Potential use of bacterial community observed during decomposition of pig (*Sus scrofa domesticus*) and sheep (*Ovis aries*) carrion in estimating the post-mortem interval during the cold season in KwaZulu-Natal Province of South Africa.

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PREFACE

The research contained in this dissertation was completed by the candidate, from June 2019 to December 2020 while based in the Discipline of Parasitology, School of Life Sciences, University of KwaZulu-Natal, Westville Campus, under the supervision of Professor S. Mukaratirwa. The research was financially supported by Prof. Mukaratirwa through his UKZN Research Productivity Incentive funds. The contents of this study represent original work by the author and have not been submitted in any form to another tertiary institution, except where the work of others is acknowledged in the text.

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DECLARATION

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ABSTRACT

This study was aimed at identifying and describing the bacterial communities present during the decomposition stages of both pig and sheep carcasses, with the potential use of these bacterial communities aiding in estimating the post-mortem interval (PMI). One pig (*Sus scrofa domesticus*) and one sheep (*Ovis aries*) carcasses were used for this study during the cold season and the study location was Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Five stages of decomposition fresh (0-1 d), bloat (2-6 d), active decay (7-12 d), advanced decay (13-51 d) and dry (5258 d) were observed and described for both the pig and sheep carcasses at the same duration from June till August 2019. Temperatures of the carcass and soil (surrounding environment) was recorded. Temperatures of the individual carcasses changed during each stage of decomposition which was influenced by the surrounding environment temperatures. Five bacterial genera (*Bacillus*, *Leclercia*, *Myroides*, *Pseudomonas* and *Providencia*) were identified using 16S rRNA gene. During the pig decomposition *Myroides* and *Pseudmonas* spp. were absent and *Leclercia* sp. was absent in the sheep decomposition process. *Providencia vermicola* was observed during the fresh till the advanced decay of pig decomposition. On a phylum level Proteobacteria was persistent from the fresh till the end of advanced decay stage of the pig carcass, while on the sheep carcass it was persistent till the end of the active stage. *Bacillus cereus* belonging to the phylum Firmicutes was observed only during the dry stage of the pig decomposition process. During sheep decomposition *Providencia* sp. were observed during the fresh till the end of active decay stage and *Bacillus cereus* was observed during the advanced decay and dry stages. Results showed that Proteobacteria was present on both carcasses at the beginning and were absent at the end of decomposition, Firmicutes was present only on the later (advanced decay and dry) stages of decomposition. The presence and absence of different bacterial species during different stages of decomposition on pig and sheep carcasses, indicates their importance in potentially estimating the post mortem interval (PMI), in forensic investigation in KwaZulu- Natal, South Africa. Although this research indicates that bacterial communities can be utilized for PMI estimations, further research is required to better understand the role of bacteria during the decomposition process. This research is especially needed in the different regions of South Africa, as results cannot be extrapolated beyond the countries/regions of study since the bacterial species spectrum vary with geographical regions and conditions.

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LIST OF ABBREVIATIONS

16SrRNA	16S ribosomal Ribonucleic acid
ADD	Accumulated degree days
Bp	Base pairs
BLAST	Basic local alignment search tool
d	Day
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Fig.	Figure
ML	Maximum likelihood
µL	Microlitre
µM	Micromolar
MEGA	Molecular evolutionary genetics analysis
NJ	Neighbour-joining
PCR	Polymerase chain reaction
PMI	Post-mortem interval
Sp.	Species
TAE	Tris – acetate – EDTA
°C	Degrees Celsius

TABLE OF CONTENTS

COVER PAGE ..... **i**
PREFACE ..... **ii**
DECLARATION ..... **iii**
ABSTRACT ..... **iv**
ACKNOWLEDGEMENTS ..... **v**
LIST OF ABBREVIATIONS ..... **vi**
TABLE OF CONTENTS ..... **vii- viii**
LIST OF TABLES AND APPENDICES ..... **ix**
LIST OF FIGURES ..... **x**
CHAPTER 1: INTRODUCTION **1**
1.1 Background **1**
1.2 Aims and Objectives **2**
1.2.2 Aim **2**
1.2.2 Specific Objectives **2**
CHAPTER 2: LITERATURE REVIEW **3**
2.1 Introduction **3**
2.2 Stages of decomposition **3**
2.2.1. The fresh stage **4**
2.2.2. The bloat stage **4**
2.2.3. The active decay stage **4**
2.2.4. The advanced decay stage **5**
2.2.5. The dry stage **5**
2.3 Epinecrotic communities (skin microflora and buccal) **5**
2.4 The role of bacterial communities in carcass decomposition **7**
2.5 The role of bacterial communities in post-mortem interval (PMI) estimations **8**
CHAPTER 3: MATERIALS AND METHODS **10**
3.1 Study site **10**
3.2 Study animals **11**
3.3 Sampling procedures **11**
3.5 DNA Extraction of buccal and skin samples **12**
3.6 Polymerase chain reaction (PCR) and gel electrophoresis **12**
3.7. Sequencing and phylogenetic analysis **13**
CHAPTER 4: RESULTS **14**
4.1. Decomposition stages **14**
4.1.1. Fresh Stage (0–1 d) **14**

4.1.2. Bloat stage (2-6 d) **14**
4.1.3. Active Stage (7-12 d) **15**
4.1.4. Advanced stage (13–51 d) **16**
4.1.5. Dry stage (51-58 d) **16**
4.2. Phylogenetic relationships of bacterial isolates from *Sus scrofa domesticus* (pig) and *Ovis aries* (sheep) based on the 16S rRNA gene **20**
CHAPTER 5: DISCUSSION **22**
CHAPTER 6: CONCLUSION **28**
References **29**
Appendices **36**
Appendix 1: Solutions **36**
1.1 Preparation of 0.1% Peptone water buffered **36**
1.2 Preparation of 60% Glycerol **36**
1.3 Preparation of Nutrient Broth **36**
1.4 Preparation of Nutrient Agar **36**
1.5 Preparation 1.8% agarose gel **36**

LIST OF TABLES AND APPENDICES

Table 4.1: Mean temperatures ^{O}C of the carcass and soil during the different stages of decomposition during the cold season.

Table 4.2: Microbial communities found on the skin and buccal cavity of pig and sheep carcasses during different stages of decomposition.

Appendix 1: Solutions

LIST OF FIGURES

Figure 3.1: The Ukulinga Research Farm near the city of Pietermaritzburg in the province of KwaZulu-Natal, South Africa.

Figure 4.1 A: Line graph illustrating the pig (*Sus scrofa domesticus*) and soil temperatures at each stage of decomposition

Figure 4.1 B: Line graph illustrating the sheep (*Ovis aries*) and soil temperatures at each stage of decomposition

Figure 4.2: Phylogenetic tree showing the relationship between the bacterial isolates obtained from pig (*Sus scrofa domesticus*) and sheep (*Ovis aries*) carcass using 16S rRNA Gene.

CHAPTER 1: INTRODUCTION

1.1 Background

Microbial communities are abundant and comprise of diverse organisms which inhabit almost every surrounding and environment. These microbial communities are found in both human and animal bodies and they have a significant role in both living and dead organisms (Finley et al., 2015). According to a study by Metcalf et al. (2013) microbial communities are divided into two different types depending on where they are located on the body parts (Dash and Das., 2020). These types include the thanatomicrobiome (Can et al., 2014; Hauther et al., 2015) and the epinecrotic communities (Pechal et al., 2013; Metcalf et al., 2016; Carter et al., 2017). Thanatomicrobiome are microorganisms (microbes) that decompose and colonize the internal organs and orifices of the host after death (Can et al., 2014; Hauther et al., 2015). The epinecrobiome community (microorganisms, insects, arthropods) reside and move on the surface of decomposing remains like on the skin or in the mouth cavity (Pechal et al., 2013; Carter et al., 2017). Both communities have a primary function in carcass decomposition (Javan et al., 2016 a, b; Dowell-Curby., 2017).

Human and animal decomposition is the breakdown of organic remains by interaction of biotic and abiotic components (Finley et al., 2015; Wescott., 2018) and is divided into multiple stages known as the fresh, bloat, decay/active, advanced/post-decay, and dry stages (Wolff et al., 2001; Goff., 2009). According to Dowell-Curby. (2017), each stage of decomposition attracts different types of organisms which in turn introduces various types of bacteria to the carcass. As a result, a decomposing body host a wide range of microbial communities which tend to change with the stages of decomposition. The ability of these bacterial communities to change over time has been regarded as a potential indicator of estimating the post-mortem interval (PMI) in forensics investigations (Hyde et al, 2013; Metcalf et al., 2013; Pechal et al., 2013). As such, there has been an increased interest in studying and identifying bacterial communities associated with different stages of decomposition (Lauber et al., 2009; Hyde et al., 2013; Pechal et al., 2013, 2014; Amaral-Zettler et al., 2015).

More research on the bacterial communities of forensic importance has been conducted in many countries in the northern hemisphere (Hyde et al., 2013, 2015; Metcalf et al., 2013, 2016; Pechal et al., 2013, 2014; Carter et al., 2015, 2017), while this research is still emerging in many regions in the southern hemisphere. Furthermore, results obtained from the northern hemisphere cannot be extrapolated beyond different countries and regions of study as bacterial species vary with geographical region and conditions. This study contributes new research and key information on the bacterial species found on both the sheep and pig carcasses during the different stages of decomposition in South Africa.

1.2 Aims and Objectives

1.2.2 Aim

1.2.1.1 To identify bacterial communities during decomposition stages of sheep and pig carcass and describe the colonization pattern of the identified communities.

1.2.2 Specific Objectives

1.2.2.1 To identify the bacterial taxa found during the different stages of decomposition of pig and sheep carcass

1.2.2.2 To determine the pattern of bacterial taxa found during the different stages decomposition of pig and sheep carcass

1.2.2.3 To assess the usefulness of identified bacteria in estimating the post-mortem interval of the pig and sheep carcass.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Decomposition is facilitated by biotic and abiotic factors such as environmental and weather conditions, as well as arthropod, scavenger, and microbial activities (Parkinson et al., 2009; Hyde et al., 2013, 2015; Metcalf et al., 2013; Lauber et al., 2014; Carter et al., 2015). High humidity and high temperature, extreme insect and microbial activity can proceed to a rapid decomposition process whereas, very low or freezing temperatures will cause a slow decomposition process (Parkinson et al., 2009; Carter et al., 2017; Zurgani ., 2018). The decomposition process is further influenced by factors such as geographical region and location, the different seasons, ante-mortem manipulation of the corpse/ carcass before death or post-mortem manipulation of corpse/carcass (Parkinson et al., 2009; Hyde et al., 2013, 2015; Metcalf et al., 2013; Lauber et al., 2014; Carter et al., 2015, 2017).

