EFFECT OF IGNATZSHINERIA INDICA (GAMMAPROTEOBACTERIA: XANTHOMONADALES) ON RATE OF DECOMPOSITION IN MICE

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by

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DEDICATION

The support system that has held my life together consists of Christ, my family; Cliff, Priscilla, Rudo and Eben; Houston assembly, the Johnsons for looking out for me. National Museums and monuments of Zimbabwe and especially Dr. Moira Fitzpatrick and Dr. T. Mukwende. Sam Houston State University especially the following; Dr. Patrick Lewis, Dr. Tamara Cook, Dr. Jerry Cook, Dr. Sibyl Bucheli. Debby Gracy of Gracy Travels. I am grateful for community, given my background, getting here and completing my masters was going to be impossible but every person I have interacted with has brought me closer to my dream. I am grateful for y'all.

ABSTRACT

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Microbes have a major role from the onset of and throughout decomposition. Studies show that a decomposing body supports a necrobiome (Pechal et al., 2013), a term coined to mean a community of living things associated with decomposition of remains, specifically with reference to microorganisms. The objective of the current study is to show how *Ignatzschineria indica*, a fly associated, and selected bacteria associated with a decomposing human body affect decomposition under controlled (laboratory) conditions. The work presented here is a laboratory experiment carried out at Sam Houston State University, Huntsville, Texas. To assess the effect of *Ignatzschineria indica* bacteria on decomposition, 3 batches of 90 mice were subjected to 9 different bacterial treatments involving 4 bacteria; A [*Ignatzschineria indica*], B [*Escherichia coli*], C [*Bacillus licheniformis*], D [*Salmonella enterica*], and combinations of *Ignatzchineria indica* and other bacteria in the following manner BA, CA, DA, PC [positive control] and NC [negative control]. 270 mice were observed throughout their decomposition process. Results from this experiment showed that the initial bacteria composition in dead mice does not affect the rate of decomposition under laboratory-controlled conditions of temperature and moisture, with the exclusion of vertebrate and invertebrate scavengers. Adding *Ignatzchineria indica* to dead mice specimens under laboratory controlled conditions does not significantly affect the rate of decomposition but instead affects the pathway of decomposition. This was evident from the different intensities of volatile organic compounds (VOCs) that were collected and assayed from

the different samples. The same experiment shows that at any given time the ambient temperature is significantly different from the subjects' temperatures during decomposition. Our findings lead us to conclude that the adition of *Ignatzschineria indica* bacteria to decomposing mice does not significantly alter the rate of decomposition. It does alter the chemical pathways of decomposition as evidenced by variant VOCs composition.

KEY WORDS: Decomposition, *Ignatzchineria indica*, Competitive exclusion, Accumulated Degree Days, Postmortem Interval

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PREFACE

A dead animal carcass in the wild interacts with a variety of factors as it decomposes. Environmental factors and a variety of scavengers, including microbes, act on it to release the rich nutrient resource back into the environment. Proteobacteria *Ignatzschineria* are present in flies' oocytes, gut, and feet and are associated with myiasis. The same bacteria rise in population density after purge during cadaver decomposition and remain consistently high throughout wet decomposition relative to other bacteria until the dry phase of decomposition. We predict that *Ignatzschineria indica* bacteria increase the rate of decomposition in human cadavers. However, a continual drawback of using human cadavers is that we are limited in our ability to carry out controlled experiments with high numbers of replicates and therefore use mice as surrogates to study the role of *Ignatzchineria indica* in decomposition.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Figure **Page**

CHAPTER I

INTRODUCTION

Forensic investigations and PMI estimates

The formula for calculating post mortem interval (PMI) is imperfect; the complexity lies in the multiplicity of factors affecting rate of decomposition. The more knowledge gained about the process, the more accurate the formula for PMI estimation and the prediction of time since death becomes (Vass, 2011; Maile et al., 2017). Therefore, more basic research in biological systems associated with the ecology of human decomposition is necessary for refinement of formulae for estimating the PMI. Forensic entomology employs knowledge of the basic biology of entomofauna, systematics, and ecology in estimating the PMI, but this comes with shortcomings, as the method is not always applicable (Benecke, 2001). For example, in cases of extreme weather conditions (i.e. cold) or other treatments that exclude insects such as enclosure or burial, the time between death and occupation of a cadaver is prolonged. In these instances, science must turn to organisms that are ever-present and less obvious: bacteria (Pechal et al., 2014).

The Process of Decomposition

One of the places you least expect to find life is on a dead body, yet cadavers represent a nutrient-rich ecosystem teeming with organisms (Carter et al., 2007; Pechal et al., 2013). Understanding the processes and roles of organisms associated with decomposition presents possibilities for more accurate reconstructions of perimortem and postmortem events. This knowledge can inform procedures, such as estimation of

postmortem interval by law enforcement, anthropologists, and pathologists (Pinheiro, 2006; Goff, 1993; Love and Hamilton, 2011; Parsons, 2009).

Decomposition is the chemical process by which the remains break down into elements for assimilation back into the ecosystem, and in forensic taphonomy, decomposition or putrefaction is the means by which a cadaver becomes a skeleton through destruction of soft tissue. The soft tissues are broken down through the action of bacteria, expelling gases, liquids, and smaller molecules (Pinheiro, 2006; Vass, 2001). Studies of forensic taphonomy are concerned with the postmortem processes of dead organisms' remains and reconstruction of the biology, ecology, and circumstances of their death (Haglund & Sorg, 1997; Metcalf et al., 2013).

The speed of reaction of chemical reactions and microbial actions occurs according to an accumulation of heat units and is calculated by the accumulated degree days (ADD). ADD is a measure of the function of time spent between the minimum and maximum heat units. The maximum and minimum demarcate the heat units at which all metabolism is still feasible (Hyde et al., 2014). The heat units are incremental and can be used to measure growth based on a 24-hour period (Gennard, 2012). The formula for ADD is

$$\mathbf{ADD} = \frac{(\mathbf{T}\boldsymbol{min} + \mathbf{T}\boldsymbol{max})}{\mathbf{2}} - \mathbf{T}\boldsymbol{base}$$

Where T is the temperature, *min* is thermal minimum; *max* is the thermal maximum and *base* is the temperature below which metabolic function ceases. Our experiment used a standard base temperature of 0 °C in ADD calculations (Ames and Turner, 2003).

A general and typical pattern of decomposition has been described based on studies of various types of remains, though the knowledge is far from complete. This

basic pattern is resultant from by both intrinsic and extrinsic factors (Wolff et al., 2001). Microbial metabolism may play a major role in the tempo and mode of decomposition, yet little is known on the mechanism of that activity on remains (Janaway, Percival & Wilson 2009; Vass 2001, Dickson et al., 2011).

Approximately 4 minutes after a human body has been declared dead, internal temperature drops. *Rigor mortis* sets in and will persist until cellular autolysis initiates, which is the digestion of the body's macromolecules by enzymes. At this point, decomposition begins and microbes start using the nutrients released during autolysis (Pinheiro, 2006; Vass 2001). Detectable changes occur on the body as it decomposes. These include visual cues such as color changes, texture changes, and expelled substances such as liquids (blood, water, and oils) and gases (Adlam and Simmons, 2007). Remains ordinarily go through five major categories of decomposition. First is the fresh stage, marked by discoloration of the flesh. Early decomposition is next, which includes the onset and cessation of bacterially induced bloat. Advanced decomposition, also known as active decay (including purge that marks transition from early to late decomposition) follows.

Post-decay, or late decomposition, is characterized by dry tissue and precedes skeletonization, the final stage. Transitions into these stages are not clearly demarcated (Hagnund & Sorg (ed), 1997; Megyesi et al., 2005, Hyde et al., 2014; Gennard, 2012; Byrd and Castner, 2010; Metcalf et al., 2013; Carter et al., 2007; Parkinson et al., 2009; Benbow et al., 2013). A single cadaver may show non-uniform decomposition rates on different body areas, (Pinheiro, 2006; Haglund & Sorg, 1997; Megyesi et al., 2005; Hyde

et al., 2014), which is a phenomenon that challenges established protocols for determining the PMI.

Subcategories of the major classifications of decomposition are also observed. These do not necessarily follow the suggested order for all individuals, as each set of remains is known to show variant patterns of decomposition depending on micro-environmental or ecological factors and other conditions. This leads to either decay or preservation. In forensic investigations, postmortem interval calculations have often been based on forensic entomology as a tool, which in turn depends on climate, geography, topography and the vertebrate and invertebrate biodiversity (Iancu et al., 2015). These differ from place to place and there are many factors affecting the pre-colonization interval (Metcalf et al., 2013).

Arid conditions have been associated with mummification in cadavers. Mummification occurs when the skin turns leathery and hard due to desiccation although underlying skin may still be soft and continue to produce an odor. Mummification is typically a result of dehydration of inaccessible tissue, usually skin. Confinement or enclosure may result in saponification, a pattern divergent from the general pattern of decomposition and mummification. Saponification refers to hydrolysis of fat by anaerobic bacteria on a decomposition resulting in adipocere, a whitish “grave wax”. (Galloway, in Haglund and Sorg, 1997; Pinheiro, 2006; Vass 2001).

The Ecology of the “Necrobiome”

Multiple factors and a variety of organisms are involved in the decomposition of human remains. Temperature, oxygen partial pressure, pH, and moisture are major factors affecting decomposition progression and rate (Vass, 2011). The same are major

factors influencing bacteria ecology (Slonczewski and Foster, 2017; Carter et al., 2008). A study on neonatal pigs arrived at a conclusion that temperature and rainfall records affect rate of decomposition significantly (Archer, 2004). Optimal conditions of high temperature and rainfall, for microbial metabolism as well as abiotic reactions on remains were present. The rate of decomposition and hence calculation of PMI have been associated with flies of forensic importance. The growth and development of larvae of these flies of forensic importance is directly affected by temperature (Slone & Gruner, 2007; Heaton et al., 2014). In the same study, temperatures of maggot masses were completely independent of ambient temperatures and the higher the maggot volume, the higher the temperatures (Peters, 2003).

A dead body represents a rich source of nutrients for many organisms; therefore, when a cadaver is decomposing, it is exposed to, and affected by a variety of elements in terrestrial ecosystems. The necrobiome is an umbrella term for the ecosystem of decomposition (Pechal et al., 2014). This includes organisms that make use of nutrients and release them into the environment. The necrobiome may include vertebrate scavengers such as birds (including vultures), carnivorous mammals, and other incidentals (Swift, Heal and Anderson, 1979; Lauber et al., 2014; Gennard 2012; Byrd & Castner, 2010). There are also invertebrate scavengers, primarily insects. It has been noted that the rate of carcass decomposition is conspicuously reduced when insects are excluded, making the presence or absence of insects the biggest influence on rate of decomposition (Simmons and Lynch-Aird, 2016; Benbow et al., 2013).

Entomofauna of cadavers primarily include the orders Diptera and Coleoptera. (Lindgren et al., 2015; Moretti and Godoy 2013; Hyde et al., 2014; Tòth et al. 2001,

Willey, Sherwood & Woolverton, 2008), but the intimate association between the cadaver and larval Diptera may be the most notable ecological interaction. Almost immediately after death, flies of the family Calliphoridae lay eggs in the moist orifices of the cadaver (Thompson et al., 2013; Hyde et al., 2014). It is because of this timing of colonization that the age of the maggot as recorded by accumulated degree-days and the time since death, or PMI, are correlated making maggot age an indispensable tool of forensic investigations.

Entomofauna ecology and biology is highly dependent on climate, geography, topography and the biodiversity in a place (Iancu et al., 2015; Simmons and Lynch-Aird 2016). Ecological factors differ from place to place.

The role insects have in decomposition, is not limited to their feeding from soft tissues but also includes their role as carriers of microorganisms such as bacteria (Byrd and Castner, 2009; Crooks et al., 2016).

These microbes are usually symbionts whether mutualistic, parasitic or protocooperants, and are significant in decomposition ecology (Hibbing et al., 2010; Willey, Sherwood & Woolverton, 2008). Dickson et al. (2011) suggested that marine microbial succession studies could provide information pertinent to PMI estimation of bodies at sea. Finley et al. (2015) also showed the potential of graveside microbes as a molecular clue for PMI estimation while Metcalf et al. (2015) suggests that more insight into microbial community assembly and metabolism has the potential to explain postmortem events of decomposition . Hyde et al. (2014) demonstrated for the first time that the microbiome of a human cadaver changes through time. Initial bacteria communities before purge are highly variable, but after purge and until the cadaver dries

out, fly-associated bacteria dominate the microbiome, and finally, once the cadaver is dehydrated, soil-associated bacteria dominate the microbiome (Hyde et al. 2014). Common during bloat and purge are two bacteria of the family Gammaproteobacteria, genus *Ignatzschineria* (Tóth et al., 2001; Tóth et al., 2006; Tóth et al., 2007), and genus *Wohlfahrtiimonas* (Tóth et al., 2008).

The bacteria genus *Ignatzschineria* (homotypic synonym *Schineria*) was described based upon the type *Ignatzschineria larvae* by Tòth and others (2001). This group of bacteria uses chitin (Tòth et al., 2007). Four strains of *Schineria* were originally extracted from the first and second larval stages of *Wohlfahrtia magnifica* (Diptera: Sarcophagidae). The same were also extracted from the gastrointestinal tract of adult flesh flies (Gupta et al., 2011).

Under transmission electron microscopy (TEM), cells of *Ignatzschineria* appear as small single rods, non-motile, with gram-negative cell wall structure and some invaginations. *Ignatzchineria* has three species: *indica, ureiclastica,* and *larvae*. *Ignatzschineria indica* colonies can be grown on Mueller Hinton (MH) agar at 37 °C, and are able to grow on nutrient agar (N/A), MacConkey agar, *Salmonella Shigella* agar, brain infusion agar, and King's medium. They are positive for catalase, oxidase reactions, phosphatase activity and phenylalanine deaminase reaction (Gupta et al., 2011). *Ignatzschineria ureiclastica* colonies also grow on MH agar, are non-pigmented and entire, and grow on the same media as *Ignatzschineria indica* (Gupta et al., 2011).

Several cases exist in the literature where bacteria of the genus *Ignatzschineria* were associated with flies of the family Calliphoridae, as well as in the laboratory with larvae of the family Sarcophagidae. In most cases, the bacteria have been extracted from

wounds with myiasis as well as the urinary tract of an unhygienic person (Gupta et al., 2011; Le Brun et al., 2015; Barker et al. 2014). When the contents of guts of larval flesh flies were cultured in the laboratory, *Ignatzschineria* were very common (Gupta et al., 2014; Tòth et al., 2005). *Ignatzschineria* have been extracted from the labellum, tarsi, and oocytes of female flesh flies and blowflies (Lynne-Bucheli Lab, in preparation).

Vass (2001), in an experiment aimed at finding methods outside of entomology to estimate the time since death, turned to the role of microbes in decomposition. He concluded that this knowledge could not be used because of the vast diversity (hundreds) of microbes he identified during decomposition. According to him because of the many microbes, it was not possible to trace any pattern that could inform a microbial clock. In 2010, Hibbing and others investigated bacterial interaction in vitro, which included *Bacillus* and *E. coli* that Vass had quoted previously as part of the hundreds of microbes of decomposition. Javan et al. (2016) explore the potential use of thanatomicrobiome, the microbiome already present in the body antemortem, in PMI estimation through microbial evidence. Follow up work by Hauther et al. (2015) profiles gut microbiome succession postmortem to identify any potential trends for use in PMI estimation.

In a study, suggesting microbes had the potential to provide a more accurate method of PMI estimation, Metcalf et al. (2013) showed that Gammaproteobacteria composition increased significantly (seemingly at the expense of other families) right before rupture (purge) during decomposition and stayed high through active decomposition. This finding seemed to suggest two things: 1) that Gammaproteobacteria thrived better than other families during active decomposition, and 2) that the family Xanthomonadaceae composition changed rapidly around rupture. Hyde et al., (2014)

demonstrated that after purge and until the cadaver dries out, fly-associated Xanthomonadaceae bacteria, specifically *Ignatzschineria*, dominate the microbiome. Their findings showed that relative abundance of *Ignatzschineria* was inversely proportional to relative diversity of other bacteria species (Hyde et al., 2014). This seemed to suggest competitive exclusion occurs among decomposition microbes.

