* remains confined to the vascular system. As demonstrated by Coles *et al*. (2015), this particular specie has ability to parasitize the brain. Although *T. vivax* has been considered primarily as a vascular parasite, some strains have been isolated in extra-vascular locations especially in late stages of infection (Osorio *et al.,* 2008; D'Archivio *et al.,* 2013).

2.1.5 Tsetse flies' habitats

A broad analysis of habitat environment (place, time, interface between host and vector) is crucial to realize the variety of situations that can be encountered in an epidemiological system (De la Rocque *et al.,* 2001). As reported by Albert *et al*. (2015), tsetse flies are sensitive to environmental conditions. They have low survival rate in areas too cold, too hot or too high. Three tsetse groups occupy three distinct ecological zones (Despommier, 2000) (Figure 2.3). These groups are: the fusca, the palpalis and the morsitans (Rayaisse *et al.,* 2011). The fusca group flies (subgenus *Austenina*) tend to occur in the lowland rainforests of West and Central Africa. The palpalis group (subgenus *Nemorhina*) is found in the riverine galleries of West and Central Africa but can extend into savannah regions between river systems; *G. palpalis* and *G. tachinoides* are important AAT vectors in this group. The morsitans group (subgenus *Glossina*) occurs in a variety of savannah

habitats lying between the forest edges and desert and includes several important vectors of AAT for instance *Glossina morsitans* sp., *G. pallidipes* and *G. austeni* (Cecchi *et al.,* 2008; Cecchi *et al.,* 2009). The ecological zone notwithstanding, their behavioural response towards the host and habitat is affected by the odour caused by volatile phenols generated from ageing mammalian urine. It has been postulated that one way of controlling the tsetse is by studying the phenols forming processes in urine (Omolo *et al.,* 2009).

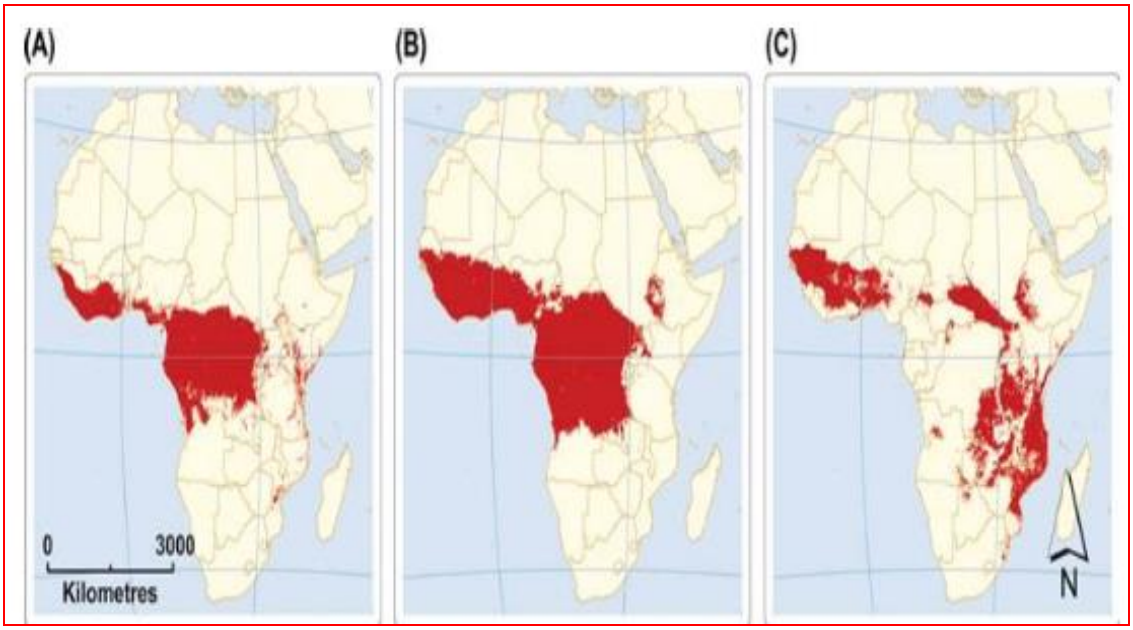

Figure 2.3: Distribution of the three tsetse groups in Africa: **A**-Fusca group; **B**-Palpalis group and **C**-Morsitans group (Adapted from Cecchi *et al.,* 2008). **Key:** The region marked red in A, B and C are tsetse habitats.

2.2 Phenols and tsetse behavioural response

Phenols or phenolics belong to aromatic class of organic compounds. They are characterized by benzene ring in which one or more of the hydrogen groups have been substituted with a hydroxyl group. This hydroxyl group dictates their character (Neveu *et*

al., 2010). Phenol, a benzene derivative, is the simplest known member of this class (Scott, 2007). Phenols are known to be synthesized artificially as well as generated naturally (Taton *et al.,* 2003). Naturally, phenols can be formed from constituents of creosote and coal tar, animal and human waste and organic materials undergoing decomposition (Le *at al*., 2005). Other natural mechanisms of phenols formation not mentioned here also exist. Synthetic production of phenolic compounds involves complex steps outside the scope of this study.

Historically, several microbial volatile organic compounds have been linked to insects' behavioural response (Ericksson *et al*., 2001; Tangtrakulwanich *et al*., 2015). Some of these compounds have critical components that attract insects to the source where they are emitted. The compounds are generated through the primary and secondary metabolism of microorganism (Ezenwa *et al*., 2014). Insects respond to emission of these volatile compounds in special forms: microbial-initiated insect attraction (Chaudhury *et al*., 2010; Tomberlin *et al*., 2012), repellence (Burkepile *et al*., 2006), as well as abnormal behaviours unfavourable to the survivorship of the insect itself (Hughes *et al*., 2011; George *et al*., 2013).

Tsetse behaviour altering compounds found in mammalian urine are varied. The compounds, for example, phenols are associated with tsetse behavioural response. The phenolic compounds are hypothesized to exist in two forms, either as conjugates or as free in state. It has been postulated that microorganisms in mammalian urine hydrolyses the conjugates to phenols (Cecchi *et al.,* 2009; Omolo *et al.,* 2009). Significant

representatives of phenolic groups include but are not limited to 3-methylphenol (m-cresol), 4-methylphenol (p-cresol), phenol and 4-ethylphenol. These phenolic compounds have been reported to increase in odour as the mammalian urine ages (Gikonyo *et al.*, 2002).

According to Leclaire *et al*.'s (2017) ageing hypothesis for the mammalian urine odour, the bacterial communities hydrolyse urine primary products into volatiles odours that influence vector behavioural response. Evidence in support of the fermentation hypothesis is derived principally from studies that link bacterial action to specific olfactory-mediated vector behaviour or to the production of certain odorants. For instance, researchers have suggested that 3-propylphenol and phenol, odorants that plays a key role in tsetse attraction to buffalo, cattle and waterbuck, is as a result of bacterial activities in ageing urine. Likewise, the characteristic odorants of Warthog urine p-cresol are projected to derive from bacterial hydrolysis of phenolic conjugates (Goodwin *et al*., 2016).

2.3 Bacterial communities and mediation of tsetse attraction to the host

Bacteria are ubiquitous and can colonize all habitats, including those occurring within animal bodies (Sapp *et al.*, 2012; Ngai *et al.*, 2013). Genetic variability, for example horizontal gene transfer and metabolic flexibility allow bacteria to adapt easily to different environments (Perterson *et al.*, 2009). Mammals live in association with a suite of microorganisms that can influence host-vector interaction traits (Fraune *et al.*, 2010; Archie *et al*., 2011). Bacteria have been suggested to play a critical role in mediation of

volatiles that elicit tsetse-host seeking behaviour. As mentioned by Wahl *et al.* (2016), bacteria profiling technologies provide a powerful tool to easily increase the understanding of microbial communities. Currently, there is little knowledge on profiles of bacterial communities at different ageing intervals of mammalian urine. There is also no information about 16S rRNA gene profiles of bacterial communities responsible for phenols formation in ageing mammalian urine. It was vital to understand the profiling of the 16S rRNA gene in urine colonizing bacteria. Based on these understanding, profiles of bacterial communities that mediate generation of tsetse attractant phenols in livestock and wildlife can be considered as strategic means in control of AAT.

2.4 16S rRNA gene sequencing

The use of 16S rRNA gene sequencing in the study of bacterial evolution has been the most ordinary genetic marker adapted for various reasons. First is that the gene exists in almost all bacteria, regularly as an operon or a multi-gene family. Secondly, the gene is highly conserved with hyper-variable regions (V1-V9). Thirdly the gene is fairly large, 1,500 base pairs, for bioinformatics functions. Lastly, the genes function has not changed over time, suggesting that random sequence changes are a more accurate measure of evolution (Patel, 2001). 16S rRNA gene has therefore been revealed to be most suited for identification and phylogenic analysis of organisms. For this reason, genes that encode 16S rRNA gene have been used expansively to establish taxonomy, phylogeny, as well as estimate bacterial species rate of divergence. Therefore, comparing the 16S rDNA sequence help in showing evolutionary relatedness among microorganisms. In standard approaches, DNA is amplified using primers binding in conserved regions allowing

partial amplification of the 16S rRNA gene of most bacteria. The 16S rRNA gene contains nine variable regions and conserved regions in between (Peer *et al.,* 1996). The conserved regions can be used in classifying very distant relatives and the variable regions are used in classifying more closely related groups of organisms and thereafter reliable phylogenetic trees constructed based on 16S rRNA gene (Pei *et al.,* 2011). In order to increase sensitivity, specificity and reliability of the 16S rRNA gene studies it is desirable to cover more than one hyper-variable region (Peer *et al.,* 1996).

2.5 Empirical review

2.5.1 Tsetse control, elimination and eradication

For advancement of solutions to the AAT problem, it is vital to understand the variables, methods and approaches associated with control and management of the parasite, vector and the disease. In this respect, researchers have sought, in their works, to study these methods, variables and approaches. For example, a study carried out by Vale *et al.* (2015) dwelt on the chemical control of riverine tsetse. The study aimed at estimating the best strategies of controlling riverine tsetse using tiny targets of about 0.06 m^2 with insecticide treated screen. In their study, they employed a model to simulate the effectiveness of many strategies of applying the targets. A deterministic model was developed using an excel sheet and it was used to simulate the movement, deaths and births of tsetse. This model suggested that use of tiny targets is the most cost effective strategy especially when used for a short period annually. It was estimated to have had a tsetse reduction of about 90 %. The authors postulated that this could have a greater impact on AAT.

Tsetse elimination, according to their model, becomes feasible when isolated areas are targeted in deployment of targets or where populations that are not self-sustaining are involved in invasion. This work was well carried out and it is important to understanding one of the chemical methods of controlling the riverine tsetse. However, this model is limited in a way that it can only be feasible when isolated areas are targeted or where non self sustaining populations are involved in invasion. As well, worth noting is that trapping and eliminating of tsetse may not effectively offer solutions to the problem of AAT. It does not consider the ecological imbalances that may be caused by killing the flies. The study is also silent on methods of controlling other species of tsetse.

Mwangi *et al.* (2008) suggested and assessed the use of δ-octalactone as a potential repellant of tsetse attack. They carried out a study where they first synthesized the δ-octalactone through an abbreviated route. The synthesized product was then assayed against three-day old starved tsetse flies, *G. m. morsitans*, in a choice wind tunnel. The testing of the results demonstrated that the racemic δ-octalactone had a potential to repel tsetse. Even though the studies demonstrated the ability of the racemic δ-octalactone to repel the tsetse fly, it also revealed that significant large amounts of the chemical were required. This thus reveals a potential weakness in large scale application of the chemical repellent.

Saini and Hassanali, (2007) attempted to demonstrate the efficacy of chemical control of tsetse. The researchers demonstrated repellence of 4-akyl-substituted analogue of guaiacol to Savannah tsetse *Glossina* spp. The analogues included: 2-methoxyfuran, 2-

methoxy-4-methylphenol, 2, 4-dimethylphenol, 4-ethyl-2methoxyphenol, 4-allyl-2-methoxyphenol, 3, 4-dimethoxystyrene and 3-4 methylnodioxy-toluene. The experiment was carried out in a two wind tunnel. Compared with the guaiacol, 4-methyl substituted derivative repellent response was found to be stronger. The other repellent showed no noteworthy repellence to tsetse. The study concluded that if the four position of guaiacol was substituted with methyl derivatives, then its ability to repel tsetse could increase. These results suggest that it is probable to come up with chemical repellents through hybridization of existing chemical compounds. The cost effectiveness of this solution was not discussed in the study. As well, the study has focused on methods of repelling tsetse and has not assessed factors that attract tsetse to its hosts.

Mechanical methods of attracting tsetse with bright coloured objects spread with adhesives were proposed and tested (Muramba *et al.,* 2013). In their study, the authors carried out field tests in Maasai Mara and Serengeti to assess the efficacy of use of modern tsetse visual traps. The research findings revealed that tsetse were attracted to blue black colours and hence can be used as cost effective means of controlling and trapping the vector. Implementation of this method would have detrimental consequences that are not restricted to tsetse and trypanosomes but will include several non-target species of conservation concern and others of great biodiversity and ecosystem service value. Some of the negative consequences, as highlighted by Ansara-Ross *et al*. (2012), include local extinction and harmful impacts on ecosystem and ecological processes and services.

Malele *et al.* (2016) conducted a study to compare the suitability of various tsetse traps across Tanzania. They tested sticky panels, code-named; NZI, pyramidal, H, biconical S3, mobile, and NGU traps across 28 locations. They reported that 52.2% of the 224 traps deployed captured at least one *Glossina* species. In total five species of *Glossina* were captured. These included *Glossina fuscipes martini*, *Glossina morsitans*, *Glossina brevipalpis*, *Glossina pallidipes* and *Glossina swynnertoni*. Biconical traps trapped flies in 27 sites, sticky panel in 20, S3 in 15, NGU in 7, pyramidal in 26, mobile in 19, H in 2 and NZI in 1. A total of 21,107 tsetses were caught. *G. swynnertoni* (55.9%) was abundantly trapped followed by *G. pallidipes* (31.1%), *G. fuscipes martinii* (6.9%) and *G. morsitans* (6.0%). The least trapped was *G. brevipalpis* (0.2%). NGU got the highest number of traps at 32.5%, sticky panel followed second at 16% while Mobile, pyramidal, biconical and S3 followed at 15.4%, 13.0%, 11.3% and 10.2% in that order. NZI and H traps managed to catch 0.9% and 0.7% correspondingly.

The researchers concluded that the most efficient trap was NGU, followed by sticky panel and mobile, in that order. Therefore, for tsetse control programmes, NGU traps could be the better choice. Conversely, of the stationary traps, biconical and pyramidal traps captured tsetse in majority of the sites, covering all the three ecosystems better compared to other traps. As a result, they would be appropriate for surveying for tsetse infestation in any given area, thus sparing the costs of making traps for each specific *Glossina* species. This study however is not ecologically sensitive since trapping of flies can lead to ecological imbalances. More so, the method can only be applied in a comparatively small area

Lelisa *et al*. (2014) carried out a cross sectional study between October, 2009 and May 2010 to estimate prevalence of bovine trypanosomiasis in Hawa-Gelan district Ethiopia. Using standard sampling and identification methods, the researchers caught three species of the genus *Glossina.* They include *Glossina morsitans submorsitans, Glossina pallidipes,* and *Glossina fuscipes*. On average, 10.5 flies per trap per day, belonging to *Glossina* species, with a higher prevalence of female flies (57.2%) were caught and recorded. Out of the total 389 cattle examined, 42 were found to be infected with trypanosomes. Three trypanosome species were identified in the study area. They included *Trypanosoma vivax* (21.4%), *Trypanosoma brucei* (23.8%) and *Trypanosoma congolense* (54.8%). They found prevalence of trypanosomiasis to be significantly ($p < 0.05$) higher in cattle with poor body condition. There was recorded association between mean packed cell volume and the occurrence of parasitaemia ($\chi 2 = 49.5$, $p < 0.05$). About 95.2% of cattle that were found to be positive for trypanosomiasis revealed a packed cell volume fewer than the lower limit for cattle.

From their study, Lelisa *et al*. (2014) concluded that the presence of *Glossina* spp. in the Hawa-Gelan district settlement areas is responsible for the bovine trypanosomiasis that is a major constraint in the agricultural sector. Therefore, application of control measures through community involvement to decrease the *Glossina* species infestation intensity is likely to boost animal productivity. The weakness of this study is that the researchers did not come up with tangible measure that can be relied upon to repel tsetse from wildlife and grazing fields.

Lord *et al.* (2017) developed mechanistic models of tsetse population dynamics that included tsetse host-seeking efficiency. They used the models to explain the effect of host density decline on observed mean monthly numbers of tsetse caught during a host elimination experiment in Zimbabwe in 1960s. Their model provided excellent fits to observed decline in catches of *G. m. morsitans* after reductions in host numbers. During the trial, the numbers of tsetse caught droped by 95% with host density reduction of 50% after 600 days. The researchers' model provides a technique of exploring the role of host density on tsetse population dynamics. Some of the findings of this study can be incorporated into models of trypanosome transmission dynamics. This can help scientists to better understand how spatio-temporal variation in host density impacts trypanosome prevalence in mammalian hosts.

It is further noted, in their study, that the modelled population went extinct. However, small numbers of tsetse were caught throughout in the host elimination exercise. This discrepancy indicates that there was still immigration of tsetse in the experimental region. This could easily lead to masking the effect of host reduction. Trypanasome transmission may decrease due to tsetse mortality resulting from low host density. However, hungrier flies may tend to bite humans, in that way increasing the risk of transmission to humans. Similarly, a reduction in host numbers results increases in the vector-to-host ratio and a density-dependent reduction in feeding success for flies that have located a host.

Studies have shown that tsetse is attracted to animals by odour. Such a phenomenon was demonstrated by Kasilagila, (2003) in a series of studies that indicated that attractiveness

of tsetse stationary traps could be altered by odour from different animals. Odours from mammals including buffalo, sheep, pigs, Oxen, donkeys, bushbuck and bush pigs were found to be attractive to tsetse. As such, with these findings, it is possible to understand the animals to target when planning for the control of spread of tsetse. Even though these results are instrumental in informing scientists about the animals with attractive odour, they fail to go further and investigate formation of phenolic volatiles that influence tsetse-host-seeking behaviour.

Harraca *et al*. (2009) carried out a study to further investigate the odour attractiveness to tsetse. They came up with antennogram records from *G. fuscipes palpalis* *G. brevipalpis fusca* and *G. pallidipes morsitans* groups. They paired them on to a chromatographic analysis. They then checked for neutral fraction, acidity and mild acidity in the animal odorous fluids. This indicated that tsetse had ability to detect phenols, aldehydes, sulphides, carboxylic acids, aliphatic, indoles, terpenes and ketones. Ratio of behavioural response from *G. pallidipes* induced by a mixture of carboxylic acids was similar to that from rumen fluid in wind tunnel. The similarities in the sensory responses demonstrated in the study are used to justify the role of phenols in tsetse attraction to hosts. These observations may provide a useful biotechnological model for controlled release of these semio-chemicals in the field. In addition, the study and its findings were well documented and can be used to further research of control of tsetse.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

To achieve the objectives of this study, the focus was laid on areas inhabited by wildlife, livestock. This focus was based on understanding that AAT affects livestock and wildlife. Two areas were selected for collection of samples to be analyzed. Sample collection was carried out at Kongoni Game Valley Ranch, in Nakuru County and Kenyatta University Cattle Farm, in Nairobi County. Authority to conduct research was approved by the National Commission for Science, Technology and Innovation (NACOSTI/P/17/73722/18052). Kongoni Game Valley Ranch is a private ranch, leased by Kenya Wildlife Services. It is situated in an agro-pastoralist semi-arid ecosystem and consists of mostly Acacia woodland with thickets along stream beds, an area favourable for Savannah and Riverine tsetse (Cecchi *et al.*, 2009). Clearance to access the ranch was obtained from the Kenya Wildlife Service. Kenyatta University Cattle Farm is characterized by a semi-intensive farming system. This farm is within the university and was easily accessible for sample collection (Figure 3.1)

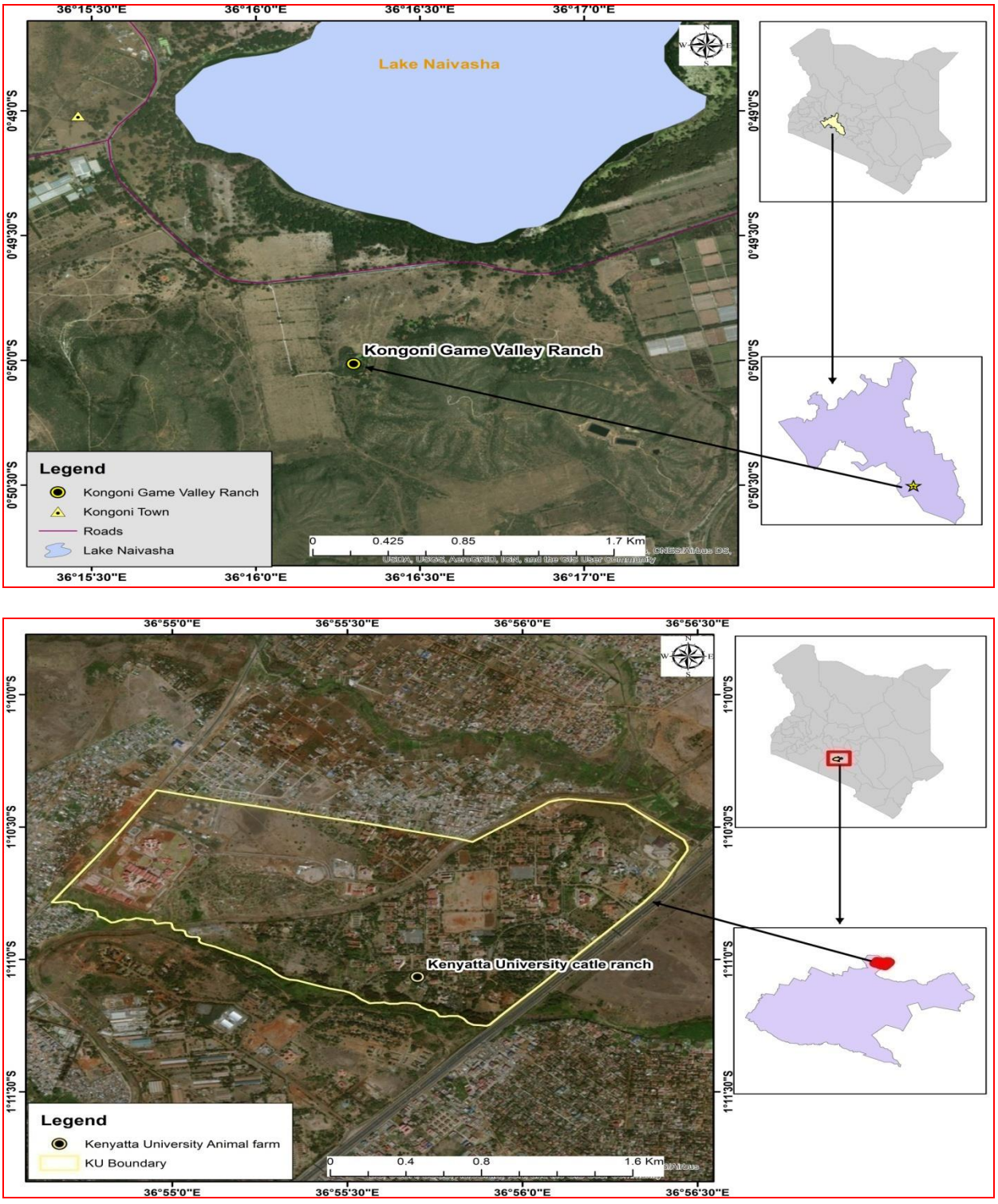

Figure 3.1: A map of Kenya showing the location of study areas; Kongoni Game Valley Ranch in Naivasha-Nakuru County and Kenyatta University cattle farm in Nairobi County

3.2 Urine sample sources

Three disease-free species were used in this study. They included male and female African buffaloes *(Syncerus caffer*), male and female domestic cattle (*Bos taurus)*, of the Freshian breed, and male elands (*Taurotragus oryx)* (Plate 3.1). The choice of these species was influenced by the reported presence of tsetse attractive phenols in Cattle and Buffalo urine (Hassanali *et al*., 1986; Madubunyi *et al.*, 1996). Eland has been shown to be resistant to trypanosomiasis (Pappas, 2002). However, it has not been known if the animal's urine has the potential to attract tsetse. It was therefore thought to be of interest to find out if there are tsetse attractant cues and their bio-agents in the urine. Buffalo and Cattle are preferred hosts by most species of tsetse (Gikonyo *et al.,* 2002). These animals were accessible for urine sample collection.

