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Patterns of Mortuary Practice Associated with Ethnoreligious Genocides of the Silk Roads: The Impact of Sub-Zero Bacterial Necrobiome on the Decay of Buried Remains

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Abstract
If body parts are buried shortly after exposure to freezing temperatures, the altered microenvironment may delay or trigger their decomposition. Using Sus *scrofa* as a human analogue, this study investigated the role of ambient temperature in the decomposition of frozen buried remains. The case study model is the third in a series of taphonomic reconstructions that focus on the role of bacteria in the decay of detached body parts. Research methods used included body part mass loss and gross morphological changes of soft tissue, soil pH, the metabolic activity of compost microbes, Gram staining, and Total Viable Count of microbes. It was hypothesised that ambient temperature would not impact the decay rates of body parts. Compost pH levels exhibited a significant difference between samples decomposing at two temperature settings. The metabolic activity of compost bacteria indicated that prior freezing may not affect microbial growth and activity. The Total Viable Count confirmed that freezing temperatures do not necessarily kill microbial decomposers by demonstrating higher microbial counts on samples exposed to a higher temperature. Gram staining showed predominantly Gram-negative bacteria potentially involved in the decay. Biomass loss used as a measure of decay was statistically significant only in the case of animal feet, suggesting a differential decomposition between body elements. The effect of the burial microenvironment on the decay of frozen remains has never been comprehensively tested. The results serve as a base for further studies focusing on the taphonomic reconstruction of mass graves in regions with continental climates.

Keywords: *Sus scrofa*, Soil necrobiome, Armenian genocide, Frozen body parts, Conflict archaeology

Introduction

Examining soil within a taphonomic framework has been a subject of longstanding inquiry, presenting an enticing avenue for understanding human decomposition (Galloway *et al*. 1989). Nonetheless, specific depositional microenvironments, notably frozen burials, have not received adequate scholarly attention, resulting in a lack of knowledge of this form of body disposal. For instance, prior freezing may alter the growth and activity of the soil necrobiome in a way that opens thawed wounds to attract decomposers. Conversely, freezing may act as a barrier to decay because body necrobiomes are killed or reduced by sub-zero temperatures. Moreover, it is not even agreed upon whether the body or soil microorganisms predominantly drive the decomposition of frozen body parts. The gap in the knowledge is complicated by the fact that most experimental case studies have been based on the decay of whole corpses, leaving the topic of detached or dismembered body parts inadequately researched (Brickley and Ferllini 2007). A handful of studies, such as Zugibe

and Costello (1993), reported freezing and thawing to favour aerobic decay for reasons stated as unknown. The authors noted that the exterior of frozen remains displayed advanced soft tissue decay compared to the core, which remained frozen for longer. Schäfer and Kaufmann (1999) suggested that freezing slows decomposition by disrupting microbial growth. In contrast, Roberts *et al.* (2017) showed freezing to accelerate decay rates. Furthermore, Stokes *et al.* (2009) reported freezing not affecting decomposition or soil chemistry but the modified burial microenvironment that impacts microbial activity in the soil.

Using Sus *scrofa* as a human analogue, the present study investigates the association between buried microenvironments and the decomposition of frozen remains in two temperature settings. This is accomplished by conducting the physiochemical analysis of compost, the metabolic activity of compost microbes, compost pH, Gram stain and Total Viable Count of microbes, and by recording biomass loss and morphological changes of soft tissue. It is hypothesised that ambient temperature will not impact the decay rates of body parts. Because commingled human remains are frequently found in clandestine burials associated with civil conflicts, the results of the study may have forensic potential in investigating mass graves located in continental climates. Historical examples include the systematic extermination of Armenians by the Ottoman authorities in eastern Anatolia, Turkey, during World War I.

The Armenians are ancient people of the Silk Roads who have inhabited the southern Caucasus for more than 3000 years (Haber *et al.* 2016). Owing to the region's strategic geographical location, they played a significant role in advancing international trade, establishing cultural and political connections, participating in currency circulation, and maintaining trade routes (Esche-Ramshorn 2021). Noteworthy commodities Armenians traded include high-quality medicinal plants, mineral dyes for silk textiles, pottery, and blacksmithing products (ibid.).