In his thesis studies, Berry (2016), investigated the nature of competition among five bacteria types associated with decomposition: *E. coli, Salmonella enterica, Bacillus licheniformis,* and the fly-borne species *Ignatzschineria indica*. These bacteria were selected because of their association with the process of decomposition in the human body and the particular strains were picked according to availability. This investigation was *in vitro* through bacterial competition assays on agar plates. Berry (2016) hypothesized that I*gnatzschineria* bacteria would produce an exponentially higher rate of growth compared to other bacteria associated with human decomposition. In his thesis, Berry conducted competition assays of one-day growth experiments where the growth of two strains of *Ignatzschineria* (*indica* and *ureiclastica*), *Bacillus licheniformis*, *Escherichia coli,* and *Salmonella enterica* were plated on differential media. The growth rates estimated through colony counts. The findings suggested more growth as indicated by the number of colony forming units (CFUs), on plates where bacteria were in competition. These results suggested cooperation among the three bacteria as opposed to competitive exclusion. He concluded that the relationship between *E. coli* and *Ignatzschineria ureiclastica* and *I. indica* was commensalism as there was peak growth of both bacteria when grown together with *Escherichia coli* (Berry, 2016). Berry's 2016

research prompted taking the study onto experimental subjects for a further look into bacterial interactions.

My master's research has investigated the role of *Ignatzschineria indica* in decomposition, expecting that the presence of *Ignatzschineria* has significant positive impact on the rate of decomposition. By introducing different strains of bacteria that have been recorded in association with human decomposition, I assessed the role that *Ignatzschineria indica* has in decomposition. I used *Ignatzschineria indica* bacteria, to test the impact of the *Ignatzschineria* community on the rate of decomposition. **I hypothesize that adding the bacteria *Ignatzschineria indica* to mice remains will affect the rate of decomposition significantly.**

Results provided more insight into bacterial biology, and ecology during decomposition as we found out that in laboratory controlled conditions, and in the absence of vertebrate and invertebrate scavengers, the rate of mouse decomposition is not significantly affected by the bacterial treatments administered. Instead, the pathway of chemical reactions is altered, and this is indicated by different composition of the VOCs emitted and the significantly different subjects' temperatures, which represent the optimum temperatures for bacteria metabolism. This experiment added to our knowledge on decomposition, which is a key component in estimating PMI during forensic investigations. Knowing how bacteria composition affects subsequent VOCs produced is useful if we are to continue looking into a possibility of using the VOC succession pattern for estimating time since death.

CHAPTER II

IGNATZSCHINERIA INDICA AND RATE OF DECOMPOSITION IN MICE

Decomposition is an assortment of processes through which a freshly dead, nutrient rich organism is broken down through nutrient cycling processes (Vass 2001). End states of decomposition can vary but usually include mummification and /or skeletonization (Janaway, Percival & Wilson 2009). Decomposition is greatly influenced by microbial metabolism, yet little is known of the bacterial mechanism of this process on human remains (Dickson et al., 2011; Pinheiro, 2006). Specific roles that particular bacteria taxa play during decomposition are yet to be fully identified (Metcalf et al., 2013). It has also been shown that environmental parameters affect community composition of most decomposers, but it is not clear how these affect the microbial community of decomposition (Hopkins, 2008; Mondor et al., 2012).

Bacteria are found in and on living bodies with a diverse composition. It is well documented that host-associated microbial communities can drive disease due to their ability to differ between healthy and disease states (Goodrich et al., 2014). This normal combination of bacteria strains found in and on the living body is called the human microbiome. An earlier study by Hyde et al. (2014) echoes the suggestion that microbes, including the bacteria family Xanthomonadaceae, to which the genus *Ignatzschineria* belongs, are key contributors to decomposition as well and experience the greatest change in relative abundance during decomposition.

Myiasis is a disease of vertebrate animals triggered by neglected open wounds, poor hygiene, and different fly larvae affecting the tissues and organs of these animals.

The etiology of myiasis is still being uncovered (Tòth et al., 2001). From sequencing bacteria from myiasis wounds, species of *Ignatzschineria* have often been found to coexist (Thompson et al., 2013). The strains *Ignatzschineria indica* and *I. ureiclastica* were extracted from the necrotic wounds of a 69-year-old sick man in France (Le Brun et al., 2015), leading to the conclusion associating *Ignatzschineria* with necrotic wounds and maggot infestation. In this situation, the wound did not heal even after removing maggots and *Ignatzschineria* was still in the system hence the suggestion that the necrosis had more to do with the bacteria than the maggots. Three other patients in whom *Ignatzschineria* was found in the urinary tract, also had maggot infested wounds, supporting this hypothesis (Barker et al., 2014).

A recent study by Berry (2016) suggests that peak growth of species of *Ignatzschineria* is obtained when they are grown together with *Escherichia coli.* This was investigated in vitro through bacterial competition assays on agar plates. In his thesis, Berry conducted competition assays of one-day growth experiments where the growth of two strains of *Ignatzschineria* (*indica* and *ureiclastica*), *Bacillus licheniformis*, *Escherichia coli,* and *Salmonella enterica* were plated on differential media. Growth rates were estimated through colony counts. The findings suggested more growth as indicated by the number of colony forming units (CFUs) on plates where bacteria were in competition. These results suggested cooperation among the three bacteria as opposed to competitive exclusion. These were possible outcomes as suggested by Majeed et al. (2011) He concluded that the relationship between *E. coli* and *Ignatzschineria ureiclastica* and *I. indica* was commensalism as there was peak growth of both bacteria when grown together with *Escherichia coli* (Berry, 2016).

I hypothesize that presence of the bacteria *Ignatzschineria indica* will affect the rate of decomposition significantly. An experiment was designed to answer the question, "Does *Ignatzschineria* bacteria affect the rate of decomposition in mice under laboratory controlled conditions?" To achieve this, mice were inoculated with different cocktails of bacteria and subsequently allowed to decompose. The rate of decomposition was assessed visually according to a standard visual scoring scale adapted from Megyesi et al., 2005 and Metcalf et al., 2013. Data were analyzed using repeated measures ANOVA and other analyses in SAS version 9.4. Results indicated that the rate of decomposition of mice remains is not significantly affected by the initial bacterial decomposition under laboratory conditions and in the absence of scavengers.

Materials and Methods

Test subjects

To investigate the effect of *Ignatzschineria indica* bacteria on decomposition, a mouse model was selected to achieve replication standards for statistical analysis. Hairless, large frozen feeder mice (*Mus musculus*) were ordered online (www.rodentia.com) and thawed prior to the experiment. All mice were acquired from the same supplier and attempts were made to ensure the same range in size was maintained. To investigate the effect of the bacteria strains on rate of decomposition, the laboratory conditions were kept as constant and uniform as possible; the only differences intended were the bacterial treatment. The skin of all mouse subjects was swabbed with ethanol at the beginning of the experiment.

Muslin chambers

Rectangular muslin bags were sewn using unbleached muslin cloth. They were designed to keep the mice free of exogenous bacteria and scavenging insects during the trial period and to prevent complete dehydration by limiting dry airflow. Each bag was fitted with a Velcro® closure to allow repeated access through time to visually assess stage of decomposition. Muslin chambers were labeled according to treatment. See **APPENDIX 2.**

Muslin chambers were made to enclose completely two medium-sized VWR® Pour-Boats Weighing Dishes, with one inverted on top of the other. For each experimental unit one hairless feeder mouse was laid on its back on a sterilized weigh boat and held in place by the feet via sterile surgical tape. Each mouse was treated experimentally and covered with another weigh boat. Then each experimental unit was sealed in its respective labeled muslin chamber.

Controls

To create the positive control (PC), sterile flytraps baited with feeder mice were placed in the woods to attract flies native to the piney-woods ecoregion of southeast Texas. These traps also served as incubation chambers for eggs laid by collected female flies and the subsequent developing larvae. I prepared sterile fly incubation chambers by autoclaving sand and immersing the chamber and weigh boats in alcohol. These were stored in an incubator, which was enclosed in a humidifier at 37 ºC for the experiment. The negative controls (NC) were treated by inoculating the mice with phosphate buffered saline solution (PBS).

Experimental bacteria cultures

Using standard microbiology laboratory techniques, four strains of bacteria, *Ignatzschineria indica, Escherichia coli, Bacillus licheniformis, and Salmonella enterica,* were cultured on individual Nutrient agar (N/A) plates for 24-36 hours, from existent pure culture stocks. The grown pure colonies were cultured in 20 mL Luria-Bertani (LB) broth, in 250 mL Erlenmeyer flasks, in an incubator shaker. The settings for the shaker were 150 revolutions per minute (rpm) at 37 °C for 24 hours. Growth cultures were transferred to centrifuge tubes and centrifuged at 4,000 rpm for 4 minutes to pelletize. The supernatant was discarded and dilutions were made with PBS to achieve 300 mL of each solution. A spectrophotometer was used to determine a 0.5 optical density (OD) at a 650-nm wavelength.

Trials

On Day 0, placement day, frozen mice were thawed; their skin was sanitized with 70% alcohol; and each was fastened onto weigh boats with sterile surgical tape. Flies were added to all PC treatments and left for an hour while bacteria dilutions were prepared. Flies were removed from the PC treatments an hour, long enough for inoculation without ovipositing. The rest of the treatments were also inoculated by pipetting a drop of the respective mixtures on each of the orifices to mimic egg-laying behavior in female flies: eyes, ears, mouth and anus. All weigh boats of inoculated mice were placed into their respective labeled muslin chambers or “body bags”.

TREATMENT	BATCH SAMPLE 1	BATCH SAMPLE 2	BATCH SAMPLE 3	*n*
A	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	10 + 10 + 10
B	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	10 + 10 + 10
C	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	10 + 10 + 10
D	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	10 + 10 + 10
BA	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	10 + 10 + 10
CA	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	10 + 10 + 10
DA	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	10 + 10 + 10
NC	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	10 + 10 + 10
PC	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	10 + 10 + 10

Figure 1. Experimental Design showing the set up for batches of mice decomposition assay. An experimental unit refers to one mouse in a weigh boat and enclosed in the muslin chamber as represented by the small numbered hexagons. N, the total number of experimental units is made up of nine "3 by 10" sets of mice subjects equal to 270.

Due to space constraints, three batches (Batches 1, 2 and 3) of 90 subjects each were set up at different times. Batch 1 was set up on the 4th of February 2017. Batch 2 on the 24th of February 2017 and batch 3 on the 23rd of March 2017. Each batch had 10 replicate subjects, bringing the total mice subjects for the whole study to 270 decomposition assays. Each batch had nine experimental treatments; A, B, C, D, BA, CA, DA, and two controls (PC and NC). Treatment A contained *Ignatzschineria indica*.

Treatment B contained *Escherichia coli* only. Treatment C contained *Bacillus licheniformis* only. D contained *Salmonella enterica* only. BA was a homogenized mixture of equal volumes of treatments B and A, CA had equal volumes of treatments C and A, DA was a homogenized mixture of equal volumes of treatments D and A. All mixtures were suspended in phosphate buffered saline (PBS) solution at 0.5 OD. See Figure 1 for the experimental design, which is a repeated measures design (Hargrave et al., 2011).

Data collection and sorting

Subjects were visited every day for 15 days, the period of time shown in pilot studies in which subjects reached the mummification stage, where gross taphonomic changes become less noticeable due to an extremely slowed rate of decomposition. During observation periods, all conducted at 10am daily, pictures of each experimental unit were taken using the standardized set up shown in the pictures in Appendix 2. An assessment of the state of decomposition was noted each day in the comments section of the data sheet (see data sheet in Appendix I), for each experimental unit, which was based on three main observable visual cues in color, skin texture, and observable "exudates" at each stage.

The rate of decomposition was quantified for each subject using a photographic time line of each of the 270 subjects (4,050 photographs were generated during the course of the experiment). I used a scoring system for the torso modified from the Megyesi et al. (2005), and Metcalf et al. 2013 systems. In this system, a series of descriptions of visual cues of decomposition are assigned a score with the early stage being one and increasing as decomposition progresses.

Temperature

Measurements of the temperatures, of the laboratory (ambient) and each subject were taken at each observation.

Bacteria swabs

During the active phase of decomposition of the mice (first 5 days), abdominal swabs were taken during sampling times as well for future metagenomics analyses.

Visual scoring system

As the trial progressed, it was obvious that we would only have 3 major visual cues of decomposition in the mice. These include the color changes, the texture change in the skin and the observable exudates, gases or liquids released as the body decomposed Table 1.

Statistical Analysis

A series of statistical analyses were employed in this study. To determine whether there was any batch effect on our results, we used 2-sample t-tests comparing means among batches 1, 2, and 3 in SAS. Once it was established that there was no batch effect among the samples, a repeated measures ANOVA was carried out on the total body score (TBS), and partial body scores for head and torso (PBS), while also considering whether it was wet decomposition (day 0 to 7) or dry decomposition (day 8 to 15). See **APPENDICES V** to **VII** for procedures and results.

Table 1. **Visual Cues in mice decomposition**

COLOUR	TEXTURE	EXUDATES
Fresh pink	Normal	Blood and liquids purged
Pale color	Stretched	Gases released
Marbling	Saggy	Oils released
Green	Sinking	
Gray	Sunken but soft	
Black	Dry and hardened	

Note. A description of visual cues observed during decomposition of mice under laboratory controlled conditions

Table 2. **Visual scoring system for the torso.**

A. Fresh	
(1pt)	1. Fresh, no discoloration
B. Early Decomposition	
(2pts)	1. Pink-white appearance (slippage/ boil-like spots) marbling (deep red blood like appearance due to veins showing)
(3pts)	2. Gray to green discoloration: some flesh relatively fresh
(4pts)	3. Bloating with green discoloration and purging of fluids
(5pts)	4. Post-bloating following release of abdominal gases. Discoloration change from green to black.
C. Advanced decomposition	
(6pts)	1. Decomposition of tissue producing sagging of flesh; caving in of abdominal cavity
(7pts)	2. Drying Out with moisture loss, abdomen still soft on the inside, sometime more gray than black
(8pts)	3. Mummification with no bone exposure (moisture loss and hardening)

Table 3. **Visual Scoring system for the head.**

Fresh	
(1pt)	Fresh, no discoloration
B. Early Decomposition	
(2pts)	Discoloration
(3pts)	Bloating of neck and/face
(4pts)	Purging of decomposition fluids out of eyes, nose mouth ears
C. Advanced Decomposition	
(5pts)	Decomposition of tissue producing sagging of flesh
(6pts)	Sinking of flesh
(7pts)	Caving in of flesh
(8pts)	Mummification

Results

Overview

Total body scores for each experimental unit ranged from 2 (fresh) to 16 (mummified) See Figure 2, showing an example of the photos during decomposition for treatment A. On each day, the photos showed different states of decomposition that varied among individuals and among the batches. Figure 3 shows the differences in distribution of scores among the different treatments daily. The mode total body score per day per treatment was used to determine the stage of decomposition for a given treatments on a given day and the series of photographs most representative of this progression were used to make the series of photographs represented by Figure 3. Photo examples for each treatment are included in **Appendix III**. Total body scores (TBS) were calculated for all 270 mice subjects throughout the 15-day decomposition period. **Appendix IV** shows a pullout of the table of raw TBS data. The series of photographs below show the state of decomposition for each of the different treatments at corresponding log (ADD) and for each day throughout the decomposition experiment.