Microbes play a vital role in decomposition (Metcalf et al., 2013) and over the years there has been a growing interest in the study of microbiome decomposition activity (Hyde et al. 2013; Metcalf et al. 2013, 2016; Pechal et al., 2013, 2014; Guo et al., 2016; Carter et al., 2017; Zurgani., 2018). Research studies by Parkinson. (2009); Hyde et al. (2013); Metcalf et al. (2013) and Pechal et al. (2013, 2014), over the years have shown that there are complex bacterial communities associated with the decomposition of carcasses.

The decomposition process is helpful in crime investigations as forensic investigators have been exploring how bacteria influence the nature and rate of decomposition. This knowledge is extremely important when estimating the post-mortem interval (PMI) or time since death, since most researchers have indicated that the analysis of bacterial communities have the potential to be used in PMI estimation (Pechal et al., 2012, 2013; Hyde et al., 2013; Metcalf et al., 2013; Lauber et al., 2014; Carter et al., 2015, 2017).

2.2 Stages of decomposition

Decomposition is a biochemical process or break down of dead matter which is controlled by different enzymatic reactions and the loss of processes within a host organism that helps to maintain cellular integrity (Hyde et al., 2013; Brooks., 2016; Carter et al., 2017; Zurgani., 2018; Shedge et al., 2020). During the decomposition process, a dead body or carcass usually passes through several stages of decomposition (Wolff et al., 2001) which include fresh, bloated, active decay, advanced decay, and dry. This characterization of each stage has been determined by the physical changes and the body temperatures of the carcass internally and externally (Wolff et al., 2001).

2.2.1. The fresh stage

The fresh stage begins with sharp and sudden decrease in the body temperature which occurs directly after the time of death (Kelly et al., 2006, 2008; Janaway et al., 2009; Brooks., 2016; Shedge et al., 2020). When an animal is dead their heart fails to beat and pump blood and this interrupts the blood circulation resulting in the appearance of purple-red discoloration on the outer areas of the body. This process is known as *livor mortis* (Kelly et al., 2006, 2008; Gonder., 2008; Finley et al., 2015; Brooks., 2016; Carter et al., 2017; Marais- Werner et al., 2017; Zurgani., 2018).

Towards the beginning of the fresh stage or just after death, the cells of the dead body release certain hydrolytic cellular enzymes, which facilitates to proceed with autolysis and decomposition (Shedge et al., 2020).

Autolysis is a self-digestion process which begins approximately 4 minutes after death and leads to decomposition (Yang et al., 2015). The release of cellular contents in the dead body (mostly internal) creates a suitable environment for microorganisms (bacterial communities) to grow and degrade the surrounding tissues (Brooks., 2016; Shedge et al., 2020). Anaerobic microbes secreted from the gastrointestinal tract and respiratory system degrade carbohydrates lipids and proteins and converts them into gases (Hyde et al., 2013; Brooks., 2016; Carter et al., 2017; Zurgani., 2018; Shedge et al., 2020).

2.2.2. The bloat stage

Bacterial putrefaction within the gastrointestinal tract, respiratory system, and other areas of the body results in the loss of body tissues (potential evidence for forensic investigation) and leads to the formation of gases like carbon dioxide, hydrogen sulphide, and methane (Hyde et al., 2013; Finley et al., 2015; Brooks., 2016; Carter et al., 2017; Zurgani., 2018; Shedge et al., 2020;). These gases are formed as a side product of anaerobiosis as the anaerobic bacteria start to digest the tissue around the abdomen and which results in inflation/bloating of the abdomen, colour changes to the outer skin (Parkinson et al., 2009; Zurgani., 2018; Shedge et al., 2020), the attraction of specific necrophagous arthropods and the release of strong odours (Finley et al., 2015; Brooks., 2016; Carter et al., 2017; Zurgani., 2018; Shedge et al., 2020). After the carcass inflates/bloats to a certain extent it ruptures (Brooks., 2016; Carter et al., 2017; Shedge et al., 2020), releasing many fluids that can give support and provide a suitable environment for the microbial biomass to grow, changing the nature of the faunal communities that are present within the soil (Hyde et al., 2013; Finley et al., 2015; Brooks., 2016; Carter et al., 2017; Zurgani., 2018; Shedge et al., 2020).

2.2.3. The active decay stage

The active decay stage, is characterized by major changes to the carcass's outer skin colour, which changes from green to blue/purple and then to black (Kelly et al., 2006; Gonder., 2008; Parkinson et al.,

2009; Marais- Werner et al., 2017). Gases that were produced in the body are no longer reserved under the skin after the rupture (Parkinson et al., 2009; Carter et al., 2017; Zurgani., 2018; Shedge et al., 2020). As the gases exit the carcass, the body temperature fluctuates and the body eventually collapses itself (Kelly et al., 2006, 2008, 2011; Carter et al., 2017; Zurgani., 2018; Shedge et al., 2020). The surface skin is observed to separate from the dermis at this stage of the decomposition (Zurgani., 2018) and the flesh on the body, is rapidly lost due to the extensive maggot and bacterial activities along with the extensive release of the fluids from the carcass. A wetness in the body and a very prominent odour is observed during this stage (Gunn., 2011; Marais- Werner et al., 2017; Carter et al., 2017; Zurgani., 2018).

2.2.4. The advanced decay stage

During this stage there is a decrease in rate of decomposition as the maximum consumption of the dead matter has already occurred. As a result, mainly dry remains such as bones, dry skin, and hair remain intact, these are the structures that are observable at the end of advanced decomposition. (Gonder., 2008; Parkinson et al., 2009; Gilbert., 2014; Finley et al., 2015). As the advanced decay stage progresses it becomes challenging to identify the sequence of transition from the advanced decay stage to the dry decay with different cadavers and carrions skin (Kelly et al., 2006; Gonder., 2008; Parkinson et al., 2009; Marais- Werner et al., 2017; Zurgani., 2018).

2.2.5. The dry stage

The dry stage represents the end of the decomposition process with no soft tissue residual. This stage occurs when the flesh, fat, layers of skin and cartilage disappear from the decomposed carcass and only bones, dry skin, and hair remain (Kelly et al., 2006; Gonder., 2008; Parkinson et al., 2009; Marais-Werner et al., 2017; Zurgani., 2018). At this point, most of the soft tissues are decomposed, and only the rigid structures of the body, like the skeleton, remain intact (Gunn., 2011; Zurgani., 2018).

2.3 Epinecrotic communities (skin microflora and buccal)

The epinecrotic microbial communities of decomposing animal carcasses and human cadavers are unique to themselves (Pechal et al., 2013). In most studies swine and mouse models are used as proxies for understanding human decomposition and the postmortem – interval (PMI) during crime investigations (Pechal et al., 2012, 2013, 2014; Metcalf et al., 2013; Yang et al., 2015; etc.,). However, there have been certain studies that were granted ethical approval to use human cadavers such as Cobaugh et al. (2015); Hyde et al., (2015). Therefore, it is important to understand both the human and animal carcass bacterial spectrum, as most studies consider animal proxies as human analogues for PMI investigations (Pechal et al., 2012, 2013, 2014; Metcalf et al., 2013; Yang et al., 2015).

Human have their own unique bacterial spectrum present on both the inside and outside of their bodies, which is called the microbiome (Hooper and Gordon., 2001). The skin is the largest organ of the human

body (Roth and James., 1988), therefore, it is a complex habitat for different bacteria (Aly et al., 1991) and has the second largest number of bacteria present (Roth and James., 1988). More than 90% of the human skin bacteria are classified into four different types: Actinobacteria (52%), Firmicutes (24%), Proteobacteria (16%), and Bacteroidetes (6%) (Grice et al., 2009). The dominant bacterial genera include *Acinetobacter*, *Corynebacterium*, *Micrococcus*, *Streptococcus*, anaerobic *Cutibacterium acnes* (formerly: *Propionibacterium acnes*) and coagulase-negative staphylococci, especially *Staphylococcus epidermidis* (Cundell., 2018; Murillo and Raoult., 2013; Andersen., 2019; Buerger., 2020). Bacteria belonging to the *Cutibacterium*, *Staphylococcus*, and *Corynebacterium* genera, are estimated to constitute 45 % to 80% of the entire skin microbiome, from isolated skin samples (Samaras and Hoptroff., 2020).

The oral cavity characteristically has some of the most diverse microbial communities, and these bacterial families include *Lactobacillaceae*, *Staphylococcaceae*, *Streptococcaceae*, and *Actinomycetaceae* (Adserias-Garriga et al., 2017). These bacterial taxa are observed to be the most dominant during the fresh stage of decomposition (Adserias-Garriga et al., 2017), and decomposition proceeds, these bacterial families begin to decrease in abundance and the phylum Tenericutes start to colonize the oral cavity (Adserias-Garriga et al., 2017). Adserias-Garriga et al. (2017), observed that the phylum *Tenericutes* was present in the oral cavity of a corpse that had reached the bloat stage. This particular phylum is found in the gastrointestinal microbiome, therefore, their presence in the bloat stage may be due to the gases purged out of the body (Adserias-Garriga et al., 2017).

A study by Gao et al. (2007), observed the interaction between bacteria and decomposition rate for both human and non-human models. Samples of the skin and buccal cavity microflora were obtained and examined. Observations indicated that every individual contained bacteria that where unique to themselves. Bacteria such as Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes were present on the skin, with Actinobacteria being predominant (Gao et al., 2007).

Javan et al. (2016 b), found that as the oral cavity decomposes, like *Clostridium* spp. from the phylum Firmicutes and other obligate anaerobes such as the genus *Prevotella* tend to dominate towards the later stages of decomposition. Javan et al. (2016 b) and Adserias-Garriga et al. (2017), observed an increase in the abundance of Gammaproteobacteria, *Pseudomonadaceae*, *Alcaligenaceae*, and *Planococcaceae* during the later stages of decomposition. These studies observed that there is a change in the original bacteria found in the oral cavity from facultative anaerobes (e.g., *Staphylococcaceae* and *Streptococcaceae*) to obligate anaerobes (*Clostridales*, *Prevotella* spp. and Pseudomonadaceae) (Javan et al., 2016 b; Adserias-Garriga et al., 2017).