In Anatolia, their presence was documented as early as the sixth century BC, predating the arrival of the Seljuk Turkmens by approximately 1,500 years (Ahmed 2006). The historic dominion over the region essentially transitioned from one empire to another, meaning that an independent kingdom of Armenia faced frequent conquests and invasions from external forces, especially by Turkic-speaking peoples, who, from the 11th century onwards, contributed to a decline in its political independence(Kaegi 2008).

Despite this, Armenians remained the largest non-Muslim community in Anatolia, which, by the 19th century, had a well-established communal identity through their language and church (Mollica and Hakobyan 2021). Nationalism and wartime radicalisation based on growing numbers and the influence of the Armenian community were arguably the causes of why Ottoman Turks led outbreaks of anti-Armenian violence (Kieser and Bloxham 2014). The atrocities culminated in the mass killings of thousands in the Sasun region in 1894, massacres in 1895 in Istanbul, and the estimated 600,000 to 1,000,000 Armenians killed on Turkish soil between 1915 and 1917, representing 50 to 70% of the population (Harutyunyan 2019). A figure comparable in proportion to the Jewish Holocaust.

The largest concentration of Armenian genocide took place in eastern Anatolia, a mountainous and rugged region with an average rainfall of 560 mm and temperatures ranging from - 43℃ in the winter to 38℃ in the summer (World Data 2024). Taphonomic research focusing on the early decomposition of frozen burials may not aid in the recovery and identification of victims of genocide from over a hundred years ago. However, it could be a valuable foundation for studies investigating the advanced stages of decay in mass graves in continental climates.

Materials

For the taphonomic studies, a total of 32 domestic pig (*Sus scrofa*) heads and feet were utilised, while 64 pork belly pieces were employed for microbiological experiments. The domestic pig was selected as an analogue as its decomposition is accepted to resemble closely that of human remains (Matuszewski *et al.*2014). All animals were sourced from a Yorkshire abattoir, John Penny & Sons in Leeds, when they reached a weight between 30 and 40 kilograms. They were slaughtered seven hours prior to the start of the experiments and stored under refrigerated conditions before being transported in a van in plastic bags to the University of Bradford Taphonomy lab. Dismemberments included five cuts to separate the head and the front and back limbs from the

torso. All pigs were adult and free from antibiotics.

Methods

Compost analyses

The Levington Organic Blend Top compost (Suregreen Ltd.) was used to replicate shallow-grave soil conditions up to 30cm. The methodologies for analysing the organic content of compost, soil particle characteristics, soil bulk density and porosity, water content, water potential and retention were adapted from established soil analysis techniques (Klute 1986; Weaver 1994; Sparks 1996; Dane and Topp 2002; Ulery and Drees 2008). The procedures for evaluating soil pH were modified from the Association of Official Analytical Chemists (Kalra 1995). A scanning electron microscope (SEM) method was used to determine the main elements present in compost. Pictures were taken at 20 kV under 30X magnification using an FEI Quanta 400 microscope. The inorganic components of the compost were identified through elemental analysis using Oxford Instruments Inca X-Sight hardware and Inca software.

Microbiological analyses

The Total Viable Count (TVC) was used for non-specific bacterial and fungi colonies on a scale of less than 100. The numbers were determined using the dilution plate method and a broad-spectrum bacterial enumeration technique (Hopkins *et al*. 2000; Weaver 1994). Gram staining was carried out on representative samples from the skin, muscle, and compost, and bacteria were observed under a microscope at 100X magnification. The Fluorescein Diacetate Hydrolysis (FDA), tailored from the study of Adam and Duncan (2001), measured the metabolic activity of compost microbes using a reduced chloroform-based solution. The study utilised three growth media types: R2A, MacConkey, and Rose Bengal agars. Observations were made at 24 hours for R2A and MacConkey agars and at 48 hours for Rose Bengal agar.

Taphonomic analyses

The taphonomic analysis included quantitative (biomass loss) and qualitative (gross morphological changes) methods. The former involved weighing each body part using an electronic scale at the beginning and end of each experiment. The latter was discussed in terms of differences in appearance associated with post-mortem history. The decomposition model was modified from Davis and Goff (2000) and Prieto *et al.* (2004) to encompass a combination of gross post-mortem changes typical for moist soil environments (Table 1).