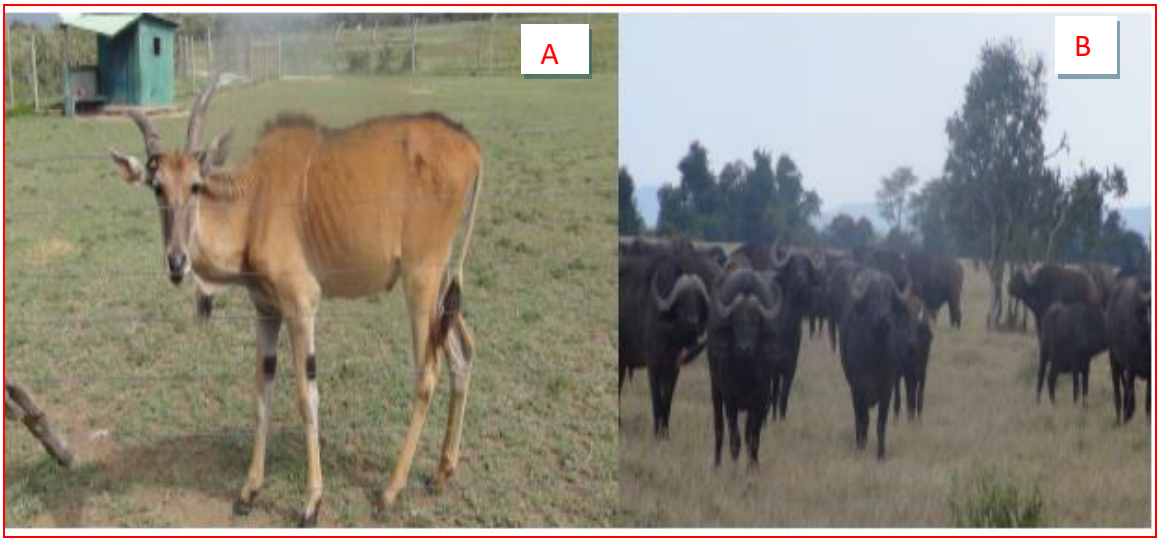

Plate 3.1: A photo showing: A-Eland and B-Buffalo whose urine samples were analyzed

3.3 Urine sample collection

Fresh buffalo and eland urine samples were collected under the guidance of Kenya Wildlife Services (KWS) Veterinary Department officers. The collection was

opportunistically, as the selected wildlife urinated naturally, during des-naring and translocation activities. Fresh cattle urine was also collected while the animals were urinating naturally. Sterile 50 ml universal bottles were used in collecting the urine samples. Samples collection was carried out for a priod two weeks. Plate 3.2 shows photos of some of the sample collection process.

Plate 3.2: Sample collection processes showing: A; urine sample collection from female cattle, B; urine sample collection from a male buffalo and C; sample collection process from eland

A white adhesive label was then placed on each bottle. Animals' name, species, location, date and time of collection was indicated on each label using a permanent waterproof marker. The urine samples were straight away placed in a cool box at 4^{o}C and then transferred to the Kenyatta University Microbiology Research Laboratory for bacteriological analysis and preparation for phenols analysis.

3.4 Sample preparation

Once in the laboratory, the 50 ml bottle urine portions for each animal species collected for the two weeks were aseptically pooled together into sterile transparent plastic bottles measuring 1000 ml. The total pooled samples for each species measured about 800 ml. These pooled samples, for each species, were then divided into two portions, each measuring 400 ml. One 400 ml portion for each animal was used for fresh urine analysis. These portions were reserved at -20^{o}C to defer any chemical transformation as advised by Kilande *et al*. (2016) and Parrah *et al*. (2013). The other 400 ml portions were left open and allowed to age at ambient conditions, throughout the study period, in the laboratory.

3.5 Enumeration and isolation of culturable bacteria in fresh and ageing urine portions

For each mammalian species, about 5 ml urine sample was drawn from the fresh urine portions for bacterial isolation and enumeration. This analysis was carried out once. As well, from each of the ageing urine sample, 5 ml urine sample was drawn for bacteriological analysis. The analysis was carried out at an ageing interval of 4 days, for 24 days. Bacterial communities were then isolated from portions of the ageing

mammalian urine fractions subjected to different ageing intervals as per the method proposed by Kilande *et al.* (2015).

For the bacterial isolation and enumeration processes, each urine sample was aseptically subjected to serial dilution. Aliquots of 0.1 ml (100 µl), making a 10^{-6} dilution factor, were aseptically pipetted and each inoculated, by spreading the inoculums evenly on the Cysteine-Lactose-Electrolyte Deficient (CLED) (Oxoid, Basingstokes, UK) medium in triplicates. The plates were subsequently inverted and incubated aerobically for 24 hours at 37°C. Colony forming units were enumerated with a colony counter and the total was computed as per the guidelines provided by Murray *et al.* (2005).

In addition, a pre-enrichment culture of each urine sample, obtained by inoculating 1 ml of urine sample into 20 ml of Nutrient Broth (Oxoid, Basingstokes, UK) and incubating at 37 ^{o}C for 24 hours, was assayed on Nutrient agar (NA) (HiMedia, Mumbai, India) plates in order to improve the chances of microbial isolation (Hoefel *et al.,* 2003). The isolated cultivable bacterial cultures were grouped into different groups based on their morphological appearance on CLED and NA.

3.6 Morphological characterization of fresh and ageing urine isolates

The bacterial isolates of fresh urine samples and the ageing urine were characterized on the basis of their morphology. This was based on the isolates ability to grow on Cysteine-Lactose-Electrolyte Deficient (CLED) and Nutrient agar (NA). Characterization was achieved by observing colony size, colony shape, colour, elevation, margin, texture,

opacity and surface of the isolates. A loop-full of bacterial cells, picked from colonies from the NA media, were Gram stained and characterized morphologically under a light compound microscope (Olympus CK, Olympus Optical Co. Ltd., Tokyo, Japan), at a magnification of 40 × 100. Preliminary characterization was carried out on the basis of Bergey's Manual of Determinative Bacteriology 9th edition (Holt *et al*., 1994). For purification, morphologically different colonies were sub-cultured on NA medium. Purification on NA was carried out using the streak plate method. The pure isolates obtained from the streak plating were then preserved on 20% glycerol stock (v v-1) at −20°C for further analysis.

3.7 Phenols production assays of mammalian ageing urine

Phenols production assays were carried out at the Kenyatta University Chemistry laboratory and the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya. In these assays, pooled urine samples for each mammalian species were analyzed for phenols at four days ageing interval for 24 days.

3.7.1 Extraction and clean up phase

Liquid-liquid extraction (LLE) method was used in the extraction of phenols. The hydrophobic organic phase of pooled urine samples, from each fermenting stage, for each animal were extracted using analytical grade dichloromethane (DCM) (Loba Chemie, Mumbai, India), three times (30 ml, 30 ml and 30 ml), at intervals of 4 days, for 24 days. During the extraction, 50 ml of the urine sample was put in a separating funnel, mounted on a stand, with the stopcock at the bottom closed. The 30 ml of DCM was then added to the urine sample, in the separating funnel, to form two layers. The contents in the

separating funnel were then shaken thoroughly, four times, with thumb held firmly on the stopper. During the shaking, the stopcock was opened periodically to vent the vapour pressure build up. The separating funnel was then mounted back on the stand. The stopper was taken off, stopcock opened, and the lower organic layer was drained into a beaker. These steps were repeated with the two more 30 ml DCM portions. The bottom organic layers for each ageing interval, of each study animal, were pooled together and put in the conical flasks. The top hydrophilic aqueous layers were also kept separate until extraction was complete and product isolated.

Further purification of the organic layers was realized using anhydrous Sodium Sulphate, a drying agent (Leonard *et al.,* 2013). Briefly, the agent was added to the pooled organic layers in the conical flask using a spatula and swirled gently. The sulphate was added until it was seen to form floating solids from initial clumps like appearance. The mixture was left to stand for about 5 minutes to ensure complete absorption of water. Complete absorption of water was determined to have been achieved after the extracted contents turned into a clear liquid, from a cloudy like a solution.

The dried pooled organic layer was then decanted and transferred in a beaker, after which it was subjected to a rotary pump at 40^{o}C to concentrate the sample. All the DCM was evaporated until organic compounds, in forms of wax-like substances, were seen formed at the walls of the rotary flask. The recovery amount was then recorded. After that, 2 ml of DCM was added into the rotary flask and swirled round to ensure collection of the wax-like substance from the walls. The substances for each sample were passed through

0.22 µm Millipore filter and the contents transferred to bijou bottles, wrapped in aluminium foils, and kept at -20^{o}C for subsequent analysis. This method was adopted, with modifications, from Das *et al*. (2015) and Silva *et al.* (2013). Plate 3.3 shows a diagrammatic illustration of some of the extraction and clean up steps.

Plate 3.3: Urine extraction and clean up steps showing; A- LLE by use of DCM, B- Concentrating the sample at 40^{o}C on a rotary pump and C-Dispensing the final extract product in a bijou bottle.

3.7.2 Gas Chromatography-Mass Spectrometry (GC-MS) analysis phase

In this phase, separation, identification and quantification of phenols were carried out at the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi Kenya. Micro-extraction technique, achieved by the use of liquid-liquid micro-extraction (LLME), was used for the characterization of complex profiles of volatile compounds found in the DCM urine extracts. Before the GC-MS analysis, derivatization was carried out. For the GC-MS chemical analysis, a stock solution was prepared. In this preparation, 1 mg of sample was separately weighed and dissolved in 1 ml DCM to make a stock of solution, 1 mg/ml, from which an experimental sample with a final concentration of 100 ng/µl was prepared. As well, 1 ml of internal standard, a control solution, was aliquoted and analysed.

The samples were analyzed by GC-MS on an ISQ 7000 (Thermo Fisher Scientific, Massachusetts USA), using the following operating conditions; (i) Inlet temperature 270°C, transfer line temperature of 280°C, and column oven temperature programmed from 35 to 280°C with the initial temperature maintained for 5 minutes then 10°C/minutes to 280°C for 10.5 minutes and the final one for 29.9 minutes 50°C/minutes to 285°C (ii) A HP-5 MS low bleed capillary column fitted GC (30 m × 0.25 mm internal diameter 0.25 µm) (Restek, Bellefonte, PA, USA) (iii) Carrier gas Helium at a flow rate of 1.25 ml/minute. A quadruple temperature of 180^{o}C and an ion source temperature of 250^{o}C maintained by the ISQ 7000 mass selective detector. MS ion source temperature was set at 230°C. Electron impact mass spectra were achieved at acceleration energy of 70 eV. (iv) A 1.0 µl aliquot of the extract was automatically injected in the split/ splitless

mode via an auto sampler. Fragment ions were analyzed over 40-550 m/z mass range in the full scan. The filament delay of 5 min was used. (v) Raw data (retention time, peak area, analyte name, % quality) files were analysed and exported to the Access database, used for storage and retrieval, using GC-MS software (Thermo Scientific's Mass Frontier, USA). As well, a wide range of deconvolution and analysis programmes were used, bundled with MS libraries which included Mass Spectral Deconvolution System (AMDIS) with National Institute of Standard Technology (NIST) MS library (version 2.0, 2011) and Tag finder.

3.8 Screening bacterial isolates for their ability to mediate production of phenolic compounds in urine samples of the selected mammal

To screen the urine-derived bacterial isolates for mediation of production of attractant phenolic cues, collected fresh urine under refrigeration was obtained and sterilized using 0.22 µm Millipore filters. Aliquots portions of 50 ml sterilized urine were aseptically transferred into sterile 500 ml Erlenmeyer flasks. A consortium of previously isolated morphologically different bacterial communities of ageing urine was inoculated in sterilized urine portion and incubation carried out for three days at ambient temperatures. Each of the isolated morphologically different bacteria of ageing urine was also dared on the sterilized urine separately. The involved bacteria were tested by inoculating aliquot portions of pooled fresh sterile urine with 10 µL of 24-hr-old cultures of the isolated bacterial cell suspension (1×10^4 CFU/ml in 0.9 % of NaCl solution) to obtain 1×10^{11} CFU/ml as the final concentration (Trocazz *et al.,* 2012). These treatments were then incubated at room temperature, under aerobic conditions.

The isolates purity in each flask was established by inoculating a portion onto NA, on each extraction day, and examining these following a period of growth. In this analysis, blanks were used for control studies. The blanks were obtained through aseptic incubation of replica sterile urine without the inoculums for three days at room temperature. For phenols assays, the 50 ml of each sample was obtained from test flasks after incubation and extracted with dichloromethane. The extracts were examined by GC-MS, and the peaks corresponding to the major phenolic components of the selected animals' urine were checked for in the chromatograms. Phenols responding to each isolate were selected on the basis of their retention time, concentration and patterns of their chromatogram peaks. This method was adapted, with modification, from Okech and Hassanali (1990).

3.9 Biochemical characterization of urine based bacterial isolates

The pure isolates for fresh and ageing urine initially preserved on 20% glycerol stock (v v-1) at −20°C were obtained and subjected to biochemical tests.

3.9.1 Triple Sugar Iron agar test (TSI)

A sterilized straight wire loop was used to touch the top of 24 hours colony. The TSI was then inoculated by first stabbing through the center of the medium to the bottom of the tube. This was followed by streaking the surface of the agar slant. The tubes were closed loosely using cotton wool and earobic incubation was carried out at 37^{o}C for 24 hours and thereafter, results were interpreted: Acid butt/alkaline slant (yellow butt/red slant) meant only glucose had been fermented and not lactose or sucrose. Acid butt/acid slant (yellow butt/yellow slant) indicated fermentation of glucose, sucrose and or lactose

fermenter. Alkaline butt/alkaline slant (red butt/red slant) implies that neither glucose nor sucrose or lactose had been fermented. Formation of CO_2 or H_2 was scored by the presence of cracks or bubbles in the agar or by separation of agar from the sides or bottom of the tubes. Formation of H_2S was indicated by blackening of the butt of the medium in the tube. This method was adapted from Kolmos and Schmidt, (1987).

3.9.2 Citrate Utilization

A loop-full of bacteria was streaked over the slant of Simmon's citrate agar as explained by Jesumirhewe *et al.* (2016). It was then incubated aerobically at 37^{o}C for 24 to 48 hours with a loose cap. A positive reaction was determined by the growth of the isolate on the slant and medium colour change to blue. The absence of growth on the slant and persisting green medium indicated negative results.

3.9.3 Methyl Red - Voges-Proskauer Test

A loop-full of bacteria taken from a 24 hours culture was inoculated in MRVP broth as per the method described by McDevitt (2009). The contents were then incubated aerobically at 37^{o}C for a period between 24 hours to 72 hours. After incubation, the results were analyzed from the broth. A clear broth indicated that the organism did not grow and cannot be tested. To test for methyl Red, 1 ml of broth was aliquoted to a sterile test tube. 5 drops of methyl red were added to the broth. A positive result was determined by formation of a red layer at the top of the broth. Negative result was determined by the formation of yellow layer. To test for Voges Proskauer, 1ml of the original broth was aliquoted to a sterile test tube. Five drops of the VP A (Napthol) reagent were then added

to the mixture followed by 5 drops of the VPB reagent (Potassium Hydroxide). The tube was shaken gently to expose the medium to atmospheric oxygen. The tube was allowed to stand for 15 minutes undisturbed and thereafter results observed. A positive result was determined by pinkish red colour at the surface. Negative results, on the other hand, were determined by the yellow colour at medium surface (Hemraj *et al.*, 2013).

3.9.4 Motility test

A sterilized wire loop was used to touch 24 hours old colony. The bacterium was then inoculated in motility medium by stabbing in the middle part of the medium. Positive motility was determined by growth of the inoculated bacteria away from the stab line. Negative for motility was determined by the growth of the bacteria along the stab line (Hemraj *et al.*, 2013).

3.9.5 Indole test

A loop-full of bacteria was inoculated into the tryptone broth and incubated aerobically at 37^{o}C for 24 hours. A few drops of Kovacs reagent were added down the inner wall of the tube with broth culture. A positive result was determined on development of a bright red colour at the interface of the reagent and broth within seconds after adding the reagent. A negative result was scored on formation of a yellow or brown layer (Jesumirhewe *et al.*, 2016).

3.9.6 Sorbital test

An inoculum from pure cultures was transferred asceptically to a sterile tube of phenol red sorbital broth. The inoculated tube was then incubated at 37ºC for 24 hours and thereafter results determined. Positive score was determined by colour change from red to yellow. Negative results were determined by colour change to pink. This method was proposed by Benard *et al.* (1990)

3.9.7 Catalase test

A tube method was used. Nutrient slants were inoculated with 24 hours bacterial culture and were incubated at 37ºC for 24 hours. After incubation, the tubes were flooded with 1 ml of 3% hydrogen peroxide. The tubes were then observed for gas bubbles. The occurrence of gas bubbles was scored positive for catalase (Reiner, 2010).

3.9.8 Nitrate reduction

The isolates were inoculated in the Nitrate broth as reviewed by Hemraj *et al. (*2013). After incubation, a dropper-full of sulfanilic acid and a-naphthylamine was then added in the broth. The medium was then observed for colour change. Red scored positive for nitrate reduction. Failure of medium to turn red, small amount of zinc dust was added. The tubes were as well observed for colour change. Red colour, after the zinc is added indicated negative nitrate reduction. Failure of the medium to turn red was scored as positive result.

3.9.9 Oxidase Test

A small amount of 24 hour old bacterial isolates were transferred from the overnight grown plate on to a moist filter paper using a sterilised wooden rod. A drop of the oxidase reagent, 10g/l solution of tetramethyl-p-phenylenediamine dihydrochloride, was placed on the culture on the moist filter paper. The results were then observed within 10 seconds. Positive score was indicated with development of dark purple colour. Negative score was recorded with no colour change (Shields and Cathcart, 2010).

3.9.10 Urease test

Urea broth in bijou bottle was inoculated with 24 hours old bacteria culture using a sterilized wire loop. The contents were incubated aerobically at 37^{o}C for 72 hours and results recorded after every 24-hour. A positive was scored on formation of bright pink colour while negative was scored on absence of colour change (Hemraj *et al.*, 2013).

3.10 Molecular characterization

Bacterial isolates tested for potential of mediating production of tsetse attractive phenols underwent further characterization, using molecular methods, based on 16S rRNA gene sequencing. Molecular characterization involved four steps. These included the extraction of genomic DNA (gDNA) from the bacterial isolates, polymerase chain reaction (PCR), Gel electrophoresis, visualization of gDNA and PCR products and lastly 16S rRNA gene sequencing.

3.10.1 DNA extraction

Genomic DNA (gDNA) was directly extracted from bacterial colonies obtained from a pure culture on agar plate as outlined by Ferdous *et al*. (2012). The 24 hours loop full of bacterial colonies were aseptically transferred in 2 ml eppendorf tubes filled with normal saline. The mixtures were vortexed and centrifuged at 13000 revolutions per minute (rpm) for 5 minutes. Washing was carried out several times in case of highly mucous bacteria. CTAB lysis buffer was added to the contents in the tubes and centrifuged at 10,000 rpm. This was then followed by draining of the supernatant and the pellet re-suspended in 450 µl Tris-EDTA buffer. The samples were then incubated in water bath at 80^{o}C for about one and half hours after which the contents were left to cool. A 500 µl solution containing Chloroform and Isoamyl alcohol, in the ratio of 24:1, was added and mixed well. The sample mixture was afterwards subjected to freezing conditions for 20 minutes. This was followed by centrifugation at 13,000 rpm for 5 minutes. At this particular point, three layers formed with the upper layer containing DNA, middle layer lipids and then the lower layer.

The upper layer was pipetted and transferred to new tube. The presence of DNA was indicated by bubble in the eppendorf tube. The upper pipette layer was then washed with 1 mL, 70% chilled ethanol and rota-evarporated for 2 minutes. The ethanol was decanted gently from the tubes and left to dry for 3 hours. After drying, the pellet was then dissolved in 40 µL DNA elution buffer and the contents incubated in water bath at 45^{o}C for 30 minutes. The tubes were drained carefully and allowed to air dry. The gDNA concentration and purity was then determined by running aliquots on 0.8% agarose gel

with 1X TAE buffer. The DNA was suspended in 50 µL TE buffer and stored at 4°C for further use.