Hyde et al. (2015), examined several skin, mouth, and rectum samples from two carcasses every day for two weeks. The oral and skin samples of both carcasses showed a similar successive trend at the phylum level, as they were colonized by similar bacterial species. In the early decomposition stages

Proteobacteria with Firmicutes was dominant, however, decreased as the decomposition progressed, and Actinobacteria became dominant towards the later stages of decomposition.

A study by Pechal et al. (2018), observed that during the fresh stage the oral cavity thanatomicrobiomes had two major phyla, Firmicutes and Actinobacteria spp., however the bloat stage saw an increase of Tenericutes spp. And a notable increase in the growth of *Ignatzschineria* spp. The dry stage saw an increase of *Firmicutes* with an abundance of *Clostridiales* and *Bacillaceae* spp. seen in the dry remains (Pechal et al., 2018).

Zurgani. (2018), conducted a study using mouse models with and without fur concentrating three regions of the mouse carrion skin, oral cavity, and interface- and- carrion, during the summer trail 2014 and spring trial 2015. During the 2014 summer trial during the active stage the mouse with fur and without fur had four bacterial phyla present Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Zurgani., 2018). Actinobacteria was only present in the skin samples. Proteobacteria was the dominant phylum in all three body regions, Bacteroidetes was seen as the second dominant phylum in the oral cavity followed by Firmicutes (Zurgani., 2018). During the spring trial 2015, similar results were noted. Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria were present on carrions with or without fur during the active decomposition stage. Proteobacteria was the dominant phylum in the oral cavity in carcasses with or without fur (Zurgani., 2018).

2.4 The role of bacterial communities in carcass decomposition

Roles that microbes play in decomposition of human corpses or animal carcasses, is not well understood although they are important for breaking down macromolecules into simpler molecules (Metcalf et al., 2016 b). Bacteria from the family *Rhizobiaceae* and *Chromatiaceae* become abundant during decomposition, as these bacteria are involved in the nitrogen cycle, catalysing processes, denitrification, and nitrate reduction (Carter et al., 2007, 2015; Zheng et al., 2013; Lauber et al., 2014; Yong et al., 2014; Jesmok et al., 2016; Metcalf et al., 2016 a,b). Furthermore, members that belong to decomposer bacterial communities contain certain genes for encoding components of pathways that breakdown the amino acids glycine, glutamate, ornithine, and lysine with the last two amino acids becoming putrescine and cadaverine and are associated with a bad odour reduction (Carter et al., 2007, 2015, 2017; Zheng et al., 2013; Lauber et al., 2014; Yong et al., 2014; Jesmok et al., 2016; Metcalf et al., 2016 a,b).

Decomposer microbial communities are rapid succession bacteria that compete for nutrients and consume each other, such as Pseudomonas which are opportunistic bacteria that are rapid growing and metabolically diverse (Metcalf et al., 2016 b). *Proteus mirabilis* from the family *Enterobacteriaceae* plays a vital role in attracting insects to decaying carcasses, and insects aid in the decomposition process. Bacteria such as *Acinetobacter* spp. break down different organic compounds and are present during the beginning of the active decay stage and are highly abundant during the advanced decay stages, and exploit multiple substrate types during decomposition (Metcalf et al., 2016 b). During the dry phase,

bacteria such as *Sporosarcina* spp. is found, which breaks down compounds such as urea (Metcalf et al., 2016 b).

Multiple decomposer bacteria can be observed in low abundance in different environments such as the human gut, skin, internal and external surfaces of insects and soil concentrations (Metcalf et al., 2016 b). *Acinetobacter* spp. are detected at low abundances in environments such as the human skin and in soils. Certain genera like *Wohlfahrtiimonas* and *Ignatzschineria* have bacteria that are carried by blow flies after death (Metcalf et al., 2016 b).

2.5 The role of bacterial communities in post-mortem interval (PMI) estimations

Researchers have combined ecological perspective and forensic science over the years to improve the understanding and knowledge of carcass decomposition. This has allowed researchers to optimize the ability to estimate the post-mortem interval (PMI) that is associated with the terrestrial ecosystems during decomposition (Metcalf et al., 2013; Carter et al., 2017; Zurgani., 2018). Several studies have utilised many models of decomposition such as; mouse, swine carcasses, and human cadavers to determine the value of these cadaver – associated microbial communities in estimating the post-mortem interval (PMI) (Pechal et al., 2012, 2013., 2014, 2018; Hyde et al., 2013, 2015; Metcalf et al., 2013, 2016 a; Finley et al., 2015, 2016; Carter et al.,2017).

The following cases by Hyde et al. (2013), Metcalf et al. (2013) and Pechal et al. (2013, 2014) were conducted on three different models to observe the relationship between bacterial communities and decomposition in estimating PMI.

Hyde et al. (2013), did an early exploratory study on the internal microbiota of human cadavers. The microbial species involved in human decomposition were recorded using metagenomics techniques, which revealed microbial signature variabilities in specific locations across the entire human body. Hyde et al. (2015), did a follow-up investigation, which revealed a shift in bacterial populations from aerobic to anaerobic bacteria in all human cadaver bodies. The right cheek, left and right biceps, and torso were found to be dominated by Proteobacteria, while Actinobacteria and Firmicutes increased at the later stages of decomposition. During the bloat and purge stages, the buccal cavity showed a high Proteobacteria abundance followed by an increase in Firmicutes and *Ignatzschineria* spp. abundance. The findings show that bacterial populations differ between carcasses and carcass regions (Hyde et al., 2015).

Metcalf et al. (2013), determined PMI using 454 pyrosequencing and regression models based on the Random Forest classifier on bacterial and microbial eukaryotic populations on mouse carcasses. Models developed on 5–10 highly predictive taxa were compared to models built on all taxa in the dataset, and similar predictions were found. At each sampling time point, samples were taken from both the surface and the interior of each corpse. The mice corpses went through all main stages of decomposition

throughout the course of the 48-day experiment (Metcalf et al., 2013). The bacteria in the soil beneath the mouse carcasses changed significantly. It revealed that the soil had six different bacterial phyla. Firmicutes was the community's predominant taxon, and was present in the abdominal cavity during the early stages of decomposition, and Proteobacteria was the most abundant taxa in the abdominal cavity during the later stages of decomposition. They were also dominant on different parts of the skin regions during the entire decomposition process (Metcalf et al., 2013). Using the models built, they were able to estimate PMI within 3.30 ± 2.52 days and obtained the highest accuracy before 34 days of decomposition.

For the first time, Pechal et al. (2014) used 454-pyrosequencing to describe the epinecrotic bacterial succession comprehensively and statistically in a terrestrial habitat on decomposing pig carcasses. In this study, bacterial communities from three pig carcasses were examined and 137,181 sequences were recovered, representing four phyla and 20 families. To distinguish the bacterial communities at each stage of decomposition, multivariate statistics were used, which showed that different bacterial communities are there at each stage of decomposition, but all the bacterial replicates found at each stage were the same. *Bacteroidaceae* and *Moraxellaceae* families were found to be significant at the fresh stage. *Bacillaceae* and *Clostridiales incertae sedis* spp. were found during the end of the bloat stage (5 days after death). Using pig substitutes for human cadavers, Pechal et al. (2013), demonstrated that the necrobiotic bacterial communities located within the skin and buccal cavity changed during the decomposition stages and along with the season.

In view of the above studies, we aim at identifying bacterial communities during decomposition stages of sheep and pig carcass and describe the colonization pattern of the identified communities. As the bacterial spectra varies with geographical regions and conditions, the current study aims to provide essential information in forensic microbiology and how these observations can be applied in research to estimate the PMI in criminal investigations and poaching of wildlife in South Africa. Bacteria are considered evidence during forensic investigations as they have individual colonization patterns during decomposition. These identified bacterial patterns could potentially be used to estimate PMI based on the appearance and disappearance of bacteria during the different stages of decomposition. (i.e., Firmicutes spp. are normally found during the later stages of decomposition). Animal species selection plays a significant role in determining bacterial species pattern during the different stages of decomposition. Therefore, it is important to identify the different bacterial species observed on the different regions of the carcasses during the different stages of decomposition by using molecular techniques. This study contributes new research on the bacterial species found on both the sheep and pig carcasses during the different stages of decomposition in South Africa.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

This study was conducted at the University of KwaZulu-Natal Ukulinga Research and Training Farm, located in Pietermaritzburg (Coordinates: -29.6627°S, 30.4050°E) (Fig. 3.1). The area has a unique climate, as the summers are considered warm to hot and mild winters with the chance of frost at certain times during winter. The mean monthly temperatures range from 13.2 °C to 21.4 °C, with a mean annual temperature of 17 °C (Kiala et al., 2017; Mills and Fey., 2004). The farm receives 680 mm of precipitation and receive rains over 106 days annually (Kiala et al., 2017; Mills and Fey., 2004). This area falls under the Southern Tall Grassveld and mostly contains herbaceous plants because of long - lasting burnings (Kiala et al., 2017; Mills and Fey., 2004). The winter season in South Africa is from June to August, however, the cold weather starts to set-in from May. This study was conducted throughout the winter months of June, July, and August over 8 weeks (58 days), and the average temperatures ranged between 18 °C and 19 °C respectively.