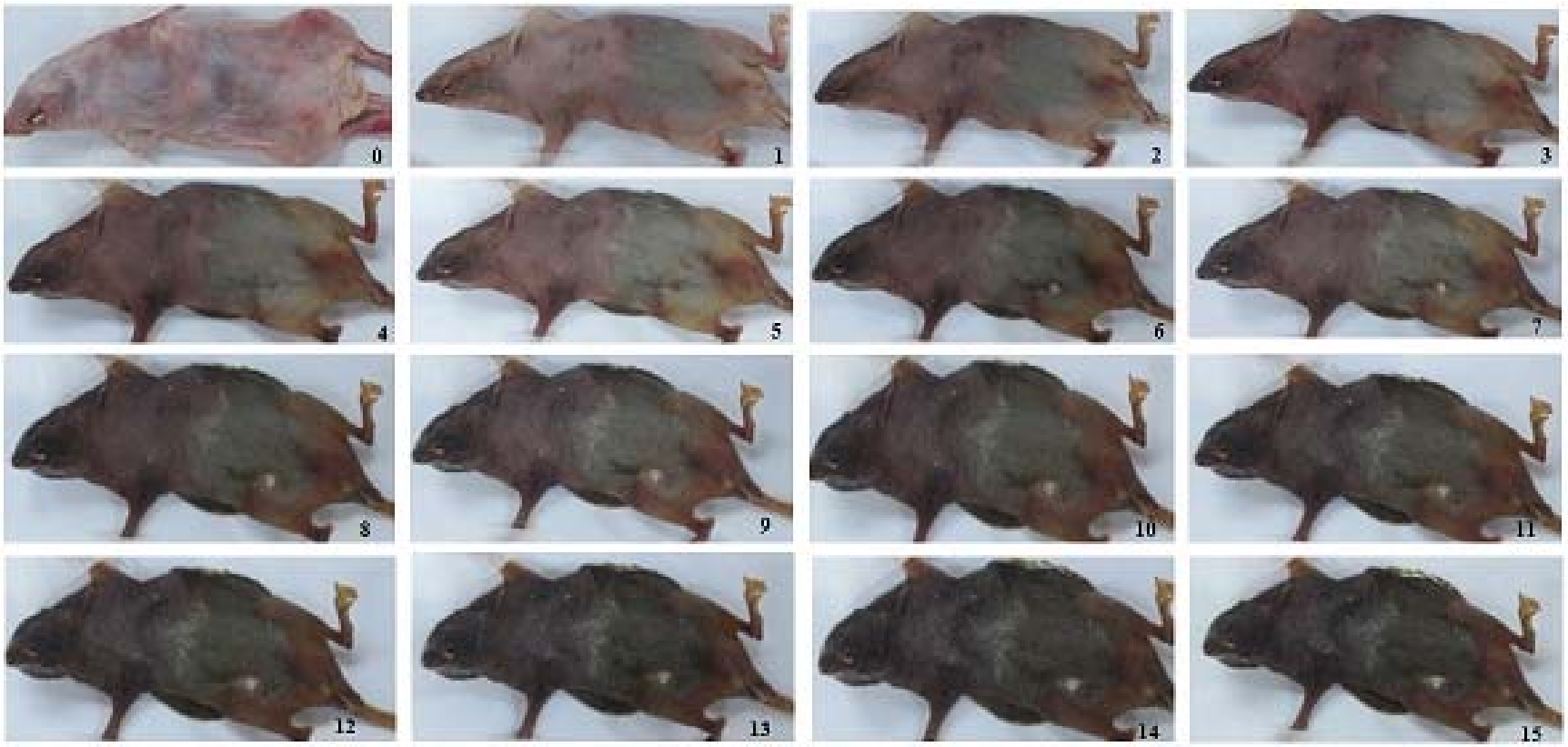

Figure 2. Progression of decomposition in mice inoculated with Ignatzschineria indica (A) under laboratory conditions over 15 days. Showing the progress of decomposition of one mouse with Ignatzschineria indica treatment as an example**.**

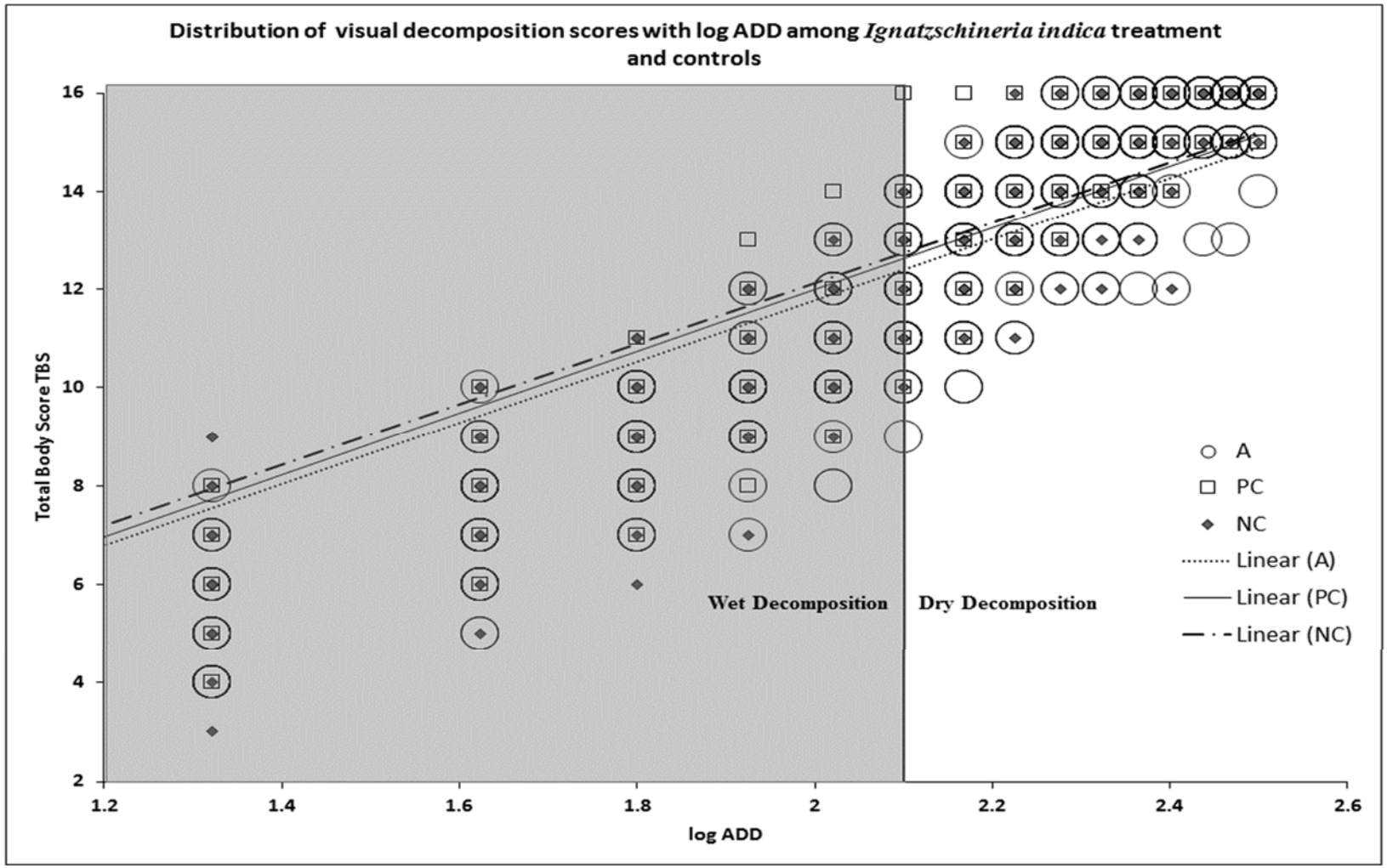

Figure 3. Distribution of TBS per log ADD for 30 specimens each of treatments A, PC and NC.

Overall rates of decomposition

Table 4 shows the number of days it took for decomposition states to change for each treatment as a means of comparing the rate of decomposition among the treatments at each stage of decomposition as shown on the scoring system. It generally took 2 days for every treatment to reach purge, with the exception of treatment DA (a combination of *Ignatzschineria indica* and *Salmonella enterica*), which prolongs the stage to day 3. Little else is noteworthy except the record that sample D seemed slower than the rest as by Day 1 progressing one visual score stage while A, C and CA had progressed 2 visual score stages in decomposition and then progressed to visual score stage 3 within 24 hours. The rest progressed to later stages in 24 hours. This visualization of the results prompts testing the significance of the viewed differences.

Table 4. **Timeline for the progression of decomposition showing for each treatment and the day each treatments moved to the next stage.**

Decomposition stage		Description of the visual cues according to the scales	A	B	C	D	BA	CA	DA	NC	PC
Fresh	A1 A1	Head Fresh, no discoloration. Torso Fresh, no discoloration	0	0	0	0	0	0	0	0	**0**
	B1 B1	Head Discoloration Torso Pink-white appearance (slippage/ boil-like spots) marbling (deep red blood like appearance due to veins showing)				1					
	B1 B2	Head Discoloration Torso Gray to green discoloration: some flesh relatively fresh		1	1		1		1	1	**1**
	B2 B2	Head Bloating of neck and/face Torso Gray to green discoloration: some flesh relatively fresh	1		1			1			
	B2 B3	Head Bloating of neck and/face Torso Bloating with green discoloration and purging of fluids	2				2		2		**2**
Early	B3 B3	Head Purging of decomposition fluids out of eyes, nose mouth ears Torso Bloating with green discoloration and purging of fluids	2	2	2	2		2	3	2	
	C1 B3	Head Decomposition of tissue producing sagging of flesh Torso Bloating with green discoloration and purging of fluids				3	3	3		3	**3**
	C1 B4	Head Decomposition of tissue producing sagging of flesh Torso Post-bloating following release of abdominal gases. Discoloration change from green to black.	3	3	3	4	4	4	4	4	**4**
	C1 B4	Head Decomposition of tissue producing sagging of flesh Torso Post-bloating following release of abdominal gases. Discoloration change from green to black.	4	4	4	4	4	4	4	4	**4**
Advanced	C2 B4	Head Sinking of flesh Torso Post-bloating following release of abdominal gases. Discoloration change from green to black.	4		4						**5**
	C2 B4	Head Sinking of flesh Torso Post-bloating following release of abdominal gases. Discoloration change from green to black.	5		5						**5**
	C2 C1	Head Sinking of flesh Torso Decomposition of tissue producing sagging of flesh; caving in of abdominal cavity	6	6	6	6	6	6	6	5	**6**
	C2 C1	Head Sinking of flesh Torso Decomposition of tissue producing sagging of flesh; caving in of abdominal cavity					7			6	**6**

(continued)

Decomposition stage		Description of the visual cues according to the scales	A	B	C	D	BA	CA	DA	NC	PC
	C2 C2	Head Sinking of flesh Torso Drying Out with moisture loss, abdomen still soft on the inside, sometime more gray than black			7		7				
	C3 C1	Head Caving in of flesh Torso Decomposition of tissue producing sagging of flesh; caving in of abdominal cavity							7		**7**
Late	C3 C2	Head Caving in of flesh Torso Drying Out with moisture loss, abdomen still soft on the inside, sometime more gray than black	7	7	8	7	8	7	8	7	**8**
	C3 C2	Head Caving in of flesh Torso Drying Out with moisture loss, abdomen still soft on the inside, sometime more gray than black	8	8	9	8	9	8	9	8	**9**
	C3 C2	Head Caving in of flesh Torso Drying Out with moisture loss, abdomen still soft on the inside, sometime more gray than black	9				10	9	10	9	
	C3 C2	Head Caving in of flesh Torso Drying Out with moisture loss, abdomen still soft on the inside, sometime more gray than black						10			
	C4 C2	Head Mummification Torso Drying Out with moisture loss, abdomen still soft on the inside, sometime more gray than black	10	9	10	9	11		11	10	**10**
	C4 C2	Head Mummification Torso Drying Out with moisture loss, abdomen still soft on the inside, sometime more gray than black	11	10	11	10	11		11	11	**11**
	C4 C2	Head Mummification Torso Drying Out with moisture loss, abdomen still soft on the inside, sometime more gray than black		11		11	11		11		**12**
Post-decay	C4 C3	Head Mummification Torso Mummification with no bone exposure (moisture loss and hardening)						11			
	C4 C3	Head Mummification Torso Mummification with no bone exposure (moisture loss and hardening)	12	12	12	12	12	12	12	12	**12**
	C4 C3	Head Mummification Torso Mummification with no bone exposure (moisture loss and hardening)	13	13	13	13	13	13	13	13	**13**
	C4 C3	Head Mummification Torso Mummification with no bone exposure (moisture loss and hardening)	14	14	14	14	14	14	14	14	**14**
	C4 C3	Head Mummification Torso Mummification with no bone exposure (moisture loss and hardening)	15	15	15	15	15	15	15	15	**15**

Two partial body scores were obtained from scoring the head and torso of each subject separately each day through the sampling period. Total body scores were calculated for each day by combining the two partial body scores for each of the 270 specimens. A total of 4 320 data points were generated. Before assessing the rate of decomposition, a two-sample t-test was done to test the hypothesis that there is no significant effect between batches for the scores obtained since the experiment was carried out in 3 identical batches due to limits of space and manpower. Results of this series of t-tests are shown in Appendix V.

Based on Pr (t) values we conclude that there is minimal batch effect. Most of the treatments did not have a significant difference [Pr (t) >0.05]. To note however, is the significant difference between Batch 1 and 3 in Samples BA (Pr (t) =0.041332), and Batch 2 and 3 in Samples DA [Pr (t) =0.030087]. Results of this test justified adjusting from a multifactorial repeated measures design shown in Figure 1 to a simple repeated measures ANOVA as shown in Figure 4.

TREATMENT	SAMPLE	*n*
A	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30	30
B	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30	30
C	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30	30
D	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30	30
BA	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30	30
CA	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30	30
DA	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30	30
NC	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30	30
PC	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30	30

Figure 4. Experimental design as analyzed using ANOVA. Note the absence of batches so sample size n=30.

Investigating the effect of bacteria treatments on decomposition

Repeated Measures ANOVA using Total body scores (TBS).

To test for the significance of the differences in TBS with ADD among the batch samples, a Repeated Measures analysis of variance (ANOVA) was carried out, the null hypothesis being that results from all 9 treatments A, B, C, D, BA, CA, DA, NC, and PC,

are not different from each other. See data tables, SAS codes and raw results for this section in **Appendix VI.**

Results of this repeated measures test conducted in Statistical Analysis Software (SAS) version 9.4 on mice total body scores (TBS), suggest that the value of log (ADD) has a significant effect on the state of decomposition as described by the scores ($p < 0.0001$). There was also a significant interaction between treatment and log (ADD) (Pr (F) = 0.0133). There was not a significant main effect of bacteria treatments on decomposition, [Pr (F) = 0.7943]; failure to reject H_0 indicates that at any given time point, the average state of decomposition among the differently treated samples is not significantly different under these laboratory controlled conditions. Table 4.

Repeated Measures ANOVA using Partial body scores (heads)

Separate analyses were conducted on head and torso scores and wet and dry decomposition stages. Overall the 9 different bacterial treatments do not show a significant difference in rate of decomposition of the heads whether throughout the entire observation period or during wet or dry decomposition periods only, as indicated by P(F) of more than 0.05 shown in Tables 4, 5 and 6. The same analyses however, show that the interaction between bacterial treatments and log ADD shows a significant difference with time hence the mice subjects PBS of heads changes with change in log ADD. Knowing that different regions of the body decompose at different rate, we analyzed the head scores and torso scores separately in search of any possible trends in rate of decomposition, among the different bacteria treatments.

Repeated Measures ANOVA using torsos PBS.

Tables 9-11 show results of repeated measures on partial body scores.

Results from these tests indicate that bacterial treatments; A, B, C, D, BA, CA, DA, PC and NC, show rate of decomposition that is not significantly different whether throughout the whole observation period or during wet and dry decomposition of the torsos, as shown by the F probabilities greater than 0.05. The same results show that log ADD affects the rate of decomposition, a confirmation of the known fact that energy units' accumulation is directly proportional to rate of decomposition.

One Way ANOVA using the time taken to reach purge for each subject

A different way of measuring rate of decomposition was applied to the same data set to verify the results obtained from the repeated measures ANOVA. For each or the 270 mice subjects, we recorded the time taken to reach purge for the head and for the torso. We subjected the two resultant data sets to a One-Way ANOVA to test the hypothesis that the different bacterial treatments do not affect the time taken to reach purge for the subjects in Statistical Analysis System (SAS) procedure general linear model. (Vass, 1992; Megyesi et al. 2005; Simmons, Adlam and Moffatt, 2010). **Appendix VII.**

Analysis of time until purge of heads

The F statistic for a One-Way ANOVA was 1.02 p=0.4245 for heads PBS. We fail to reject H_0 and conclude that the treatments have no significant effect on time until purge of the heads (Figure 5).

Analysis of time until purge of torsos

Figure 6 shows mean distributions among the 9 treatments, the null hypothesis that there are no significant differences among the means of the 9 treatments for the torsos. The F statistic for a One-Way ANOVA was 1.26 p=0.2648 for torsos.

