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Characterization of the soil microbial community associated with the decomposition of a swine carcass

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ABSTRACT

Identification of bacterial species and their metabolic activity may be useful in differentiating soil samples associated with forensic analyses of large animal carcasses. In this preliminary study, lipolytic and proetolytic soil bacteria were enumerated over the time course of decomposition of a swine carcass. A 60-kg pig was used because it resembles a human body in its fat distribution and cover of hair. Soil core-samples (grave-soil) were taken underneath the carcass every three days until day 15, and then sampled on days 28, 43, 57, and 71 between September and December 2008. Results indicated that lipolytic bacterial counts were initially the lowest at day 0 (before exposure) and increased to their highest between days 9 and 12 (active decomposition); they then decreased and leveled at days 15 through 71. Conversely, the proteolytic bacterial counts initially were the highest at day 0, slowly decreased at days 3 (fresh) and 6 (bloat) with a rapid decline at day 9 (active) followed by a second major decline at day 28 (advanced); they then leveled through the remaining time period. Of the 33 isolates that could be identified, 18 were in the genus *Acinetobacter*. The qPCR results using Group I lipase-specific primers followed a similar pattern as the lipolytic CFU observed.

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1. Introduction

Microorganisms greatly modify the characteristics of the ecosystem in which they live by causing chemical changes to take place by their metabolic activities (Stahl and Tiedje, 2002). Even though the past few decades have focused on describing metabolic diversity, relatively little is known about the diversity of microorganisms, their metabolic activity levels in nature, and how they affect ecosystems. In this regard, little information has been reported in the literature regarding the microorganisms or their metabolic activity associated with the decomposition of large vertebrate carcasses above ground (i.e., cadaver decomposition islands). Most studies regarding microbial diversity and community structure are associated with profiling soil microbial populations (Horswell et al., 2002; Heath and Saunders, 2006; Lerner et al., 2006; Meyers and Foran, 2008), clandestine graves (Pfeiffer et al., 1998; Hopkins et al., 2000; Tibbett and Carter, 2008), or artificial situations using small quantities of tissue in the laboratory (Tibbett et al., 2004; Carter and Tibbett, 2006), rather than whole organisms in the natural environment.

The majority of decomposing remains of humans (and poached wildlife) of forensic interest are recovered above ground and often placed directly onto vegetation, leaf litter, soil surface, etc. Furthermore, soil microbes could be of particular forensic importance in later stages of decay when insect abundance has declined. From an investigative and evidentiary point of view, the more information one has, the stronger the case. Thus, this pilot study addressed the need for gathering a more realistic and comprehensive database of microbial diversity, community structure, and succession by conducting field research using an adult swine carcass (i.e., human representative and reference model for forensic entomology research) throughout carrion decomposition.

Given that the primary mediators of nutrient cycling of decaying remains are presumably microbial, an analysis of the microbial communities of both the carcass and site is essential to fully understand the carrion habitat. The major nutrients for bacterial degradation contributed by a swine carcass would presumably be protein and lipid. Therefore, the purpose of this study was to characterize the microbial diversity and succession of soil microbes based on genomic sequences, as well as to determine enumeration of lipolytic and proteolytic bacteria, isolation and identification of cultivable microbes, and lipolytic activity of the soil bacterial community associated with one swine carcass throughout decomposition.

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2. Materials and methods

2.1. Sampling

The study plot consisted of one intact, fresh adult swine carcass (47 kg) placed directly on the ground surface within a 2-m × 2-m sampling plot in a grass field in Hammond, LA, and monitored throughout decomposition (i.e., fresh, bloat, active decay, advanced decay, and putrid/dry remains stages). These time intervals correspond with the different stages of decay (Vass, 2001). Soil from the field plot was analyzed to determine bulk density and soil texture as described by Sylvia et al. (2005). Two soil cores (2 cm in diameter × 2 cm in depth, 12.6 cm^3 each) were collected beneath the carcass at the torso every three days until day 15 of decomposition, and then sampled every two weeks until day 71. Soil cores (2-cm depth) comprised of predominantly aerobic microorganisms associated with decaying detritus and organic-rich topsoil were mixed to form a composite sample for further analysis. To minimize vertebrate scavenging, the carcass was surrounded by a 1-m-high, 0.64-cm^2 hardware screen enclosure secured to wooden stakes.