3.10.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was perfomed to amplify the 16S rRNA gene fragments from the pure isolates (Haas *et al*., 2011). PCR amplification of the small sub unit (SSU), 16S rRNA gene was carried out using 27-Forward (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492-Reverse (5'- CGGTTACCTTGTTACGACTT-3') primers. These primers span hyper variable regions 1 to 3 (V1-V3), 3 to 6 (V3-V6) as well as (V1-V9). Due to the nearly infinite number of different bacteria, it was desirable for the study to cover more than one hyper-variable region. Covering more than one hypervariable region increases sensitivity, specificity and reliability of the 16S rRNA gene studies since taxonomic classification of all regions is compared. The reaction was done in a 50 µL volume, containing 10 × PCR buffer (100 mM Tris [pH 8.3], 500 mM KCl, 4 mM MgCl2, 0.1% gelatin) (5 µL), 2 µL each of primers, 2 mM dNTP mixture (2.5 µL), Taq DNA polymerase (1 µL), PCR water (Dho), up to 50 µL, and template DNA (1 µL). The PCR reaction was carried out in Techgene thermocycler FTGENE5D model (Techne-UK). The PCR amplification was performed at the following conditions: pre-denaturation at 94^{o}C for 5 minutes, 36 cycles of denaturation at 94^{o}C for 1 minute, annealing at 54^{o}C for 1 minute and extension at 72^{o}C for 2 minutes, and finally extension at 72^{o}C for 10 minutes. After the PCR reaction, PCR products, 5 µL each, were inspected for quality by gel electrophoresis. The remaining portion of PCR products were then stored at -20^{o}C for further use.

3.10.3 Gel electrophoresis and visualization of Genomic DNA and PCR products

Genomic DNA samples extracted successfully and the PCR products were fractionated and observed using agar rose gel electrophoresis. A 1X TBE buffer was made by adding 10 mL of 5X TBE buffer into 90 mL of distilled water. A 0.8% agar rose gel for gDNA and 1.2% for PCR products TBE gel was made to which 1µL of ethidium bromide was added. The solution was mixed by swirling and poured into the acrylic gel tray with the two ends tapped up to make a reservoir. A 14-well comb was set in place and the gel was left to set for 30 minutes. When the gel was solidified, the comb and tapes were removed and the gel tray placed in the buffer tank. The gel was submerged to a depth of about 5 mm by pouring 0.5X TBE into the tank.

On a strip of parafilm, 2 µL of loading dye Bromophenol blue (BPB) was mixed with 7 µL in the case of gDNA sample and 3 µL in the case of the PCR amplified 16S rRNA gene products. The mixtures were then, at separate event, carefully loaded into the well of the gel. The 100 bp DNA ladder (size range; 100 bp-2,000 pb) for PCR products, and 1Kb DNA ladder (size range; 250 pb-10,000 pb), for gDNA (ThermoFischer Scientific, UK) were loaded along with samples on their respective gels as molecular weight markers. Gel electrophoresis was done at 80 volts potential difference for about 30 minutes for gDNA sample and 60 minutes for PCR amplified 16S rRNA gene products. When the run was complete; the gel trays were removed from the buffer tanks. The products were then visualized under UV trans-illuminator light. The gels were then photographed using a digital photograph as advised by Kumar *et al*. (2015).

3.10.4 16S rRNA gene sequencing

Before sequencing, purification of the PCR products was carried out by use of Exo-SAP mix (Exonuclease-Shrimp Alkaline Phosphatase) reagent. A 7 µL of PCR product from each of three was added in 3 µL Exo-SAP mixture in the tube. This was transferred into a thermal cycler and purified using two-cycle steps. Step one was carried out at 37^{o}C for 15 minutes, while the second step was carried out at 80^{o}C for 15 minutes. After purification, the PCR products were sequenced. Sequencing reactions were realised using a BigDye Terminator cycle sequencing kit (Applied Biosystems, USA). The primers used for sequencing were 27F and 1492R for 16S rRNA gene. The sequences were generated by Sanger (Capillary) sequencing, using the AB1 3730 DNA Sequencer (Applied Biosystems, USA).

3.11 Data analysis

3.11.1 Analysis of isolates ability to mediate phenols production

For statistical analysis of chemical data, specific phenolic compounds in urine samples were identified based on retention time as well as the absorbance spectra on GC-MS. The concentration of each phenolic compound was determined based on the peak area. To analyze the potential of production of phenols, mean comparison of total phenolic compounds (TPC) abundance in different mammalian urine, at the different ageing interval, were subjected to one way Analysis of Variance (ANOVA). To evaluate the relationship between phenols, mammalian animal and ageing interval, two way factorial ANOVA was used to generate the mean concentrations of TPC. These were followed with significant difference determination among means using Tukey's Honesty Significant Difference (HSD) test. The statistical analysis was carried out using Statistical

Analysis System (SAS), version 9.1 (SAS Institute, Cary, NC). Differences were considered significant at $P \leq 0.05$.

Data for bacterial morphological diversity was subjected to Paleontological Statistics Software Package (PAST) and isolates similarity analysed using the Jaccard index (Cole *et al.,* 2013). Bacterial diversity across ageing intervals was then determined using the Shannon and Simson 1-D diversity indices (Keylock, 2005). Richness estimator, Chao 1, was performed to estimate the bacterial richness of urine samples across ageing intervals (Kamika *et al.,* 2016). Data obtained from bacteria inoculated in fresh urine was as well subjected to analysis of variance (ANOVA) using SAS (2010). Means pair-wise comparison was carried out using Tukey's HSD (Honestly Significant Difference) at 5% level.

3.11.2 Phylogenetic analysis of sequence data

Sequence data (ab1) were assembled and edited using Finch Tv chromatogram viewer software (Mishra *et al*., 2010). Consensus (contiq) building and analysis for forward and reverse sequences was carried out using DNA Baser software (Zhang *et al*., 2012). The sequences obtained were compiled and compared with sequences in the GenBank (http://www.ncbi.nlm.nih.gov/) databases using Basic Local Arrangement Search Tool (BLAST) program (www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al*., 1997**)**. The 16S rRNA gene sequences of strains closely related (highest total score, identity and query cover) to the study isolates were retrieved from National Centre for Biotechnological Information (NCBI) sequence data repository. For describing the isolates phylogenetic

relationship, the 16S rRNA gene multiple sequences retrieved from NCBI and those of the isolates were aligned by using Clustal X version 2 software (Larkin *et al*., 2007). Comparison 16S rRNA gene sequences were used to show evolutionary relatedness among the bacterial communities. The evolutionary history of the isolates was then presented in the form of a phylogenetic tree. The 16S rRNA gene nucleotide sequence for *Methanocarsina barkeri* (Accession number: NR025303.1) obtained from NCBI Genbank was incorporated in the tree as an out group. *Methanocarsina barkeri* is an archea belonging to Methanogens and they inhabit ruminant gut as commensals. The tree was inferred by the maximum likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969), at 1000 iterations, using Molecular Evolutionary Genetics Analysis version 7 (MEGA 7) (Kumar *et al.*, 2016). The SSU 16S rRNA gene sequences of the study isolates were added into the NCBI GenBank database manually.

CHAPTER FOUR: RESULTS

4.1. Isolation and characterization of cultivable bacteria in mammalian fresh urine

A total of ten, morphologically different, bacteria were isolated from fresh urine. Four of the isolates (B1, B2, B3 and B4) were from cattle urine sample, three were revealed in buffalo urine sample (b1, b4 and b3) and the remaining three in eland (E1, E2 and E3) urine sample. When grouped based on colour change of CLED medium, B1, B2, B3, E1, E2, E3, b1 and b3 turned the medium blue whereas B4 and b4 turned the medium yellow. Based on the morphological traits of the ten isolates; colony shape were either circular or filamentous. Elevation was either raised or flat. The colonies' margins observed were either irregular or entire. Some colonies revealed rough surface while others had smooth and shiny surfaces. Colony sizes were varied from large, medium to small. The cell arrangement was either single, chains or pairs. Texture varied among the colonies from mucoid, dry to moist. When analysed for translucent and opacity on NA medium, most colonies were found to be opaque with only one colony being transparent. The isolates when sub-cultured on NA medium showed cream, white or orange colours (Table 4.1; Plate 4.1).

Table 4.1: Morphological characterisation of bacterial isolates from fresh urine samples of cattle, buffalo and eland

Isolate	**Isolates Laboratory Designation Number**									
characteristic	**B1**	**B2**	**B3**	**B4**	**E1**	**E2**	**E3**	**b1**	**b4**	**b3**
colony shape	flmnts	circular	circular	circular	circular	bacillus	flmts	circular	circular	flmts
Elevation	raised	flat	flat	raised	flat	flat	flat	raised	raised	flat
margin	irregular	entire	entire	entire	entire	irregular	irregular	entire	entire	irregular
Surface	rough	ss	ss	ss	rough	rough	rough	ss	ss	rough
colony size	large	small	large	small	medium	large	large	medium	medium	medium
cell size	large	small	large	large	small	small	small	small	small	large
cell arrangement	singly	singly	chains	singly	singly	singly	chains	pairs	singly	singly
texture	mucoid	dry	dry	wet	mucoid	mucoid	wet	moist	moist	mucoid
colour CLED	blue	blue	blue	yellow	blue	blue	blue	blue	yellow	blue
colour NA	cream	cream	cream	white	white	cream	white	cream	orange	cream
Opacity	opaque	opaque	opaque	tp	opaque	opaque	opaque	opaque	opaque	opaque
Urine source	Cattle	Cattle	Cattle	Cattle	Eland	Eland	Eland	Buffalo	Buffalo	Buffalo

Key: ss=smooth and shiny; tp=transparent; smg=swarming; flmnts=filamentous; B=Cattle, b=buffalo, E= eland Probable organism**:** B1**=** *Proteus* spp., B2= *Serratia* spp., B3= *Enterobacter* spp., B4= *Staphylococcus* spp., E1= *Enterobacter* spp., E2= *Klebsiella* spp., E3= *Proteus* spp., b1=*Streptococcus* spp., b4= *Enterococcus* spp., b3= *Enterobacter* spp.,

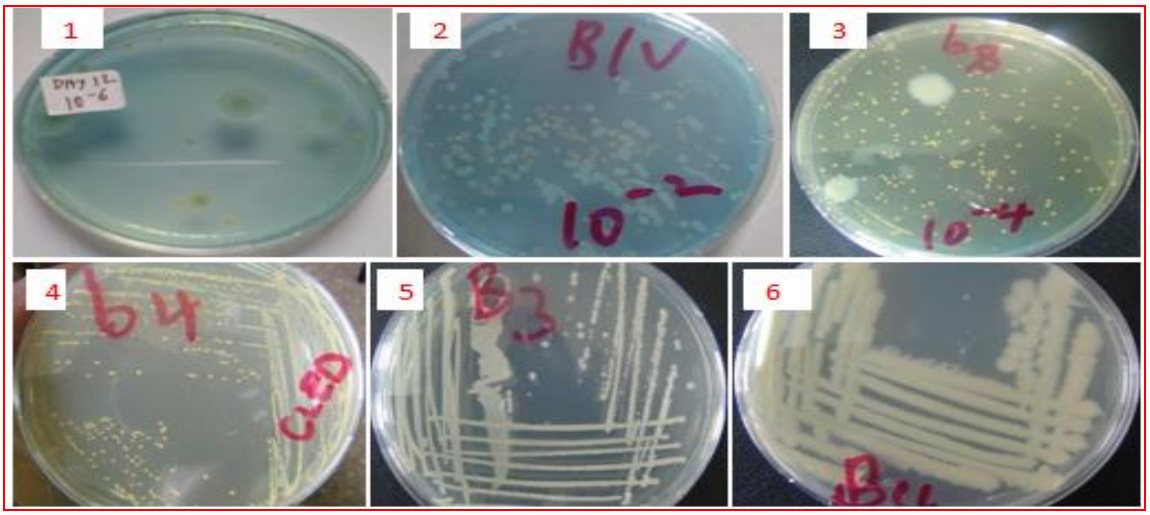

Plate 4.1: Some of the bacterial isolates from urine samples of the three study animals on culture plates. 1; Blue and yellow bacterial colonies of elands' day eight urine cultured on CLED medium 2; Blue and yellow bacterial colonies of cattles' day eight urine cultured on CLED medium. 3;Blue and yellow bacterial colonies of buffalos' day eight urine cultured on CLED medium 4; Bacterial isolate from buffalo urine sample streaked on CLED agar 5; Bacterial isolate from cattle urine sub-cultured on NA. 6; Bacterial isolate from buffalo urine sub-cultured on NA

In the Gram staining procedure, 30% (3) of the isolates were Gram positive cocci. The remaining 70% (7) were Gram negative and all were rods. Biochemical identification of these isolates revealed that the bacteria tentatively belonged to seven different genera (Table 4.2). Isolates B1 and E3 presented morphological and biochemical characteristic similar to *Proteus* spp., while isolates E1, B3 and b3 closely resembled *Enterobacter* spp. On the other hand, B2, E2, B4, b1 and b4 revealed characteristics similar to *Serratia* spp., *Klebsiella* spp., *Stapylococcus* spp., *Streptococcus* spp. and *Enteroccoccus* spp. respectively. *Enterobacter* spp. was common in urine samples from all animals. *Proteus* spp. occurred in urine sample belonging to eland and cattle only. The rest of the isolates only occurred in urine samples belonging to one animal's species.

Table 4.2: Biochemical profiling of bacterial isolates from cattle, buffalo and eland fresh urine

Test	**Isolates Laboratory Designation Number**									
	B1	B2	B3	B4	E1	E2	E3	b1	b4	b3
Gram stain	-	-	-	+	-	-	-	+	+	-
Cell Shape	R	R	R	C	R	R	R	C	C	R
Indole	-	+	-	+	-	-	-	-	+	-
Citrate	+	+	-	+	-	+	+	+	-	-
Urease	+	-	-	-	-	+	+	-	-	-
Motility	+	+	-	+	-	-	+	+	-	-
MR	+	-	+	+	+	-	+	-	-	+
VP	-	-	+	+	+	-	-	-	-	+
Oxidase	-	-	-	-	-	-	-	-	-	-
Glucose (TSI)	+	+	+	+	+	+	+	-	-	+
Lactose (TSI)	-	-	+	+	+	+	-	-	+	+
Sucrose (TSI)	-	+	+	+	+	+	-	-	-	+
H_2S (TSI)	+	-	-	-	-	-	+	-	-	-
Gas (TSI)	+	-	+	+	+	+	+	-	-	+
Catalase	+	+	-	+	-	+	+	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	S.+	-	S.+	-	S.+	-	-	-	-

Key: TSI=Triple Sugar Iron, VP=Voges Proskauer, MR=Methyl Red, += positive, - =negative, S. += slightly positive, R=rod, C=cocci, B=cattle, E= eland, b=buffalo. Probable organism**:** B1**=** *Proteus* spp., B2= *Serratia* spp., B3= *Enterobacter* spp., B4= *Staphylococcus* spp., E1= *Enterobacter* spp., E2= *Klebsiella* spp., E3= *Proteus* spp., b1=*Streptococcus* spp., b4= *Enterococcus* spp., b3= *Enterobacter* spp.,

4.2 Progressive transformation of bacterial populations in fresh and ageing urine of the study animals

Across ageing intervals, cattle urine registered the higest bacterial counts followed by buffalo and lastly eland (Figure 4.1). All the recorded bacterial count was significantly different across ageing intervals, at $P \leq 0.05$. Comparison of cattle urine across ageing intervals indicates that there is no statistical difference in bacterial load of fresh urine (7.07×10^5 CFU/ml), day four old urine (1.27×10^6 CFU/ml), day eight old urine (9.27×10^6 CFU/ml) and day twenty four old urine (2.08×10^6 CFU/ml) (P=0.0001). Bacterial count for days sixteen (8.20×10^7CFU/ml) and twenty (9.40×10^7 CFU/ml) were not significantly different in cattle urine at P=0.0001. At day twelve of urine ageing intervals, bacterial load was 5.57×10^7 CFU/ml. The highest and lowest values for bacteria counts were observed at days twenty and four in that order (Figure 4.1).

Comparison of buffalo urine across ageing intervals revealed the highest (8.60×10^7CFU/ml) bacteria count at day twenty old urine. The comparison showed this value to be significantly different from the rest of the ageing intervals at P=0.0001. The lowest value (4.50×10^5CFU/ml) was revealed in fresh urine. However, this value was not significantly different with that recorded at days four (5.23×10^5 CFU/ml), eight (1.21×10^7CFU/ml), twelve (2.67×10^7CFU/ml) and twenty four (2.08×10^7CFU/ml) ($P \leq 0.0001$). At day sixteen, bacteria count of buffalo urine was 6.00×10^7 CFU/ml (Figure 4.1).

Comparison of eland urine sample across ageing intervals recorded highest (2.32×10^7 CFU/ml) bacterial load at day twenty. However, this value was not significantly different from that of day sixteen (2.23×10^7 CFU/ml) at P=0.0001. The bacteria count at day; twenty four (6.01×10^6 CFU/ml) and day eight (1.66×10^6 CFU/ml) showed no statistical difference, at P=0.0001. As well, fresh urine (4.36×10^5 CFU/ml) and day four old urine bacterial load (5.55×10^5 CFU/ml) had no significance difference (P=0.0001). There was a gradual increase in bacterial load from fresh urine up to day twenty and thereafter a decline in day twenty four across all animals. The highest bacterial concentrations (cattle=9.40×10^7 CFU/ml; buffalo=8.60×10^7 CFU/ml; eland=2.32×10^7 CFU/ml) were recorded at day twenty in all the animals. Continued ageing of cow, buffalo and eland urine was identical, with increased bacterial colonies up to day twenty (Figure 4.1).

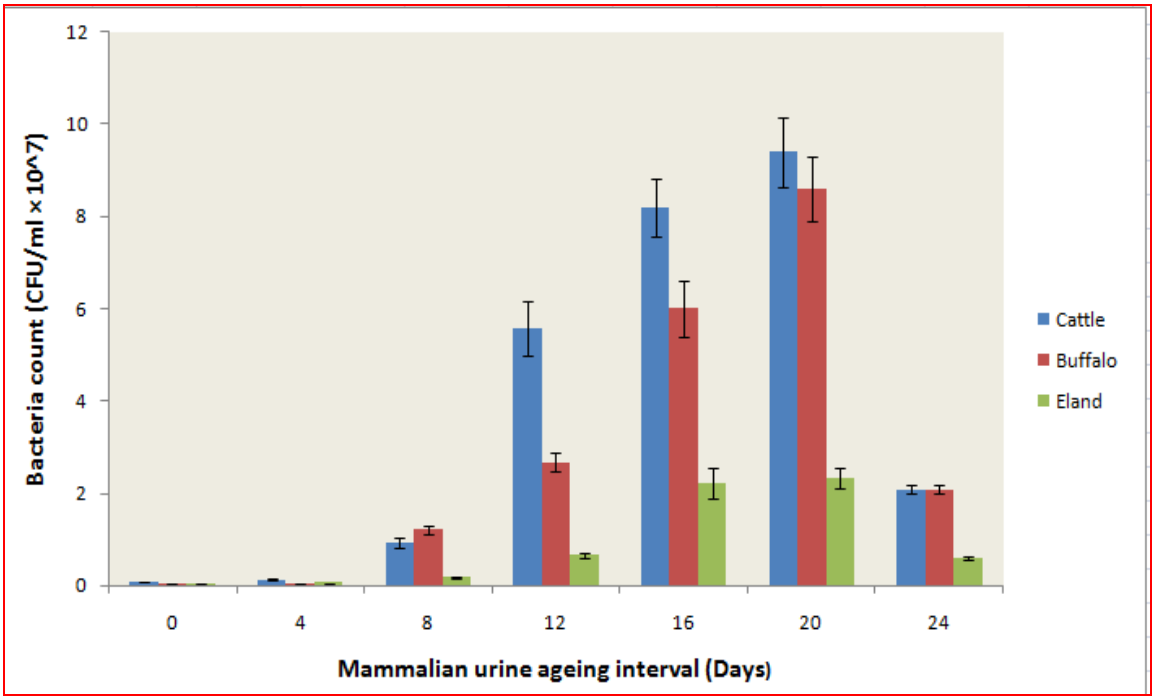

Figure 4.1: Progressive transformation of bacterial populations in cow, buffalo and eland fresh and ageing urine samples

4.3 Progressive transformation of morphologically diverse bacterial isolates in ageing mammalian urine

A total of 151 isolates colonising ageing mammalian urine were isolated. Of the isolates, 41% originated from cattle urine samples, 32% were from buffalo urine samples and 27% were from eland urine samples. The 151 isolates were grouped into 19 groups, based on their similarity in morphology (Table 4.3).

Bacterial communities' dissimilarities increased with fermenting period, up to day twenty, when they started depreciating. Bacteria belonging to group iii, iv, v, vii, xi, xii, ix, xvii, xviii and xix were revealed to dominate urine habitat towards the end of fermentation period. Members belonging to viii, x, xiii, xv and xvi groups increased with ageing interval up to day twenty. Members belonging to group i were at least present throughout the fermenting period. Group ii bacterial isolates were observed only at days 4, 8 and 12 and later on they disappeared with increased ageing days. Members of group xiv and vi, unlike other groups, showed fluctuation in diversity during the fermenting period.

Table 4.3: Morphological grouping of bacteria isolated in ageing urine samples of cattle, buffalo and eland

Isolate characteristic	**Ageing urine isolate groups**																		
	i	**ii**	**iii**	**iv**	**v**	**vi**	**vii**	**viii**	**ix**	**x**	**xi**	**xii**	**xiii**	**xiv**	**xv**	**vvi**	**xvii**	**xviii**	**xix**
colony shape	c	fm	c	fm	fm	c	c	c	c	c	c	fm	c	c	fm	c	c	fm	c
Elevation	r	r	f	r	f	r	f	r	r	f	f	f	f	r	r	f	f	r	f
margin	e	i	i	e	i	e	i	e	e	e	i	i	e	e	e	e	e	i	i
Surface	ss	ss	sth	rg	sth	ss	ss	ss	sth	sth	sth	rg	ss	rg	ss	sth	sth	rg	rg
colony size	m	m	l	sm	l	m	l	pp	m	m	sm	sm	pp	m	sm	pp	sm	l	m
texture	mo	mo	d	d	mu	mu	mu	mo	wt	wt	mu	wt	mo	d	mo	mo	mu	mu	mo
cled colour	y	y	b	b	b	b	b	y	b	b	b	b	b	b	y	y	b	y	y
na colour	o	w	cw	cm	w	w	cm	w	w	w	cm	cm	w	cm	w	cm	cw	cm	cm
Opacity	op	op	op	op	op	tp	op	tp	op	tp	op	op	tp	op	tp	tp	op	op	op
RI	b2	b5	b7	b10	b12	b22	E8	E11	E48	E49	E51	E53	B25	B34	B42	B70	B71	B73	B74

Key: RI=representative isolate; c=circular; fm=filamentous; r=raised; e=entire; rg=rough; ss=smooth and shiny; sth=smooth; m=medium; wt=wet; mo=moist; mu=mucoid; y=yellow; b=blue; cw=creamish white; o=orange; cm=cream; w=white; tp=transparent; op=opaque; i=irregular; d=dry; f=flat; l=large; sm=small; pp=pin-point.