Table 5. **Results of between and within subjects' effect repeated measures ANOVA on TBS of 270 Mice subjects' decomposition assays.**

Source	F Value	p
Bacterial treatments	0.58	0.7943
log ADD	6774.93	<.0001
log ADD* **Bacterial treatments**	1.32	0.0133

Table 6. **Results of between and within subjects' effect repeated measures ANOVA on PBS on heads of 270 Mice subjects' decomposition assays.**

Source	F Value	p
Bacterial treatments	1.48	0.1654
log ADD	4280.04	<.0001
log ADD* **Bacterial treatments**	1.85	<.0001

Table 7. **Results of repeated measures ANOVA on head PBS during wet decomposition**

Source	F Value	p
Bacterial treatments	1.24	0.2760
log ADD	3756.27	<.0001
log ADD* **Bacterial treatments**	1.68	0.0014

Table 8. **Results of repeated measures ANOVA on head PBS during dry decomposition**

Source	F Value	p
Bacterial treatments	1.92	0.0593
log ADD	313.09	<.0001
log ADD* **Bacterial treatments**	1.69	0.0013

Table 9. **Results of repeated measures ANOVA on torsos PBS.**

Source	F Value	p
Bacterial treatments	1.02	0.4202
log ADD	6237.75	<.0001
log ADD* **Bacterial treatments**	1.23	0.0477

Table 10. **Results of repeated measures ANOVA on torsos PBS (during wet decomposition)**

Source	F Value	p
Bacterial treatments	1.02	0.4245
log ADD	1977.00	<.0001
log ADD * **Bacterial treatments**	1.18	0.1915

Table 11. **Results of repeated measures ANOVA on torsos PBS (during dry decomposition)**

Source	F Value	p
Bacterial treatments	1.38	0.2035
log ADD	376.37	<.0001
l log ADD * **Bacterial treatments**	1.04	0.3971

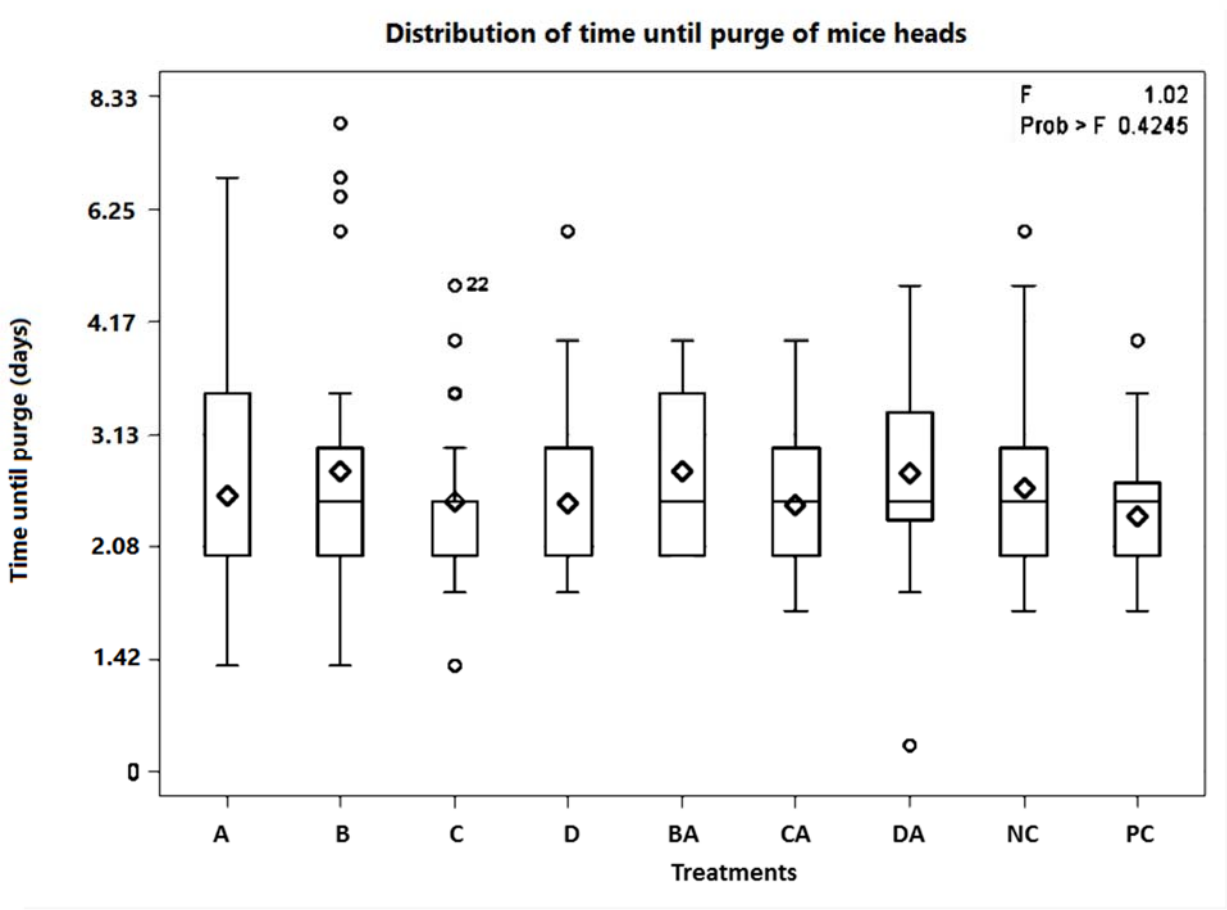

Figure 5. The distribution of sample means of time until purge in the samples across all treatments in the heads.

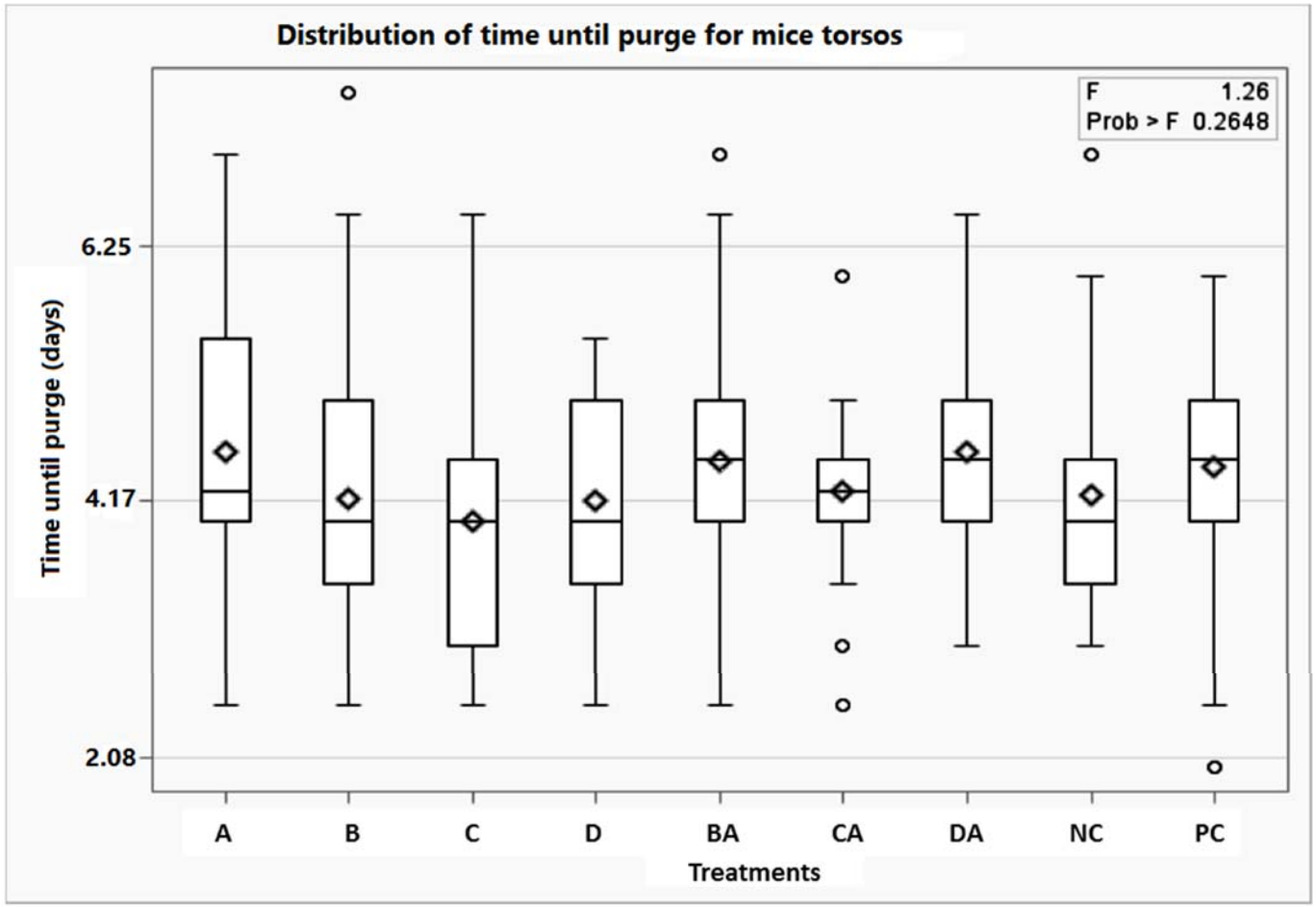

Figure 6. Showing the distribution of sample means of time until purge in the samples across all treatments in the torsos.

Overall rates of decomposition

In the two-sample t-tests that we subjected our scoring data initially, there was an anomaly, i.e. the significant difference between Batch 1 and 3 in Samples BA (Pr (t) =0.041332), and Batch 2 and 3 in Samples DA (Pr (t) =0.030087). This is possible if any one of the batches had a contaminated bacteria sample.

Overall, visual scores and odors did not appear the same during sampling, leading us to expect rates of decomposition to be different across the bacterial treatments, but the statistical tests gave a different result.

Repeated Measures ANOVA results

Results of all repeated measures ANOVA agreed on the result of the time effect as represented by log ADD, rejecting the H_0 that at different log ADD, the TBS or PBS means are the same. It has been shown in previous studies that as thermal heat units (ADD) accumulate, the total body score increases (Megyesi et al., 2005; Slone and Gruner 2007; Metcalf et al., 2013). Decomposition is largely driven by bacteria and arthropod process, each of which are dependent on accumulated heat units (as well as other factors). All repeated measures ANOVA results support the result that the differential bacterial treatments **DO NOT** affect the change in the visual decomposition score with time and change in ADD. **This answered our research hypothesis because it allows us to conclude that at uniform laboratory controlled conditions, introducing bacteria to a dead mouse does not alter the rate of decomposition in any significant way.** However, this was unexpected because we could observe differences visually during the experiments like time until mummification differences. This led to the idea that maybe a visual score may not be the best method of estimating the rate of

decomposition, hence a follow up One-Way ANOVA was implemented using the time until purge.

One Way ANOVA using the time taken to reach purge

The null hypothesis for this test was that there is no significant difference in time until purge among the 9 treatments. We failed to reject this null and concluded that the different treatments produced similar time until purge. This failure to reject null may be due to a possibility that bacteria composition may not be responsible for the rate at which decomposition takes place. This may be the most plausible explanation. Since all other factors affecting decomposition were held constant except the bacterial composition, we may conclude that bacteria composition affects the path taken by decomposition. This conclusion ties in with the different odors detected from the differently treated samples. The smells are from volatile organic compounds (VOCs), which escape as gaseous bi-products of the action of bacteria on decomposing material. This finding is further discussed in Chapter III.

CHAPTER III

VOLATILE ORGANIC COMPOUNDS AND DECOMPOSITION ODOURS

Introduction

Volatile organic compounds (VOCs), produced during bacterial metabolism, are responsible for many unpleasant odors given off antemortem (i.e., body odor) or postmortem (i.e. decomposition) (Brown, 2012). Scientists have explored the utility of VOCs during forensic investigations. A classic example is the Casey Anthony case where VOCs were used to trace the previous location of a dead body that had been discovered. Forensic investigator Vass found evidence that the dead body had been placed inside a car and hence associated a suspect with the murder because of traces of 5 human cadaver associated VOCs discovered on a piece of carpet from the trunk of that car (Ensminger, Ferguson and Papet, 2016). Microorganisms are the main producers of VOCs on human remains through metabolic processes including fatty acid fermentation, amino acid fermentation, and other forms of anaerobic fermentation (Brown, 2012). For VOCs to be used in locating human remains and identification of the stage of decomposition, it is critical to know how bacteria affect their composition (Deyne, 2016).

VOCs are part of the chemical communication signals used by invertebrates to locate suitable food or other ecological necessities (Mathis & Philpott, 2012) An established composition of common mammalian VOCs is responsible for attracting necrophagous entomofauna to dead bodies (Kasper, Mumm, and Ruther, 2012; Forbes, 2012). The bi-products of microbial metabolism and therefore the chemical attractants of flies may be affected by many factors including the antemortem diet of the deceased. Patterns of arthropod succession on a decomposing cadaver can be matched with

combinations of VOCs present during the different stages of decomposition (Paczkowski and Schutz, 2014). Because of this succession pattern observes in VOCs, a profile of VOCs may be useful in predicting PMI.

During this research, as the decomposition assays progressed, noticeably distinct odors emanated from each of the treatments. These odors prompted me to question their origin and the degree to which it may be due to differences in the bacteria treatments. To explore this question further, an experiment was designed to test qualitatively the volatile organic compounds exuded during the wet decomposition stage for each of the experimental treatments. This experiment was designed to test **the hypothesis that experimental treatment administered affects VOC composition** during the active wet decomposition stage; however, at no point were the results tested statistically.

The bigger question in this case is if pattern of succession of VOCs changes with changes in initial bacteria composition. For the purposes of this study, we sought to investigate any potential differences in VOC abundance in three different treatments (A, PC and NC), during the active stage of decomposition on day 6 of the decomposition assays. In our setup, mice of similar weight, size, age and antemortem diet were held under the same controlled laboratory conditions to standardize the experiment such that the only noticeable difference was the treatment (Goodrich et al., 2014). Previous literature suggests that the differences would affect the composition of VOCs but that the composition of insect attractants would still be comparable for each stage (Kasper, Mumm, and Ruther, 2012).

Materials and Methods

During the third batch of the decomposition assays, on day 6, six subjects were sampled for volatile organic compounds. These were from two replicate samples of each of the following experimental treatments; *Ignatzschineria indica* (A), the positive control (PC), and the negative control (NC). The second set of three samples was set up the same way as the experiment described in chapter II, as extras, but the mice were not disturbed or opened from set up day until day 6 when sampling of VOCs occurred. We adopted the sampling regime to ensure a stronger VOC intensity based on a thesis study by Deyne (2016). We used solid-phase micro extraction (SPME) in this laboratory experiment in a headspace extraction. This method was selected because of its ease and speed of use (Deyne, 2016; Frederickx et al., 2012).

Polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fibres (65 μm coating) in field samplers were used for VOC sampling. Conditioning of SPME fibres, i.e. heating them to purge packaging contaminants, was carried out for 30 minutes. The quantity of analyte extracted is proportional to its sample rations (Mester et al., 2000), allowing us to assess the relative abundance of different volatile substances in the sample. During the 30 to 40-minute extraction phase, the SPME fibre was inserted into the sample set up and suspended in the air trapped between the two weigh boats, without touching any solid material for 30 minutes at room temperature, 21 $^{\circ}$C. See illustration of experimental set up in Figure 7.

The fibre was drawn back into the protective case and covered for transportation to the Texas Research Institute for Environmental Studies (TRIES) laboratory.

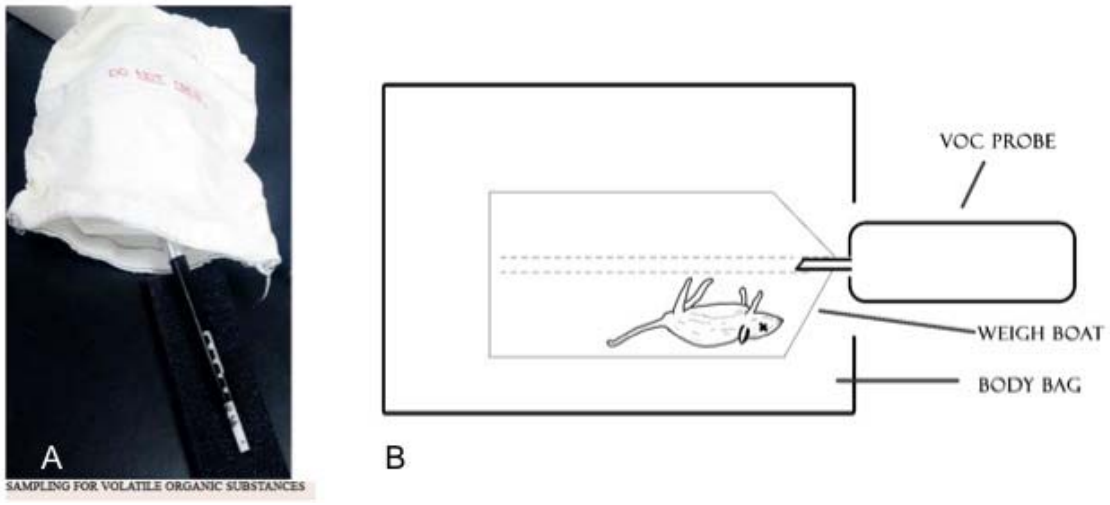

Figure 7. A. Experimental set-up for VOC sampling; B. Schematic diagram showing experimental set up.