2.2. Media and culture conditions

The number of cultivable aerobic lipolytic and proteolytic bacterial counts for each sampling event was determined using the pour plate method. This method included diluting 1 g of soil from each homogenized sample in 99 ml of sterile saline solution (0.9% (w v^{-1}) NaCl) and mixed thoroughly. Standard serial dilutions followed and a 1-ml aliquot of each dilution was plated for enumeration. For enumeration of lipolytic bacteria, Spirit Blue Agar (Difco Laboratories, Detroit, MI) supplemented with Wesson oil (3% v v^{-1}) was used as the differential medium. Lipase activity was visualized by halos around lipolytic colonies. Proteolytic bacteria were enumerated using a gelatin agar (consisting of nutrient agar [Difco Laboratories, Detroit, MI] and 3% [w v^{-1}] gelatin [Sigma, St. Louis, MO]) as the differential medium. Protease activity was visualized by clearing zones around proteolytic colonies. Plates were incubated at 28 °C. All counts were made in triplicate.

2.3. DNA isolation

DNA from soil samples was extracted (0.25 g) using the MOBIO Power Soil DNA (Solano Beach, CA) extraction kit as per the manufacturer's instructions. The DNA was suspended in 50 μl sterile TE and stored at −80 °C. Bacterial isolate genomic DNA was extracted using the MOBIO UltraClean Microbial DNA (Solano Beach, CA) extraction kit as instructed by the manufacturer. The DNA was suspended in 50 μl sterile TE and stored at −80 °C.

2.4. qPCR of Group I lipase from soil samples

Forward primer (5′-GYTGTTRTAGCAGTYGAAGTTC-3′) (Tm 59.9 °C) and reverse (5′-GASAKCCAGGTSGGAAT-3′) (Tm 58.4 °C) primers were designed by our laboratory using Beacon Designer Software (Palo Alto, CA). Quantification of the Group I lipase copy number in the extracted DNA was performed using the I-Cycler (Bio-Rad Laboratories, Hercules, CA). SYBR green (Bio-Rad Laboratories, Hercules, CA) was used as a double-stranded DNA (dsDNA) binding dye, whereby the fluorescence intensity of the dye increases with the amount of amplified dsDNA. Baseline and threshold calculations were performed with the I-Cycler software (version 4).

Amplification was performed with SYBR green Super Mix (Bio-Rad Laboratories, Hercules, CA) and the same set of primers used for the Group I lipase gene at a final concentration of 0.2 μM. The amplification consisted of 35 cycles: denaturation (95 °C for 3 min), annealing (55 °C for 30 s), and elongation (72 °C for 1 min).

The standard curve was calculated on the basis of a Group I lipase purified PCR product of known copy number. The standard curve was used to determine the copy number per gram of sediment. The qPCR measurements were performed in triplicate. The amplification efficiency (E) was calculated from the slope of the standard curve using the formula: percent $E = 10-1/\text{slope} \times 100$. Since SYBR green might also bind to nonspecific dsDNA, a melting curve was performed to assure the specificity of the PCR.

2.5. qPCR calibration curves

Calibration curves (gene copy number versus the cycle number at which the fluorescence intensity reaches a set cycle threshold value) were obtained using serial dilutions of pure-culture genomic DNA, *Pseudomonas chlororaphis*, and/or plasmid carrying a single, Group I lipase gene, *pueA* (Stern and Howard, 2000). Standard curves were generated using triplicate 10-fold dilutions of plasmid DNA, and the plasmid DNA concentrations ranged from 1×10^{-8} to 1×10^{-2} ng of DNA per reaction. Target copy numbers for each reaction were calculated from the standard curves (Pfaffl, 2001). Equation (1) was used to calculate the number of gene copies in a known amount of DNA.

$$\begin{aligned}\text{Gene copies} = {} & (\text{DNA concentration } [\text{ng } \mu\text{l}^{-1}]) \\ & \times (1 \text{ copy}/10003 \text{ ng}) \\ & \times (1 \text{ mol bp DNA}/660 \text{ g DNA}) \\ & \times (6.023 \times 10^{23} \text{ bp mol}^{-1} \text{ bp}) \\ & \times (1 \text{ copy/genome or plasmid (bp)}) \end{aligned} \quad (1)$$