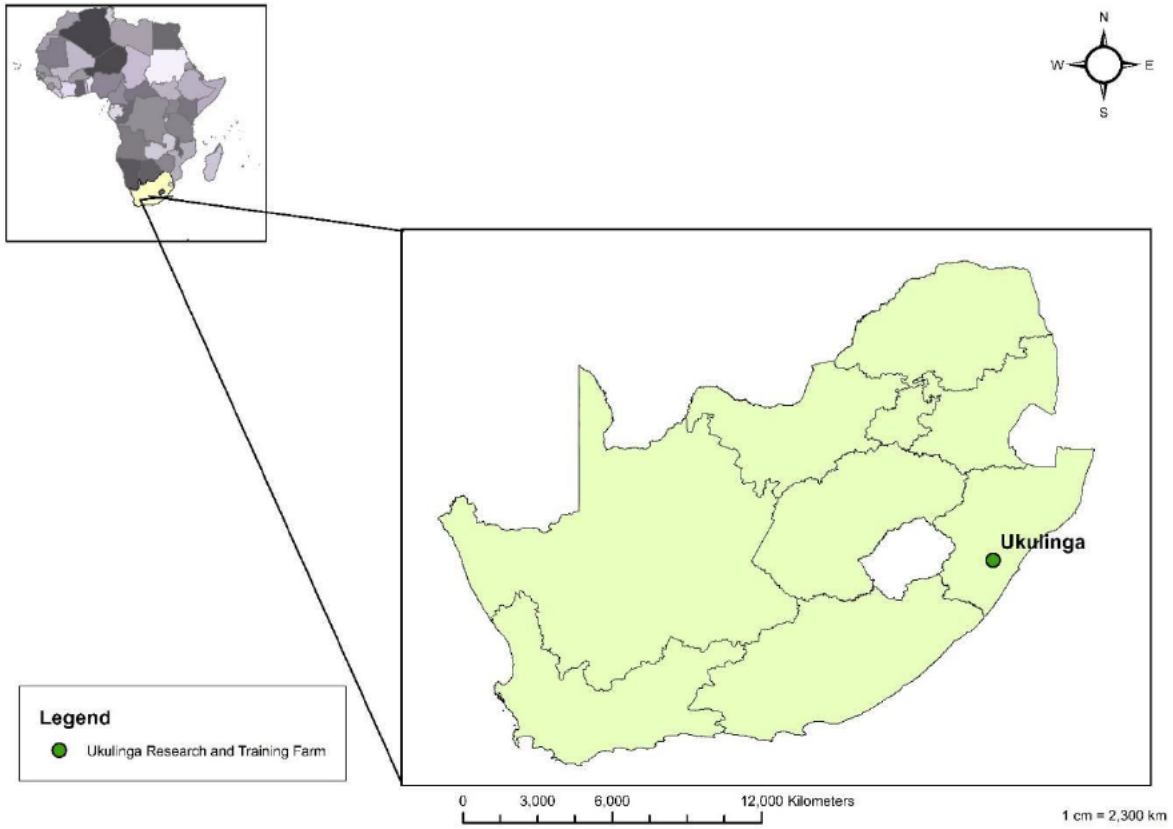


Figure 3.1: Map showing location of the study area in Pietermaritzburg, KwaZulu-Natal, South Africa (Tembe and Mukaratirwa., 2021)

3.2 Study animals

Two domestic farm animals were used for this study. A Large White adult pig (*Sus scrofa domesticus*) with an average live weight of 80kg donated by Hmb School Trust piggery in Greytown, South Africa (approximately 60 km from the Ukulinga Research Farm). A Merino adult large sheep (*Ovis aries*) with a live weight of 80 kg donated by the Ukulinga Research Farm, Pietermaritzburg, South Africa. The pig and sheep were humanely euthanized, and two stab wounds were created around the neck and abdomen region to mimic cases of illegally killed animals.

3.3 Sampling procedures

The carcasses were immediately placed 210 m part along transects with anti- scavenging cages (100 cm × 100cm × 100cm) to prevent disturbances by large scavengers such as rats and to allow free movement of insects. Sampling was conducted from day 0 till day 58 of the decomposition process. The stages of decomposition were determined as described in previous studies (Wolff et al., 2001; Brooks., 2016; Tembe and Mukaratirwa., 2021). Data was collected on a daily schedule throughout the course of sampling through photographic observations of both the carcasses and the surrounding environment (soil and grass areas), also the carcasses physical changes and odour changes were recorded during each stage, day, and time of decomposition. The temperatures of the carcasses and surrounding soil were measured daily using a MAC-AFRIC infrared thermometer (Adendorff machinery mart - temperature range −50~380°C, ± 2%) between 09:00 am – 11:00am. Sampling of each carcass was approximately one hour each.

Bacterial communities were sampled daily after the carcasses were exposed to the environment (0 hrs), for 58 days using sterile swabs. Each carcass was sampled for approximately 60 seconds from the buccal cavity (top area of the mouth and under the tongue) and the two different skin regions (head and torso) on the carcasses using sterile swab (Metcalf et al., 2013 and Pechal et al., 2013). The swabs were then transferred into 10 ml centrifuge tube containing 5ml of 0.1% buffered peptone water (Sigma-Aldrich) (Appendix. 1.1). The tops of the swabs were cut-off with a pair of scissors to fit in the centrifuge tube. Samples collected were placed in a cooler box containing ice and immediately transported to the Genetics laboratory at the University of KwaZulu-Natal, Westville campus. Once the samples were in the laboratory 5 ml of 60% glycerol (Sigma-Aldrich) (Appendix.1.2) was pipetted into each centrifuge tube containing buccal and skin samples under the fume hood. Samples were then stored at – 80°C until further processing. The samples were aseptically inoculated (500µl) on nutrient broth (Sigma- Aldrich) (Appendix.1.3) in test tubes (5ml each) and incubated at 37°C for 24˚C. Samples were then cultured on nutrient agar medium (Sigma- Aldrich) (Appendix.1.4) and then incubated in aerobic conditions in an incubator (Scientific Series 2000, USA) at 37˚C for 24h (Uthayasooriyan et al., 2016). Single colonies were collected and used in DNA extraction.

3.5 DNA Extraction of buccal and skin samples

The Quick - DNA Tissue Miniprep Kit (ZYMO RESEARCH CORPORATION), was used to extract DNA from the samples, this procedure was conducted using the manufacturer's instructions. The cultured media was removed before the cells were pelleted at ~ 500 x g for 2 minutes and the supernatant was discarded. The cell pellets (1-5 x 10^6 cells) were resuspended in 200 µl DNA Elution Buffer. Approximately 200 µl sample was added to a microcentrifuge tube along with 200 µl BioFluid and Cell Buffer (Red) and 20 µl Proteinase K. The microcentrifuge tube was then vortexed thoroughly and incubated at 55 ºC for 10 minutes. One volume of Genomic Binding Buffer was added to the digested sample and vortexed thoroughly (e.g., 420 µl Genomic Binding Buffer was added to 420 µl digested sample). The mixture was then transferred to a Zymo-SpinTM IIC-XLR Column in a collection tube and centrifuged (≥ 12,000 x g) for 1 minute. The collection tube with the flow was discarded. Next, 400 µl of DNA Pre-Wash Buffer was added to the column in a new collection tube and centrifuged (≥ 12,000 x g) for 1 minute. The collection tube was then emptied. Thereafter, 700 µl g-DNA Wash Buffer was added and centrifuged (≥ 12,000 x g) for 1 minute and the collection tube was emptied. Next, 200 µl g-DNA Wash Buffer was added and centrifuged (≥ 12,000 x g) for 1 minute. The collection tube with the flow through was then discarded. The DNA was then transferred to a new microcentrifuge tube for elution. DNA Elution Buffer (≥ 50 µl) was added and incubated for 5 minutes, and then centrifuged at maximum speed for 1 minute. The eluted DNA was then stored at ≤ 20 ºC for future usage.

3.6 Polymerase chain reaction (PCR) and gel electrophoresis

Conventional PCR protocol was used to amplify the 16S rRNA gene with the universal primers: Forward primer- 63f (5'-CAG GCC TAA CAC ATG CAA GGC-3') and Reverse primer- 1387r (5'-GGG CGG WGT GTA CAA GGC-3') were designed to amplify approximately 1,300 bp of a consensus 16S rRNA gene (Marchesi et al., 1998). PCR amplification was performed using the above – mentioned primers in a 25 µl reaction volume. Each reaction consisted of 12.5 µl DreamTaq Green mastermix (Thermo Fisher Scientific, Cat: K1081, Thermo fisher Scientific Baltics UAB, Vilnius, Lithuania).1.0 µl of each primer (forward and reverse), 5.5µl of distilled water, and 5 µl DNA. PCR was performed in a thermal cycler (BioRad, California, USA) under the following conditions: 3 minutes at 95°C, 30 cycles of 30 seconds denaturation at 94°C, 1-minute annealing at 55°C and 1.5 minutes polymerization at 72°C, and a final polymerization step for 5 minutes at 72°C to finish replication on all templates. One cycle at 4ºC (indefinite period) to allow storing of the sample prior to further analysis. The PCR products and 1kb DNA ladder molecular marker (GeneRuler DNA Ladders, Thermo Fisher Scientific Inc., MA, USA) were separated by electrophoresis on a 1.8% agarose gel containing ethidium bromide at 70V for 40 minutes (Powerpac Basic Power Supply, Bio-Rad, Hercules, CA, USA). A Uvitec UV transilluminator was used to visualize the DNA bands and the image was captured using a Uvitec digital camera.

3.7. Sequencing and phylogenetic analysis

Five µL of purified PCR products for 16S rRNA gene were sent to Central Analytic Unit at Inqaba Biotechnical Industries (Pty) Ltd. for DNA sequencing. DNA fragments were sequenced in the forward and reverse directions using the primers used in the initial amplification. Sequences were returned as ABI files for subsequent analyses. Sequences obtained were edited and aligned using the Clustal W algorithm implemented in BioEdit v.7.0.9.0 to remove minor inconsistencies (Hall., 1999).

Sequences were then aligned with reference sequences obtained from GenBank in BioEdit. A BLAST search was performed to identify the closest matches from GenBank and these sequences were included in the alignment (Altschul et al., 1997). MEGAX was used to determine the substitution model before trees were constructed (Kumar et al., 2016). The aligned sequences were subjected to phylogenetic analyses using Neighbour-joining (NJ) and Maximum Likelihood (ML) in MEGAX to explain the phylogenetic relationships between experimental samples and the closest matches identified by blast searches downloaded from GenBank (Kumar et al., 2016). Bootstrap analysis was performed using 1000 bootstraps (100 replicates).

CHAPTER 4: RESULTS

4.1. Decomposition stages

Throughout the sampling period there were five stages of decomposition observed in both the pig and sheep carcasses during the cold winter season namely: fresh, bloat, active, advanced, and dry stages. The results indicated a correlated relationship between the carcass temperature and soil temperature regardless of the carcass type as shown in Fig. 4.1A; B and Table 4.1. Observations of both the pig and sheep carcasses were similar during the duration of the decomposition stages.