The fibres from the 6 samples were individually inserted into the GC Mass spectrometer for analysis. The GC/MS was an Agilent Technologies 7890A GC with a 5975C mass-selective detector. The GC had a HP5/MS capillary column that was 30 m in length with a 0.25 mm inner diameter and 0.25 μm film thickness. The injection port was lined with a 0.75 mm inner diameter SPME injection port liner and was held at a constant 250 °C. The carrier gas was hydrogen. The GC oven was initially held at 30°C for three minutes, increased to 80 °C at 3 °C per minute, then to 120 °C at 10 °C per minute, and finally to 320 °C at 40 °C per minute. At each time point, the mass spectrum was collected from m/z 20 to 600.

A link to raw data is provided in the results section. The numerical equivalent for the abundance, i.e. the area under the wave peaks, was used to qualitatively assess the

differences among samples from *Ignatzschineria indica* (A), negative control (NC) and the positive control (PC).

Results

During analysis of the compounds adsorbed to the fibres, a library search report was generated using the National Institute of Standards and Technology (NIST) Mass Spectral library. Various volatile organic compounds were observed from the selected samples. A full report on all results from the analysis, including some test samples, are accessible online[1].

Qualitative assessment of the results of these peaks showed variable intensity across the three samples; hence, we singled out 15 compounds for further assessment. The resulting normalized peak area represented a relative amount of each compound of interest and this figure was used in plotting the bar graphs used for a qualitative assessment. From the resultant assessment, only about 24 of them varied interestingly across samples A, NC and PC and 15 were selected for further qualitative analysis.

Figure 8 describes some of the differences. Especially striking in terms of strong pattern and presumably resulting from anaerobic fermentation of glucose, amino acids, fatty acids by microbes were: 3-octanol, indole, xylene, pentanoic acid ethyl ester, “Possibly Pentanoic acid, 2−hydroxy−4−methyl−, methyl ester”, methionol, and phenethyl alcohol. (Brown, 2012; Forbes & Perrault, 2014). They map very strongly to PC samples as shown by the bigger peaks under the rectangle to the extreme right of figure 8 around the 25th minute. Some compounds like 3-octanol and xylene appear to be lower in PC samples than in the others so profiling them would help us diagnose what is happening in

[1] https://github.com/DonovanHaines/MadambaThesis

each sample in terms of the chemical reactions occurring at that point of sampling. Other compounds with interesting patterns included toluene, 2,-3-butanediol, 2-butanone, 2-pentanol, benzene acetaldehyde, hexane and naphthalene. See Figure 9 for additional supporting evidence.

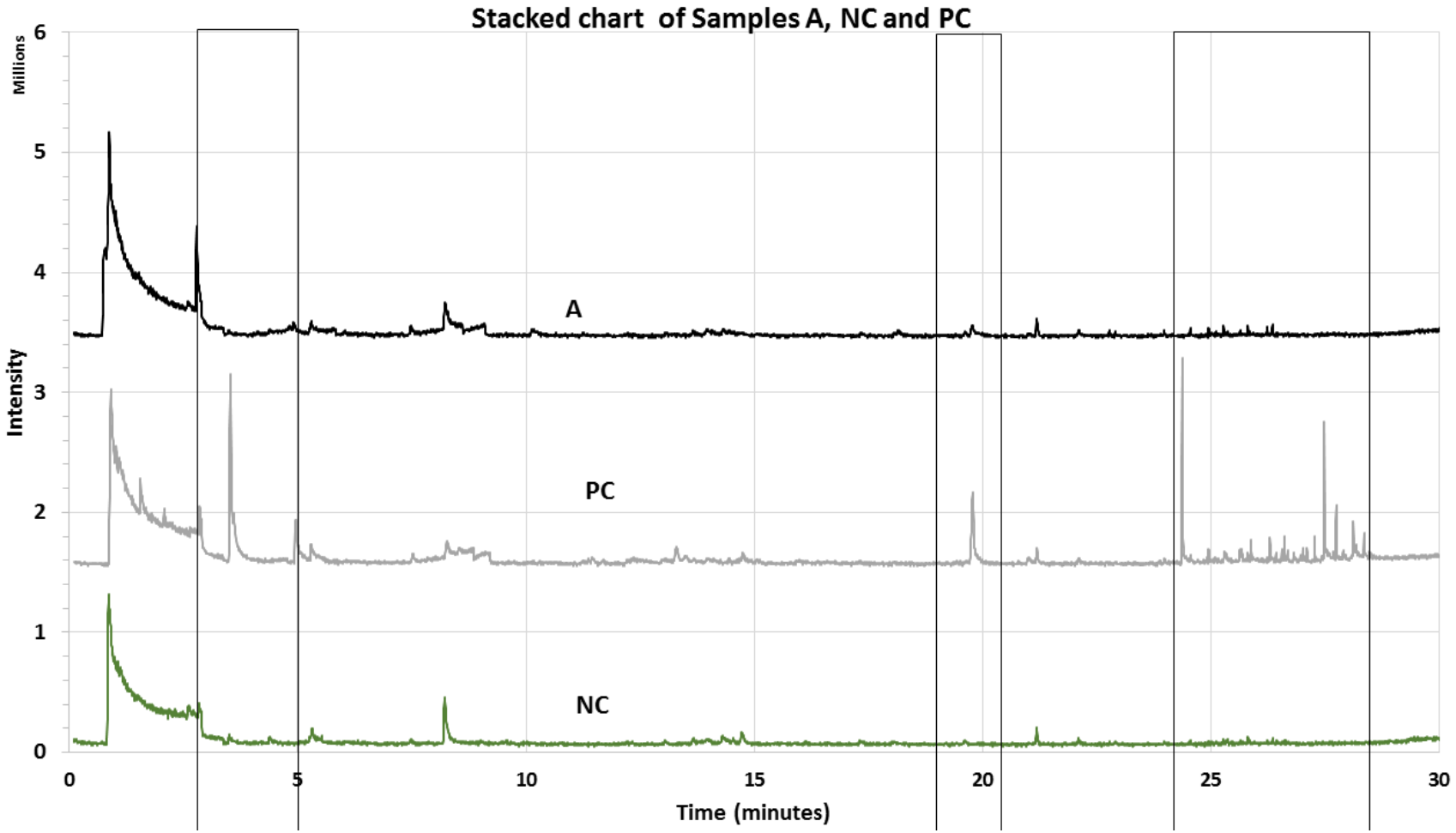

Figure 8. Qualitative assessment of gas chromatogram peaks across samples A, NC and PC. Note the different peaks in each sample under the boxed regions indicating the different compounds composition for each sample.

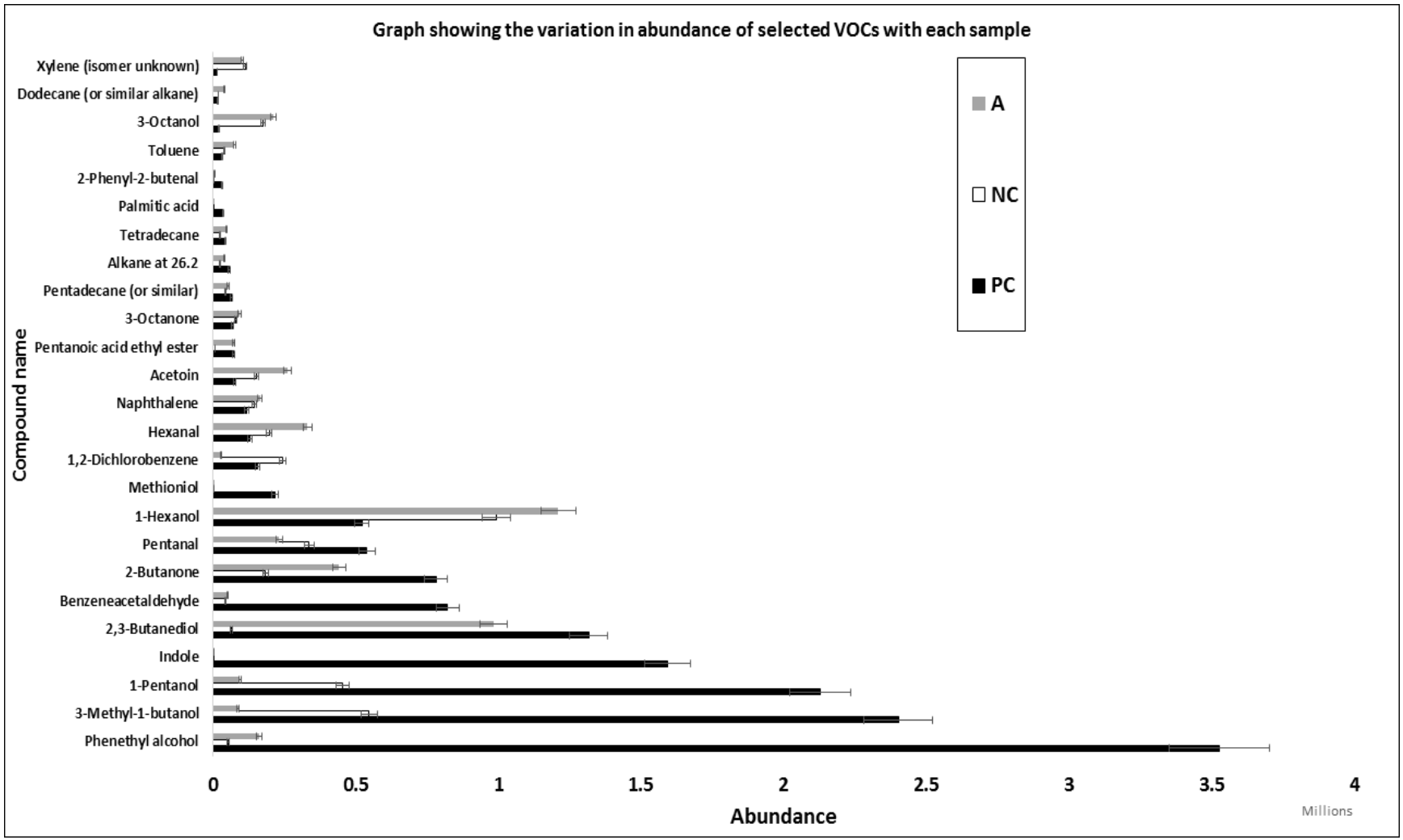

Figure 9. Relative abundance of notable VOCs across the three samples A, NC, and PC.

Profile of selected VOCs collected in the samples

A qualitative assessment of the similarities and differences among the peaks from the different peaks in samples A, NC and PC was made. Figure 10 shows the relative abundance of some of the VOCs represented in each of the treatments. The qualitative assessment influenced a literature search to profile some of the compounds of interest. Table 4. 3-octanol is a known fly attractant. In our samples it was lowest in PC where we added flies. 2-butanone and 2,3-butanediol are both products of glucose fermentation by bacteria and they exhibit the same pattern of composition in all 3 samples. 1- pentanol, a product of yeast fermentation showed its highest composition in PC and least in A. If any such trends can be further analyzed for any statistical significance, they may inform the tracing back of events of chemical decomposition and the action of bacteria during decomposition.

The results demonstrate fluctuations in VOCs composition likely influenced by community composition and abundance of living organisms (bacteria or fungi) on decomposing material hence the difference in odors (Perrault, Stuart and Hobbs, 2014). Profiles of the VOCs produced during decomposition vary according to the 3 treatments investigated (A, NC, and PC) suggesting that bacteria community structure plays a role in odor emanation during the decomposition process.

2-Butanone (*methyl ethyl ketone*) C_4H_8O

This pungent, colorless volatile liquid compound is also called methyl ethyl ketone, a natural product of some trees and fruits. It is used as a solvent and exposure to nose and eyes produces irritation. In our results, the PC had a very high peak for 2-butanone while the least peak was in the NC, with almost a quarter of the relative

abundance shown in PC. It is a glucose fermentation product known to have been detected during active decay in pigs (Brown, 2012; PubChem website).

1-Hexanol $CH_3 (CH_2)_5OH$

Listed in literature among the volatiles of postmortem vertebrate decomposition. (Paczkowski & Schutz, 2011, Vass, 2008) In our results, A has the highest peak while PC lowest has the lowest.

2, 3 Butanediol $C_4H_{10}O_2$

A product of microbial glucose fermentation by *Klebsiella oxytoca*, *Bacillus subtilis*, *Aeromonas hydrophilia* and several species of *Serratia* (Brown, 2012). In our experiment, this compound peaked the highest in the PC and the lowest in the NC.

2-Pentanol $C_5H_{12}O$

This product is a by-product of microbial breakdown of the amino acids threonine, leucine, isoleucine, and valine (Brown, 2012). In our experiment, 2-pentanol peaked in the PC but A had the smallest peak.

3-Octanol $C_8H_{18}O$

This gas is a product of linoleic acid (octadecanoic acid) breakdown, which is an essential fatty acid component of cell membranes. Mathis and Philpott (2011) associate the compound with attraction of flies in the family Phoridae hence suggesting a possible role in host location and acceptance by the fly. In our experiment, 3-octanol was most abundant in treatment A, followed by NC, and least in PC. Perhaps in the presence of invertebrate scavengers, one sample (possibly A) is likely to attract more scavengers than the others therefore resulting in a higher rate of decomposition.

Toluene C_7H_8

Vass et al. (2008) listed toluene as a product of decomposition burial events. In our experiment, the compound peaks highest in A, and the least in the positive control. Toluene was identified by Vass et al., 2008 as one of 30 compounds most important in human decomposition.

Naphthalene *$C_{10}H_8$*

Vass et al. (2008) also identified naphthalene the 30 burial gases generated during human decomposition. In this experiment, naphthalene peaks highest in treatment A, although it is almost uniformly represented in all three samples.

Indole *C_8H_7N*

Indole was identified by Vass et al in 2008 as one of 30 compounds most important in human decomposition and is known to regulate bacteria physiology. This compound markedly peaks in PC and is almost negligible in A and NC.

Xylene (xylol or dimethylbenzene) $(CH_3)_2C_6H_4$ and ***Pentanoic acid ethyl ester***

Information related to decomposition on these two VOCs was hard to find but both had notable trends across the samples. Xylene, a common solvent, was least abundant in the PCs and most abundant in NC while pentanoic acid ethyl ester is abundant in both A and PC and almost absent in NC. Pentanoic acid ethyl ester is an ester associated with active decay (Forbes & Perrault, 2014).

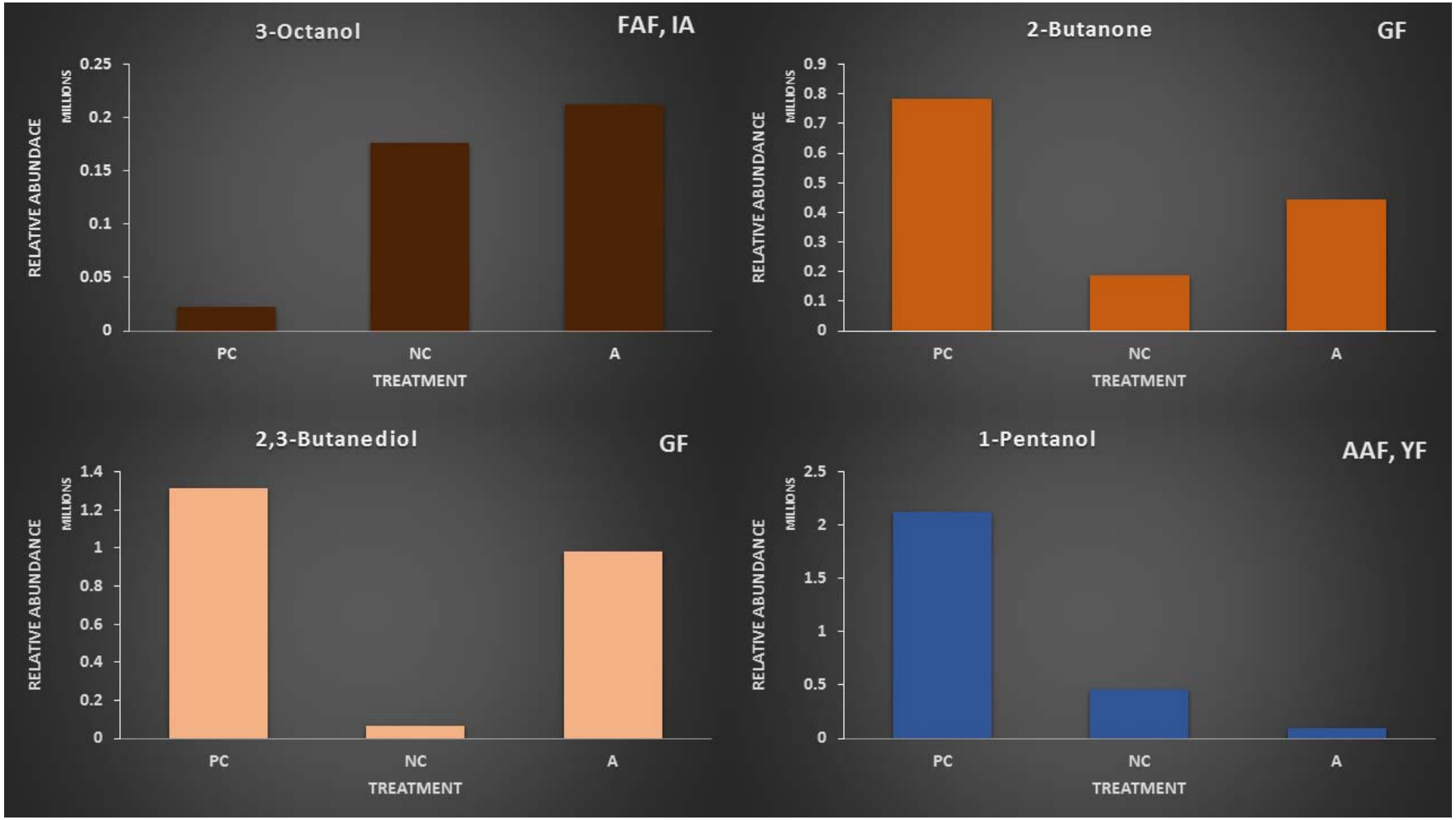

Figure 10. Relative Abundances of selected VOCs showing variations among treatments A, PC, and NC.