Total number of groups identified at each fermenting intervals of urine samples was also documented (Figure 4.2). Compared across the samples from the three animals, cattle urine had the highest number of bacterial groups across the fermentation period. Buffalo and eland revealed equal number of morphologically different bacterial groups in ageing urine at days four, eight and twenty, each with 3, 5 and 8 isolates, respectively. In the same ageing intervals, cattle urine had 8 isolates for day four and 10 for both days eight and twenty. At day sixteen, isolates from cattle and buffalo urine were equal, 14 each. In all the three animals, there was positive shift in bacterial density with ageing interval up to day sixteen, when the density decreased gradually. Some of the bacteria isolated early in urine were seen to disappear with ageing period. As well, when compared, there was morphologically diverse bacterial isolates in ageing than fresh urine of the study mammals.

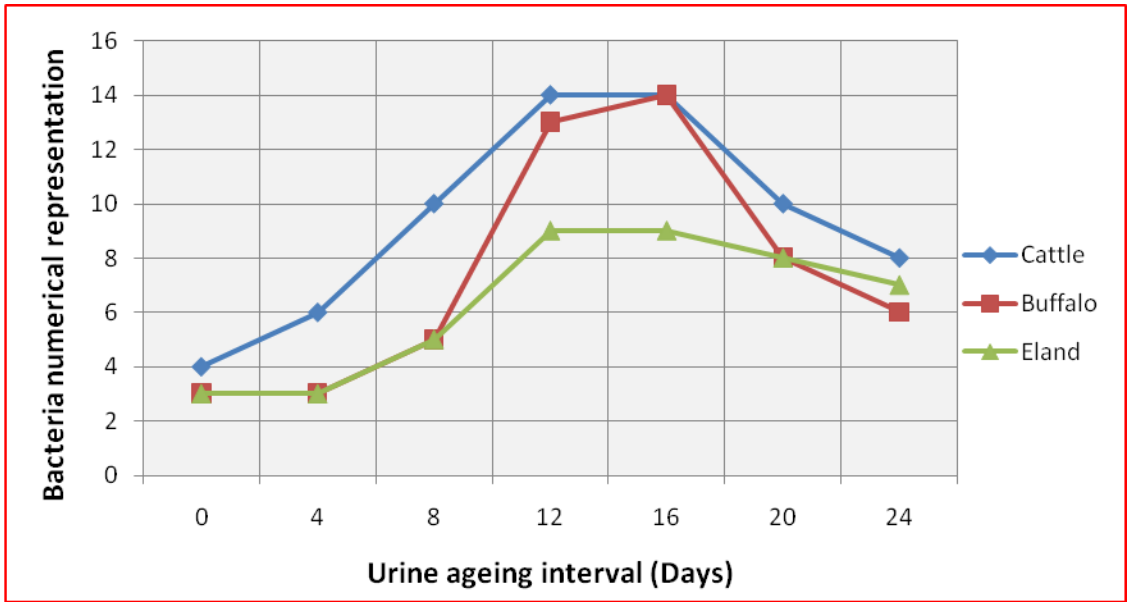

Figure 4.2: Numerical representation of morphologically different bacterial isolates in fresh and ageing urine samples of cattle, buffalo and eland

The nineteen morphological grouping, as shown in table 4.3 were coded and run through Paleontological Statistics Software Package (PAST). The cluster analysis revealed 8 phenotypic clusters for bacterial isolates of cattle, buffalo and eland fermenting urine. The clusters were separated at distance/similarity index of 4.0. These clusters were arrived at based on the phenotypic traits of the isolates and clustering was not dependent on the source of the urine sample from which the bacteria were isolated. Figure 4.3 shows the clusters.

Clusters G, K and N are each composed of three representatives of the isolate groups. The isolates E11, B70 and E49 belong to cluster G separated at similarity index 3.9. Isolates E48, B71 and E51 belong to phenotypic cluster K separated at similarity index 2.5, while isolates b5, B42 and B25 shared phenotypic cluster N separated at similarity index of 3.3. The other five phenotypic clusters were shared with two isolates at different separation points: Isolates B74 and b2 shared phenotypic cluster L at similarity index 3.6, b22 and B34 shared phenotypic cluster M at similarity index 3.4, b12 and E8 shared phenotypic cluster J at similarity index 2.2 while E53 shared phenotypic cluster I, with B73 at similarity index 2.0 (Figure 4.3)

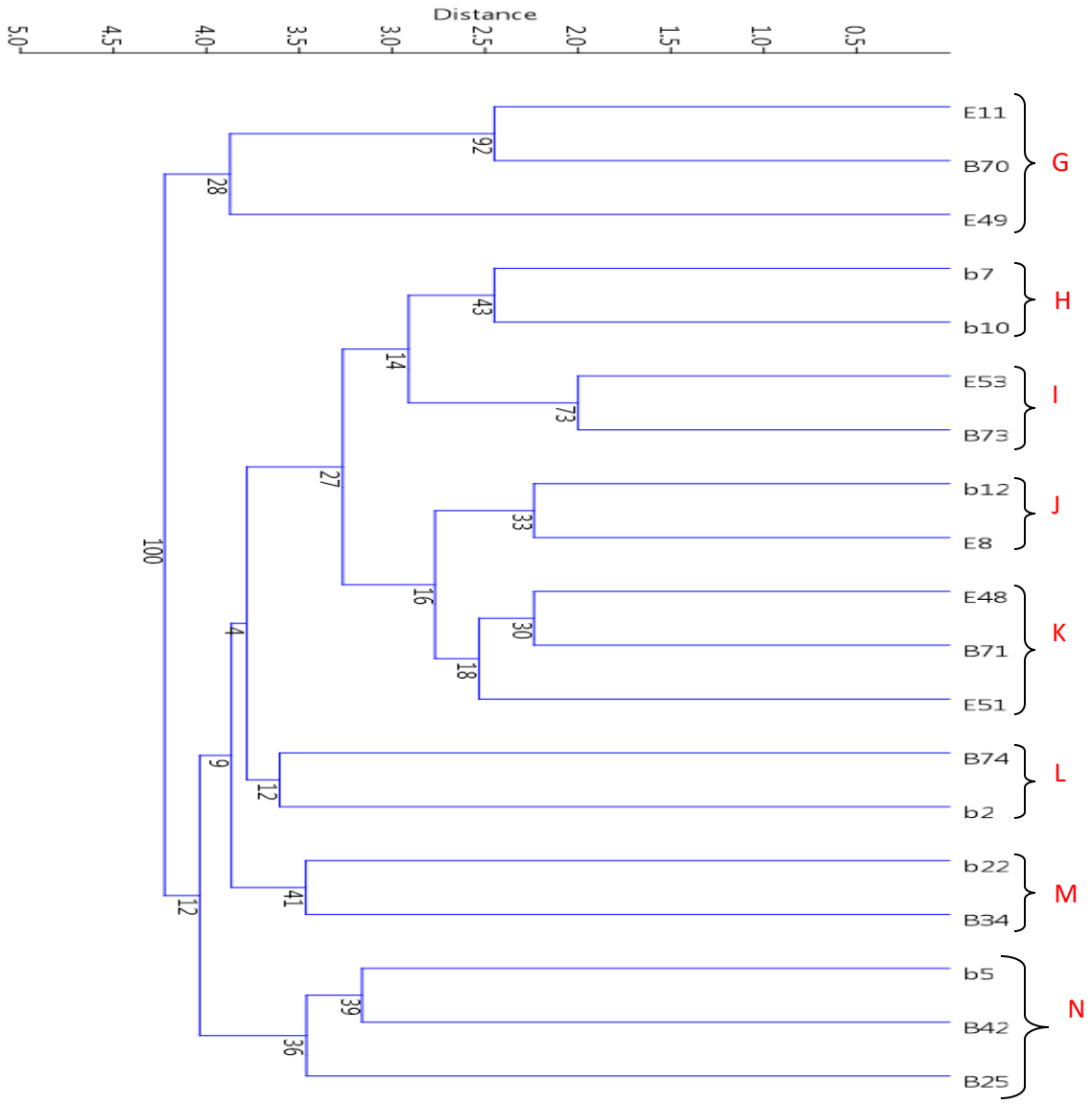

Figure 4.3: Distance dendogram showing morphological relationship of bacterial isolate in fermenting urine of cattle, buffalo and eland. The distance dendogram was drawn based on Neighbour-joining method and Bray-Curtis similarity index. Numbers shown at the nodes of the dendogram indicate the percentage Bootstrap support for 1000 iterations. The scale bar at the top represents the number of substitutions per isolates.

According to Shannon Weiner (*H*) diversity index, day twelve and day sixteen had the highest diversity of bacterial isolates, both at 2.639 while day zero (fresh urine) had the lowest diversity of bacterial isolates, at 1.099. Similar to *H*, Simpson 1-D index revealed

highest bacterial diversity at day twelve and day sixteen, both at 0.9286 while lowest bacterial diversity at day zero (fresh urine) showed an index of 0.6667. The lowest bacterial dominance index was observed at days twelve and sixteen, at 0.07143 while the highest bacterial dominance index was revealed at day zero (fresh), at 0.3333. Bacterial evenness diversity was equal across ageing intervals (Table 4.1).

Table 4.4: Morphological diversity profile of the nineteen bacterial isolates from the pooled ageing urine samples of the study animals

Diversity indices	**Ageing interval (Days)**						
	0	**4**	**8**	**12**	**16**	**20**	**24**
Dominance D	0.3333	0.1667	0.1	0.07143	0.07143	0.09091	0.125
Simpson 1-D	0.6667	0.8333	0.9	0.9286	0.9286	0.9091	0.875
Shannon H	1.099	1.792	2.303	2.639	2.639	2.398	2.079
Evenness_e^H/S	1	1	1	1	1	1	1
Brillouin	0.5973	1.097	1.51	1.799	1.799	1.591	1.326
Menhinick	1.732	2.449	3.162	3.742	3.742	3.317	2.828
Margalef	1.82	2.791	3.909	4.926	4.926	4.17	3.366
Equitability-J	1	1	1	1	1	1	1
Fisher-alpha	0	0	0	0	0	0	0
Berger-Parker	0.3333	0.1667	0.1	0.07143	0.07143	0.09091	0.125
Chao-1	6	21	55	105	105	66	36

4.4 Generation of phenols in different mammalian urine at different stages of fermentation

4.4.1 Identification of phenolic compounds in DCM extracted urine

Gas Chromatography analysis of DCM urine extracts showed several peaks in the chromatogram. The peaks indicated presence of phenolic and non phenolic compounds. Through the study, a total of nine volatile phenolic compounds were identified in the

urine headspace. Upon comparison of the mass spectra of the nine volatiles with NIST library data, the specific compound names were established with a similarity threshold of >80%. The compounds were identified as: P-cresol, o-cresol, m-cresol, phenol, 3-ethylphenol, 3-n-propylphenol, 2-methyloxyphenol, 4-ethylphenol and 4-propylphenol (Fugure 4.4 and Figure 4.5).

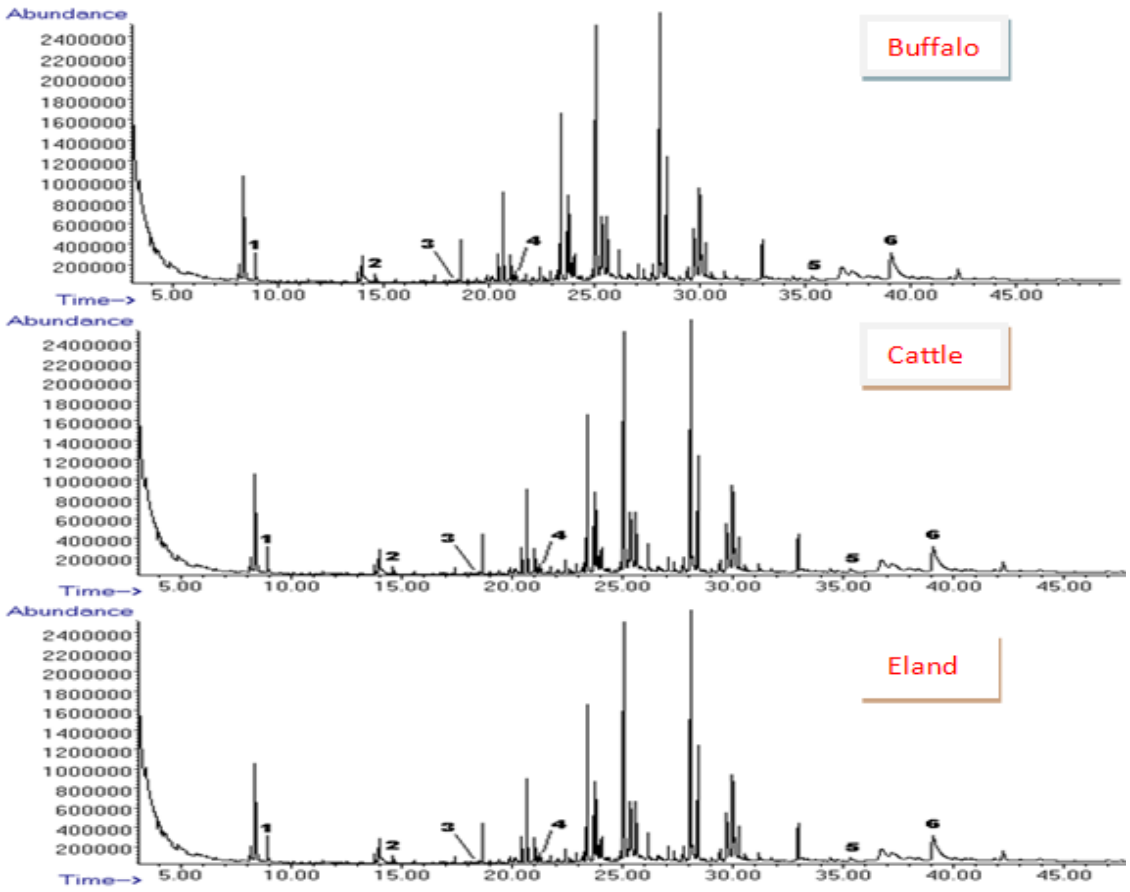

Figure 4.4: Representative GC-MS chromatogram for phenolic extracts of buffalo, cattle and eland urine samples at day four. Peaks identities: (**1**) P-cresol (**2**) Phenol (**3**) O-cresol (**4**) 3-methylphenol (**5**) 4-ethylphenol (**6**) 3-ethylphenol

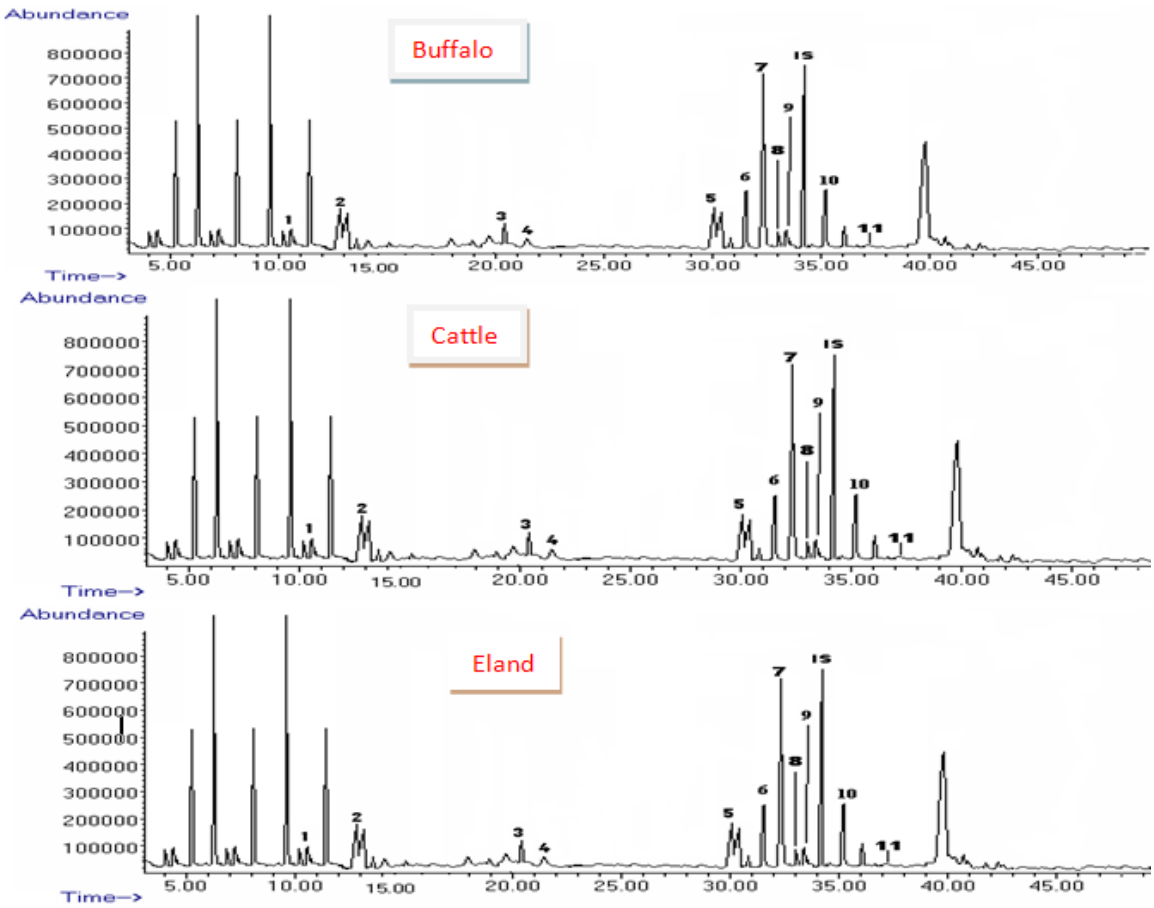

Figure 4.5: Representative GC-MS chromatogram for phenolic extracts of Buffalo, cattle and eland urine samples at day twenty. Peaks identities: (**1**) P-cresol (**2**) Phenol (**3**) O-cresol (**4**) m-cresol **(5**) 4-ethylphenol (**6**) 3-ethylphenol (**7)** 3-propylphenol (**8**) 4-propylphenol (**9**) 2-methyloxyphenol (**10**) 3-n-Propylpyridine (**11**) Decanal, **IS**-internal standard

Most notably, there was important qualitative difference among ageing intervals for the identified phenolic compounds. The ion spectra of Phenol, the parent compound, P-cresol, o-cresol and m-cresol were found to be present in all of the investigated samples (Peaks, 2, 1, 3 and 4 in that order). The 2-methyloxyphenol, 3-propylphenol, and 4-propylphenol, at peaks 9, 7 and 8, were found in the samples between days eight and twenty only. The three phenols had varied retention time. Two phenolic compounds, 3-ethylphenol and 4-ethylphenol, were found in all samples except in the day twenty four

samples. The two had different retention time. For each animal urine sample, there was qualitative difference in identified phenols across the fermentation period. At day four, 6 phenolic compounds were detected. At days eight, twelve, sixteen and twenty, 9 phenolic compounds were detected. Four phenolic compounds were detected on day twenty four (Figure 4.6). However, as illustated in figure 4.6, the qualitative difference was revealed to be the same for urine samples from all the three animals. Besides phenolic compounds, other organic compounds (3-n-Propylpyridine and decanal) were also identified. This is represented in peaks 10 and 11, respectively, in figure 4.5. Even so, in a complete scan mode, several unidentified compounds were observed with a strong m/z whose MS fragmentation spectra indicated an assortment of product ions (data not shown).

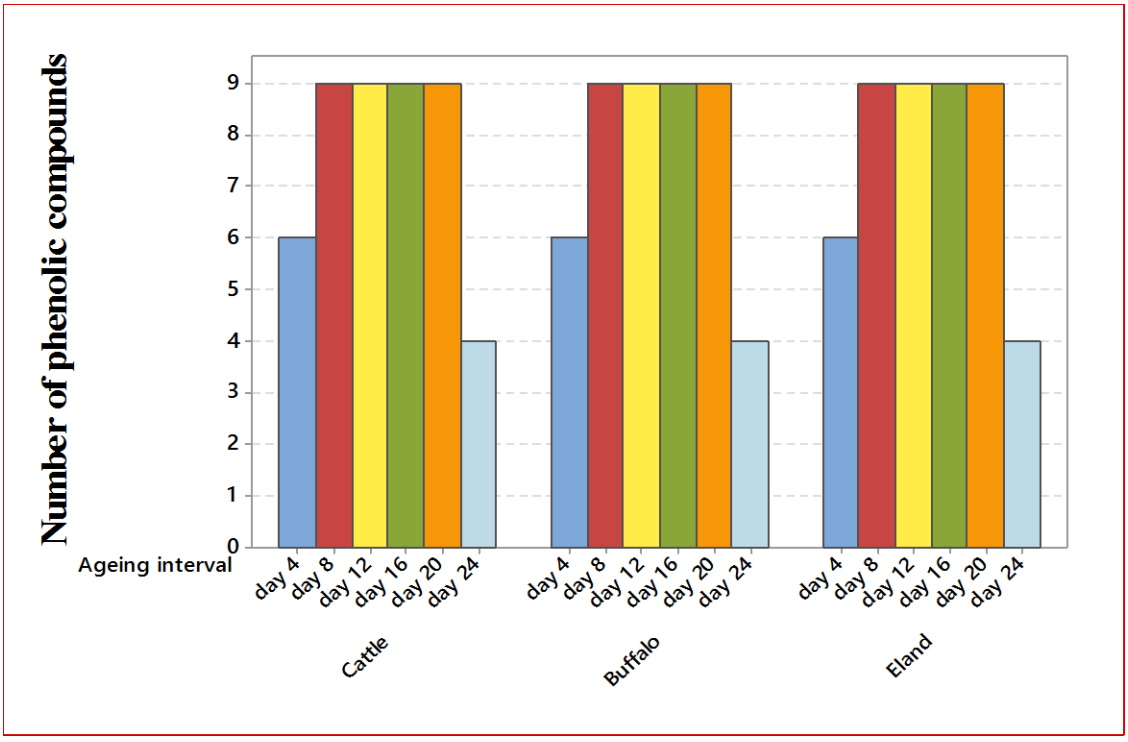

Figure 4.6: Qualitative distribution based on the number of phenolic compounds characterized at different ageing intervals across study animals

4.4.2 Quantitative analysis of detected phenolic compounds in cattle ageing urine

Concentration of total phenolic compounds (TPC) in cattle urine extracts were compared across ageing intervals as presented in table 4.5. Based on these results, phenolic compounds concentration was statistically different, at $p \leq 0.05$ across ageing period. Phenolic compounds concentration increased with ageing urine up to day 20 as illustrated in table 4.5. However, in day 24, the mean phenolic concentration was seen to depreciate. All the phenolic compounds observed at days four and eight were significantly different, at p=0.0001. There was no significant difference in mean concentration of 3-ethylphenol (265004.00 mg/l) and para-cresol (263477.33 mg/l) in day 12, at p=0.0001. Similarly, the results showed no significant difference in mean concentration for 3-ethylphenol (280481.33 mg/l), ortho-cresol (286834.33 mg/l) and para-cresol (294575.67 mg/l) at day 16. At day 20, the mean concentrations of 3-ethylphenol (344200.33 mg/l) and ortho-cresol (345332.33 mg/l) were not significantly different, at p=0.0001. 2-methyloxyphenol, 3-propylphenol and 4-propylphenol were not detected in day four old urine, whereas 2-methyloxyphenol, 3-ethylphenol, 3-propylphenol, 4-ethylphenol, 4-propylphenol fell below detection limit, in the 24 days' old urine. Throughout the ageing intervals of urine, 3-propylphenol consistently registered the highest concentration.