4.1.1. Fresh Stage (0–1 d)

The fresh stage of decomposition of the pig carcass was characterised by soft torso and flexible limbs, however, there was no foul odour. There was a decrease in the body temperature from 25.4°C to 15.4°C during this stage the mean temperature was 20.5°C and the soil temperature showed no significant decrease 16.5°C to 16.0°C (Fig. 4.1A and Table 4.1). The sheep carcass had no physical changes or foul odour observed, the body temperature ranged from 26°C to 16°C and the mean temperature was 21°C and the soil temperature showed a slight decrease from 16.5°C to 16.0°C (Fig. 4.1B and Table 4.1).

Two bacterial taxa were recorded during this stage of decomposition of the pig (Table 4.2) and comprised of *Leclercia* species and *Providencia vermicola*. *Leclercia* species were only recorded in this stage of decomposition on the skin of the carcass. These species were first to colonize the skin and buccal cavity of the pig carcass within a few hours of death during the cold season (Table 4.2). One bacterial taxon was recorded during this stage of decomposition of the sheep carcass (Table 4.2) and comprised of the bacterial species *Providencia vermicola*. This species was the first to colonize both the skin and buccal cavity of the sheep carcass within a few hours of death during the cold season.

4.1.2. Bloat stage (2-6 d)

The pig carcass was characterized by a darkened body colour and a foul odour was perceived. Bloating of the pig carcass caused an increase of the body temperature in the first two days from 26.5°C to 28.7°C (Fig. 4.1A). The temperature then decreased, to 22.7°C on the third day, which was then followed by an increase to 24.3°C on the fourth day (Fig. 4.1B). The body temperature slightly increased to 24.8°C on the last day of the bloat stage (Fig. 4.1A). The body temperature was higher than that of soil temperature (Fig. 4.1A). The soil temperature continued to increase from day two until the last day of the bloat stage (16.1°C – 22.6°C) (Fig. 4.1A). The sheep carcass was associated with swelling of the abdomen and foul odour. A different trend was observed in the sheep body temperature as it showed a steady increase in temperature from day two till day six (23.7°C – 30.4°C), which increased greatly compared to the last day of the fresh stage which was 16°C. The soil temperature at the beginning of the bloated stage was 18.8°C, followed by a decrease to 16°C n the second day of this stage (Fig. 4.1B).

The temperature increased to 17.5°C on the third day of this stage and proceeded to increase until the end of this stage (22.8°C) (Fig. 4.1B). During the bloat stage, the soil temperature was slightly lower than the body temperature. Swelling in the abdomen and a foul odour were associated with the sheep carcass.

Providencia vermicola was the only bacterium present in both the buccal cavity and on the pig carcass (Table 4.2). This species persisted in the buccal cavity from the fresh stage till the end of the bloat stage. Two bacterial taxa were recorded during this stage of decomposition of the sheep and comprised of *Myroides species* and *Providencia vermicola*. *Povidencia vermicola* was the bacterium present on the skin of the carcass from the fresh stage till the end of the bloat stage (Table 4.2).

4.1.3. Active Stage (7-12 d)

The active stage of decomposition of the pig carcass was associated with the commencement of skin peeling and an intense foul odour especially towards the last day of this stage. During this stage the pig carcass had a body temperature of 22.8°C on the first day (Fig. 4.1A). The temperature slightly declined to 22.2°C on the second day of this stage, then increased to 25°C on the third day. By the fourth day the temperature had sharply decreased to 20.3°C and sharply increased to 31°C on the last day of this stage (Fig. 4.1A). The soil temperature had the same pattern as the body temperature on the first day of this stage 12°C and on the last day the temperature was 19°C (Fig. 4.1A). Throughout this stage of decomposition, the soil temperatures were much lower compared to the body temperatures (Fig. 4.1A and Table 4.1).

The physical changes noted in the sheep carcass comprised significant loss of tissue and deflation of the carcass. The foul odour was still present and much more prominent. The sheep body temperature showed a different trend when compared to the pig body temperature (Fig. 4.1A and B). The carcass body temperature was 21.8°C on the first day of this stage, which then slightly declined to 21.2°C on the second day. Thereafter, the body temperature steadily increased throughout this stage, until the last day of this stage 30.2°C. The temperature on the last day of this stage was 8.4 °C higher than the first day (Fig. 4.1B). The soil temperature exhibited an increase from 14°C – 23.4°C throughout this stage (Fig. 4.1B), however, the soil temperature was still lower than that of the body temperature (Fig. 4.1B).

Bacterial phylum Proteobacteria, genus *Providencia*, was dominant in the active stage of the pig carcass, as *Providencia alacalifaciences* and *Providencia vermicola* were present on the carcass (Table 4.2). This showed that the genus *Providencia* was persistent from the bloat stage of decomposition till the end of the active stage (Table 4.2). The bacterial taxon recorded during this stage for the sheep carcass were *P. alacalifaciences* and *Pseudomonas parafulva* (Table 4.2). The genus *Providencia* was persistent from the bloat stage to the active stage (Table 4.2).

4.1.4. Advanced stage (13–51 d)

During the advanced decay stage (13-51 days), the decomposition process slowed down as the temperatures became colder during the winter months. Extreme peeling of the carcass and dry skin were seen throughout the whole pig carcass. An extreme loss of soft tissue was observed in the pig carcass and the foul odour was still present. The pig body temperature was similar to that of the surrounding soil temperature for most of the days during this stage (Fig. 4.1A). No exact trend was observed as the temperatures of the body and soil both increased and decreased, however, on days: 1, 19, 20, 24,25, 26, 27, 28, 29, 32, 37, 43, 44 and 46 the body temperatures were slightly lower than the soil temperature (Fig. 4.1A). The pig body temperatures ranged from 16.8 °C to 34.9 °C, with a mean temperature of 24.3 °C and the soil temperature ranged from 16.3°C – 31.7°C, with a mean temperature of 23.2 °C (Fig. 4.1A and Table 4.1) throughout this stage.

The physical changes of the sheep carcass observed were the significant loss of body tissue, as a result the bones on the legs and rib cage were visible. The limbs on the sheep carcass had collapsed and bent into a resting position. There was still a slight odour still present. The body temperature was similar to that of the surrounding soil temperature (Fig. 4.1B). The body temperature ranged from 11.3°C – 32.1°C, while the soil temperature ranged from 11.6°C – 30.0°C (Fig. 4.1B). The body temperature had a slightly wider range when compared to the soil temperature, however, the soil temperature was higher than the body temperature on days 16, 17, 19, 21, 24, 26, 27, 29, 36 and 44 (Fig. 4.1B).

During the advanced stage of decomposition *P. vermicola* species was recorded on both the buccal cavity and skin of the pig carcass (Table 4.2). *Providencia* species were present in the buccal cavity from the fresh stage till the end of the advanced stage of decomposition, whereas, on the skin *Providencia* species were present from the bloat stage till the end of the advanced stage (Table 4.2). *Providencia* species were persistent throughout the first four stages of decomposition on the pig carcass except in the buccal cavity during the fresh stage (Table 4.2). There was a change in the bacterial phylum during the advanced stage of decomposition of the sheep carcass. *Bacillus cereus* which belongs to the phylum Firmicutes, was present in both the buccal cavity and on the skin of the sheep carcass (Table 4.2).

4.1.5. Dry stage (51-58 d)

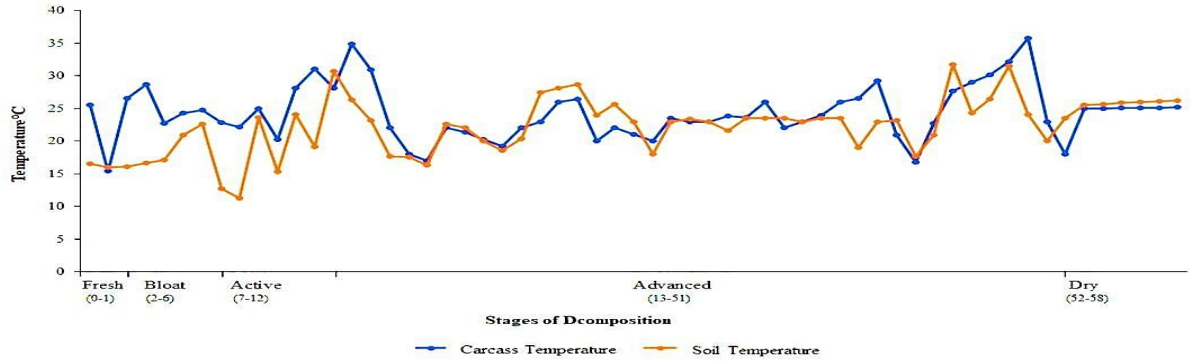
Only a few noticeable changes were recorded during this stage of decomposition of the pig carcass. There was only dry skin with no signs of moisture on the carcass. There was no odour detectible from the carcass. During this stage a similar pattern of the pig body temperature was observed as in the advanced stage, where body temperature was similar to soil temperature (Fig. 4.1A). However, the soil temperatures were slightly higher than the body temperature throughout the dry stage (Fig. 4.1A). Body temperatures ranged between 18.0°C – 25.2°C with a mean temperature of 24.1°C and the soil temperatures ranged between 23.5°C – 26.2°C with a mean temperature of 26.6°C (Fig. 4.1A and Table

4.1). During the advance stage of decomposition of the sheep carcass, changes observed that the gut contents, the skin, and the wool all dried out and there was no foul smell present. The wool also increased the decompositional process as the body temperature on the inside increased. The dry stage had comparable pattern to that of the advanced stage, where body temperature was similar to soil temperature, however, the temperatures in the dry stage were opposite to the observations in the advanced stage (Fig. 4.1B). The soil temperature was lower than that of body temperature throughout the dry stage (Fig. 4.1B). Body temperatures ranged between 21.0°C – 27.0°C with a mean temperature of 24.8°C and the soil temperatures ranged between 18.7°C – 25.0°C with a mean temperature of 21.6°C (Fig. 4.1B and Table 4.1).

During the dry stage of pig decomposition, there was a change in the bacterial phylum. *Bacillus* sp. which belongs to the phylum Firmicutes, was present in both the buccal cavity and on the skin, *Providencia* species were not seen during this stage (Table 4.2). *Bacillus cereus* was present in the buccal cavity and *Bacillus pumilius* on the skin of the sheep carcass (Table 4.2). *Bacillus cereus* was recorded only during the dry stage in the buccal cavity and on the carcass skin of the pig (Table 4.2).