Table 12. **Profile and trends for selected VOCs recorded during the experiment and bibliography.**

Identifier	IUPAC Functional Group and priorities	FAF	AAF	GF	YF	IA	MCP	Observations on peaks	Reference
2-Butanone (Methyl ethyl ketone)	Ketone			Yes *alkanes				Highest peak in PC, lowest in NC	[1] [2] [3]
1-Hexanol	Alcohol	Yes			*fungal degradation			Highest peak in A, lowest in PC and NC in between.	[2] [3]
Dodecane	Alkane		Yes					Highest peak in A, low in both PC and NC	[1] [3]
1-Pentanol	Alcohol		Yes		Yes			PC had the highest peak. A, had the lowest (1/15 of PC). NC was about 1/5 of PC.	[1] [3]
3-Methyl-1-butanol (Isoamyl alcohol)	Alcohol		Yes		Yes			The highest peak was in PC Lowest in A (1/18 of pc) NC peak was about 1/5 of PC.	[3]
3-octanol	Alcohol	Yes				Yes		Highest peak in A, NC peak slightly lower than A but much higher than PC, PC very low	[5] [3] [2]
Indole	Amine		Yes					Highest in PC but negligible in A and NC.	[4]
Xylene (xylol or dimethyl benzene)								Highest peak in NC, lowest in PC, Slightly lower than NC much higher than PC	

(continued)

Identifier	IUPAC Functional Group and priorities	FAF	AAF	GF	YF	IA	MCP	Observations on peaks	Reference
Pentanoic acid ethyl ester	Ester	Yes	Yes	Yes	Yes			Almost absent in NC but almost equal amounts in A and PC.	[3]
2−hydroxy−4 −methyl ester	Ester	Yes	Yes	Yes	Yes			PC about 5X higher than both A and NC. Lowest in NC.	
Phenethyl alcohol	Alcohol							Most abundant in PC, A about 1/12 of PC, NC the least, about 1/20 of PC	
Toluene	Reagent							Highest in A, NC about half of A, PC slightly lower than NC	[4] [3]
2, 3 Butanediol				YES				Most abundant in PC almost 10X NC which is the least, A has about a 12^{th} of PC	[1] [3]
2-Butanone	acetone							Highest in PC, A was almost half of PC and lowest in NC almost half of A.	
Benzene acetaldehyde	Aldehyde								
Hexanal	Aldehyde							Highest in A, almost 2X NC, lowest in PC	
Naphthalene								**Highest in A lowest in PC**	**[7] [4]**

***FAF**: Fatty Acid Fermentation **AAF**: Amino Acid Fermentation **GF**: Glucose fermentation **YF**: Yeast Fermentation **IA**: Insect Attractant **MCP**: Maggot Cuticle Product*. [1]Kim et al., 2015; [2] Forbes and Perrault, 2014; [3] Brown, 2012; [4] Vass et al., 2008; [5] Mathis and Philpott, 2012; [6] Paczkowski & Schütz, 2011[7] Deyne, 2016; [8] Boumba, 2008;

CHAPTER IV

TEMPERATURE VARIATIONS DURING MICE DECOMPOSITION WITHIN AND AMONG BACTERIA TREATMENTS

Introduction

Temperature changes impact bacteria activity and consequently the progression of decomposition (Campobasso et al., 2001). They also directly affect ADD or the accumulated heat units within a 24-hour period. At low temperatures, decomposition occurs at a slower pace than it does at high temperatures. During the course of this experiment, there was difference between ambient and subject temperatures daily. Although not the focus of the research, this observation drew our attention and sparked curiosity regarding the effects of the added bacterial treatments relative to predictable temperature trends. We recorded the temperatures of each subject through time. Temperature of ambience was recorded. **We set out to test the hypothesis that daily temperatures of subjects among different bacterial treatments varied.**

Materials and Methods

In the same experimental design, temperature of ambience was recorded every day before recording the decomposition data using a mercury bulb thermometer. Additionally, temperatures of each subject were recorded using a handheld infra-red laser thermometer.

Statistical Analysis

Subjects temperature data were subjected to repeated measures ANOVA for significant differences among means as affected by the bacteria treatment. This test was conducted in SAS version 9.4. See **APPENDIX IX** for procedure and results.

Results

The ambient temperature fluctuated slightly (with no statistical significance) around an average of 21.5°C. Ambient temperature was lower than subjects' temperatures at all times. The daily average subjects' temperatures all differed significantly from each other depending on treatment ($p<0.001$). For all treatments and for all days, the differences among the daily average subjects temperature among treatments were statistically significant, i.e. different stages of decomposition showed differences in the daily subjects' temperature among all treatments (Table 13).

When comparing the daily average temperatures of the subjects per treatment, average temperatures for the PCs were generally the highest of the nine treatments, while CA had the lowest daily average temperatures (Figure 12). Of treatments A, PC, and NC, treatment A had the lowest daily averages, while PC had the highest at most times. The temperatures of the subjects, though higher than the ambience, seem to follow the general pattern of change as recorded in the ambient temperatures, as indicated by the similar patterns of all graphs.

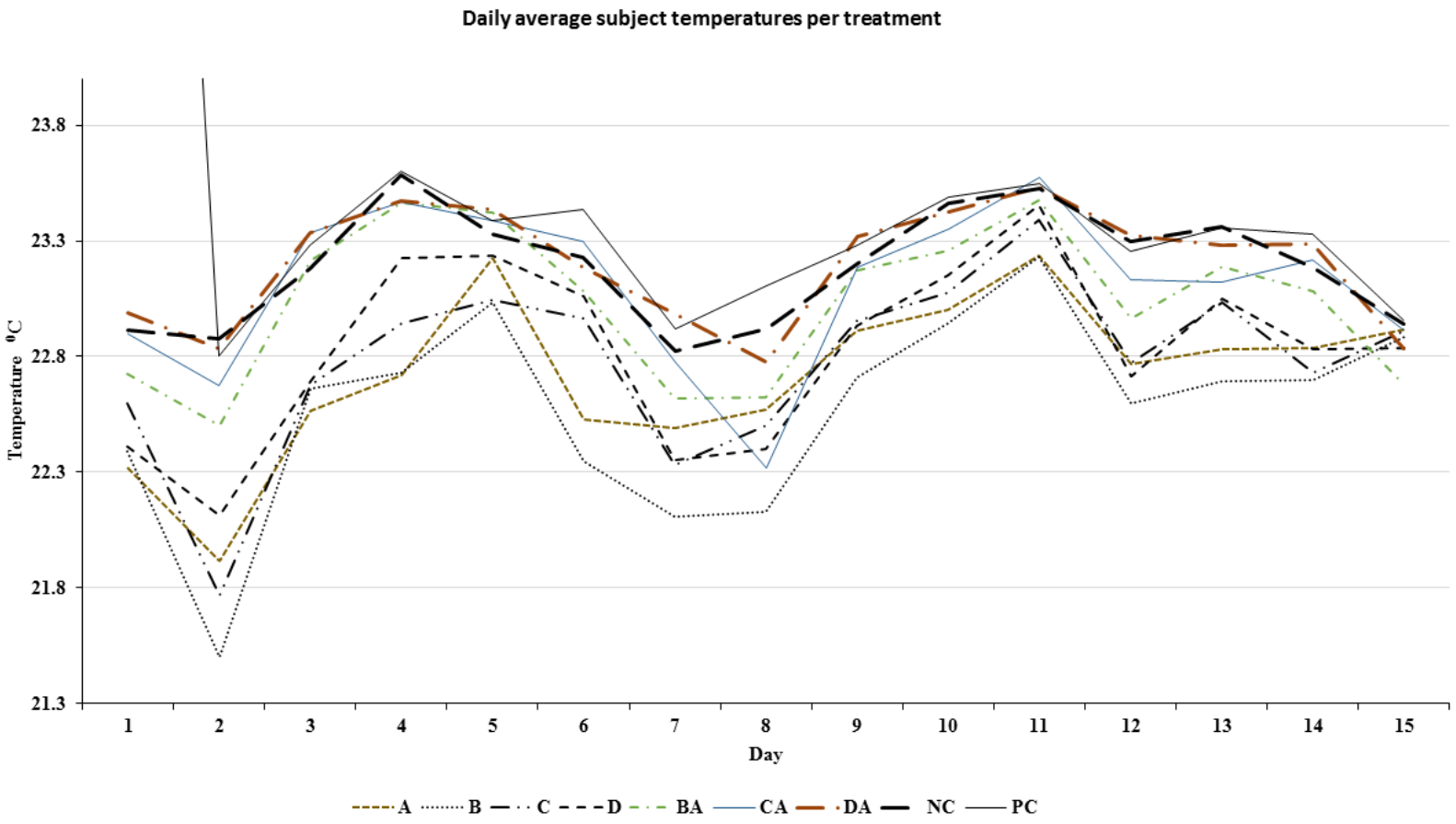

Figure 11. Average temperatures of subjects in all 9 experimental treatments through time.

Results of the repeated measures ANOVA on subjects' temperatures.

Table 13. **Results of Repeated Measures ANOVA analysis on subjects' temperature data.**

Source	p
Bacterial treatments day 1-day 15	<.0001
Time	<.0001
Time* **Bacterial treatments day 1-15**	<.0001

Interpretation of Results

The temperature of the subject is always higher than the ambient temperature in our experiment. This suggests that heat units (ADD) are not only accumulating from the environment but also from the metabolic reactions occurring on the subject, this observation requires further testing. Different compositions of bacteria are undergoing different chemical reactions of metabolism and therefore have different temperatures as indicated by the significantly different means for the subjects' temperatures of the nine bacteria treatments.

CHAPTER V

DISCUSSION

Ignatzschineria and Rate of Decomposition

Method development and preliminary studies

Decomposition is a complex multifactorial process and takes different pathways depending on antemortem and postmortem conditions. Different parts of the same body may show different stages or levels of decomposition at any given time due to antemortem and postmortem factors. Before carrying out this research, a trial experiment was conducted during the summer of 2015. Several observations were made during these trial experiments, leading to fine tuning of several aspects of the methodology as well as development of a key for visually scoring decomposition that was specific to mice under local experimental conditions.

Why a 15-day experiment? Determining the end point.

When Megyesi et al. (2005) established the visual scoring system for decomposing bodies, they used data from different human bodies. When Metcalf et al. (2013) conducted experiments on grave soils using mice in the laboratory, they were hydrated from time to time and hence the entirety of decomposition took 42 days. The decision to exclude soil in this experiment was based on Lauber et al. (2014) observation that soil microbiome affects decomposition rate. In the preliminary experiment, several bodies under laboratory conditions were observed from the onset to the cessation of decomposition, in the absence of scavengers, additional moisture and humidity, and with greatly reduced microbial communities. I was able to establish a general pattern of decomposition. In my experiment, I observed that decomposition always resulted in

mummification rather than soft tissue removal. This preservation consistently happened by day 15. To verify that no additional changes happened to the bodies after day 15, the first batch was kept and observed for 50 days. There were no further observable changes on the bodies so for the sake of time, 15 days was the experimental end-point.

Visual decomposition scoring system

The visual scoring system for the mice bodies shown in **Table 2** was adapted from the Megyesi et al. (2005) system with the assessment of skeletonization omitted. I did not observe skeletonization as the mice subjects were not exposed to scavengers and bacteria were limited. This general trend was made with comparison to observations on mice left in the field for fly collection liquefy as they were exposed to weather conditions and invertebrates.

The head scoring scale was adapted from Metcalf et al. (2013). Our experiment showed that bloat of neck and face occurred before the purge of liquids from eyes and ears justifying the use of two stages in the evaluations as shown in **Table 3**. In this key, the stages are in order and the stages progress in one direction, with no stage recurring.

In this experiment, the application of the scoring system was necessarily subjective. To overcome this challenge, the scores applied were the average taken from three researchers assessing the same picture (Dabbs et al., 2016).

In applying the Megyesi et al. (2005) system, the experiment echoes sentiments from earlier researchers that the system is not a “one size fits all” scoring system but tends to require adjustments depending on the subject and the conditions of decomposition (Keough et al., 2017). The scoring system presented in this study applies to dead mice under laboratory-controlled condition, in the absence of soil and any

additional moisture. I observed a predictable pattern in the stages of decomposition in this scenario, which led to the scoring system presented. I also confirmed that even in small vertebrates like mice, rate of decomposition was not uniform across the body. We focused on the head and the torso; limbs were excluded because of the difficulty in detecting visual cues of decomposition from them.

About decomposition

Factors involved in decomposition include, but are not limited to, the composition of the substrate, bacterial community structure, presence of fungi, vertebrate and invertebrate scavengers, weather elements like temperature and rainfall, and condition of placement. In this study, the condition of the initial substrate was controlled, mice were ordered from the same supplier, and therefore reared under the same conditions. They were likely fed and watered according to a standardized method. All mice selected for the experiment were of similar in weight and age as well (Easton and Smith, 1970).

All subjects were swabbed with alcohol to reduce and standardize the initial skin bacteria populations before adding treatments. The initial composition of bacteria in the mice bodies was assumed to be uniform because of the uniform treatment as well as the uniform diet and living environment. The only major difference among the mice subjects was the 9 different treatments administered to them. From an in-vitro study by Berry (2016), when *Escherichia coli* and *Ignatzschineria* bacteria were grown in the same petri dish, they both showed higher growth than when separated.

All vertebrate and invertebrate scavengers were excluded from subjects with the exception of the positive control where there was 1-hour exposure to adult flies found in Southeast Texas or their larval stages. Because this was laboratory work, the temperature

was kept constant and there was no exposure to water. This was done in an effort to observe the effect of adding only *Ignatzschineria indica* bacteria on the rate of decomposition of a body with additional complications as limited as possible. Rate of decomposition refers to a measure of the advancement of decomposition of a mouse through time (Lynch-Aired et al., 2015).

Bacteria Interactions

The main hypothesis and question of this study was to investigate the role of *Ignatzschineria indica* in decomposition. Having observed a noticeable increase in *Ignatzschineria* during the active stage of decomposition, (Hyde et al., 2014), I assumed that these bacteria must play an important role in decomposition, which should thereby influence the rate of decomposition as measured by a body score, total or partial, relative to accumulated heat units. From our experimental results, *Ignatzchineria* does have an important role but this role does not translate to significantly increasing the rate of decomposition, compared to other common bacteria.

We thought we might detect a different pattern from the torsos of the mice during both wet and dry decomposition, so we carried out three tests whose results are shown in Tables 9, 10 and 11. These results again showed no significant influence of bacterial treatment on rate of decomposition. This may mean that the initial composition of bacteria does not have a significant impact on how fast the decomposition progresses especially in this laboratory controlled experiment where we have excluded vertebrate and invertebrate scavengers. We have left the decomposition process to a number of anaerobic fermentation processes that seem to progress at similar rates regardless of what the initial composition of bacteria was.