Copy number estimates assume an average molecular weight of 660 for a base pair in double-stranded DNA and one gene copy per 1.5-Mbp-sized genome (Ritalahti et al., 2006). The number of target genes per gram of sample was determined with Eq. (2). The number of gene copies per reaction mix was determined from the appropriate standard curve based on the cycle number at the set threshold fluorescent intensity. This value was then multiplied by the volume (in μls) of extracted DNA obtained from each sample and divided by both the number of microliters of DNA used per reaction mix (e.g., 5 μl) and the amount of sample from which the DNA was extracted.

$$\text{Gene copies per gram sample} = \frac{(\text{gene copies per reaction}) \times (\text{volume of DNA } [\mu\text{l}])}{(5\ \mu\text{l DNA per reaction mix}) \times (0.25 \text{ g of sample})} \quad (2)$$

2.6. DNA sequencing

Nucleotide sequences were determined by the Pennington Biomedical Research Center (Baton Rouge, LA) and analyzed using the BLAST program in GenBank (Altschul et al., 1997).

3. Results

3.1. Cultivable heterotrophic bacteria in samples from topsoil

This preliminary study was conducted between 24 Sept. and 3 Dec., 2008. Soil from the field plot was determined to have a bulk density of 1.51 g cm^{-3} and a soil texture of a sandy loam. Ambient temperatures for the first 15 days of decomposition were observed to have a maximum averaging 30 °C and a minimum averaging

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23 °C (Table 1), whereas by days 28 through 59 of decomposition, the average maximum and minimum temperatures were 25 and 17 °C, respectively. The lowest daily maximum (17 °C) and minimum (10 °C) ambient temperatures of the study were observed on Day 71.

Cultivable aerobic heterotrophic bacterial counts as $\log_{10}$ colony forming units (CFU) per gram of soil sample from each time interval throughout decomposition are shown in Fig. 1. The response of the soil lipolytic bacterial population due to the presence of the decomposing pig differed from that of the proteolytic bacterial population. As noted in Fig. 1A, the lipolytic bacterial counts were initially the lowest at day 0 ($5.5 \times 10^3 \pm 0.781$) and increased to their highest between days 9 and 12 ($123.67 \times 10^3 \pm 3.06$ and $117 \times 10^3 \pm 4$, respectively), then decreased and leveled at day 15 through 71. The proteolytic bacterial counts (Fig. 1B) initially were the highest at day 0 ($53 \times 10^5 \pm 5.8$), then slowly decreased at days 3 and 6, displayed a rapid decline at day 9 ($11.33 \times 10^5 \pm 2.52$), followed by a second major decline at day 28 ($1.933 \times 10^5 \pm 0.153$), and then leveled throughout the remaining decay process (i.e., until day 71).

During active decay, advanced decay, and putrid/dry remains stages (days 9–59), bacteria were isolated and identified from the differential lipolytic and proteolytic media. Isolates were randomly selected and purified by the streak plate method three times on the medium from which they originated before they were further identified. Identification initially consisted of Gram staining, catalase testing, oxidase testing, and Biolog system analysis (Hayward, CA) followed by 16s rDNA sequencing. Thirty-three isolates were identified from the above procedures (Table 2). Of these 33 isolates, 18 were identified in the genus *Acinetobacter* and 7 in the genus *Bacillus*. Representatives in *Bacillus* were identified throughout the study, while *Acinetobacter* isolates were observed between days 9 through 28. Additional isolates of interest include: *Kurthia*, isolated during days 9 and 12; *Brevibacterium* and *Arthrobacter*, isolated during days 28 through 43; and *Serriatia*, observed on days 43 and 59.