Methods of data analysis

One-way ANOVA was used to measure the overall differences in mass loss of the analogues. Friedman's test was employed to compare the distribution of pH levels in two different temperature settings. TVC and Gram staining are qualitative microbiological methods unsuitable for statistical analyses; therefore, data were collected and discussed in terms of approximate numbers and characteristics to support or contrast the statistically analysed results.

Research design for pork belly experiments

Pork belly samples were exposed to two burial microenvironments: category C (20°C) and category E (30°C). A total of 64 pieces of pork belly (Table 2; Table 3) were used for compost pH measurements (24 samples), TVC and Gram stain analyses (22 samples), and measurements of soil microbe activity (18 samples).

Pork belly pieces cut to measure 5cm width x 5cm length were placed in autoclaved clear glass jam jars with proportions of 228ml, height 85mm, diameter 63mm with lid/neck of 63mm. Approximately 3cm of Levington Organic Blend Top compost was filtered through a 5mm sieve to the bottom of the jar, under the meat slices, and filled to the top. Jar lids were pierced to allow oxygen access. Pork belly tissues were frozen at -18°C for 72 hours and placed in the incubator without thawing. The duration of the experiments was 28 days.

The compost moisture level was established prior to the experimentation and was not treated; therefore, ambient temperature was used as the main variable. Compost pH levels were determined prior to the experiment, at weekly intervals, and the end of the experiment for each category. Controls included pH measurements of compost with no contact with the meat at the start, at weekly intervals, and at the end of the experiment.

Once pH was established, samples were discarded, and the new samples were tested for each category.

Table 1: Description of morphological appearances for buried body parts

Decay category	Description
Fresh	**No discolouration or signs of lividity, intact skin**
Putrefaction	The pinkish appearance of the skin; cream to light brown discolouration of the skin with slight skin slippage
Early disintegration	Ash white with green and black stripes visible under the skin, further skin slippage
Active decay	The skin colour is dark red and the skin texture, crispy. Skin is sagging and flaking from most parts. Skin structure is leathery to stringy with evident fatty tissues
Advanced decay	Substantial greasy substance, decomposed tissue, cartilage and tendons exposed. Formation of moisture, thin greyish substance on bone trauma ends. Bone exposure of most of the samples with greasy substances and decomposed tissue
Skeletonisation	Complete bone exposure with no tissue left

Table 2: Breakdown of sample size for pork belly samples

Compost pH of frozen pork belly

Category	*Harvest 1*	*Harvest 2*	*Harvest 3*	*End of trial*
C	3	3	3	3
E	3	3	3	3

TVC of aerobic bacteria for frozen buried pork belly

Category	*Harvest*	*End of trial*
C	3+1 ctrl sample	3+1 ctrl sample
E	3+1 ctrl sample	3+1 ctrl sample

TVC of anaerobic bacteria for frozen buried pork belly

Category	*End of trial*
C	3
E	3

Soil metabolic activity of microbes for frozen pork belly

Category	*Start*	*Harvest*	*End of trial*
C	3	3	3

Table 3: Summary of experiment set ups for pork belly samples

Duration	Microenvironments/Category	Tested variables	Applied methods	Control
28 days	1. 20^0C and 40% soil moisture (cat C) 2. 30^0C and 40% soil moisture (cat E)	Ambient temperature/soil moisture	1. Compost pH 2. Total Viable Counts 3. Gram stain 4. Metabolic activity of compost microbes	Surface decay

Research design for body part experiments

A total of 32 body parts (16 animal heads and 16 animal feet) formed part of the taphonomic trials (Table 4). The original sample size for body parts was 16; however, to ensure the accuracy of the results, the trial was repeated. The duration of the experiments was 28 days. Animal heads and feet were exposed to two micro-environmental settings. Category C body parts were kept at an average room temperature of 20.8°C, and category E remains decayed in a heated room at an average of 26.1°C. The compost moisture was not treated. Control was surface decay.

Body parts were weighed and photographed after delivery then inspected for visible signs of skin decay or insect succession. The average mass for sixteen animal heads was 4.7 kilograms, and for animal feet, 0.88 kilograms. After freezing for 72 hours at -18°C, the remains were deposited individually into IKEA fabric storage boxes (33 x 38 x 33 cm) in approximately five centimetres of compost, with the rest covering up to one-third of the bag.