The mixed treatments BA, CA and DA were to investigate whether we would have the same effect on actual mice subjects. Our statistical tests show that there is no significant increase in rate of decomposition in these treatments. On the plates, we count number of colony forming units (CFU) in a period of 36 hours whereas in decomposition on mice, we are waiting for the visual effect of the breaking down of macromolecules on the body then we score it and watch the change with time. We are unable to measure the rate of bacteria growth on our mice subjects and therefore cannot make comparisons between the bacteria growth experiments and the decomposition assays.

Volatile Organic Compounds

Statistical tests revealed that decomposition in mice takes about the same time to reach the end point under laboratory controlled conditions whether one adds bacterial treatments or not. These results support findings from other studies that show the important role that scavengers, especially invertebrates play during decomposition (Payne 1965; Gennard, 2012). From the different composition of VOCs, it is possible that if invertebrates were included in the experiment, one treatment could have attracted more than the other resulting in faster breakdown of the remains due to mechanical action and additional microbes.

The non-uniformity of decomposition on the same body may be linked to the different tissue composition ratios on each body part as well as the different microbial composition. Brown (2012) extracted VOCs from different parts or tissue of pigs and though there were common VOCs across all, there were some that seemed specific to muscle tissue for example. This difference is probably because of the different chemical pathways involved in breaking down macromolecules of the different tissues, and

variation in rate and the end-point of decomposition. Initial bacteria composition did not affect rate of decomposition however but affected the pathway of chemical decomposition by microbes.

Temperature findings

While ANOVA results showed there was no significant difference in the rate of decomposition among the different mice using total body scores for all mice subjects in all bacteria treatments, it was visually evident that samples with different bacterial treatments looked and smelled different especially during the wet stages of decomposition. This study is the first to show consistent and statistically significant differences among subjects' temperatures during decomposition, that they vary with different bacterial treatments. Sharma et al. (2013) suggest an effect of the maggot mass on decomposition; that the size of a maggot mass is proportional to its temperature. This experiment supports the above findings by suggesting that the action of bacteria associated with these maggots is responsible for the temperature increases. Microbes contribute to a localized temperature increase on the body, which in turn may affect ADD value. Therefore, we may need to factor in localized temperatures where possible to get a better estimate of PMI.

In our ADD calculation quoted in Chapter 1, T_{min} and T_{max} both refer to ambient temperatures. According to our results, the more localized the temperatures, the closer we get to a more refined PMI. For example, instead of using a very general weather site for Texas temperatures for example, temperatures from a local weather station closer to the area of study would be more accurate, better still if it is an experiment, the subject's temperature may give a better representation of ADD.

The study agrees with findings that ADD is directly proportional to rate of decomposition (Megyesi et al., 2005). The results indicated for all treatments that the higher the total ADD, the more decomposition occurred.

Future directions

In the absence of vertebrate and invertebrate scavengers and weather elements, fungi and bacteria are the main driving force behind decomposition. Our experiment was no exception as shown by the fungal growth in Figure 13. Fungal growth on a cadaver can provide evidence that contributes towards calculation of PMI (Al Na'imi, 2007). The composition of fungi on a body depends also on the bacteria and fly population present. These can contribute to the composition of fungi present. A possible experiment here would be to have one sample exposed to flies as a treatment as in PC, another as a control not exposed to flies as in NC, and then observe the fungi species and their abundance.

In our experiment, some samples developed mold growth and some were completely covered by it as the experiment progressed. It is possible that we may have had species specific to certain to a particular treatment but that is subject to further investigation. The exclusion of scavengers had a huge role to play in this finding and a follow up experiment must include scavengers especially flies. A different result is expected when flies are introduced because of the dynamics among fungi, bacteria and flies in the ecosystem. More research is required to gain better understanding and be better able to predict the timeline of events.

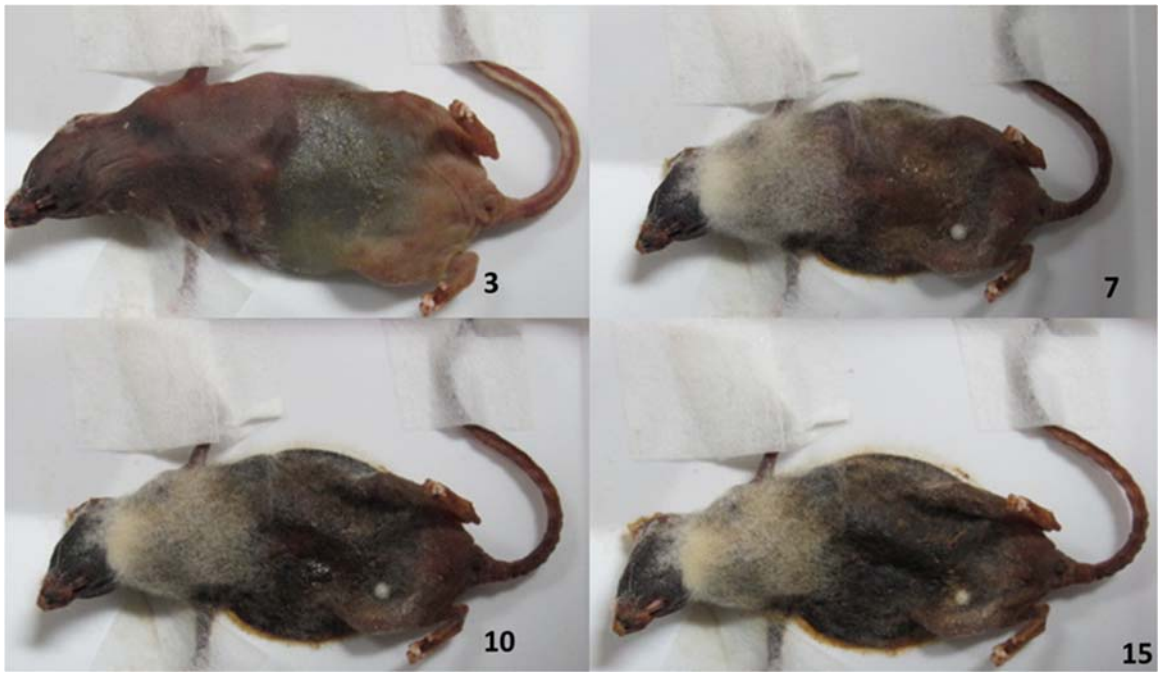

Figure 12. Fungal growth on a mouse body showing decomposition days 3, 7, 10 and 15.

Conclusions

The rate of decomposition among treatments was measured through two methods, time until purge and the visual scoring system. Neither test resulted in a significant difference in rate of decomposition among the treatments under laboratory-controlled conditions. In a different light, this result highlights the huge impact that the mechanical action of scavengers both vertebrate and invertebrate have on the rate of decomposition. However, I have demonstrated that there are consistent and statistically significant differences between ambient and subjects' temperatures during decomposition, also varying with different bacterial treatments.

When using the Megyesi et al. (2005) methods for predicting PMI range, ADD plays an important role. The currently used temperature in calculating ADD is the temperature of ambience. My study has demonstrated that there is need to design an

experiment to look into the possibility that the ambient temperature is significantly lower than the subjects' temperature and therefore may not fully capture the accumulation of heat units. This experiment would be aimed at suggesting that given an option between general temperatures and localized temperatures, the localized temperatures may give a closer estimation of what is happening.

VOC readings from this experiment established that VOC composition is dependent on bacteria composition (Dekeirsschieter et al., 2009). This is important knowledge as it demonstrates that once we come up with a microbial clock for decomposition, a VOCs clock may subsequently follow as well

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APPENDIX I: Data sheet for Photography, Temperature and Scoring Notes for Mice Decomposition Assays.

Recorder(s) _____

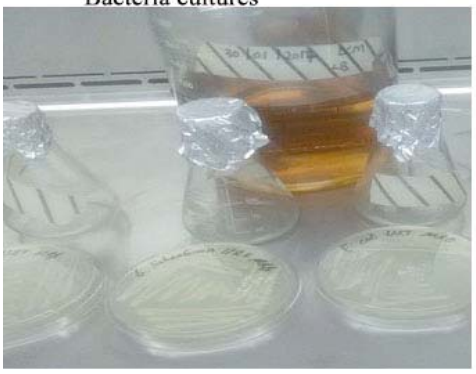
Date_____**Time**_____

Batch No.	Unit ID	Photo	Ambient temperature °C	Subject's temperature °C	Swab	Comments/notes