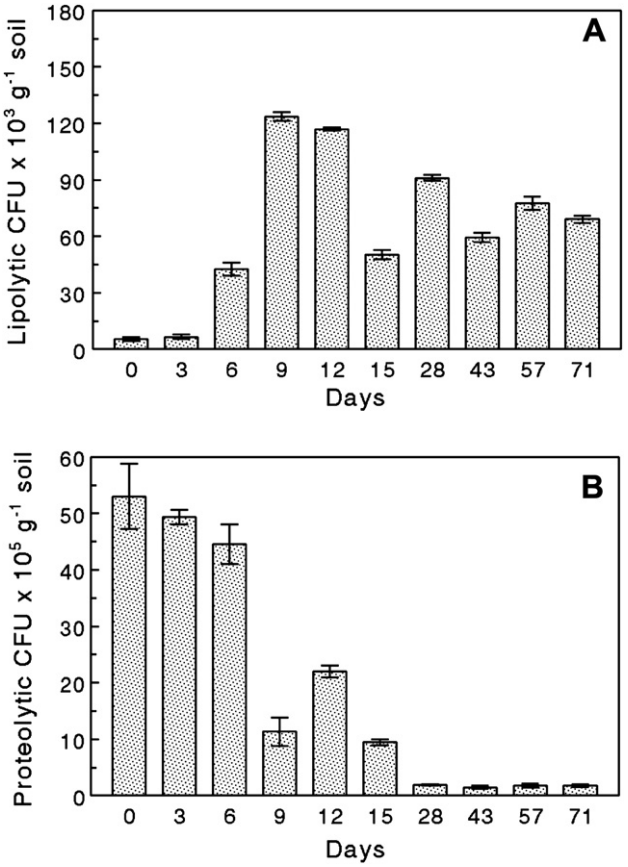

Fig. 1. Bacterial colony counts as determined by the pour plate method for each sample time interval taken during decomposition. (A) Colony forming units (CFU) detected for lipolytic bacteria. (B) Colony forming units (CFU) detected for proteolytic bacteria.

3.2. qPCR of Group I lipase

The qPCR primer set was applied to *P. chlororaphis* pure cultures and for the cloned PueA gene (Group I lipase) from *P. chlororaphis*. Results were similar for the cloned PueA gene or chromosomal DNA as templates. For all qPCR assays, there was a linear relationship between the log of the plasmid DNA copy number and the calculated threshold cycle value across the specified concentration range ($R^2 > 0.99$). The PCR efficiency for all reactions was ≥92.2%

Standard curves were constructed for qPCR regression lines generated by plotting the cycle number versus dilution (Fig. 2A) and cycle number versus gene copy number (Fig. 2B). Using this information, the copy number of Group I lipases per gram of soil was elucidated for each time interval of decomposition (Fig. 3). The qPCR results followed a similar pattern as the lipolytic CFU obtained (Fig. 1A). The gene copy number was the lowest at day 0 (125), slowly increased until day 9 (2078), and then was the highest on days 12 and 15 (268,850 and 101,805, respectively). The gene copy number decreased at day 28 (5412) and leveled from days 43–71 (1825).

Table 1
Temperature recordings for the highs and lows for each sample day period.[a]

Sample days	Stage of decay	High (°C)	Low (°C)	Daily average (°C)
0	Before exposure	31	25	28
3	Bloat	30	25	27.5
6	Active	30	23	26.5
9	Active	26	20	23
12	Active	30	23	26.5
15	Advanced	30	25	27.5
28	Putrid/dry	26	16	21
43	Putrid/dry	25	20	22.5
58	Putrid/dry	25	14	19.5
71	Putrid/dry	17	10	13.5

[a] Daily ambient temperature and precipitation were recorded at the Hammond Airport, Hammond, LA weather station of the Louisiana State Office of Climatology.

Table 2
Bacteria isolated from soil samples during active, advanced, and putrid/dry decay stages (days 9–59) identified by 16s rDNA sequencing.

Bacterial isolates	Enzyme phenotype	Cell morphology
Acinetobacter baumannii	lipolytic	Gram$^-$ coccobacillus
Acinetobacter haemolyticus	lipolytic	Gram$^-$ coccobacillus
Acinetobacter johnsonii	lipolytic	Gram$^-$ coccobacillus
Acinetobacter junii	lipolytic	Gram$^-$ coccobacillus
Acinetobacter sp. P4	lipolytic	Gram$^-$ coccobacillus
Arthrobacter cumminsii	proteolytic/lipolytic	Gram$^+$ coccobacillus
Bacillus cereus	proteolytic	Gram$^+$ rod
Bacillus mycoides	proteolytic	Gram$^+$ rod
Brevibacterium sp.	proteolytic	Gram$^+$ rod
Kurthia gibsonii	proteolytic	Gram$^+$ rod
Pseudomonas aeruginosa	lipolytic	Gram$^-$ rod
Serratia marcescens	lipolytic	Gram$^-$ rod