Table 4.5: Quantitative analysis of detected phenolic compounds in cattle ageing urine samples

Phenolic compound	Mean±SE (mg/L)					
	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24
2-methyloxyphenol	ND	436199± 1451.97^{c}	461701.67 ±2891.08^{c}	492479.33 ±1157.01^{c}	561583 ±2902.8^{c}	ND
3-ethylphenol	275437.67 ±2889.93^{b}	260967.67 ±3460.93^{d}	265004.00 ±1732.63^{e}	280481.33 ±2310.85^{f}	344200.33 ±1157.88^{e}	ND
3-propylphenol	ND	548431.33 ±1157.3^{b}	633464.33 ±10474.9^{b}	646006.33 ±1731.19^{b}	702896 ±882.36^{b}	ND
4-ethylphenol	86644.33 ±1733.5^{g}	126983.33 ±2310.56^{h}	196400.33 ±1735.23^{g}	201009.67 ±575.93^{g}	243238.67 ±1730.32^{g}	ND
4-propylphenol	ND	243435.67 ±1151.53^{e}	300869.67 ±291.67^{d}	328502 ±1727.43^{d}	398625 ±187.51^{d}	ND
Cresol-meta	168101.67 ±2312^{d}	134247.33 ±2887.91^{h}	91816.33 ±1204.3^{i}	94385.67 ±1455.03^{i}	97531.33 ±1528.94^{i}	226981 ±2027.86^{e}
Cresol-ortho	154050.33 ±2079.1^{e}	197696.67 ±882.48^{f}	238506 ±2401.57^{f}	286834.33 ±2599.7^{ef}	345332.33 ±2029.56^{e}	401901.33 ±881.67^{c}
Cresol-para	177544.33 ±1766.17^{c}	234896.33 ±2026.27^{e}	263477.33 ±2336.05^{e}	294575.67 ±2334.38^{e}	333066 ±2335.57^{f}	325992.33 ±3212.32^{d}
Phenol	134832.33 ±2250.82^{f}	155794 ±2602.16^{g}	163919.00 ±2083.51^{h}	184944.33 ±1766.86^{h}	205754 ±2311.42^{h}	755528 ±1766.23^{b}
Control	798180 ±2.89^{a}	798180 ±2.89^{a}	798180 ±2.89^{a}	798280 ±2.89^{a}	798600 ±2.89^{a}	798180 ±2.89^{a}
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Each value is expressed as mean (mg/l) of three replication ±standard error, (n=3). All compounds listed above had (match percent) percentage quality of >80%. Means followed by the same letter within a column are not significantly different according to Tukey's Honest Significance Difference (HSD) at 5% level. ND (not detected) represents values that fell below the detection limit

4.4.3 Quantitative analysis of detected phenolic compounds in buffalo ageing urine

The concentrations of detected phenolic compounds across ageing interval were statistically different, at $p \leq 0.05$ (Table 4.6). However, comparison among phenolic compounds at each day of fermentation revealed diverse outcomes. For instance, at day four, there was no significant difference in concentrations for meta-cresol (163582 mg/l), para-cresol (159542.33 mg/l) and ortho-cresol (165767.33 mg/l), at p=0.0001. At the

same day, 3-ethylphenol registered the highest concentration (273865.33 mg/l), while 4-ethylphenol (87753.67 mg/l) registered the lowest concentration. Both 3-ethylphenol and 4-ethylphenol were statistically different from the rest of the compounds in this particular day. The 2-methyloxyphenol, 3-propylphenola and 4-propylphenol did not meet the detection limits at day four.

At day eight of fermentation, the 3-n-propylphenol (545419.67 mg/l) was observed to be highly concentrated, compared to the rest of the compounds. 4-ethylphenol registered the lowest concentration (130318.67 mg/l). This value was not significantly different from meta-cresol (134549.67 mg/l) (P=0.0001). 3-propylphenol (621715.33 mg/l) and cresol-meta (91868.67 mg/l) were revealed to have the highest and lowest concentrations, individually, at day twelve. The two were significantly different from the rest of the compounds at this particular day. Tukey's pair-wise comparison revealed significant difference among compounds screened at day twelve old buffalo urine sample. Nonetheless, there was no significant difference in 3-ethylphenol (265463.33 mg/l) and para-cresol (263838.33 mg/l) concentrations of day twelve, at p=0.0001. Elsewhere, 3-ethylphenol (284305.00 mg/l) and ortho-cresol (285541.00 mg/l) showed no significant difference in their concentration at day sixteen (p=0.0001). 3-propylphenol (645103.33 mg/l) and meta-cresol (95367.33 mg/l) registered the highest and lowest concentration respectively, at day sixteen. The concentrations were statistically different from the rest of the compounds (Table 4.6)

Table 4.6: Quantitative analysis of detected phenolic compounds in buffalo ageing urine samples

Phenolic compound	Mean±SE (mg/L)					
	Day 4	**Day 8**	**Day 12**	**Day 16**	**Day 20**	**Day 24**
2-methyloxyphenol	ND	434093.67 ±2336.33^{c}	462946.67 ±1520.98^{c}	492305.67 ±2605.47^{c}	559775.33 ±1448.45^{c}	ND
3-ethylphenol	273865.33 ±2029.37^{b}	266316.33 ±1455.80^{d}	265463.33 ±1195.84^{e}	284305.00 ±1733.50^{f}	343967.33 ±1456.37^{e}	ND
3-propylphenol	ND	545419.67 ±2904.71^{b}	621715.33 ±4407.76^{b}	645103.33 ±2303.63^{b}	702534.33 ±1859.60^{b}	ND
4-ethylphenol	87753.67 ±879.40^{e}	130318.67 ±2332.33^{i}	195333.33 ±1772.34^{g}	203413.67 ±1454.99^{g}	243787.00 ±1766.98^{g}	ND
4-propylphenol	ND	245822.33 ±1448.03^{e}	305167.00 ±2905.21^{d}	326836.00 ±2190.11^{d}	397421.33 ±1523.49^{d}	ND
Cresol-Meta	163582.00 ± 1766.23^{c}	134549.67 ±1527.53^{i}	91868.67 ±1204.35^{i}	95367.33 ±1762.76^{i}	96813.00 ±2188.28^{i}	42106.67 ±884.75^{d}
Cresol-Ortho	165767.33 ±1767.30^{c}	197072.67 ±1764.84^{g}	239834.67 ±1759.74^{f}	285541.00 ±1766.61^{f}	344432.67 ±2335.67^{e}	57144.67 ±1455.83^{c}
Cresol-Para	159542.33 ±1735.23^{c}	214026.33 ±1733.21^{f}	263838.33 ±1588.33^{e}	298202.00 ±580.82^{e}	332538.67 ±1733.78^{f}	72797.33 ±1152.10^{b}
Phenol	141549.33 ±1735.51^{d}	157734 ±1530.25^{h}	162112.67 ±1765.03^{h}	184691.33 ±2083.75^{h}	203549.00 ±1155.28^{h}	75952.33 ±2030.30^{b}
Control	798122.33 ±2.33^{a}	798128.00 ±2.65^{a}	798120.00 ±2.89^{a}	798300.00 ±2.89^{a}	798420.00 ±2.89^{a}	798420.00 ±2.89^{a}
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Each value is expressed as mean (mg/l) of three replication ±standard error; (n=3). All compounds listed above had (match percent) percentage quality of >80%. Means followed by the same letter within a column are not significantly different according to Tukey's Honest Significance Difference (HSD) at 5% level. ND (not detected) represents values that fell below the detection limit

At day twenty, there was no significant difference between 3-ethylphenol (343967.33 mg/l) and ortho-cresol (344432.67 mg/l) concentrations. The 3-propylphenol (702534.33 mg/l) still registered the highest concentration whereas meta-cresol (96813.00 mg/l) recorded the lowest concentrations at day twenty (p=0.0001). At day twenty four, only four phenolic compounds were within the detection limits. They include: Cresol derivatives and phenol, the parent compound. On the contrary, 2-methoxyphenol, 3-

ethylphenol, 3-propylphenol, 4-ethylphenol and 4-propylphenol did not meet the detection limit. Phenol registered the highest concentration (75952.33 mg/l) at day twenty four, even though there was no statistical difference in the concentrations of this compound and para-cresol (72797.33 mg/l) at p=0.0001. Meta-cresol (42106.67 mg/l) was observed to be lowest in concentration at day twenty four.

Comparison of each phenolic compound across urine ageing interval revealed an interesting trend; levels of TPC concentration were lowest at day four of ageing urine and generally increased, except for 4-propylphenol, from day four up to day twenty. The 4-propyphenol showed increased concentration in day twenty when compared to day sixteen. All the concentrations were observed to drastically depreciate at day twenty four of ageing urine. Similar to cattle urine, the most abundant phenolic compound in buffalo urine was 3-propylphenol. However, it was only detectable between days eight and twenty.

4.4.4 Quantitative analysis of detected phenolic compounds in eland ageing urine

Volatile phenols concentrations across ageing intervals were statistically different, at $p \leq 0.05$. When compared among ageing days, phenols were observed to gradually increase from day four up to day twenty and thereafter, decreased in concentration at day twenty four, save for the 3-ethylphenol and cresol-meta, which did not obey the trend explained (Table 4.7).

Table 4.7: Quantitative analysis of detected phenolic compounds in eland ageing urine samples

Phenolic compound	Mean±SE (mg/L)					
	Day 4	**Day 8**	**Day 12**	**Day 16**	**Day 20**	**Day 24**
2-methyloxyphenol	ND	412507.33 ±1155.57^{c}	461366.67 ±2603.99^{c}	492800.67 ±580.81^{c}	562102.67 ±1155.28^{c}	ND
3-ethylphenol	268123.33 ±1155.86^{b}	268626.67 ±1155.28^{d}	265047.67 ±2308.54^{e}	284947.33 ±2308.82^{f}	344299.0±1 734.07^{e}	ND
3-propylphenol	ND	517531.33 ±1157.01^{b}	620138.00 ±1733.21^{b}	645547.00 ±2887.62^{b}	702200.33 ±575.33^{b}	ND
4-ethylphenol	88013.00 ±577.06^{g}	126096.33 ±2311.42^{h}	196538.00 ±578.51^{g}	204018.00 ±1731.76^{g}	243118.67 ±2307.67^{g}	ND
4-propylphenol	ND	240035.00 ±1733.50^{e}	301349.00 ±575.91^{d}	328599.33 ±579.66^{d}	398426.00 ±579.67^{d}	ND
Meta-cresol	159363.33 ±1731.47^{d}	134520.67 ±1734.36^{h}	91147.33 ±577.93^{i}	94567.00 ±58.04^{i}	98481.00 ±579.95^{i}	35845.00 ±904.25^{e}
Ortho-cresol	145254.00 ±2309.98^{e}	198273.00 ±576.49^{f}	240210.33 ±2027.75^{f}	286345.67 ±575.91^{f}	345100.67 ±1157.01^{e}	458286.67 ±1154.99^{c}
Para-cresol	169734.67 ±578.51^{c}	238588.33 ±1732.34^{e}	261093.67 ±5457.64^{e}	298913.00 ±579.38^{e}	332545.67 ±577.65^{f}	119933.33 ±2315.18^{d}
Phenol	114602.33 ±2889.35^{f}	156556.00 ±3462.66^{g}	163333.33 ±879.08^{h}	185239.67 ±1734.65^{h}	204555.00 ±1734.36^{h}	756730.0 ±642.26^{b}
Control	798163.33 ±1.45^{a}	798179.00 ±3.06a	798161.33 ±1.86^{a}	798349.00 ±2.08^{a}	798419.00± 2.08^{a}	798162.00 ±0.58^{a}
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Each value is expressed as mean (mg/l) of three replication ±standard error; (n=3). All compounds listed above had (match percent) percentage quality of >80%. Means followed by the same letter within a column are not significantly different according to Tukey's Honest Significance Difference (HSD) at 5% level. ND (not detected) represents values that fell below the detection limit

Based on the results of this study, 2-methyloxyphenol, 3-propylphenol and 4-propylphenol did not meet the detection limit at day four fermenting urine. The 3-ethylphenol (268123.33 mg/l) and 4-ethylphenol (88013.00 mg/l) were observed to have higher and lower concentration, correspondingly. At the same day, these compounds were significantly different from the rest of the compounds, at P=0.001. Nonetheless, all the compounds at day four were statistically different, at p=0.0001. At day eight, 3-

propylphenol (517531.33 mg/l) had the highest concentration whereas 4-ethylphenol (126096.33 mg/l) recorded the lowest concentration. Conversely, 4-ethylphenol concentration was not significantly different from meta-cresol (134520.67 mg/l), at p=0.0001. Elsewhere, there was no statistical difference in the concentrations of 4-propylphenol (240035.00 mg/l) and Para-cresol (238588.33 mg/l), at this particular day (p=0.0001).

The data revealed that at day sixteen, 3-propyphenol (645547.00 mg/l) concentration was significantly higher than the rest of the compounds, at p=0.0001. Alternatively, Meta-cresol (94567.00 mg/l) registered the lowest concentration. According to Tukey's pair-wise comparison, the two compounds were significantly different from the rest of the compounds observed at day sixteen. There was no significant difference between 3-ethylphenol (284947.33 mg/l) and ortho-cresol (286345.67 mg/l) (p=0.0001) in day sixteen and twenty. The 3-propylphenol (702200.33 mg/l) and meta-cresol (98481.00 mg/l) were observed to have higher and lower concentration respectively, at day twenty. The concentration of 3-ethylphenol (344299.0 mg/l) and ortho-cresol (345100.67 mg/l) were not significantly different at day twenty (p=0.0001).

At day twenty four, only four compounds were observed. Phenol (756730.0 mg/l) was statistically high in concentration than the rest of the compounds (p=0.0001). It was followed by ortho-cresol (458286.67 mg/l), then Para-cresol (119933.33 mg/l) and lastly, meta-cresol (35845.00 mg/l). Five compounds that were detected in early days of ageing

urine did not meet the detection limit, at day twenty four. They include 2-methyloxyphenol, 3-ethylphenol, 3-propylphenol, 4-ethylphenol and 4-propylphenol.

4.4.5 Effect of different mammalian urine and their fermentation period on generation of phenols

In this study, specific phenolic compounds in urine fraction were quantified in all samples across ageing interval. Factorial analysis was used to analyse interactions between main factors, that is, the ageing days and the animals under study, and their effect on phenols concentration. The relation of specific phenolic compounds with each animal was also analysed and the interaction between main factors determined (Table 4.8).

The results revealed different concentrations of specific phenolic compounds at different ageing times. Comparison between main factors and their interaction revealed that there was significant difference in amount of phenols emitted among ageing days ($P \leq 0.05$). This was so except for o-cresol that showed no statistical difference across urine ageing intervals ($P=0.2439$). The data shows that P-cresol was in high concentration (332717 mg/L) at day twenty, followed by day sixteen (297230 mg/L), day twelve (262803 mg/L), day eight (229170 mg/L), day twenty four (72908 mg/L), day four (168940 mg/L) and lastly day four (168940 mg/L) ($P=0.0001$). Based on Tukeys' pair-wise comparison there was no shared significance in mean concentration among the days.

Table 4.8: An evaluation of bacterial generation of phenols in different mammalian urine at different stages of fermentation

DAYS	Phenolic compounds M±SE (mg/L)									
	2-MP	**3-EP**	**3-PP**	**4-EP**	**4-PP**	**m-cresol**	**O-cresol**	**P-cresol**	**phenol**	**Control**
4	ND	272475.44 ±1545^{c}	ND	87470.33 ±623^{e}	ND	163682.33 ±1596^{a}	155023.89 ±3145^{a}	168940.44 ±2707^{e}	130328.00 ±4215^{f}	798255.22 ±9^{a}
8	427600.00 ±3882^{d}	265303.56 ±1604^{d}	537127.44 ±5012^{d}	127799.44 ±1325^{d}	243097.67 ±1114^{d}	134439.22 ±1069^{b}	197680.78 ±618^{a}	229170.33 ±3932^{d}	156694.67 ±1356^{e}	798262.33 ±9^{a}
12	462005.00 ±1230^{c}	265171.67 ±905^{d}	625105.89 ±3928^{c}	196090.56 ±759^{c}	302461.89 ±1096^{c}	91610.78 ±532^{e}	239517.00 ±1072^{a}	262803.11 ±1826^{c}	163121.67 ±870^{d}	798253.78 ±9^{a}
16	492528.56 ±843^{b}	283244.56 ±1275^{b}	645552.22 ±1185^{b}	202813.78 ±815^{b}	327979.11 ±871^{b}	94773.33 ±677d^{e}	286240.33 ±946^{a}	297230.22 ±980^{b}	184958.44 ±938^{c}	798209.67 ±10^{a}
20	561153.67 ±1055^{a}	344155.56 ±736^{a}	702543.33 ±625^{a}	243381.44 ±982^{a}	398157.44 ±509^{a}	97608.44 ±825^{d}	344955.22 ±963^{a}	332716.78 ±861^{a}	204619.33 ±953^{b}	798279.67 ±30^{a}
24	ND	ND	ND	ND	ND	101644.22 ±31355^{c}	764064.67 ±481072^{a}	172907.67 ±38889^{e}	529403.44 ±113366^{a}	798254.00 ±42^{a}
Animals										
Cattle	325327.17 ±56565^{a}	237681.83 ±26650^{a}	421799.67 ±73176^{a}	142379.39 ±19846^{a}	211905.39 ±37987^{a}	135510.56 ±10932^{a}	270720.17 ±20547^{a}	271592.00 ±18133^{a}	266795.28 ±53290^{a}	798266.67 ±37^{a}
Buffalo	324853.56 ±56475^{a}	238986.22 ±26730^{a}	419128.78 ±72746^{a}	143434.39 ±19861^{a}	212541.11 ±38003^{a}	104047.89 ±9192^{a}	214965.50 ±22151^{a}	223490.83 ±21240^{a}	254264.78 ±9782^{a}	798251.72 ±33^{a}
Eland	321462.89 ±56170^{a}	238507.33 ±26708^{a}	414236.00 ±72267^{a}	142964.00 ±19902^{a}	211401.56 ±37965^{a}	102320.72 ±9354^{a}	508055.28 ±240739^{a}	236801.44 ±17671^{a}	263502.72 ±53922^{a}	798238.94 ±25^{a}
P-Values for main factors and their interaction										
Day	≤0.0001	≤0.0001	≤0.0001	≤0.0001	≤0.0001	≤0.0001	≥0.2439	≤0.0001	≤0.0001	≥0.2431
Animal	≥0.0913	≥0.4593	≥0.0604	≥0.5072	≥0.2721	≥0.07351	≥0.2636	≥0.0801	≥0.1021	≥0.3631
Day * Animal	≤0.0001	≥0.0572	≤0.0001	≥0.8272	≥0.0855	≤0.0001	≥0.2143	≤0.0001	≤0.0001	≥ 0.5081

ND=not detected**;** each value is expressed as mean ± standard error (mg/l); All compounds listed above had (match percent) percentage quality of >80%. 2-MP=2-Methyloxyphenol; 3-EP=3-ethylphenol; 3-PP=3-propylphenol; 4-EP=4-ethylphenol; 4-PP=4-propylphenol. Means with the same letter within columns are not significantly different according to Tukey's Honest Significance Difference (HSD) at 5% level.

The results show significant difference in phenol, concentration across ageing intervals (P=0.0001). Day twenty four recorded the highest concentration (529403.44 mg/l) for this compound followed by day twenty (204619.33 mg/l), day sixteen (184958.44 mg/l), day twelve (163121.67 mg/l), day eight (156694.67 mg/l) and lastly day four (130328.00 mg/l). The highest concentration (764064.67 mg/L) for o-cresol was observed at day twenty four (P=0.2439). This was followed by 344955.22 mg/l at day twenty, 286240.33 mg/l at day sixteen, 239517.00 mg/l at day twelve, 197680.78 mg/l at day eight and finally 155023.89 mg/l at day four. Means concentrations for o-cresol across ageing intervals were not significantly different as revealed by Tukey's pair-wise comparison. The m-cresol at P=0.0001 was highly concentrated (163682.33 mg/L) at day four. This was followed by 134439.22 mg/l at day eight, 101644.22 mg/l at day twenty four, 97608.44 mg/l at day twenty, 94773.33 mg/l at day sixteen and 91610.78 mg/l at day twelve. Mean concentrations at day twelve and sixteen were not significantly different based of Tukey's HSD pair-wise comparison.