APPENDIX II: Experimental Procedure in pictures

MAKING BAGS AND TRAPPING FLIES

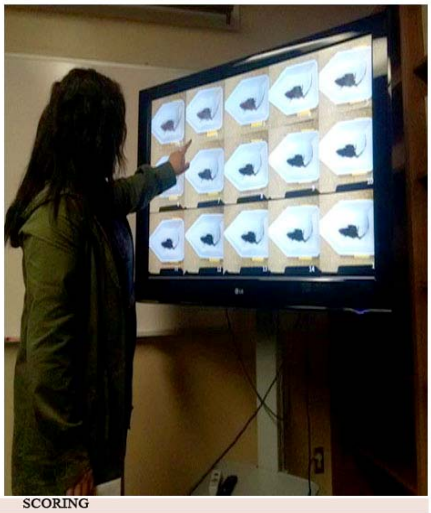
Bacteria cultures



Inoculating

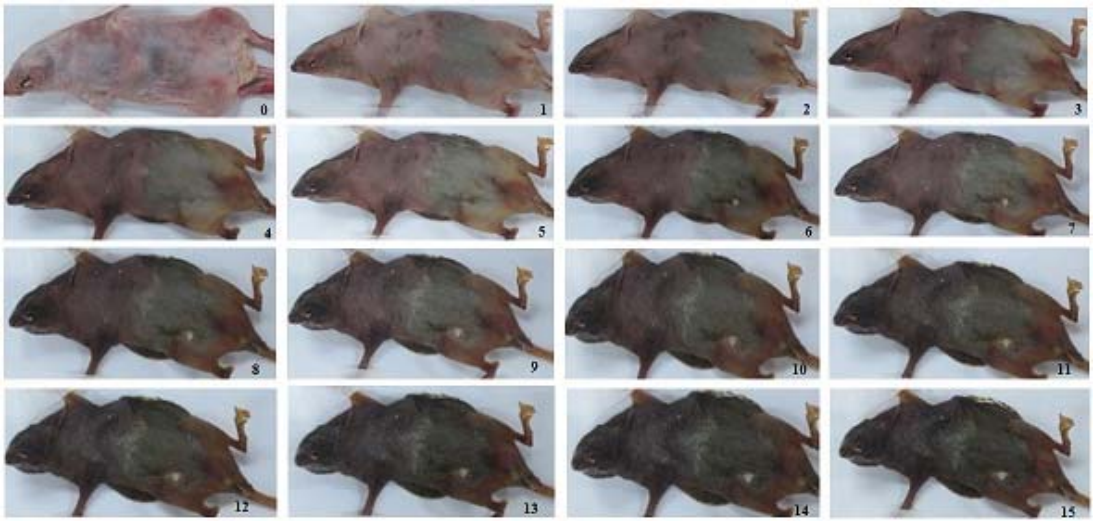
SETTING UP EXPERIMENTAL UNITS

PHOTO-DOCUMENTING

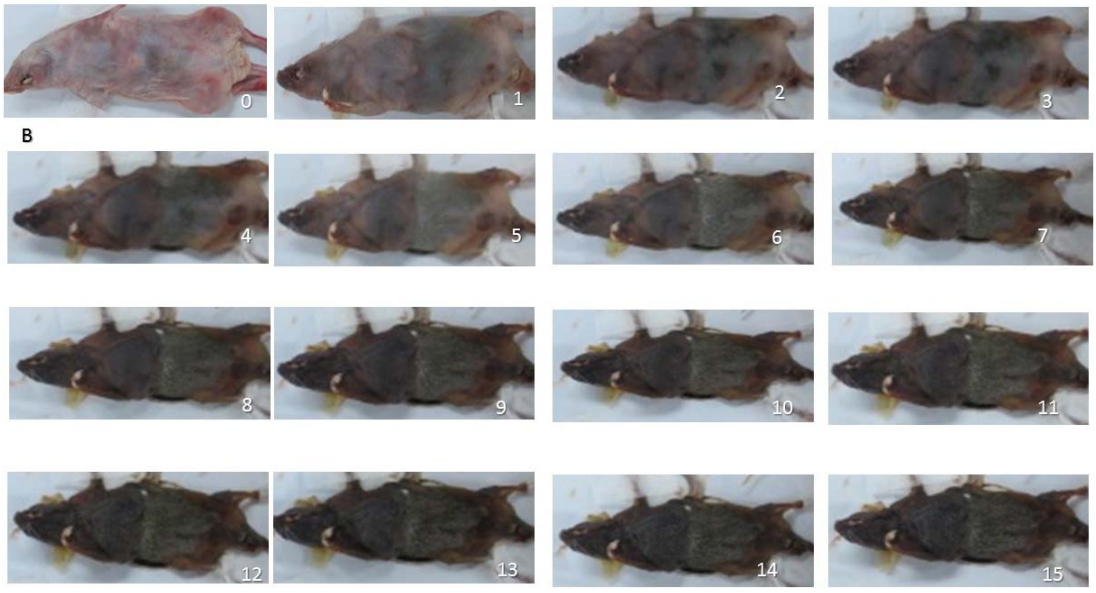


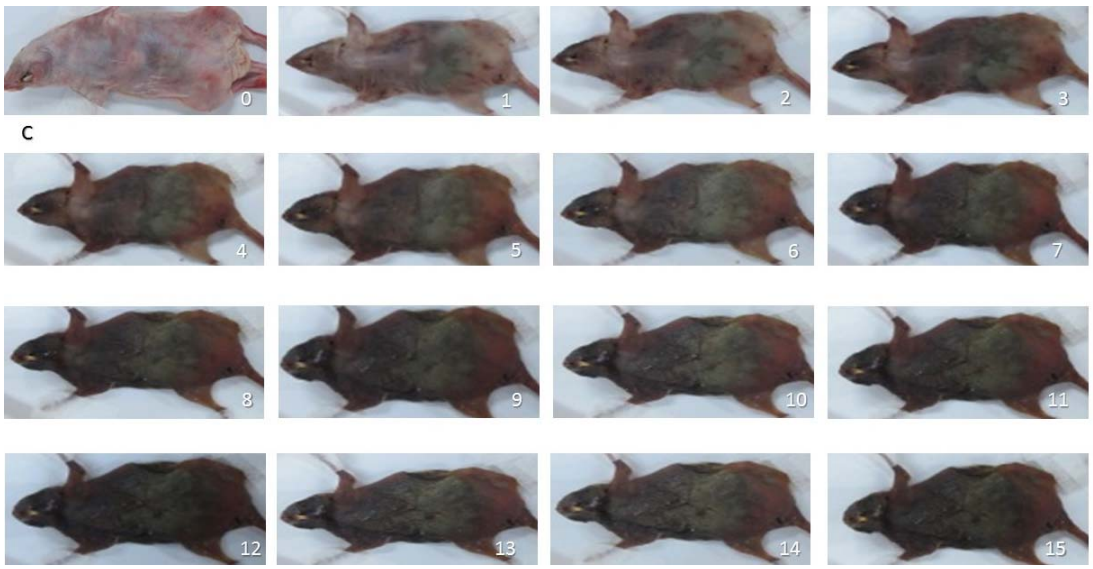
SCORING

APPENDIX III: Mice Daily progression of Decomposition per treatment examples



Picture represents treatment A

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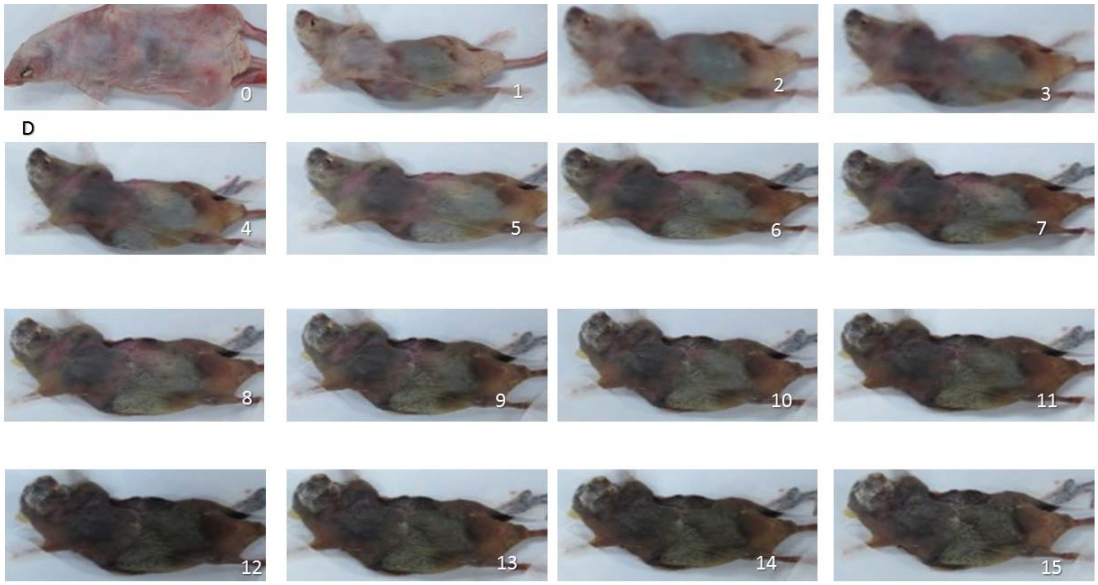
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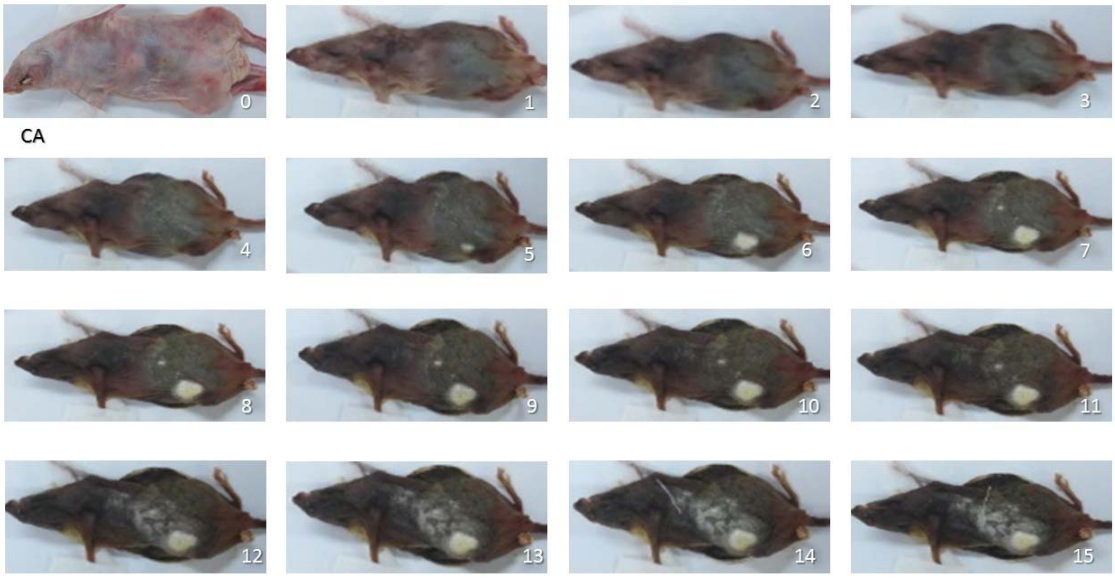
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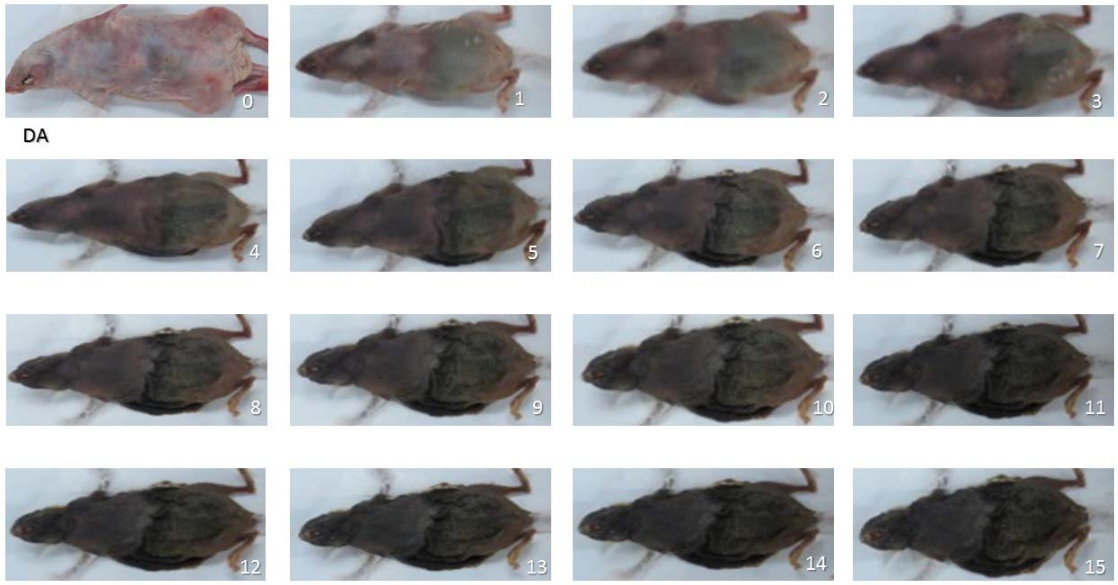
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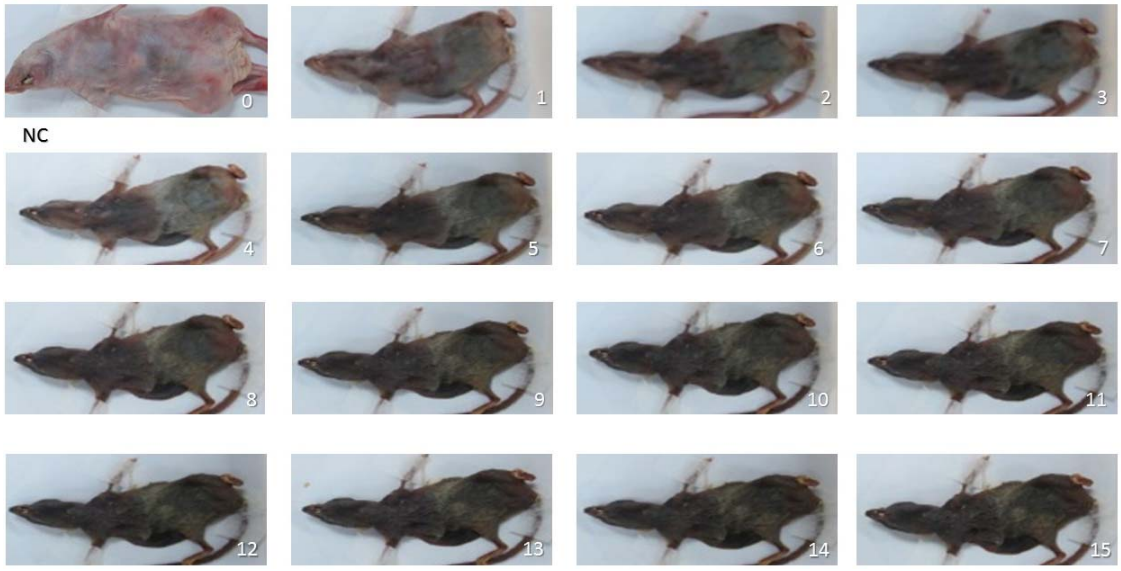
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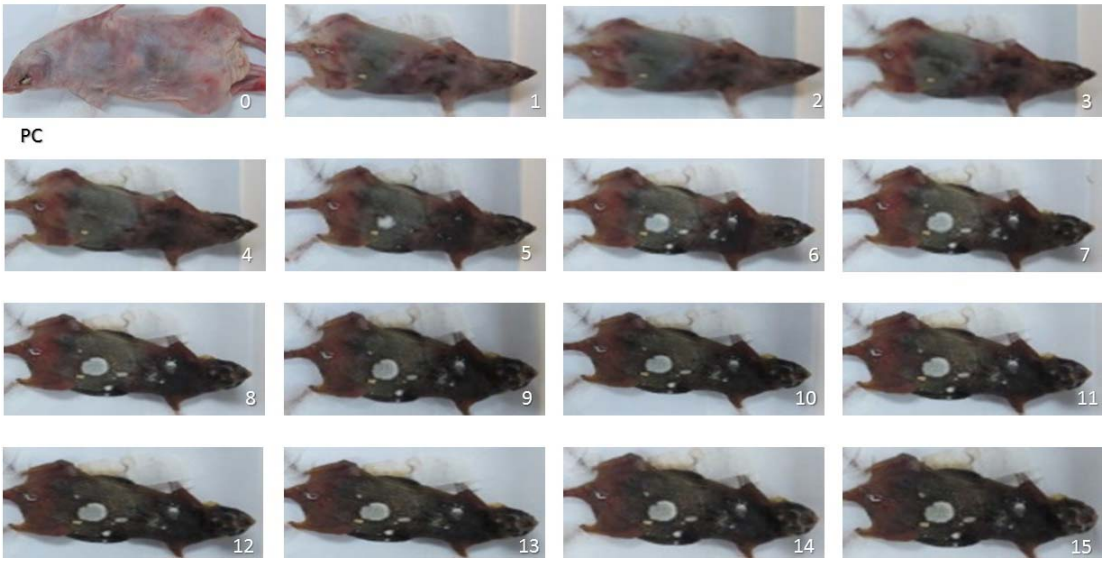
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APPENDIX IV: Table of Partial and Total Body Scores

This data is available online at the URL provided below:

https://github.com/Dorothy-Madamba/Masters-Thesis-Appendices

APPENDIX V: t-test results

Are batches 1 2 and 3 TBS significantly different from each other for the different treatments?

a. *Ignatzschineria* samples (A)

Groups tested	Pr (t) unpaired	conclusion (unpaired)
1vs 2	0.845507	not significant
2vs3	0.496196	not significant
1vs3	0.627308	not significant

b. *Escherichia coli* samples (B)

Groups tested	Pr (t) unpaired	conclusion (unpaired)
1vs 2	0.158938	not significant
2vs3	0.938459	not significant
1vs3	0.147955	Not significant

c. *Bacillus licheniformis* samples (C)

Groups tested	Pr (t) unpaired	conclusion (unpaired)
1vs 2	0.094106	not significant
2vs3	0.863076	Not significant
1vs3	0.06933	Not significant

d. *Salmonella enterica* samples (D)

Groups tested	Pr (t) unpaired	conclusion (unpaired)
1vs 2	0.336669	not significant
2vs3	0.770692	Not significant
1vs3	0.210886	Not significant

e. Samples BA

Groups tested	Pr (t) unpaired	conclusion (unpaired)
1vs 2	0.94467	not significant
2vs3	0.054536	Not significant
1vs3	0.041332	significant

f. Samples CA

Groups tested	Pr (t) unpaired	conclusion (unpaired)
1vs 2	0.611006	not significant
2vs3	0.203415	Not significant
1vs3	0.435813	Not significant

g. Samples DA

Groups tested	Pr (t) unpaired	conclusion (unpaired)
1vs 2	0.781455	not significant
2vs3	0.030087	significant
1vs3	0.055341	Not significant

h. Samples NC

Groups tested	Pr (t) unpaired	conclusion (unpaired)
1vs 2	0.614162	not significant
2vs3	0.475313	Not significant
1vs3	0.207171	Not significant

i. Samples PC

Groups tested	Pr (t) unpaired	conclusion (unpaired)
1vs 2	0.559531	not significant
2vs3	0.342559	Not significant
1vs3	0.697881	Not significant

APPENDIX VI: Repeated Measures ANOVA results for rate of decomposition based on bacteria treatments

This data is available online at the URL provided below:

https://github.com/Dorothy-Madamba/Masters-Thesis-Appendices

APPENDIX VII: SAS input for One Way ANOVA

This data is available online at the URL provided below:

https://github.com/Dorothy-Madamba/Masters-Thesis-Appendices

APPENDIX VIII: VOCs results

This data is available online at the URL provided below:

https://github.com/DonovanHaines/MadambaThesis

APPENDIX IX: Repeated Measures ANOVA for temperatures

This data is available online at the URL provided below:

https://github.com/Dorothy-Madamba/Masters-Thesis-Appendices

VITA

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Sam Houston State Natural History Collection
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2015 to 2017

EDUCATION:

2017 Sam Houston State University- Master of Science Biology
Thesis: Towards a microbial clock: Effect of *Ignatzschineria* (Gammaproteobacteria: Xanthomonadales) on rate of decomposition in mice.

2008 National University of Science and Technology, Zimbabwe- BSc. Hon. Forestry Resources and Wildlife Management.
Undergraduate Thesis: "Habitat Disturbance and its Impact on Insect diversity. A Case study of NUST Campus"

PROFESIONAL EXPERIENCE:

09/2015-Present	**Graduate Research Assistant**, Sam Houston State Natural History Collections
02/2012-Present	**Head of Entomology Department**; Natural History Museum, Bulawayo (National Museums and Monuments, Zimbabwe.
02/2009-Present	**Founder and Administrator**; Reach A Young Soul (RAYS) Trust.
11/2008-11/2011	**Ecologist**; Chipangali Carnivore Research Institute
11/2008-11/2011	**Education Officer**; Chipangali Wildlife and Environmental Education Program.

TRAVEL GRANTS AND FUNDING:

2012 Travel grant for second Africa World Heritage Fund (AWHF) UNESCO World Heritage nomination training course, Mbale, Uganda.

2013 Travel grant for third Africa World Heritage Fund (AWHF) UNESCO World Heritage nomination training course, Entebbe, Uganda.

2013 Travel grant for Biodiversity Informatics Training Curriculum (BITC) Data capture course, Accra Ghana.

2014 Travel grant for BITC Data diagnoses course, Entebbe, Uganda.

2016 Office of Research and Sponsored Programs Travel grant Biodiversity Informatics Research KU, BITC.

2016 Office of Research and Sponsored Programs Travel grant75th Annual Fall Meeting for the American Society for Microbiology. Texas Branch Meeting, Dallas, TX

2017 Graduate Studies SHSU travel grant to the Annual Meeting of the Entomological Society of America, Denver, Colorado 4-8th November 2017

Research Internship:

08/2006-07/2007 **Trainee Assistant field Ecologist;** Chipangali Carnivore Research Institute, Bulawayo, Zimbabwe.

04/2013-06/2013 **Intern;** Centre for Heritage Development in Africa, Mombasa, Kenya.

PUBLICATIONS:

Pickering J. **Madamba D. C.,** Staples T., Walcott R., 2016, Status of Moth Diversity and Taxonomy: A comparison Africa and North America North of Mexico. *Southern Lepidopterist' News*, 38:241-248.

PRESENTATIONS:

2010 Wildlife Environment Zimbabwe, "Chipangali CWEEP, achievements, successes and challenges."

2010/11 Schools outreach program Children's Wildlife and Environmental Education Program.

2013 Wildlife Environment Zimbabwe, "The Museum Children's Wildlife Conservation Club".

2013 Africa World Heritage Fund 2nd and third World Heritage nomination training course, "Available information and gaps in UNESCO World Heritage Nomination Dossier for Ziwa National Monument"

2014 Natural History Museum of Zimbabwe, "Natural History at the Centre" a place for Natural History and collections.

2015 Mafuwe K. and Madamba D. C., TDWG annual conference, Nairobi Kenya.

2016 The effect of *Ignatszchineria* (Gammaprotea: Xanthomonadales) bacteria on the rate of decomposition in hairless feeder mice at the 75th Annual Fall

Meeting for the American Society for Microbiology. Texas Branch Meeting, poster presentation.

2017 A preliminary study of the Evolutionary relationships among the species of genus *Mengenilla* (Strepsiptera: Mengenillidae), including insights on taxonomy of *Mengenilla chobauti*. 120th Annual Texas Academy of Science Meeting, oral presentation.

2017 The effect of *Ignatszchineria* (Gammaprotea: Xanthomonadales) bacteria on the rate of decomposition in hairless feeder mice at the First Tree Minute thesis competition at Sam Houston State University. Huntsville, Texas.

2017 The living dead: biodiversity of human remains in Southeast Texas. Annual Meeting of the Entomological Society of America. Denver Colorado.

GRANTSMANSHIP:

- Office of Research and Sponsored Programs, Sam Houston State University, Masters in Biology Study grant, Huntsville, TX, United States.

PROFESSIONAL MEMBERSHIPS:

Since 2015 Texas Academy of Science

Since 2015 Biological Sciences Graduate Students Organization (BSGSO)

Since 2015 Entomological Society of America

Since 2016 American Society for Microbiologists

Since 2017 Honors Society