Proteobacteria was the persistent phylum observed during the first four stages of the pig decomposition and during the first three stages of the sheep decomposition (Table 4.2). Firmicutes was the persistent phylum observed during the dry stage of the pig decomposition (Table 4.2) and during the advanced and dry stages of the sheep decomposition (Table 4.2).

A).



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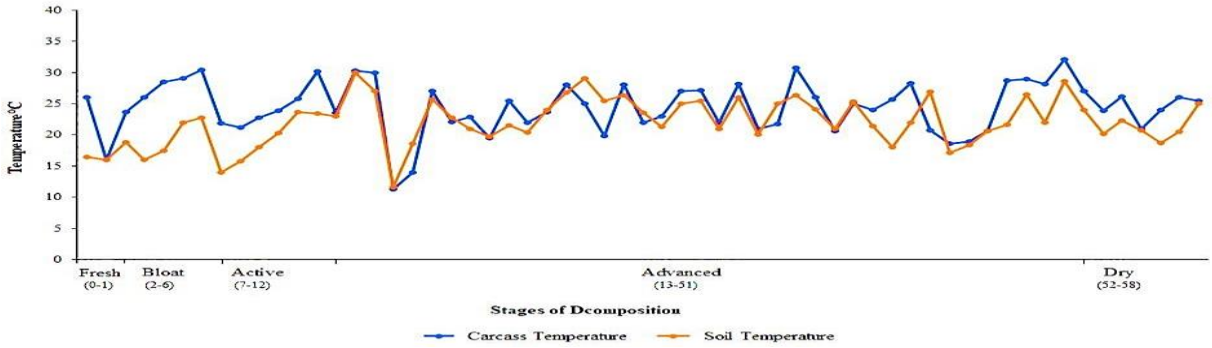

Figure 4.1: Comparison between body temperature of the carcass **(A)** pig carcass with soil temperature (Tembe and Mukaratirwa., 2021) **(B)** sheep carcass with soil temperature (Tembe et al., 2021) during the different stages of decomposition during the cold season in KwaZulu-Natal province of South Africa.

Table 4.1: Mean temperatures ^{O}C of the carcass and soil during the different stages of decomposition during the cold season.

Stage of decomposition	**Mean Temperatures of Carcass and Soil (°C)**			
	Pig Carcass	**Soil**	**Sheep Carcass**	**Soil**
Fresh	20.5	16.3	21	16.5
Bloat	25.4	18.7	27.5	19.4
Active	24.9	17.7	24.3	19.2
Advanced	24.3	23.2	24.1	23.1
Dry	24.1	25.6	24.8	21.6

Table 4.2. Microbial communities found on the skin and buccal cavity of pig and sheep carcasses during different stages of decomposition.

Phylum	Family	Genus/ species	Presence/absence of microbial community at different stages of pig and sheep carcass decomposition				
			Fresh (0-1 d)	Bloated (2-6 d)	Active (7-12 d)	Advanced (13-51 d)	Dry (52-58 d)
Proteobacteria	*Morganellaceae*	*Providencia vermicola*	+ *	+ *	*	+ *	
Proteobacteria	*Morganellaceae*	*Providencia alcalifaciens*			+		
Proteobacteria	*Pseudomonadaceae*	*Pseudomonas parafulva*			+ *		
Bacteroidetes	*Flavobacteriaceae*	*Myroides phaeus*		+			
Proteobacteria	*Enterobacteriaceae*	*Leclercia sp.*	*				
Firmicutes	*Bacillaceae*	*Bacillus cereus*				+ *	
Firmicutes	*Bacillaceae*	*Bacillus pumilus*				+ *	+ * +

Keys: * = skin; + = buccal cavity; d = days; ●——► = pig; ●- - -► = sheep

4.2. Phylogenetic relationships of bacterial isolates from *Sus scrofa domesticus* (pig) and *Ovis aries* (sheep) based on the 16S rRNA gene.

The phylogenetic tree analysis involved 22 nucleotide sequences, and there were 700 positions in the final data set. The topology of the 16S rRNA tree based on samples collected from both pig and sheep carcasses (Figure 4.2) showed well resolved nodes with congruency between Maximum likelihood and Neighbour – joining tree values. The in-group structure was well supported by Neighbour – joining/ Maximum Likelihood (NJ/ML) (100/98). The in-group formed a supported monophyletic clade with a strong bootstrap (100/68) with respect to the outgroup sequence (*Helicobacter pylori*). Within the ingroup five well defined clades were formed on a family level: *Morganellaceae* (100/99), *Enterobacteriaceae* (100/99), *Pseudomonadaceae* (100/100), *Bacillaceae* (100/97) and *Flavobacteriaceae* (100/100).

Each of these five main family clades contain bacteria isolates from both pig and sheep carcass during decomposition. These family clades formed smaller clades consisting of individual bacterial species from both pig and sheep carcass. From the clade *Morganellaceae* isolates P-AD_{sk}1, S-B_{sk}1, P-AD_{b}2 and P-B_{sk}1 formed a strong bootstrap support (99/98) for the neighbour-joining and maximum likelihood analysis with *Providencia verimcola* MT263027.1/ KF781538.1. Isolate S-A_{b}1 formed a monophyletic clade (99/98) with *Providencia alacalifaciences* AB682262.1/ NR115879.1. Isolates S-Fsk2 and S-Fsk3 formed a well-defined clade that was well supported (100%) in NJ/ ML. Isolate S-F_{b}1 formed a sister clade (63/49) with S-A_{b}1. From the *Bacillaceae* family, isolates S-D_{b}2, P-AD_{sk}2, P-D_{b}2 and SAD_{b}2 formed a well-defined clade with *Bacillus cereus* KI437489.1/ MG733919.1/ MT256066.1. Isolate S-D_{sk}1 formed a well-defined monophyletic clade (100/100) with *Bacillus pumilus* KI438145.1. In the *Pseudomonadaceae* clade only one isolate was observed S-A_{sk}1 and it formed a well-defined monophyletic clade (100/100) with two GenBank derived isolates *Pseudomonas parafulva* MH972190.1/ JQ059356.1. Enterobacteriaceae isolate P-F_{sk}2 formed a bootstrap support (86/57) for the neighbour-joining and maximum likelihood analysis with *Enterobacter* sp. MH127571.1 and *Leclercia* sp. MN082055.1. *Flavobacteriaceae* had two isolates S-Fsk2 and S-F_{sk}3 that formed a monophyletic clade (100/100) with each other and formed a bootstrap support (57/59) for the neighbour-joining and maximum likelihood analysis with *Myroides* sp. KP453997.1.

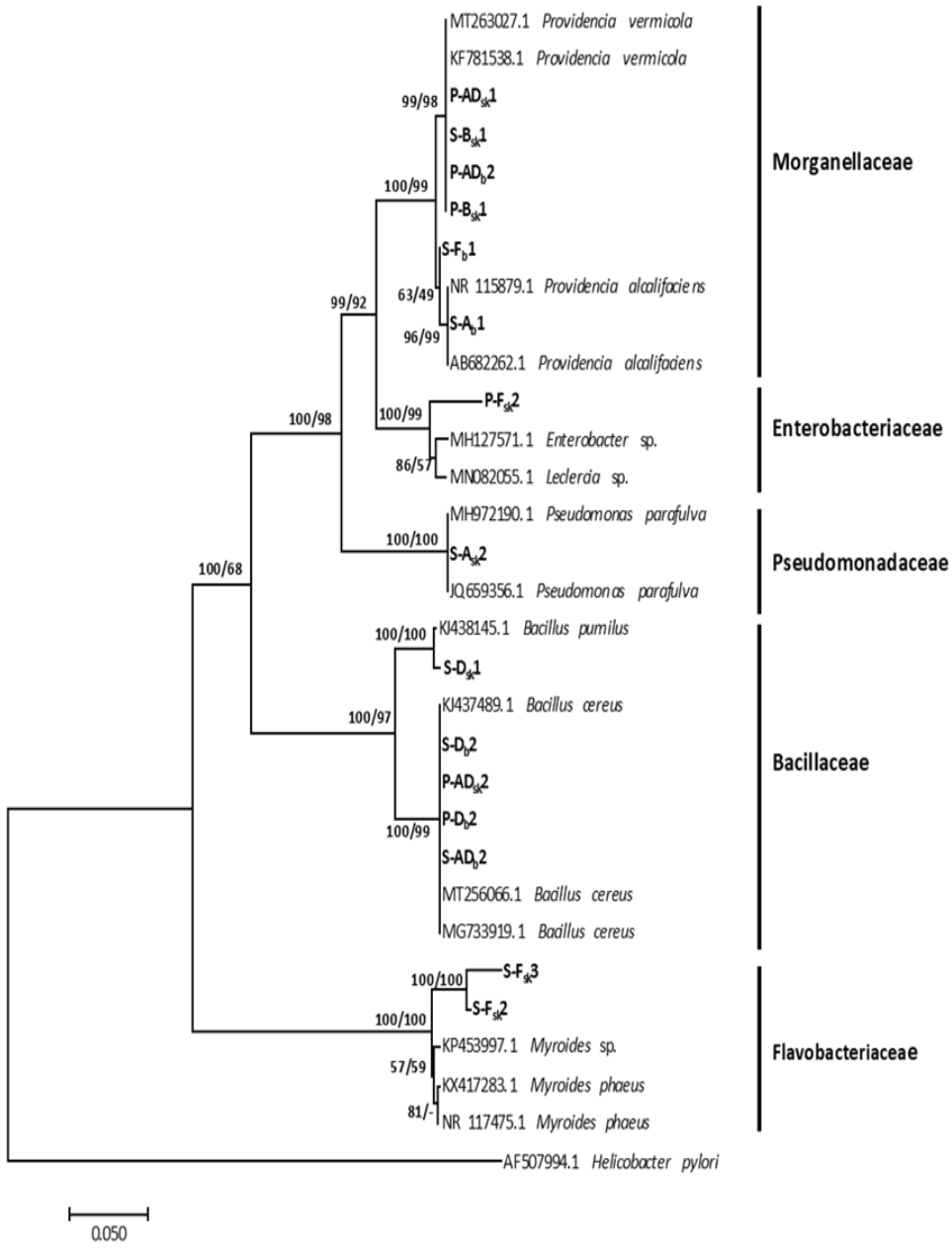

Figure 4.2: Phylogenetic tree showing the relationship between the bacterial isolates obtained from pig (*Sus scrofa domesticus*) and sheep (*Ovis aries*) carcass using 16S rRNA Gene. Nodal support is indicated as Neighbour-joining/ Maximum likelihood (bootstrap). Taxon labels show the species name strain/accession number. Helicobacter pylori is the outgroup. The evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA X. P = pig; S = sheep; b = buccal; sk = skin; F = fresh stage; B = bloat stage; A = active stage; AD = advanced stage; D = dry stage; - = No bootstrap values

CHAPTER 5: DISCUSSION

Although bacteria have been recognized as a significant factor for decomposition, there have been few studies describing the microbial ecology of decomposition (Hyde et al., 2013, 2015; Metcalf et al., 2013; Pechal et al., 2013; Carter et al., 2015, 2017). Bacteria are essential for carcasses , both on the internal and external surfaces during decomposition (Pechal et al., 2012, 2013; Hyde et al., 2013; 2015; Metcalf et al., 2013; Carter et al., 2015).