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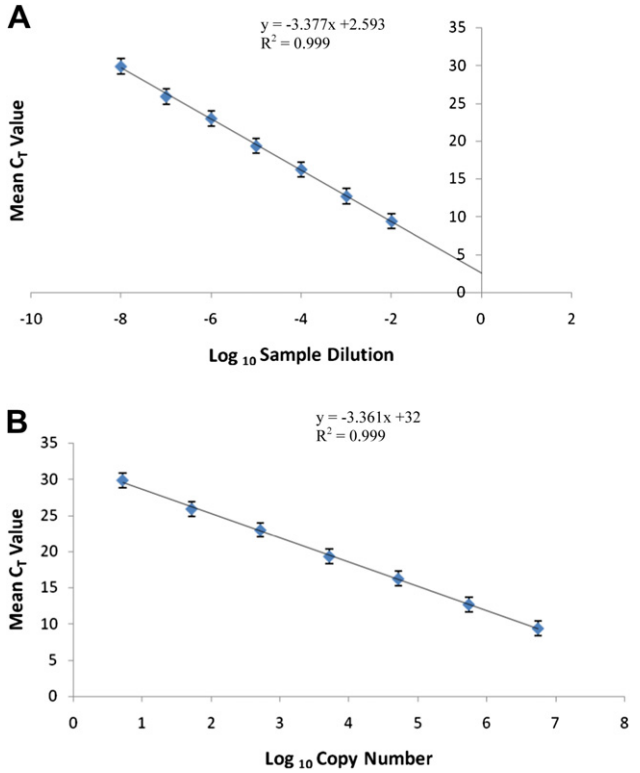

Fig. 2. Standard curves for qPCR reactions for Group I lipase genes. (A) Standard curve showing cycle number versus dilution of reaction volume of qPCR reaction using the Group I lipase-specific gene primer set with pPU2 (Stern and Howard, 2000) containing a Group I lipase gene fragment as the template. (B) Standard curve showing cycle number versus log of the copy number of Group I lipase genes.

4. Discussion

Ambient temperature is one of the most important environmental factors affecting decomposition, and thus, a pivotal parameter affecting both insect and bacterial activities. Precipitation, ambient temperatures, and insect species diversity observed during this study were typical for fall in southeastern Louisiana.

The overall bacteria counts for lipid-degrading microbes increased from day 0 to day 15, and then decreased and leveled through the remaining time intervals. On the other hand, the overall bacteria counts for protein-degrading microbes slowly decreased from day 0 to day 15, then leveled through the remaining time intervals. These results indicate that the lipid content of the carcass was contributing more as nutrients to the soil community rather than the protein content and the lipolytic bacteria were likely responding to an increase in lipid. Another factor in the decrease in bacterial numbers may be the decrease in average daily temperature after day 28.

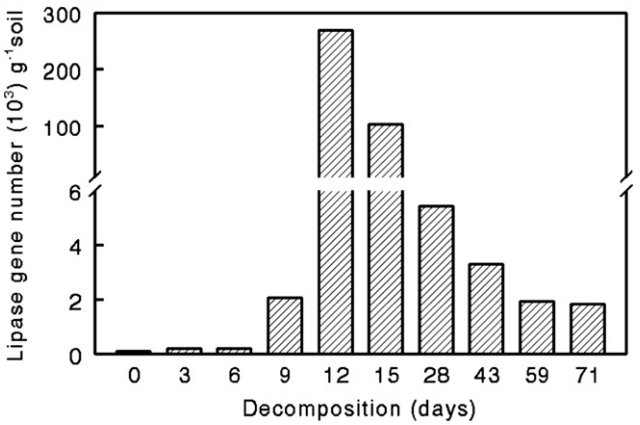

Fig. 3. Analysis of the number of Group I lipase genes containing samples by use of the specific qPCR primers. The bar graphs depict the number of copies of gene per gram soil for each sample time interval. The figure is in three subsets for easier viewing due to scaling.

The qPCR analysis targeting Group I lipases followed a similar pattern as observed for the lipolytic bacteria counts. As anticipated, the changes in copy number of Group I lipases in the topsoil beneath the swine carcass correlated with insect activity and tissue degradation typically associated with the five stages of decay (fresh, bloat, active decay, advanced decay, and putrid/dry remains). For example, changes in copy number of Group I lipases during the bloat stage (day 3) and initial days of the active decay stage (day 6) were minimal in comparison to soil samples collected prior to the placement of the fresh swine carcass (day 0) in a grass field (Fig. 3). These results were probably directly related to the activities of blow fly adults and larvae (family Calliphoridae) at the carcass.