The highest concentration of 4-ethylphenol at 243381.44 mg/L, was recorded at day twenty while the lowest concentration (87470.33 mg/L) at day four. Mean concentrations for day eight twelve and sixteen were 127799.44 mg/l, 196090.56 mg/l and 202813.78 respectively. Tukeys pair-wise comparison showed concentrations were significantly different across the days (P=0.0001). At $P \leq 0.0001$, 344155.56 mg/L was the highest concentration for 3-ethylphenol. This was observed at day twenty. The lowest concentration (265171.67 mg/L) was recorded at day twelve. The concentration 283244.56 mg/l, 272475.44 mg/l, 265303.56 mg/l were observed at days sixteen, four

and eight correspondingly. Mean concentrations recorded at days eight and twelve were not significantly different as revealed by Tukey's HSD, at P=0.0001. 3-propylphenol, 4-propylphenol and 2-methoyloxyphenol were not observed in days four and twenty four. The highest concentration (702543.33 mg/l) of 3-propylphenol was observed at day twenty. This was followed by day sixteen (645552.22 mg/l), day eight (537127.44 mg/l) and day twelve (625105.89 mg/l). The 4-propylphenol was highly concentrated (398157.44 mg/l) at day twenty four followed by day sixteen (327979.11 mg/l), day twelve (302461.89 mg/l) and lastly day eight (243097.67 mg/l). The 2-methyloxyphenol concentration increased with ageing intervals from day eight (427600.00 mg/l) today twenty (561153.67 mg/L). Days twelve and sixteen recorded mean concentrations of 462005.00 mg/l and 492528.56 mg/l respectively.

Statistical comparison of specific phenolic compounds concentration among animals showed no significance difference at $P \leq 0.05$. The highest mean concentration (325327.17 mg/l) of 2-methyloxyphenol was observed in cattle urine headspace. This was followed by 324853.56 mg/l and 321462.89 mg/l for buffalo and eland, respectively (P=0.0913). The highest mean concentration value for 3-ethylphenol was recorded in buffalo (238986.22 mg/l) followed by eland (238507.33 mg/l) and lastly, cattle (238986.22 mg/l) at P=0.4593. 3-propylphenol was highest in concentration in urine sample belonging to cattle (421799.67 mg/l) followed by that from buffalo (419128.78 mg/l) and then eland (414236.00 mg/l) (P=0.0604). Buffalos sample at P=0.5072 recorded the highest 4-ethylphenol concentration (143434.39 mg/l) followed by eland and cattle at 142964.00 mg/l and 142379.39 mg/l respectively.

At P=0.2721, 4-propylphenol was observed to be high in concentration (212541.11 mg/l) in buffalo samples, then cattle samples (211905.39 mg/l) and lastly eland samples (211401.56 mg/l). Ortho-cresol at P=0.2636 was highly observed in eland samples (508055.28 mg/l), followed by cattle (270720.17 mg/l) and then buffalo (214965.50 mg/l). M-cresol (P=0.07351) p-cresol (P=0.0801) and phenol (P=0.1021) were highly observed in cattle urine sample with means concentrations of 135510.56 mg/l, 271592.00 mg/l and 266795.28 mg/l, correspondingly. In buffalo, m-cresol was observed to be 104047.89 mg/l, while the concentration in eland was recorded at 102320.72 mg/l. Eland samples showed the second highest concentrations for p-cresol and phenol at 236801.44 mg/l and 263502.72 mg/l respectively. The lowest concentration of p-cresol and phenols was noted in buffalo samples, at 223490.83 mg/l and at 254264.78 mg/l, correspondingly.

The effect of interaction of main factors on phenols generation varied. The interaction revealed that the phenols concentrations for 3-ethylphenol, (p=0.0572) 4-ethylphenol (p=0.8272), 4-propylphenol (p=0.0855) and o-cresol (p=0.2143) were not significantly different. On the other hand, the interaction between main factors showed that there was significant difference among the phenols concentrations for 2-methylphenol, 3-propylphenol, m-cresol, p-cresol and phenol, all at P=0.0001.

4.5 Potential of production of tsetse attractive cues by urine based bacterial communities in the selected mammals

4.5.1 Screening bacterial isolates for their ability to mediate production of phenols in urine samples of the selected mammals

Urine fermentation with mixed bacteria (m.b) revealed presence of eight phenolic compounds (Table 4.9). The resultant phenolic concentrations were similar, higher or lower compared with the ageing urine sample, at $P \leq 0.05$. A comparison between individual isolates incubated in sterile fresh urine samples and that of mixed culture samples showed similarities in the type of phenolic compounds detected. Out of 19 bacteria previously isolated, only 8 were observed to have significant association with volatile phenols. These included: b2, b22, E11, E49, B25, B34, B42, and B70. The volatile phenols detected in urine sample incubated with these bacteria were also detected in naturally colonized mammalian urine samples.

Para-cresol was significantly present in urine sample incubated with E11 (425989.00 mg/l), E49 (170206.67 mg/l), B25 (333066.00 mg/l), B34 (104538.67 mg/l) B70 (432655.67 mg/l) and m.b (159542.33 mg/l), at (P=0.0001). O-cresol was only observed in urine sample incubated with b2 (98100.67 mg/l) and m.b (141549.33 mg/l), at P=0.0001. The m-cresol was revealed in presence of B34 (35703.33 mg/l) and m.b (165767.33 mg/l), at P=0.0001. The 3-ethylphenol was recorded when urine was subjected to b22 (273865.33 mg/l) and m.b (163582.00 mg/l) at P=0.0001. The 4-ethylphenol was revealed in urine incubated with b22 (216816.33 mg/l) and m.b (87753.67 mg/l), at P=0.0001. Elsewhere, 3-propyphenol was significantly associated

with E11 (543339.67 mg/l), B25 (97421.33 mg/l), B42 (27813.33 mg/l), B70 (546006.33 mg/l) and m.b (273865.33 mg/l), at P=0.0001. The 4-propylphenol was significant in m.b (98887.67 mg/l), E11 (13797.3 mg/l) and B70 (12797.33 mg/l), at P=0.0001. Phenol was significant, when fresh sterile urine was inoculated with E11 (133811.3 mg/l), B70 (127144.67 mg/l), m.b (42106.67 mg/l), B25 (122922.33 mg/l) and E49 (14589.0 mg/l), at P=0.0001. Sterile urine without inoculums as well as those inoculated with b5, b7, b10, b12, E8, E48, E51, E53, B71, B73 and B74 did not show any evidence of the formation of phenols.

Table 4.9: Quantitative and qualitative analysis of phenolic compounds using cattle, buffalo and elands' sterile fresh urine incubated with various bacterial isolates

Bacterial	Phenols forms identified mg/L (mean±SE)							
isolate	**p-cresol**	**o-cresol**	**m-cresol**	**3-E-phenol**	**4-E-phenol**	**3-p-phenol**	**4-p-phenol**	**Phenol**
m.b	159542.33 ±1735.23^{d}	141549.33 ±1735.51^{a}	165767.33 ±1767.30^{a}	163582.00 ±1766.23^{b}	87753.67 ±879.40^{b}	273865.33 ±2029.37^{b}	98887.67 ±304.80^{a}	42106.67 ±884.75^{c}
b2	19.00 ±3.51^{f}	98100.67 ±1527.20^{b}	20.33 ±2.28^{c}	ND	ND	ND	ND	21.67 ±7.22^{e}
b22	28.67 ±5.46^{f}	ND	28.00 ±2.89^{c}	273865.33 ±2029.37^{a}	146816.33 ±23331.60^{a}	12.67 ±3.48^{e}	14.00 ±4.51^{c}	17.33 ±3.33^{e}
E11	425989.00 ±3293.56^{a}	26.33 ±3.84^{c}	ND	16.33 ±4.10^{c}	20.00 ±3.0^{c}	543339.67 ±2844.94^{a}	13797.33 ±574.75^{b}	133811.33 ±3481.30^{a}
E49	170206.67 ±2848.00^{c}	42.67 ±4.45^{c}	32.33 ±3.31^{c}	49.00 ±12.12^{c}	42.00 ±11.14c	27.33 ±4.06^{e}	218.33 ±63.44^{c}	14589.00 ±883.89^{d}
B25	333066.00 ±2335.57^{b}	33.67 ±1.86^{c}	ND	49.33 ±3.48^{c}	44.33 ±2.60^{c}	97421.33 ±1523.49^{c}	35.33 ±3.89^{c}	122922.33 ±580.56^{b}
B34	104538.67 ±2514.82^{e}	ND	35703.33 ±48.42^{b}	ND	ND	38.67 ±1.86^{e}	ND	ND
B42	24.00 ±1.53^{f}	24.00 ±0.58c	14.33 ±0.33^{c}	ND	15.33 ±2.91^{c}	27813.33 ±11766.87^{d}	25.33 ± 1.76^{c}	16.67 ±2.82^{e}
B70	432655.67 ±178.79^{a}	16.67 ±2.19^{c}	ND	21.33 ±1.41^{c}	22.00 ±2.89^{c}	546006.33 ±1731.19^{a}	12797.33 ±1152.10^{b}	127144.67 ±1455.83^{b}
Blank	17.67 ±0.88^{f}	17.33 ±1.2^{c}	17.33 ±1.20^{c}	15.67 ±1.33^{c}	13.00 ±0.58^{c}	14.33 ±1.20^{e}	16.67 ±1.45^{c}	13.33 ±0.88^{e}
P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

Each value is expressed as mean (mg/l) of three replication ±standard error; (n=3). All compounds listed above had match percent (percentage quality) >80%. Blank= sterile fresh urine incubated at 37^{o}C for 3 days; m.b=mixed bacteria for the 19 isolates. ND (not detected) represents values that fell below the detection limit. Means followed by the same letter within a column are not significantly different according to Tukey's Honest Significance Difference (HSD) at 5% level. Isolates b5, b7, b10, b12, E8, E48, E51, E53, B71, B73 and B74 (not shown in the table) did not register any detectable amounts of phenols when incubated in sterilized fresh urine of the study mammals.

4.5.2 Diversity profile of bacterial communities with potential to mediate production of phenols in mammalian urine across ageing interval

Diversity indices of bacterial communities with ability to mediate production of phenols in mammalian urine were determined across ageing intervals. These indices are illustrated in table 4.10

Table 4.10: Morphological diversity profile of bacterial communities with potential to mediate production of phenols in pooled mammalian urine samples across ageing interval

Diversity indices	**Ageing interval (Days)**						
	0	**4**	**8**	**12**	**16**	**20**	**24**
Dominance D	0.3333	0.2	0.125	0.125	0.125	0.125	0.3333
Simpson 1-D	0.6667	0.8	0.875	0.875	0.875	0.875	0.6667
Shannon H	1.099	1.609	2.079	2.079	2.079	2.079	1.099
Evenness_e^H/S	1	1	1	1	1	1	1
Brillouin	0.5973	0.9575	1.326	1.326	1.326	1.326	0.5973
Menhinick	1.732	2.236	2.828	2.828	2.828	2.828	1.732
Margalef	1.82	2.485	3.366	3.366	3.366	3.366	1.82
Equitability-J	1	1	1	1	1	1	1
Fisher-alpha	0	0	0	0	0	0	0
Berger-Parker	0.3333	0.2	0.125	0.125	0.125	0.125	0.3333
Chao-1	6	15	36	36	36	36	6

According to *H*, the highest diversity of these bacterial communities was observed at days eight, twelve, sixteen and twenty, all at 2.079. On the other hand, day zero (fresh urine) and day twenty four had the lowest diversity of the bacterial isolates, all at 1.099. Similar to *H*, Simpson 1-D index revealed highest bacterial diversity at days eight, twelve, sixteen and twenty, all at 0.875. At these ageing intervals is where most of the phenolic compounds were expressed. The lowest bacterial diversity as revealed by

Simpson 1-D index was at day zero (fresh urine) and day twenty four, all at 0.6667. The lowest bacterial dominance index was observed at days eight, twelve, sixteen and twenty, all at 0.125. Conversely, the highest bacterial dominance index was revealed at day zero (fresh) and twenty four, all at 0.3333. Bacterial evenness diversity was equal across ageing intervals.

4.6 Biochemical characterization of bacterial isolates with and without potential to mediate production of phenols

On inoculating the isolates on each urine extraction day on NA, they showed morphological similarity with the ones illustrated in table 4.3 above. On Gram staining, 7 isolates were cocci in shape while 12 isolates were rods. Of the 19 isolates, 5 were identified as Gram negative. They include b5, b22, B25, B42 and B74. The rest of the isolates (b2, b7, b10, b12, E8, E11, E48, E49, E51, E53, B34, B70, B71 and B73) were found to be Gram positive. Gram staining was followed by biochemicals tests and results documented as shown in table 4.11.

Table 4.11: Biochemical profiling of bacterial isolates screened for generation of phenolic compounds in mammalian urine

Test	**Isolates Laboratory Designation Number**																		
	b2	b5	b7	b10	b12	b22	E8	E11	E48	E49	E51	E53	B25	B34	B42	B70	B71	B73	B74
Gram stain	+	-	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	+	-
Shape	C	R	R	R	R	C	R	C	R	C	R	R	R	C	R	C	R	R	C
Indole	+	+	-	-	-	-	-	+	+	+	-	+	+	+	+	+	-	+	-
Citrate utilization	+	+	+	+	+	-	+	-	-	-	+	-	-	-	-	-	+	+	+
Urease	-	+	+	-	+	-	+	-	+	-	+	+	+	+	-	-	+	+	-
Motility	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	+	+
MR	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-
VP	-	-	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+
Glucose (TSI)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Lactose (TSI)	+	+	-	-	-	+	-	+	-	+	-	-	+	+	-	+	-	-	-
Sucrose (TSI)	-	-	+	-	+	-	+	+	-	+	+	-	+	-	-	+	+	+	-
H_2S (TSI)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Gas (TSI)	-	-	+	+	+	+	+	-	+	+	-	-	-	-	+	-	-	+	-
Oxidase	+	-	-	+	-	+	-	-	+	-	-	+	-	+	+	-	-	+	+
Catalase	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Nitrate	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
Sorbitol	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	+

Key: TSI=Triple Sugar Iron, VP=Voges Proskauer, MR=Methyl Red += positive, - =negative, R=rod, C=cocci, Probable organism: b2= *Planococcus* spp., b5=*Providencia* spp., b7=*Bacillus* spp., b10=*Bacillus* spp., b12=*Bacillus* spp., b22=*Psychrobacter* spp., E8=*Bacillus* spp., E11=*Enterococcus* spp., E48=*Bacillus* spp., E49=*Streptococcus* spp., E51=*Bacillus* spp., E53=*Bacillus* spp., B25=*Morganella* spp., B34=*Micrococcus* spp., B42=*Ochrobactrum* spp., B70=*Enterococcus* spp., B71=*Bacillus* spp., B73=*Bacillus* spp., B74=*Alcaligenes* spp.,

Based on the morphological and biochemical traits, the isolates were grouped into ten different genera. Isolates b7, b10, b12, E8, E48, E51, E53, B71 and B74 presented traits similar to *Bacillus* spp. Members of *Bacillus* sp. were indistinguishable from each other by biochemical assays, contrary to morphological study. E11 and B 70 closely resembled *Enterococcus* spp. Isolates b2, b5 and b22 had characteristics similar to *Planococcus* spp., *Providencia* spp., and *Psychrobacter* spp. respectively. Isolates E49 resembled *Streptococcus* spp., whereas B25, B34, B42 and B74 resembled *Morganella* spp., *Micrococcus* spp., *Ochrobactrum* spp., and *Alcaligenes* spp. respectively. Interestingly, some of the bacterial genera which were present in fresh urine were detectable but their counts diminished to zero with increased urine ageing intervals. As expected, majority of the bacteria linked to production of volatiles in urine were found to dominate urine samples with increased days of urine fermentation, although at different times.

4.7 Molecular characterization of bacterial isolates with and without potential to mediate production of phenols

Morphological and biochemical studies were further confirmed with molecular analysis. Molecular characterization based on 16S rRNA gene confirmed the bacteria to belong to different species and strains. Molecular analysis of 16S rRNA gene of bacteria associated with phenols production further authenticated the existence of varied population of bacteria in mammalian urine at different ageing intervals. Genomic DNA extracted from pure bacterial cultures showed intact band when loaded to 0.8% agar rose gel run at 80 volts for about 30 minutes. The 1500 bp amplified region of 16S rRNA gene revealed different bands intensities when visualized in 1.2 % agar rose gel run at 80 volts for one

hour. At approximately 1500 bp, all PCR products showed definite and appropriately sized band in all lanes (Plate 4.2).

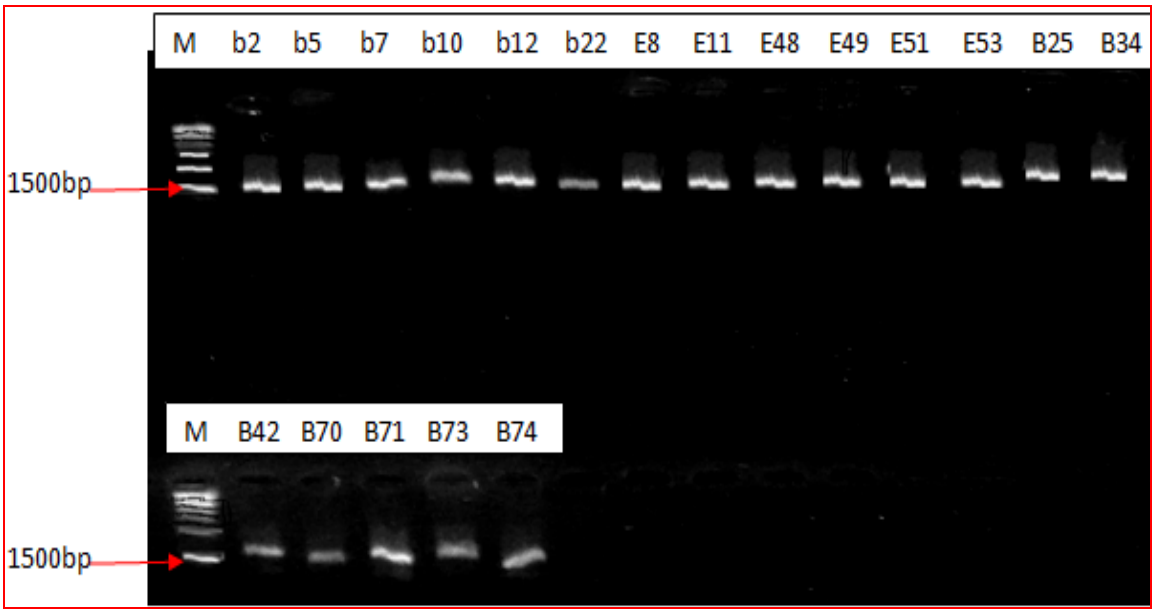

Plate 4.2: Gel electrophoresis image of PCR amplified 16S rRNA gene, of bacterial isolates of mammalian ageing urine, on 1.2 % agarose gel (M=100 bp DNA ladder).

Alignment and analysis of sequences obtained from the isolates showed that they closely related to known bacterial leneages. Presumptive identification of isolated bacteria tested for potential to mediate tsetse attractive phenols was confirmed with molecular characterization. On sequencing the 19 bacterial isolates, 14 different species belonging to different strains were revealed. The query sequence in NCBI gene bank database, using BLAST based on 16S rRNA gene sequencing, showed the relatedness of the tested organism with same identity within different genera. This is indicated in table 4.12.

Table 4.12: Bacterial strains showing significant similarity with mammalian urine bacterial isolates tested for ability to mediate production of phenols

Laboratory designation	**Spp/strain. identification[a]**	**Accession No. of the nearest neighbour**	**16S rRNA gene similarity (%)**	**Associated phenolic compound[b]**
b2	P*lanococcus massiliensis strain ES2*	NR 144714.1	99	o-cresol
b5	P*rovidencia rettgeri* RB151	CP017671.1	99	ND
b7	*Bacillus cereus* ISSFR-3F	CP018931.1	99	ND
b10	*Bacillus pumilus* GLB197	CP018574.1	99	ND
b12	*Bacillus cereus* MLY1	CP024655.1	99	ND
b22	*Psychrobacter alimentarius* PAMC 27889	NZCP014945.1	99	3-ethylphenol/4-ethylphenol
E8	*Bacillus cereus* ATCC 4342	CP009628.1	99	ND
E11	*Enterococcus faecalis* KUB3006	AP018538.1	90	P-cresol/3-propyphenol/4-propylphenol/ Phenol
E48	*Bacillus megaterium* YC4-R4	CP026736.1	99	ND
E49	*Streptococcus agalactiae* 2603V/R	NC004116.1	100	P-cresol/ Phenol
E51	*Bacillus cereus* M3	CP016316.1	99	ND
E53	*Bacillus safensis* U17-1	CP015611.1	99	ND
B25	*Morganella morganii* subsp. *morganii* KT	NC020418.1	99	P-cresol/3-propyphenol/ Phenol
B34	*Micrococcus luteus* NCTC 2665	NC012803.1	99	p-cresol/m-cresol
B42	*Ochrobactrum pituitosum* AA2	CP018782.1	92	3-propyphenol
B70	*Enterococcus faecalis* OG1RF	CP002621.1	99	P-cresol/3-propyphenol/4-propylphenol/ Phenol
B71	*Bacillus cereus* CMCC P0021	CP011151.1	99	ND
B73	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5033	HG328253.1	99	ND
B74	*Alcaligenes faecalis* JQ135	CP021641.1	99	ND

ND (not detected**)** is indicated against bacterial isolates that were negative for mediation of phenols when inoculated in sterilizd fresh urine of the study animals**.**

[a] Best match in NCBI GeneBank database.

[b]phenols detected in fresh urine samples inoculated with specific bacteria for 3 days at ambient conditions.

Phylogenetic relationship of the isolates revealed the presence of P*lanococcus massiliensis strain ES2* (99 % 16S rRNA gene sequence similarity with isolate b2); *Psychrobacter alimentarius* PAMC 27889 (99 % 16S rRNA gene sequence similarity with isolate b22), P*rovidencia rettgeri* RB151 (99 % 16S rRNA gene sequence similarity with isolate b5); *Enterococcus faecalis* OG1RF (99 % 16S rRNA gene sequence similarity with isolate B70); *Enterococcus faecalis* KUB3006 (90 % 16S rRNA gene sequence similarity with isolate E11); *Morganella morganii* subsp. *morganii* KT (99% 16S rRNA gene sequence similarity with isolate B25); *Micrococcus luteus* NCTC 2665 (99% 16S rRNA gene sequence similarity with isolate B34); *Ochrobactrum pituitosum* AA2 (92% 16S rRNA gene sequence similarity with B42); *Alcaligenes faecalis* JQ135 (99% 16S rRNA gene sequence similarity with isolate B74) and *Streptococcus agalactiae* 2603V/R (99.9% 16S rRNA gene sequence similarity with isolate E49) (Table 4.11).