For this study, the pig a non-human model was considered an acceptable proxy for humans and game animals respectively. Pig models are considered good proxies to study the decomposition of human corpses because they have similar physiology, have similar bacteria in their gastrointestinal tract (GI) tracts, and have a thin hair covering (Schoenly et al., 2006; Metcalf et al., 2013; Pechal et al., 2013; Carter et al., 2015, 2017).

The results from this study observed a correlated relationship between the soil temperature and the carcass temperature for both the pig and sheep, as it was seen that the carcass temperature was directly influenced by the soil temperature. Both the pig and sheep carcasses followed the pattern of the soil temperature irrespective of the stage of decomposition or the different type of animal carcasses used, as the soil temperature decreased or increased so did the carcass temperature. The duration and pattern of decomposition taken by both the carcasses during the winter season were similar. This was unexpected because the sheep carcass still had its wool on it. Kelly et al. (2006), observed differences between clothing and wrapping swine carcasses across all four seasons.

Kelly et al. (2006), observed that during the winter season, the clothing may have restricted the loss of body heating. Little evaporation occurred once the carcasses were wrapped and slowed the release of decomposition fluids into the soil during the active stage of decomposition. Therefore, during the winter season the carcass remained in the active stage as there was little change in the decomposition rate (Kelly et al., 2006). During summer trials Kelly et al. (2006), showed that wrapping of the carcasses delayed the drying of the tissues and caused changes to the decomposition of the carcass.

The results from our study indicated that the duration of each decomposition stage was similar to other studies (Galloway., 1997; Wolff et al., 2001; Kelly et al., 2006, 2008, 2011; Martinez et al., 2007, Gilbert., 2014; Marais – Werner et al., 2017; Probst et al., 2020); and the bacterial taxon recorded were similar to those recorded in various studies (Hyde et al., 2013, 2015; Metcalf et al., 2013, 2016 a, b, 2019; Pechal et al., 2013, 2014). However, the difference between our study and other studies is that we terminated the dry stage earlier. Therefore, we were not able to record results from the completed dry stage up to skeletisation of the carcasses, hence we were only able to compare the results obtained in the first few days of the dry stage to results from other related studies. Nevertheless, the bacterial species isolated in our study were comparable to those observed in previous studies.

During the fresh stage of decomposition, the pig carcass, had a soft torso with flexible limbs, however both the pig and sheep carcasses had no foul odour present, similar observations were observed in other studies (Kelly at al., 2006; Gilbert., 2014; Marais-Werner et al., 2017; Probst et al., 2020). Surprisingly the observations recorded in the fresh stage of our study were extremely similar to the decomposition pattern of a wolf carcass recorded by Gonder. (2008). Studies during the winter by Kelly et al. (2006); Parson. (2009); Gilbert. (2014) had comparable results as it was observed that the body temperatures of the pig carcasses (*Sus scofa Linneaus*) decreased during the fresh stage.

Bacterial from the phylum Proteobacteria and class Gammaproteobacteria were seen as the first colonizers in both the buccal and skin regions of both carcasses. *Providencia vermicola* from the family Morganellaceae was the first bacterium to colonize both regions of the sheep carcass and in the buccal cavity of the pig during the fresh stage. However, *Leclercia* sp. from the family Enterobacteriaceae was the first bacterium to colonize the skin regions on the pig carcass. A study by Pechal. (2012), observed during the initial field placement of swine (*Sus scrofa*) carcasses, Gammaproteobacteria was the dominant taxon for insect exclusion (62%) carcasses. Results obtained from Pechal. (2012), showed that during the first day of sampling Gamamaproteobacteria decreased in abundance but still remained dominant for insect exclusion (33%) and access (39%) carcasses, with *Providencia* species (16%) also present A follow -up study by Pechal et al. (2013), indicated similar results that the early stages of decomposition was dominated by Proteobacteria, followed by Firmicutes, this trend was observed in carcasses with and without insect exposure. The results observed in our study correspond to the results recorded by Pechal et al. (2012, 2013), as Gammaproteobacteria namely *Providencia* species were found during the early stages of decomposition and Firmicutes namely *Bacillus* species were found during the later decomposition stages.

In (2015), Hyde et al. conducted a study on the bacterial succession during the decomposition of human corpses human. This study recorded that Proteobacteria was the dominant species during the initial samplings of the skin sites, which consisted of right cheek, left and right biceps, and torso. During the fresh stage of decomposition Proteobacteria comprised of 60 to over 80 % of the bacteria present. When compare of to our study the buccal cavity (top area of the mouth and under the tongue) and the two skin regions (head and torso) were sampled and Proteobacteria was found to be dominant during the initial sampling stages as well.

The class Gammaproteobabteria consist of many bacterial species such as Chemotrophs which generate energy by consuming organic compounds and are often referred to as decomposers and aid during the decomposition process. Proteobacterial species are essential in carcass decomposition, especially during the early stages of the decomposition process. Proteobacteria have a predictable colonization sequence during decomposition and therefore, they are valuable as potential indicators of the post-mortem interval (PMI) during carcass decomposition (Pechal et al., 2012, 2013, 2014; Finely et al., 2014).

The bloated stage of the pig carcass was associated with the discolouration of natural flesh on the body, face, and a foul odour, however, in the sheep carcass swelling or bloating of the abdomen region was observed with a foul odour released. Similar observations were made in many studies such as Kelly. (2006) and Gonder. (2008)., where during the bloated stage the body colour turned dark pink and marble. Many studies have observed that the bloat stage of decomposition is mainly characterized by a foul odour that is released (Galloway., 1997; Allaire., 2002; Kelly et al., 2006, 2008, 2011; Gonder., 2008; Gardner and Bevel., 2009; Parsons., 2009; Gunn., 2011; Pechal et al., 2012; Gilbert., 2014; Marais-Werner et al., 2017; Probst et al., 2020). During the bloat stage a sharp increase in the body temperature of both carcasses were observed. According to many studies such as Gardner and Bevel. (2009) and Gunn. (2011), as the bacteria decompose the body, release a foul odour of gases that causes the body to bloat and forces a purging fluid out of the body and attracts flies. The attraction of flies is seen greatly during g the warmer season because of the intensity of the smell, whereas, in the colder season this observation is barely evident (Gardner and Bevel., 2009; Gunn., 2011). However, this purging of liquids was seen in our study. It was further observed that during both carcasses body temperature was slightly higher than that of the soil temperature The pig had a mean temperature of 25.4°C, whereas the soil had mean temperature 18.7°C. Similar results were observed in the sheep carcass which had a mean temperature 27.5°C compared to the soil which had a mean temperature 19.4°C.

Our results showed that *Providencia. vermicola* was persistent on both sampling regions of the pig and the skin of the sheep region from the fresh stage of decomposition. Hyde et al. (2015), observed a similar trend during the bloat stage of the human cadavers where the microbial community from the buccal cavity had an abundance of Proteobacteria which was followed by an increased abundance of Firmicutes. Proteobacteria was dominant after the purging of fluids until the later stages of decomposition where Firmicutes became more abundant (Hyde et al., 2015).

A study by Pechal. (2012), also observed a similar bacterial pattern, where Gamamproteobacteria represented 59% of the bacterial community with insect exclusion and represented 44% of the bacteria community with insect access to the carcasses. *Providencia* species were dominant during the third day of sampling and different bacterial species belonging to Gammaproteobacteria were present on the fifth day. Our study corroborates with the findings of Pechal. (2012), where *Providencia* species were also recorded during the bloat stage of decomposition with the exception of *Myroides* sp. which was only present in the buccal cavity of the sheep carcass during the bloat stage. These findings correspond with the observations made by Choudhary et al. (2019), where the first outbreak of pneumonia caused by *M. odoratimimus* (*Myroids* species) in immunocompromised postweaning piglets was reported. Samples were collected from nasal swabs and pneumonic lung samples from the piglets and cultured. *Myroides* sp. are considered as low-grade opportunistic pathogens. These bacterial species are known to cause fatal infections occasionally and are isolated from soil and water sources (Choudhary et al., 2019), therefore, the presence of *Myroids* species in our study maybe directly related to the soil.

The active decay stage of the pig carcass, was characterized by the peeling and slippage of the carcass skin and tissues, and also a foul odour, whereas, the sheep carcass was associated with deflating of the carcass, tissue loss and foul odour. The carcass skin was ruptured and the purging of fluids were seen, this was followed by deflating of the carcass and drying of the skin. Slippage and peeling of the skin were seen as the skin became drier throughout the remaining decomposition process, these results correspond to studies by Kelly. (2006, 2008), and Marais- Werner et al., (2017). The foul odour was still present from the bloat stage; however, the intensity of the smell was much more pungent in both carcasses. Our results correspond with observations recorded from other studies Kelly et al. (2006, 2008), Gardner and Bevel. (2009), Parson., (2009) and Gilbert., (2014).