Adult blow flies are highly attracted to a fresh carcass and are referred to as the "first wave" of insects due to their ability to locate a body and begin depositing eggs (oviposition) within minutes of death. Blow fly larvae mechanically break down tissues with their mouth hooks and preferentially consume skeletal muscle of vertebrates; thus, they typically leave behind a "collapsed" carcass consisting of connective tissues (bone, adipose, cartilage, ligaments, tendons), internal organs, and hair/fur as they migrate away to undergo pupation in the surrounding soils and leaf litter. In this study, the blow fly larval masses rapidly increased in number and size (i.e., they went through three immature life stages called "instars") throughout active decay (days 4–14), resulting in accelerated decomposition and nutrient loading to the topsoil beneath the carcass. The mass migration of post-feeding third-instar larvae away from the carcass began on day 15 (and continued for 2–3 days), marking the beginning of the advanced decay stage.

After the departure of the Calliphoridae larvae the rate of nutrient loading decreased, as evidenced by the decline in Group I lipase gene copy number during advanced and putrid/dry remains stages of decay. Furthermore, the gene copy number remained higher (days 28–71) than prior to carcass placement (day 0) due to continuing tissue degradation by microorganisms and numerous fly and beetle species, such as Diptera (flies): Fanniidae, Muscidae, Piophilidae, Sarcophagidae, Stratiomyidae; and Coleoptera (beetles): Cleridae, Dermestidae, Nitidulidae, and Trogidae.

As a human corpse or animal carcass decomposes, the soft tissues of the body (particularly the dependant side) will become "coated" with a grayish-white waxy substance called adipocere (or "grave wax"). Adipocere (long-chain hydroxyl fatty acids) is the end-product from the saponification of lipids (adipose tissue) in the presence of water and bacteria (Gill-King, 1997). According to previous studies (Pfeiffer et al., 1998; Horswell et al., 2002), the composition of adipocere and the bacteria isolated from adipocere samples (*Pseudomonas*, *Serratia*, and *Bacillus*) have shown lipolysis is involved in its decomposition. These bacterial genera were isolated in our study as well; however, *Acinetobacter* was found to be the predominant organism isolated in our study.

Lipase genes can be grouped into one of eight families (Jaeger et al., 1994). The lipolytic bacteria identified in this study are known to contain a Group I lipase. This family of lipases and other serine hydrolases are characterized by an active serine residue that forms a catalytic triad in which an aspartate or glutamate and a histidine also participate (Jaeger et al., 1994; Persson et al., 1989; Winkler et al., 1990). Sequence analysis of these genes reveals that encoded proteins contain serine hydrolase-like active site residues

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(G-H-S-L-G) and a C-terminal nonapeptide tandem called repeat in toxin (RTX), (G-G-X-G-D-X-X-X) repeated three times. Group I lipases lack an N-terminal signal peptide but instead contain a C-terminal secretion signal. The secretion of these enzymes occurs in one step through a three-component, ATP-binding cassette (ABC) transporter, Type I secretion system (Arpigny and Jaeger, 1999). Our study focused on targeting only Group I lipases for qPCR analysis. If the soil community harbors additional family of lipases in their genomic content, this may explain why our measurements for the number of copies of lipase genes were less than that of the lipolytic bacteria numbers observed.

Lastly, our results from this pilot study have highlighted some important and interesting findings about how a decaying animal carcass above ground influences the diversity and succession of soil microorganisms beneath the carrion. However, additional experiments including more sites and animal species, references to remote soil composition and community structure, and a variety of geographical locations are necessary before we can truly understand the species interactions and ecological impacts of large vertebrate carrion above ground on the soil microbial community.

5. Conclusion

The results observed in this study for the bacterial enumeration, identification of bacterial isolates and qPCR indicate that lipid biodegradation is more prevalent than protein biodegradation by soil bacteria involved in the degradation of swine carrion. Furthermore, information gained from this pilot study suggests that if we are able to identify discrete assemblages, and/or specific groups of microorganisms (such as Group I lipase genes, *Acinetobacter spp*., etc.), over time, then we could potentially predict known microorganisms associated with a particular stage of decay.

Forensic science is a continuously evolving field in which numerous disciplines intertwine to answer a particular question. The use of soil microbes to “age” an above-ground crime scene would strengthen and add rigor to a postmortem estimation by combining novel information about the carrion habitat with the more traditional methods of pathology, entomology, and anthropology.

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