Partial 16S rRNA gene sequence of isolated bacteria analyzed with BLAST search also revealed presence of different species and strains belonging to *Bacilli* spp. They include; *Bacillus pumilus* GLB197 (99% 16S rRNA gene sequence similarity with isolate b10); *Bacillus cereus* MLY1 (99 % 16S rRNA gene sequence similarity with isolate b12); *Bacillus cereus* CMCC P0021 (99 % 16S rRNA gene sequence similarity with isolate B71); *Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5033 (99% 16S rRNA gene sequence similarity with isolate B 73); *Bacillus cereus* ATCC 4342 (99% 16S rRNA gene sequence similarity with isolate E8); *Bacillus cereus* ISSFR-3F (99% 16S rRNA gene sequence similarity with isolate b7); *Bacillus cereus* M3 (99% 16S rRNA gene sequence similarity with isolate E51); *Bacillus megaterium* YC4-R4 (99%16S rRNA

gene sequence similarity with isolate E48) and *Bacillus safensis* U17-1 (99% 16S rRNA gene sequence similarity with isolate E53) (Table 4.11). The 16S rRNA nucleotide sequences for the isolates in this study have been deposited in NCBI GenBank under Accession Numbers MK123487-MK123505.

Evolutionary relatedness of the nineteen bacterial isolates with and without potential to mediate production of phenols in mammalian urine was evaluated based on their 16S rRNA gene. A phylogenetic tree based on these isolates is shown in figure 4.7. The bacteria grouped into seven clusters. The clusters grouped based on isolates similarity in relation to mediation of urine volatile phenols. Furthermore, some bacterial communities clustered depending on when they were isolated from urine wheraeas others grouped along their genotypic relatedness.

Clusters 1 and 2, exclussively contain bacterial communities that showed negative results for phenol mediation. Also, worthy to note is that bacteria that fell under these clusters were isolated between days, sixteen and twenty four of ageing mammalian urine. The two clusters were supported by bootstrap values of 100% and 78% respectively. Cluster 1 comprised of isolates b7, E51, E8, b12 and B71 whereeas cluster 2 was represented by isolates E48, B73, b10, and E53. The closed neghbours for all the isolates in these two clusters were dominated by different members of the *Bacillus* spp.

Bacteria having ability to mediate formation of phenols in mammalian urine were grouped in closely related phylogenetic positions. They grouped together in clusters 3, 4,

6 and 7. The clusters were supported by bootstrap values of 99%, 100% , 87% and 91% correspondingly. Cluster 3 was represented by isolates E11 and B70. These isolates were closely related to different strains of *Enterococcus faecalis*. Cluster 4 and cluster 6 were represented by only one isolate each, B34 and B42 repectively. The closed neighbour for isolate B34 was *Micrococcus luteus* NCTC 2665 while that for B42 was *Ochrobactrum pituitosum* AA2. Cluster 7 comprised of three isolates, B25, b2 and E49, whose close neighbours were *Morganella morganii* subsp. *morganii* KT, *Planococcus massiliensis strain ES2* and *Streptococcus agalactiae* 2603V/R in that order.

Cluster 5, supported by a bootstrap value of 91%, comprised of isolates b22, b5 and B74 represented by closest neighbours; *Psychrobacter alimentarius* PAMC 27889, *Providencia rettgeri* RB151 and *Alcaligenes faecalis* JQ135 respectively. Nevertheless, a unique trend was noted with this cluster. Not all the representative isolates were positive for mediation of phenols production. Isolates b5 and B74 showed negative results when tested for potential to mediate phenols production in mammalian urine (Table 4.7). In terms of genotypic relatedness, these isolates were distantly related to their counterparts in clusters 1 ansd 2.

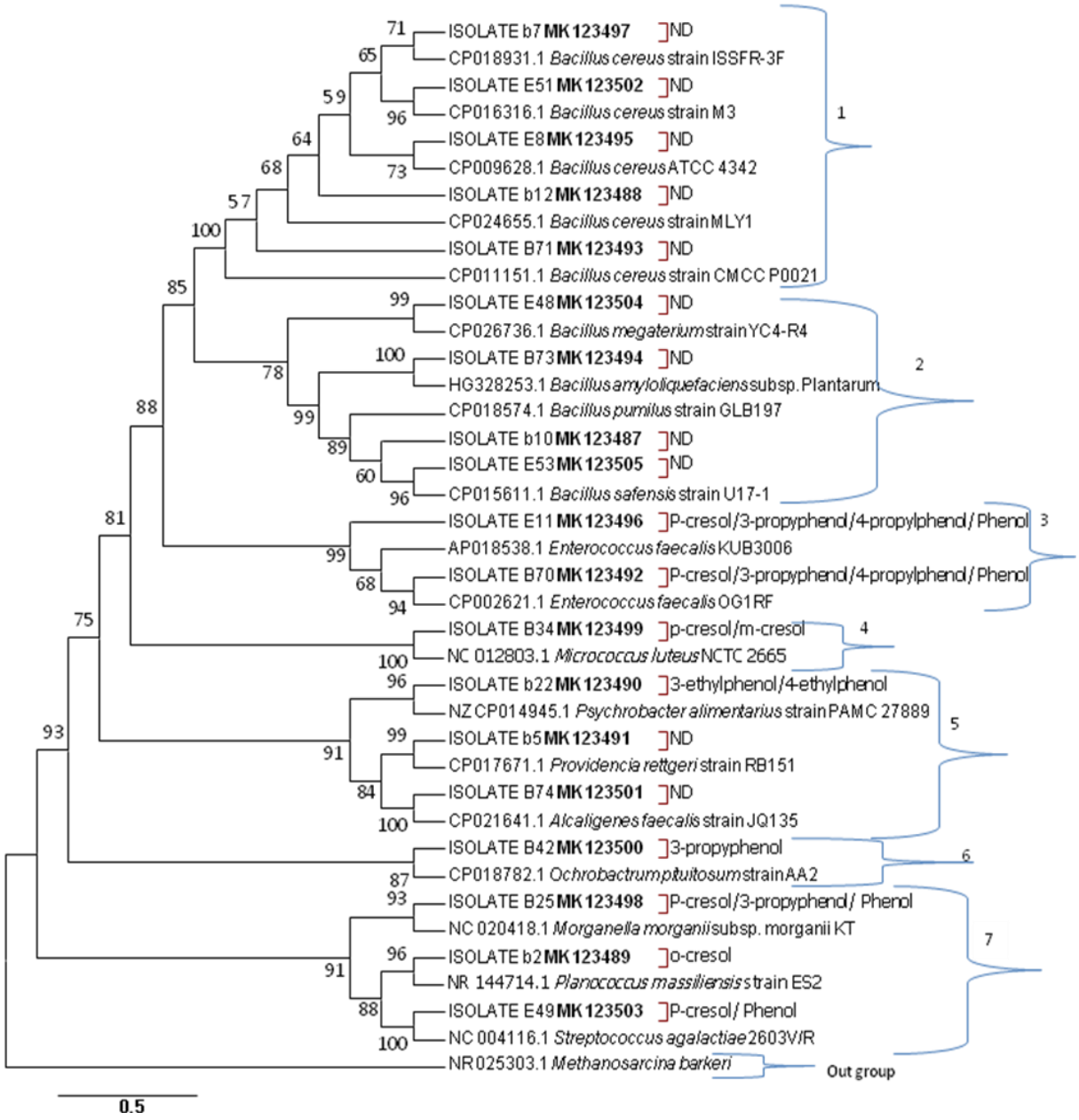

Figure 4.7: Molecular phylogenetic analysis of bacterial communities with and without potential to mediate production of phenols in mammalian urine by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 39 nucleotide sequences. There were a total of 1494 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2015). ND (Not detected**)** is indicated against isolates that were negative for phenols production. Bacterial isolates with potential to mediate phenols production are indicated with their respective phenols in brackets.

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1 Isolation and characterization of cultivable bacteria in fresh mammalian urine

A microbial study carried out on fresh mammalian urine isolates revealed the existence of bacteria in the samples tested. Presence of bacteria in the fresh urine of mammals has been previously reported (Jana *et al.,* 2016), where diverse microbial communities were recovered. Furthermore, the results of this study support previous studies, which revealed that bacteria are omnipresent with adaptability to different environments (Sikuguchi *et al.,* 2002; O'Malley, 2007; Sapp *et al.,* 2012; Ngai *et al.,* 2013). Presence of bacteria in fresh urine in all the study animals could be due to interaction of urine with facultative anaerobic bacteria, colonizing the epithelial cells lining the urethra, at the moment of urine discharge (Madigan and Brock 2009; Hilt *et al.,* 2014; Engelhaupt and Erika, 2014). The bacterial isolates potential to survive in fresh urine samples of the study animals suggests that urine is a suitable growth medium for bacteria. Similar suggestions were expressed by Andreev *et al.* (2017), Daley and Midodzi, (2018) who reported presence of bacteria in fresh urine.

Eland urine was observed, on culturing, to support least population of bacteria, compared to the cattle and buffalo urine. Presence of bacteria in fresh cattle and fresh buffalo urine suggests potential contamination with faecal bacteria during sample collection from female animals. It is worth noting that male elands were used for this study, reducing the possibility of faecal contamination, because of anatomy of their urinary system. The female buffalo's and cattle's urinary system is susceptible to faecal contamination. This explanation is supported by earlier studies by Tarre *et al.* (2004), Kilande *et al.* (2016)

and Andreev *et al.* (2017). They all noted possibilities of faecal contamination during urine sample collections from female animals.

Characterization of fresh urine associated bacterial isolates revealed presence of *Proteus* spp. *Serratia* spp, *Enterobacter* spp, *Klebsiella spp*, *Staphylococcus spp.*, *Streptococcus spp.* and *Enterococcus* spp. The same bacteria were previously identified in mammalian urine samples by Zboromyrska *et al.* (2016). The source of Enterobacteriaceae (*Proteus* spp., *Serratia* spp., *Enterobacter* spp., *Klebsiella* spp., and *Enterococcus* spp.) in fresh urine could be linked to gut flora of the study animals. Many members of Enterobacteriaceae exist as normal part of the gut flora in the intestine of animals, according to findings that were reported by Sikirov *et al.* (2010), Schierack *et al.* (2007) and Drzewiecka *et al.* (2016). The source of *Staphylococcus sp.,* and *Streptococcus sp.* in fresh urine could be from the skin surface of the animals. More details about the existence of such bacteria on animal skin surface can be found in a study carried out by Council *et al.* (2016). Other studies, for instance, Kosecka *et al.* (2018), Foysal and Lisa, (2018), have reported existence of these bacteria in nature including air, soil, water and plants. It is therefore, possible that the animals, in the current study, may have picked them horizontally during foraging activities.

All the bacteria identified in fresh urine have facultative anaerobic traits. Evidence of presence of facultative anaerobes in urine has been presented before, for example, in studies carried out by Chaurasia *et al.* (2015) and Brook, (2004). Facultative anaerobic trait of these isolates suggests that they can survive in different environments. The ability

of facultative anaerobes to survive in different environments was previously reported by Martin, (2017) and Mix *et al.* (2018). It is also possible that the fresh urine bacteria thrived in these conditions because they were primary colonizers of the habitat. It can be postulated that they achieve this growth as the primary successors. This therefore, demonstrates the proficiency of the isolated bacterial communities at same level and condition.

5.1.2. Progressive transformation in bacterial populations and diversity in ageing mammalian urine

The current study was able to cultivate 19 morphologically different bacteria in ageing urine samples when compared to the study carried out by Íñigo *et al.* (2016) who reported 336 correctly identified bacterial species in the mammalian urine samples. This difference in number of bacteria identified could be attributed to the variation in the species under study or in the methods used to culture and identify the isolates. Whereas the findings in this study were drawn from culture depended procedures, the study by Íñigo *et al.* (2016) used culture independent methods to identify bacteria directly from urine samples. Culture-dependent procedures used in this study were based on studying each bacterium in isolation. These methods are limited in nature (Videira *et al*., 2013; Kilande *et al*., 2016; Goel *et al*., 2018).

Evaluation of temporal changes in patterns of cultivable bacterial populations and diversity in fermenting urine revealed a gradual change in these patterns. These findings agree to a study carried out by Zhao *et al*. (2013), who observed shift in population and

diversity of urine associated bacteria. The gradual shift in population and diversity of the bacterial population as urine fermented suggests the dynamics of primary and secondary succession among bacterial communities with differing growth conditions. Previous studies by Chandna *et al.* (2013), Dini-Andreote *et al.* (2014) and Moitinho *et al.* (2018) reported similar findings.

Bacterial communities' dynamics in fermenting urine can be related to many factors (Mohanty *et al.,* 2014). One of the factors is pair-wise interaction occurring in different species in the community, as suggested by Stubbendieck *et al.* (2016). Interactive processes that involve competition are thought to trigger selection pressure, in response to resource conditions, which eventually shapes microbial community structure (Maitra and Dill, 2015; Stingl, 2018). Observed disappearance of some of the earlier urine colonizers as urine fermented could be related to exploitation as well as interference competitions. For instance, in interference competition, persisting urine bacteria may have created inhospitable zones for competitors by releasing diffusible antagonists (toxins and antibiotics). These interactions have been explained by Stubbendieck and Straight (2016). Bacteria, for example *Bacillus pumilus,* which was isolated in ageing urine in this study, have been found to produce compounds antagonist to fungal and bacterial pathogens (Banerjee *et al*., 2007). In addition to exploitation and interference competitions, bacteria that were able to persist in urine with ageing intervals may be highly adapted to growth with their neighbours. This type of adaptation is supported by literature published by Freilich *et al.* (2011).

Bacteria, for example *Enterococcus* spp, that were found to be consistent throughout the ageing period may have relied on developmental functions to survive throughout. Habbing *et al.* (2010) stated that such developmental functions could be used by some microbes to exclude their competitors. This can be achieved through the exclusion of latecomers by bacterial strains already present, (priority effects) in the habitat (Shade *et al*., 2013). In the exclusion of late comers, bacterial communities can change abruptly in response to small perturbations, linked to changing conditions or the presence of multiple stable states (Salonen *et al.,* 2014).. Related to these findings, elimination of coliforms during the composition of animal waste material has also been reported (El Fels *et al*., 2015).

It is also important to note that, acquisition of more nutrients from urine by interacting urine associated bacterial communities may have favoured increase in population and diversity of some bacteria in ageing urine (Ponomarova and Patil, 2015). Related suggestions were documented by Fierer *et al.* (2010). Through "metabolic syntrophism" (Mutalistic metabolism) some bacterial cells can facilitate growth of others. This creates an environment characterised by multispecies biochemical reliance (Prokopenko *et al*., 2013). Morris *et al.* (2013) suggested that metabolic syntrophism is achieved as diverse species posses complementary biochemical pathways needed to liberate nutrient from the environment.

Bacterial communities' dynamics in fermenting urine in this study can as well be attributed to abiotic and microenvironment variables. Stewart and Frankline, (2008)

reported on the influence of microenvironment on the population and diversity of bacterial communities. Hutchison *et al.* (2016) also demonstrated that bacteria are social organisms and interact expansively between and within species while responding to external stimuli from the environment contemporaneously. In the current study, at each fermenting interval of mammalian urine, bacteria were exposed to different environmental growth conditions. In cases where the environment was harsh to colonising bacteria, different growth strategies may have been adapted to fit in the complexities of community structures at different scales resulting in bacterial shift with ageing urine. This suggestion was previously forwarded by Liu *et al.* (2011).

As well, Anandham *et al*. (2015) reported elimination of some bacterial communities colonising cow dung by fermentation process during organic formulation preparation. Based on this analogy, bacteria, for example, *Enterococcus faecalis* and *Planococcus* which were observed to persist in urine with changing conditions can be said to undergo constant growth (nutrient-insensitive). Such bacteria are highly favoured with the changing urine conditions against their counterparts whose growth rate is dependent on the external environment (nutrient-sensitive). Changes in external conditions after that, triggers a change in microbial community state, an occurrence that has been previously explained (Costello *et al.*, 2012; Mao *et al*., 2015).

Biochemical products of fermenting urine could be another possible cause of a shift in bacterial community structures of the present study. Sting, (2018) reported that microorganisms respond to changing environmental conditions by modifying their

number or altering their community diversity. Fermenting urine releases volatile organic compounds (VOC) that may constitute an important regulatory factor in determining the interrelationship between organisms in microbial ecosystems (Humphris *et al*., 2002). This is supported by Schmidt *et al.* (2015), who observed that microbe-microbe interactions are mediated via secondary metabolites. Volatile phenolic compounds generated in urine are believed to alter the physiochemical urine environment causing ecological niche modification.

5.1.3 Generation of phenols in different mammalian urine at different stages of fermentation

Presence of phenolic compounds in DCM urine extracts of the current study confirmed that mammalian urine has a large range of phenolic compounds. These findings are in agreement with previous studies that identified phenols in animal urine (Tangtrakulwanich *et al*., 2015; Alkhaldy *et al*., 2018; Spiehs *et al*., 2018). The presence of tsetse attractant cues in eland ageing urine provides more information on which animals to target when controlling tsetse. Eland has been shown to be resistant to trypanosomiasis (Pappas, 2002). However, it has not been known if the animal's urine has the potential to attract tsetse. Some of the phenolic compounds detected in this study for instance phenol, the parent compound, p-cresol and 3-propylphenols have been previously linked to tsetse behavioural response (Hassanali *et al.*, 1986; Bursel *et al*., 1988; Owaga *et al*., 1988; Madubunyi *et al.*, 1996; Lane and Fraser, 1999).

In the phenol analysis of urine samples of the three animals across fermentation days, this study observed dissimilarities in the types of phenol present. These results highlight the influence that urine ageing period can exert on phenols occurrence. For instance, on day four and day twenty-four, 4-propylphenol, 3-propylphenol and 2-methyloxyphenol were not detected and this trend was observed across the urine samples from the three animals. These dissimilarities can be explained on the bases of various factors, which work either in synergy or independently, during urine fermentation. These factors may include biochemical activities, ageing period, kinetics in bioactivities of urine and natural variability of precursors from which phenols originate. However, this is a confounding finding that needs further investigation.

This study observed that there was an equal distribution of phenolic compounds in urine samples from the three study animals. This result indicates that the type of the animal under study did not determine the variation in phenols detected. Such a trend was observed by Madubunyi *et al.* (1996), while studying buffalos, waterbucks and cattle. This observation showed no differences in type of phenols identified per animal urine sample. The nature of these phenolic compounds is of great interest because they are responsible for some functional properties of tsetse, for example, the behavioural response (Vale *et al.,* 1988; Brightwell *et al*., 1991).

Quantitative analysis of the detected phenols showed their existence in different study animals in about the same ratio. However, these ratios varied across the ageing periods. Therefore the type of mammalian species did not seem to influence phenols concentration. These results can be used to reinforce study findings of Madubunyi *et al.*

(1996) which showed that phenol ratios did not depend on the animals whose urine sample was under study but varied with ageing urine. Limited quantitative differences among specific phenols, at same fermentation period among study animals potentially indicate that there is no inter-individual variability of phenols metabolism.

Phenols concentration variations in urine varied with fermentation days. Therefore, phenols concentration in urine samples was observed to depend on the fermentation period. These results are in line with a study carried out by Alkhady *et al.* (2018), which reported an increase in the volatile organic compounds in urine with increase in fermentation period. In another related study, Williams and Evans (1981) reported an increase in concentrations of phenol, p-cresol during the accumulation of pig manure in a store. Shift in biochemical activities as urine ferments could be linked to variation in phenols concentration observed in the current study. The shift in biochemical activities is a possible indication that there is a shift in bacterial communities some which could be crucial in the mediation of phenols in mammalian urine. This observation might serve as case report on the influence of ageing period on phenols amounts of mammalian urine.

Even though this study observed an increase in phenols concentration with increase in fermentation days, this trend was reversed on day 20 to day 24, when the amounts reduced drastically. This observation has also been recorded by Spoelstra (1980) whose study on decomposing urine indicated that phenol concentration increased with increased measuring period while p-cresol concentration increased initially but decreased with increased measuring period. Overall, the lowest concentration for phenols was found at

initial and last stages of urine fermentation. Although phenols are present in low concentration at these stages, the compounds may still have an impact on the overall odour impression of the samples and may influence, accordingly, tsetse host-seeking behaviour.

5.1.4 Effect of different mammalian urine and their ageing interval on generation of phenols

This study sought to evaluate bacterial generation of phenols in urine samples of study animals at different stages of fermentation. In this evaluation, total numbers of phenols identified across the study animals, and their concentrations, were not significantly different. This revealed that animal specificity had no influence on volatile phenols of bacterial origin detected in this study. A similar observation was made by Lemfack and Piechulla, (2016). These findings further confirm a study reported by Schenkel *et al.* (2015) which stated that habitat specificity has almost no influence on VOC produced by microorganisms. Several factors can be attributed to these findings. Among them is the similarity in dietary components of the study animals. Studies carried out by Martin, (1982), Jung *et al.* (1983), Ha and Lindsay (1991), Lane and Fresar, (1999) revealed dietary proteins as precursors for phenols. A portion of dietary proteins which is not used for growth and other production activities of animals are excreted via urine and faeces. The excretion is normally in the form of; (1) urea, glucuronides and sulphate in urine, (2) non-digested proteins in faeces, and (3) bacterial proteins in faeces. When exposed to bacteria in the environment, the excreted proteins and their metabolites act as precursors for the formation of volatile phenolic compounds.

At different stages of fermentation, there was a significant difference in the total number of phenolic compounds generated and this was observed across all the mammals tested. These results indicate a potential of fermentation period to dictate emission of phenols from mammalian urine. This could be related to many variables that occur during urine fermentation. For example, as reported by Ara *et al.* (2016), shift in bacterial communities with their biochemical activities as urine ages could be a determining factor in the emission of urine phenols. Bacterial communities with the potential to generate the non-detected phenols may not have colonized urine at that moment. The absence of such bacteria in mammalian urine medium at any one particular moment implies that there are no biochemical activities that may lead to the formation of associated phenols. The absence of the phenols and probably the mediating bacteria needs further investigation.

Phenols precursors and their interaction with potential bacterial candidates for phenols generation in urine may have influenced the varied release of volatile phenols as observed in this study. The studies carried out by Le *et al*. (2005) and Hammond *et al*. (1989) which established that p-cresol, phenol and other volatile phenols are released as a result of microbial degradation of amino acids L-tyrosine and L-tryptophan support this suggestion. These amino acids act as precursors for the phenols. According to their study, L-tyrosine is deaminated to 4-hydroxy-phenylpropanoic acid which is later decarboxylated to 4-ethylphenol or oxidized to 4-hydroxyphenylacetic acid. The 4-hydroxyphenylacetic acid is further de-carboxylated to phenolics, for instance, phenol and P-cresol.