During the first two days of this stage the pig body temperature indicated a slight decrease in the beginning of this stage and sharply increased on the last day. During the cold season the pig carcass temperature decreased in the beginning and subsequently increased until the last day of the active stage. Similar results with the body temperatures were seen in other studies by Martinez et al. (2007), where the body temperature was increased towards the later days of this stage. The sheep body temperature steadily increased until the last day of decomposition, the soil temperatures were lower than that of the body temperature throughout the decomposition process. After the purging of liquids there was a steady increase observed in the carcass temperature which may be related to the microbial and arthropod larval activity within the carcass body. All bacteria found during this active stage of decomposition belonged to Proteobacteria phylum and the class Gammaproteobacteria and were persistent on all sampling regions of both carcasses. *Providencia* species where persistent on both the pig and sheep carcass sampling regions, with the difference on the sheep skin where *Pseudomonas* species was present.

A study was conducted by Zurgani. (2018), on carcass transformations using rabbit carcasses as decomposition models during the summer and spring seasons. During the active stage of decomposition of the summer trial, Actinobacteria, Bacteroides, Firmicutes, and Proteobacteria were observed in both carcass conditions with and without fur (Zurgani., 2018). Our results corroborate with the results recorded by Hyde et al. (2015) and Zurgani. (2018), with Proteobacteria as the dominant bacteria during the active stage of decomposition.

Zurgani. (2018), observed that during the active stage Proteobacteria was the dominant phylum and the most abundant phylum in the carrions with fur and without fur. Proteobacteria was the dominant taxon in all the three-body regions i.e., oral cavity (66%), skin (55%) and interface-sand (81%), our results were similar as all the sampling regions of both the pig and sheep had Proteobacteria present. The 2015 spring trials showed a similar trend as all four bacterial phyla: Actinobacteria, Bacteroides, Firmicutes, and Proteobacteria were present under both conditions, also Proteobacteria was the dominant phylum in the oral cavity during the active stage of decomposition (Zurgani., 2018), when compared to our winter

results only Proteobacteria was present during this stage. In our study bacteria from Bacteroides and Firmicutes were present during the bloat, advanced and decay stages.

The advanced stage of decomposition was characterized by four main observations. The decrease in the pungent odour, the excessive peeling and slippage, the drying of out skin and tissue levels and the significant loss of body mass and soft tissue, whereas the sheep carcass had a significant loss of body tissue, collapsed limbs, visible bone structure. Both carcasses still had a slight odour present. Our observations were similar to results by Kelly. (2006, 2008), Gonder. (2008), Marais- Werner et al., (2017). The body temperatures were extremely similar with that of the soil temperatures surrounding the carcasses. The pig carcass had a mean temperature of 24.26°C, whereas, the soil had a mean temperature of 23.16°C. Our trend of the body temperatures was similar to the results observed Wolff et al. (2001) and Kelly et al. (2006).

During the advanced stage the bacterial taxon found on the sheep carcass was different. The bacterial taxon on the pig carcass was *P. vermicola* which occurred on both sampling sites but persisted on the pig skin from the bloat stage till the end of the active stage of decomposition. The sheep carcass had a different bacterial taxon, *Bacillus cereus,* which was present in the buccal cavity and on the skin regions. Studies by Pechal et al. (2012, 2013) and Hyde et al. (2015), noted that Proteobacteria was dominant in the early stages of decomposition, however, was replaced by Firmicutes in the later stages of decomposition in both human and swine models. The bacterial taxon found during the advanced stage of decomposition from the sheep carcass correspond with the results from Pechal et al. (2012, 2013) and Hyde et al. (2015). However, in the advanced stage of decomposition of the pig carcass, Proteobacteria was still persistent.

During the dry stage of decomposition *Bacillus* species was observed to be dominant in all sampling regions of the pig and sheep carcasses. Our results observed corroborate with the results recorded by other studies as, Firmicutes increase towards the later stages of decomposition (Pechal et al., 2012, 2013, 2014; Hyde et al., 2015). Firmicutes such as *Bacillus* spp are usually decomposers found in a variety of environments including the skin, mucosae, and gastrointestinal tract (Carter et al., 2017). Numerous Bacilli species produce extracellular hydrolytic compounds that breakdown complex polymers, and hence they can deteriorate remains (Carter et al., 2017).

Grice et al. (2009), was able to classify that the skin and the inner mucosal surfaces of humans contain symbiotic bacteria belonging to Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Three of these phyla Actinobacteria, Firmicutes, and Proteobacteria are able to survive in environments with low moisture content, like dry human skin (Grice and Segre., 2011). Our results are consistent with previous studies as the three main bacterial phyla were found in our study.

Guo et al. (2016) observed that Proteobacteria, Firmicutes and Actinobacteria were the dominant phyla in the buccal cavities of live Sprague Dawley rats, whereas, the dominant rectal phyla were Bacteroidetes

and Firmicutes. Results from Guo et al. (2016), showed that the relative abundance of Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, showed differences and variations during the stages of decomposition which could assist in the estimations of PMI. Furthermore, additional results Gammaproteobacteria were the main bacterial class found in the buccal cavity and the rectum of Sprague Dawley rats during the later decomposition stages (Guo et al., 2016). The results observed by Guo et al. (2016), supports previous studies that Gammaproteobacteria has an important role to play in the decomposition process (Guo et al., 2016).

Phylogenetic tree based on 16S rRNA gene formed five clades, which resolved the interspecific variation among the study samples within each clade (Fig 4.2). Molecular results indicated that the majority of bacterial isolates obtain from both pig and sheep buccal and skin samples were observed to be similar and closely related. All bacterial isolates from both carcasses indicated a common pattern during the decomposition stages.

Molecular analysis of isolates P-AD_{sk}1, S-B_{sk}1, P-AD_b2 and P-B_{sk}1 confirmed the presence of Proteobacteria (*Providencia verimcola)* found during the first four decomposition stages of the pig and during the first two decomposition stages of the sheep, these results corroborate with the findings of Pechal et al. (2012, 2013, 2014); Hyde et al.(2015). Isolates S-D_b2, P-AD_{sk}2, P-D_b2 and SAD_b2 confirm the presence of Firmicutes (*Bacillus* spp.) observed during the later decomposition stages in both the pig and sheep carcass, these results correspond to results recorded by Pechal et al. (2012, 2013, 2014); Hyde et al.(2015). These bacterial species are of forensic value because of their predictable appearance and disappearance patterns. Therefore, the bacterial succession and decomposition pattern maybe useful in forensic microbiology to estimate the PMI.

For our study we used the culture method to for the bacterial isolates, however, other studies by Metcalf et al. (2013) and Pechal et al. (2014), have shown promising results by using next generation sequencing and regression models for estimating the PMI using swine and mouse models. Each of these experiments were conducted under environmental or research laboratory conditions (Metcalf et al., 2013; Pechal et al., 2014). These studies were able to model the community succession to predict which microbial taxa during decomposition for estimating PMI (Metcalf et al., 2013; Pechal et al., 2014). Although the methods used to identify the bacterial taxa were different between these studies and ours, the bacterial taxa succession pattern found from these studies corresponds with the bacterial community succession found during our study. Bacteroidetes, Firmicutes, and Proteobacteria were the three main bacterial phyla observed in these studies and ours indicating that they play a major role during decomposition and estimating the PMI.

CHAPTER 6: CONCLUSION

The duration and interval of the decomposition stages between both carcasses during the winter season were similar to each other. The body temperature of the carcasses changed during the different stages of decomposition, and peaked during the bloat stage especially in the sheep carcass. Thereafter, the temperatures began to decline and stabilized between the active, advanced, and dry stages of decomposition. However, the change in the carcass temperatures was influence by the soil temperature of the surrounding environment. Both carcasses were considered good models in determining bacterial succession and decomposition pattern during the winter season in KwaZulu-Natal Province of South Africa, as both carcasses had similar bacterial succession patterns to each other and to other studies which were conducted in different countries and regions. During the initial stages of decomposition, the predominant species were Proteobacteria, mainly consisting of *Providencia* spp. These were replaced by Firmicutes (*Bacillus* spp.) from during the latter dry stages of decomposition. The bacterial succession and decomposition pattern observed might be potentially valuable in forensic investigations involving PMI estimate. Although, forensic microbiology has become a useful tool during crime investigations in the northern countries, it is still emerging in the southern African countries. Most studies completed in southern Africa have mainly focused on the use of the insect species found within and on carcasses during decomposition in forensic entomology as a tool to estimate PMI. However, little regard has been given to the role those bacterial taxa play during carcass decomposition and the potential insights into determining PMI. Future studies on the application of bacterial communities to determine PMI, in various crime investigation cases and poaching of wildlife animals in southern Africa are recommended. Therefore, it is recommended that research on the bacterial succession during decomposition be conducted also during the summer season in southern Africa for a better comparison of results from both seasons. Future studies should be conducted at different geological locations within southern Africa during the different seasons.

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Appendices

Appendix 1: Solutions

1.1 Preparation of 0.1% Peptone water buffered

0.1% peptone water buffered was prepared and autoclaved for 15 minutes at 121°C. A laminar flow cabinet was used as a sterile environment to pour 5 ml of peptone water into 10 ml centrifuge tubes, and the lids were closed.

1.2 Preparation of 60% Glycerol

The 60% glycerol solution was prepared using 600 ml of Glycerol Gilcerina and 400 ml of distilled water and autoclaved for 15 minutes at 121°C.

1.3 Preparation of Nutrient Broth

16 g of Nutrient Broth powder was suspended in one litre of distilled water; it was heated gently to dissolve all the medium, after that, it was dispensed into a bottle and sterilized by autoclaving the solution at 121°C for 15 minutes.

1.4 Preparation of Nutrient Agar

26 g of Nutrient Agar powder was suspended in one litre of distilled water; it was heated and agitated until the medium boiled and dissolved, after that, dispensed into a bottle and sterilized by autoclaving the solution at 121°C for 15 minutes. After cooling for a few minutes, the Nutrient agar was dispensed into standard Petri dishes (60 x 15mm) and allowed to solidify in the fume hood.

1.5 Preparation 1.8% agarose gel

1.8g of agarose powder was measured, 100ml of 1X TAE buffer was added and dissolved completely in the microwave. Once cooled a little, 2µl of ethidium bromide was added to the solution prior to the casting of the gel to allow visualization of the DNA bands by the transilluminator with UV lights. The was poured into the gel tray; the combs were placed to create the wells and left in the fume hood until it solidified. The gel was removed from the gel tray and placed in the gel tank.