Nutrients availability as urine fermented may be associated with variation in total phenolic compounds observed in this study. Microorganisms release particular VOC in a nutrient-rich environment that corresponds to stress-free growth. However, under conditions of competition, limited nutrients or a stressed environment, a different set of microbial VOCs may be produced as the metabolic pathways shift to enable adaptation to resource availabilities (Davis *et al*., 2013). Alternatively, as decomposition progresses, some microbes may not possess the metabolic pathways necessary to utilize the changing resource. These bacteria are replaced by species that possess the necessary metabolic pathways, thus resulting in a fluctuating microbial community structure and with it, a shifting volatiles profile. This argument is supported by previous studies carried out by Schultz and Dickschat (2007), Zhang *et al*. (2009), Kalinová *et al.* (2009) and Von Hoermann *et al.* (2011),

The ability of some bacteria colonising urine to biodegrade phenols is a likely reason why some phenols were not detected at some stage of urine fermentation. Previous studies, for example, Zhang *et al.* (2013) and Banerjee and Ghoshal, (2010), have demonstrated the ability of several bacteria, including *Bacillus* spp., to degrade or oxidize phenolic compounds into other compounds. For that reason, absence or presence of bacteria species at a particular stage of urine fermentation can be related to the presence or absence of the associated phenolic compounds. This may be the reason why some phenols were identified throughout the ageing intervals while others were absent at particular ageing interval.

Just like total phenolic compounds, phenols amounts varied significantly, at different levels of fermenting urine samples. This shift in phenols concentration can be attributed to the dynamics of the release of volatile secondary metabolites by urine colonizing bacteria. During the early stages of urine colonization, the bacteria were in an environment rich with nutrients. In this condition, most bacteria might have adopted primary metabolism to promote growth and facilitate replication which is priority functions in prokaryotes. However, with depleting nutrients in fermenting urine, the colonizing bacteria may have resulted to the utilization of other strategies that activate lower priority pathways useful to metabolize alternative substances as energy sources and produce unique secondary metabolites.

Some phenols concentrations decreased during the fermentation period but were not completely eliminated. This trend was similar in all the animals, across the fermentation period. This observation is in line with the hypothesis that biodegradation of phenols by microorganism has limitations, as explained by Kang *et al*. (2005). This limitation varies with the microorganism involved in biodegradation, their count and conditions (aerobic or anaerobic) (Kang and Kondo, 2002). Kang *et al.* (2005) reported a study on biodegradation in which about 90% of the isolated bacteria in their study could degrade bisphenol to a certain degree, but only 15% were able to do complete bisphenol biodegradation. The combined effect of the main factors on the amounts of phenolic compounds showed a mixed trend. This mixed trend can be related to bacterial communities involved in the generation of specific phenols, and their interaction with other micro biota of urine.

5.1.5 Potential of production of tsetse attractive phenols by urine-based bacterial communities in the selected mammals

When the sterilized fresh urine was fermented with a mixture of isolated bacteria, the amounts of detected phenols were varied. Some were higher; some lower while others were similar in concentration when compared to that of the original urine samples. In a study that looked at mixed bacterial cultures and microbes in the soil, Kai *et al.* (2009) observed that the mixed cultures had potential to produce volatile organic compounds that were higher or lower in concentrations compared to that produced by individual soil microbe. Elsewhere, Troccaz *et al.* (2013) revealed that incubation of sterile urine with a bacterial mixture of *Enterococcus faecalis*, *Escherichia fergusonii*, *S. agalactiae*, *Citrobacter koseri*, and *M. morganii* produced a representative aged urine odour.

The differences in concentration, in the current study, can be attributed to multivariate factors. For example, there could have been competition among the mixed bacteria, where some inhibited activities of their neighbours who might have been responsible for the mediation of phenols production. This suggestion is supported by Andreev *et al*.s' (2017) study which revealed that lactic acid bacteria inhibit activities of some bacteria in urine that breed significant VOC. Some of the bacteria could also not be producing any volatile but are essential for the existence of others that mediate phenols production. In related studies, Huang *et al.* (2004) demonstrated that a mixture of bacteria derived from a natural larval habitat, that included *Pseudomonas, Stenotrophomonas, Enterobacter, Pantoea , Klebsiella, Acinetobacter, Aeromonas,* and *Bacillus* reduced oviposition in

gravid A. *gambiae.* The researchers concluded that communities, and not individual bacteria species, are essential for releasing the VOC complexes that elicit oviposition.

Variation in amounts of detected phenols can as well be attributed to the presence of microbes which naturally prevent the formation of phenols. This is likely achieved through enzymatic activities of the associated bacteria which work against the production of phenols by other species. A similar phenomenon has been reported by Trocazz *et al.* (2016) who noted that lactic acid fermentation of urine resulting from activities of lactic acid bacteria was associated with reduced levels of phenols in urine at some stage. Urine incubated with mixed bacteria may also be comprising bacteria that produce detected phenols and others that break them down once they are formed. This finding suggests that different bacterial communities co-existing in urine are essential determinants for the release of volatile organic compounds complexes needed to elicit tsetse behavioural response.

When the sterilized urine samples were inoculated with individual bacteria for three days, the results revealed a potential of 8 isolates to mediate production of volatile phenols (Table 4.9). These findings are in conformity with a number of studies that include Troccaz *et al.* (2013), Zhang *et al.* (2013), Lie *et al*. (2016), Schulz and Dickschat (2007). In their study, Schulz and Dickschat (2007) associated about 50% to 80% of bacteria investigated for microbial volatile organic compounds with the production of the volatiles. As well, the role of volatiles in determining the behaviour of insects has been reported (Tasin *et al.*, 2018). In the current study, specific phenols were detected in urine

samples inoculated with the specific bacterial isolates. This observation may suggest that species-specific difference exist in the metabolization and excretion of these phenolic compounds.

The presence of volatile phenols from specific individual bacteria in urine samples may be attributed to multiple factors. First, it might be due to the difference in nutritional activities of different bacterial species inoculated in urine samples. These suggestions are supported by previous studies by Teira *et al.* (2011) and Tasin *et al*. (2012) who demonstrated through a nutrients manipulation experiment that bacterial phylogenetic groups respond differently to change in nutrients. Secondly, individual bacterial metabolism could be linked to differences in phenols profiles observed. By use of metabolic pathways of glycolysis, or degradation of amino acids, bacteria can generate diverse aromatic compounds (Schulz and Dickschat, 2007). According to Todar (2012), many VOCs are generated during primary and secondary metabolism in microorganisms. Phenols are products of secondary metabolic processes. Therefore, the production of secondary metabolic phenols in the current study is believed to be species-specific or restricted to a limited phylogenetic group.

Some of the isolated bacteria (b5, b7, b10, b12, E8, E48, E51, E53, B71, B73, and B74) did not record any significant or detectable amounts of phenols (data not shown). The inability of these isolates to mediate phenols production indicates that volatile organic compounds are limited to certain group of microorganisms as suggested by Vherulst *et al.* (2009). This is further supported by an earlier study carried out by Lemfack *et al.* (2016)

which reported significant variation in VOC profiles of some bacteria. Failure by isolates, in the current study, to mediate production of phenols may be linked to different variables in the urine micro biota profile or functionality, a phenomenon which has also been observed by Alkhady *et al.* (2018). One of the variables could be the ability of the inoculated bacteria to act on the phenols precursors and conjugates in urine thus degrading them. Many bacteria can grow on several aromatic compounds as well as their derivatives as the only source of energy and carbon (Basha *et al.,* 2010; Hupart-kocurek *et al.,* 2012). In another study, Pradeep *et al.* (2015) stated that various aspects including microorganism determine the efficiency of phenol biodegradation in environments.

Another variable for absence of phenols in urine samples incubated with individual bacterial species could be insufficient or low levels of bacterial enzymes responsible for activities that trigger biosynthesis of phenols. Waszewska *et al.* (2014) illustrated that staphylococcal and corynebacterial communities co-habiting on human skin do not metabolise substrates using the same enzymatic activities. These observations can be used to describe why the number of VOC generated by E11 from eland urine and B70 from cattle urine studied here was higher than that of b2 from buffalo urine and B42 from cattle urine. These study findings suggest that phenolic compounds associated with tsetse chemoreception are likely to be varied, based on the bacteria involved in the production of that volatiles.

5.1.6 Characterization of bacterial isolates with and without potential to mediate phenols production in ageing mammalian urine

The significant similarity of bacterial isolates, that were positive for mediation of production of phenols, with *Enterococcus faecalis* KUB3006, *Psychrobacter alimentarius* PAMC 27887, *Streptococcus agalactiae* 2603V, *Morganella morganii* sub.sp. *morganii* KT, *Micrococcus luteus* NCTC2665, P*lanococcus massiliensis strain ES2, Ochrobactrum pituitosum* AA2 and *Enterococcus faecalis* OGIRF (Table 4.10), suggests the potential of these bacterial communities to mediate production of phenols in mammalian urine. These findings are supported by earlier studies by Bokkenheuser *et al.* (1987), Schneider *et al.* (1999), Schneider and Blaut, (2000), Schoefer *et al.* (2003), Trocazz *et al*. (2016), and Alkhaldy *et al.* (2018) who demonstrated the ability of different bacteria to metabolise phenols and other volatiles.

In the current study, bacteria having the ability to mediate formation of phenols clustered in closely related phylogenetic positions. This clustering suggests that bacterial communities with potential to mediate production of phenols are restricted to a phylogenetic group. Therefore, these bacteria may have evolved to colonize mammalian urine and mediate production of volatile phenols that influence tsetse-host seeking behaviour. As well, biochemical pathways that are responsible for the production of phenols by bacterial species may be conserved across taxonomic groups of these bacteria. Isolate b2 which closely related to *Psychrobacter alimentarius* PAMC 27887, may have retained critical genes that code for mediation of production of phenols. Unlike her

counterparts in that cluster, the isolate showed potential for mediation of production of phenols.

Enterococcus faecalis strains showed potential to mediate production of p-cresol, phenol and 3-propylphenol. These phenols have been previously reported to attract tsetse to their host animals (Gikonyo *et al*., 2000; Hassanali *et al.,* 1986; Bursel *et al.,* 1988). This bacterium was observed to persist in ageing urine, of all the study animals, with changing conditions, which suggests its ecological importance in tsetse-host seeking behaviour and transmission of AAT. These results closely relate to a study carried out by Trocazz *et al.* (2016) who documented the role of *Enterobacteriaceae* in the production of p-cresol, phenol and other volatiles in human urine. The origin of *Enterococcus faecalis* varies from animals, environmental and human sources. However, the intestinal tract of humans and animals is the natural habitat of this bacterium (Klein, 2003). In a study carried out in Germany on samples of animal origin reported that *Enterococcus faecalis* were the most dominant (72%) of all the *Enterococcus* spp. isolated (Peters *et al*., 2003). As reviewed by Fisher and Philips (2009), this bacterium is tolerant to a wide range of stress and hostile environmental conditions and can colonise a wide range of niche. Features that make this bacterium tolerant include among others their malleable genomes, which may contribute to their adaptation to harsh environments and increase the ability of certain lineages to colonize the ageing urine. It is further reported that the bacterium can survive for a long time outside their natural intestinal hosts (Arias and Murray, 2012). This may explain why, in the current study, the bacterium was able to persist in the conditions of

ageing mammalian urine. This long interaction with ageing urine provides a platform for the generation of urine phenols as observed in this study.

Morganella morganii sub.sp. *morganii*, just like *Enterococcus faecalis* strains showed potential to generate p-cresol, phenol and 3-propylphenol. These phenols have an ecological role in the attraction of tsetse vector to the host (Bursel *et al.,* 1988). Ability of this bacterium to survive in ageing urine samples of all the study animals suggests that it is of immense influence on tsetse-host seeking behaviour and transmission of AAT to livestock and wildlife. These findings compare to those of a study carried out by Marshall *et al.* (2016) who established that *Morganella morganii* produces phenol as sex pheromone of the New Zealand grass grub (*Costelytra zealandica*). Their study further highlighted that this phenol is biosynthesised from the amino acid tyrosine in the colleteral gland, by this bacterium. *Morganella morganii* is often found in human and animals gut (Jones-Dias *et al*., 2016). The bacterium has also been found to be common in livestock environment (Fischer *et al*., 2016; Rodrigues *et al*., 2017; Wang *et al*., 2017). Through its interaction with animals, it is likely that this bacterium gains access to mammalian urine where it mediates production of associated urine phenols.

In this study, *Streptococcus agalactiae* 2603V bacterium, isolated from ageing urine of the study animals, showed potential to mediate production of phenol and 3-propylphenol which are known to attract tsetse. The incidences of this bacterium to mediate production of volatiles, in the study samples, compares well with other findings. For example, production of 3-methylbutanoic acid volatiles in sterilized milk strongly correlated with

growth of S*taphylococcus aureus* when mixed with S*taphylococcus agalactiae* (Chen *et al*., 2018). S*taphylococcus agalactiae* has been isolated in different bovine environments, and reported by researchers in studies such as Reyes *et al.* (2017), Svennesen *et al.* (2018), Holmøy *et al.* (2018), Skjstrup *et al.* (2018) and Miranda *et al.* (2018). Jørgensen *et al.* (2016) reported that strains belonging to this bacterium differ in their ability to survive in the environment. This may explain the presence of this specific bacterium strain in the urine of the study animals. The presence of this bacterium in the bovine environment creates an opportunity for the bacterium to colonise mammalian urine and thus mediate the formation of phenols as observed in this study. Once phenols are generated for example, in grazing fields, they influence tsetse behavioural response towards their host animals which increases opportunity for AAT transmission.

Micrococcus leteus, NCTC 2665, in the current study, showed the ability to generate p-cresol and m-cresol. Previous studies, for example, field experiments involving tsetse behavioural response by Brightwell *et al.* (1991) detected the presence of p-cresol in buffalo urine samples. Mediation of the production of phenols by this bacterium in the current study may explain earlier reports of the involvement of skin bacteria in the production of volatiles that determine host-seeking behaviour of some insects (Verhulst *et al*., 2009; Verhulst *et al*., 2011). *Micrococcus* spp. primary natural habitat is mammalian skin; secondary habitats include meat, dairy products, soil and water (Grice *et al*., 2009; Weyrich *et al.,* 2015). In related studies that targeted the analysis of mosquito attractive cues in animals and human skin bacteria, Busula *et al.* (2017) reported the potential of a mixture of volatiles released by skin bacteria of cow as well as

human skin bacteria to attract mosquito species. It is, therefore noteworthy to suggest that this bacterium potentially plays a role in phenols production in wildlife and livestock when urine comes in contact with animal urine.

This study established that *Psychrobacter alimentarius* PAMC 27889, constitute a proportion of ageing mammalian urine flora. The bacterium showed the ability to generate 3 and 4-ethylphenols. These phenols have been previously attributed to the attractiveness of cattle urine to AAT vector (Bursell *et al.,* 1988). A study carried out by Broekaert *et al.* (2013) established, sea food spoilage, volatile compounds associated with *Psychrobacter* spp. In a related study, Trexeler *et al*., (2003) reported that gravid *Aedes abopictus* oviposited more frequently in water inoculated with a member of *Psychrobacter spp*. The author's findings potentially suggest that the bacterium plays a role in the attraction of the vector to the preferred oviposition sites. Members of *Psychrobacter* spp. have been previously isolated in different environments. For instance, pig and dairy manure (Hamm *et al.,* 2016), soil, decomposing bovine materials, meat, dairy products and poultry (Gonza´lez *et al*., 2000; Bjorkevoll *et al*., 2003; Fjellheim *et al*., 2007; Meziti *et al*., 2010; Yang *et al*., 2017). Association of *Psychrobacter* spp. with bovine environment could be favourable prospect for *Psychrobacter alimentarius* PAMC 27889 to colonise mammalian urine, leading to formation of the urine phenols.

Ochrobactrum pituitosum AA2 bacterium was found to have the potential of mediating production of 3-propylphenol which is a critical compound of tsetse attraction to the host. A study involving rhizospheres profiled this bacterium as having the ability to produce

VOC that promote plant growth (Baysal *et al*., 2017). Members of *Ochrobactrum* spp. are omnipresent and have been isolated from several ecological niches for instance water, soils, plants animals and humans (Kulkami *et al*., 2017; Dini-Andreote *et al*., 2018). Even though there is no much information about this bacterium concerning volatiles generation, interaction of *Ochrobactrum* spp. with several ecological niches presents an opportunity for *Ochrobactrum pituitosum* AA2 to colonise mammalian urine in the environment which likely mediates formation of 3-propylphenol.

Planococcus massiliensis strain *ES2* in this study was found with potential to generate o-cresol when inoculated in sterilized urine. Currently, it is not clear if the o-cresol has the potential to attract tsetse like its cresol counterparts. Brega *et al.* (1990) documented the presence of this particular phenolic compound in human urine even though the presence was not linked to this bacterium. This bacterium was previously isolated in the human gut, sea water, clamp prawns and marine (Seck *et al*., 2016; Too *et al*., 2017). Ability to survive in different environments may be related to their presence in fermenting urine of mammals where they are associated with the production of o-cresol as established in this study.

Providencia rettgeri RB151 *and Alcaligenes faecalis* JQ135, on the other hand, were negative for phenols production. These members clustered together with *Psychrobacter alimentarius* PAMC 27887 which showed potential for phenols generation. This could be an indication that potential to mediate production of tsetse attractive phenols have evolved independently in bacterial communities. It may as well suggest that *Providencia*

rettgeri and Alcaligenes faecalis have lost, through mutation, critical genes that are essential in the mediation of formation of phenols in mammalian urine. A previous study (Heibuth *et al*., 2015) has showed the ability of members of the genus to degrade phenol in activated sludge tanks. Other bacteria with the ability to biodegrade phenols have also been isolated from human gut, animal manure and cattle milk (Chander *et al*., 2006; Savage *et al*., 2017).

In the current study, *Providencia rettgeri* RB151 *and Alcaligenes faecalis* JQ135 are likely to have broken down phenolic compounds, in the ageing urine, into non-volatile simpler elements through biodegrading. The phenolic compounds, in this case, can be considered as the bacteria food source or substrate, deriving carbon and energy from the compounds. Alternatively, it could be that the bacteria naturally lack biochemical activities that contribute to the generation of phenols in mammalian urine. *Providencia* spp. *and Alcaligenes* spp. have been isolated from nature, clinical, human and animal samples (Blaiotta *et al.*, 2016).

Members belonging *Bacillus* spp. were negative for phenols production when tested in this study. Previous studies, involving members of this genus showed ability of some members to biodegrade phenols and other organic compounds (Felshia *et al*., 2017; Iqbal *et al*., 2018; Zhang *et al*., 2018; Eragat *et al*., 2018). *Bacillus* spp. comprises an assorted group of bacteria extensively distributed in nature (Parvathi *et al*., 2009; Gopal *et al*., 2015; Singh *et al*., 2016). The members belonging to this genus have been isolated in many areas that include but are not limited to dairy farms (Owusu-Kwarteng *et al*., 2017),

fresh water (Larrea-Murrel *et al*., 2018), soil (Xavier *et al*., 2017) and wildlife (Meyer *et al*.,2014). The species adapt easily to diverse habitat. The ability of these bacteria to biodegrade could have played a role in the suppression of phenolic compounds towards the end of urine ageing intervals.

Likewise, members of *Bacillus* genus produce a large number of bioactive metabolites that have been revealed to exhibit antibacterial and antifungal activities (Tameiro *et al*., 2002). This phenomenon may be associated with a decrease in other bacterial species colonising urine towards the end of fermentation period when there was a high abundance of *Bacillus* spp. *Bacillus amyloliquefaciens* subsp. *plantarum,* for example, is known for its bio control against plant pathogens (Borris *et al*., 2011; Borris *et al*., 2015; Chowdhury *et al*., 2015). Further analysis of this bacterium has revealed its impressive capability to synthesise a diverse spectrum of secondary metabolites that suppress microbes (Chen *et al*., 2009a; Borris *et al*., 2013). Therefore, it is alluring to hypothesize that the occurrence of members of *Bacillus* genera in mammalian urine could have played a part in the suppression of phenols formation. This may have happened directly by degrading phenols or indirectly by eliminating bacterial communities in urine habitat associated with phenols formation.

5.2 CONCLUSIONS

The current study sought to profile bacterial communities' in mammalian urine that mediate production of tsetse attractive phenols and posit important concerns that would be used in control against trypanosomiasis.

i. There are bacterial communities in fresh and ageing cattle, buffalo and eland urine. Cattle fresh urine revealed 4 bacterial isolates. Buffalo and eland fresh unrine samples revealed 3 bacterial isolates each. The bacteria identified in fresh urine based on morphological and biochemical characterization includes *Proteus* spp., *Enterobacter* spp., *Serratia* spp., *Klebsiella* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp.

ii. Nine phenolic compounds: phenol, meta-cresol, para-cresol, ortho-cresol, 3-ethylphenol, 3-n-propylphenol, 2-methyloxyphenol, 4-ethylphenol and 4-propylphenol, were detected in ageing urine samples of cattle, buffalo and eland. Phenols generation in cattle, buffalo and eland urine varied depending on the day of fermentation with highest expression levels observed between days, 16 and 20 where highest diversity of urine-based bacteria was reported. However, the species of the three study animals did not affect the generation of the phenols. This study indicates that fermentation period and bacterial communities influence the production of certain volatile phenols in mammalian urine.

iii. The bacteria identified that appear to mediate production of phenolic compounds in ageing cattle, buffalo and eland urine include: *Psychrobacter alimentarius* PAMC 27887, *Enterococcus faecalis* KUB3006, *Streptococcus agalactiae* 2603V, *Morganella morganii* sub.sp. *morganii* KT, *Micrococcus luteus* NCTC 2665, *Ochrobactrum pituitosum* AA2 and *Enterococcus faecalis* OGIRF and P*lanococcus massiliensis* strain ES2.

5.3 RECOMMENDATIONS

i. This study provides insight into the influence of bacterial communities in the formation of urine phenols in wildlife and livestock. On the basis of this information, biotechnological models should be developed to reduce the emission of odorous phenols in grazing fields and reduce the attraction of tsetse to livestock and wildlife.

ii. The identified bacteria that mediate production of tsetse attractive phenols in mammalian urine should be incorporated in AAT control operations. This will open potential pathways of reducing the attraction of tsetse to the host animals and enhancing “push-pull” models in vector control and trypanosomiasis elimination interventions

iii. This study hypothesizes that tsetse attraction to livestock can be controlled significantly by achieving optimum solution in reduction of bacteria that mediate production of phenols. However, more investigation needs to be done to significantly specify this optimum reduction.

iv. The current study focused only on cultivable bacteria. Further bacteriological studies of ageing urine by use of culture independent procedures can help reveal the uncultivable bacteria in urine involved in the generation of phenols.

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