Skin and muscle tissues were swabbed at the start and end of all experiments to ensure body flora and microbial numbers (TVC and Gram stain) were in line with the parallel study of pork belly slices. All samples were left to decompose undisturbed. Tinytag Gemini PT100 loggers were used to monitor ambient temperatures hourly. Compost was sprinkled with sterilised water regularly to keep the moisture level steady. Control samples decomposed with no contact with compost approximated surface decomposition. At the end of the experiment, samples were again visually observed for any signs of insect succession. They were photographed, weighed, and cleaned before being categorised into decomposition stages.

Table 4: Breakdown of sample size for body parts

Category	Sample	Sample size
C	Head	6
C	Foot	6
C ctrl	Head	2
C ctrl	Foot	2
E	Head	6
E	Foot	6
E ctrl	Head	2
E ctrl	Foot	2

Results

1. Qualitative and quantitative assessments before burial

Compost condition

The physical and biochemical characteristics of compost indicated a suitable decomposing microenvironment (Table 5). The high percentage of silicon allowed soil mineral formatting, which, together with favourable pH, controlled the chemical and physical properties of compost, compensating for a lower rate of organic content. The Fluorescein Diacetate Hydrolysis revealed a similar metabolic activity of bacteria between the three samples, with the fluorescein release ranging from 0.06mg to 0.08mg. TVC isolated from R2A agar also showed a similar trend of bacterial numbers,

and Gram staining indicated predominantly Gram-positive bacteria.

Table 5: Compost analyses prior to experimentation (sieved 5mm)

Compost characteristics	Analysis results
Bulk density	1.44g/cm^3
Particle porosity	45.6%
Soil structure and type	Medium loan
Soil texture	58.9% sand, 23.7% silt and 17.9% clay
Soil water content	27%
Soil water retention	40%
Soil water potential	-0.033MPa
Percentage of organic content	12%
Soil pH	7
Main inorganic content	Oxygen 48% Silicon 34.20%

Pork belly and body part analyses

Mostly Gram-negative bacteria were associated with animal tissue (Table 6). TVC was low, presumably because of sub-zero temperatures, which, however, did not kill microbes. No animal parts showed any signs of insect activity or skin condition. The heads and legs of the animals, based on the stage of decomposition, were classified as 'Fresh'. The average mass for sixteen animal heads was 4.7 kilograms, and for sixteen animal feet, 0.88 kilograms.

Table 6: TVC and Gram stain analyses prior to burial

Sample	Gram stain /aerobic conditions Muscle	Gram stain/aerobic conditions Skin	TVC Muscle	TVC Skin
Pork belly 1	G-	G-	2.0 X 10^5	3.1 X 10^5
Pork belly 2	G-	G-	1.5 X 10^5	4.0 X 10^5
Pork belly 3	G-	G-	2.5 X 10^5	2.6 X 10^5
Head 1	G-	G+	3.2 X 10^5	3.4 X 10^5
Head 2	G-	G-	3.2 X 10^5	3.5 X 10^5
Head 3	G-	G-	2.7 X 10^5	2.8 X 10^5
Foot 1	G-	G+	2.0 X 10^5	2.5 X 10^5
Foot 2	G-	G-	3.0 X 10^5	2.7 X 10^5
Foot 3	G-	G-	2.9 X 10^5	3.1 X 10^5

2. Micro-environmental monitoring during the decay

Compost pH

Friedman's ANOVA yielded a significant difference between the two categories (X^2=16.20, $p < 0.05$), showing that compost pH varied according to the temperature levels. pH levels of samples incubated at 20°C (category C) ranged between 8.10 and 8.92 during each sampling interval and dropped to between 7.00 and 7.99 at the end of the experiment. The samples incubated at 30°C (category E) had higher pH that varied between 8.66 and 9.87 during harvests and lowered to 7.10 and 8.04 at the end of the trial.

Metabolic activity of soil microbes

Friedman's ANOVA yielded significant results for the metabolic activity of compost bacteria in two different temperature settings (X2=10.33, $p < 0.05$). The lowest difference between the two groups was at the start of the experiment. During harvest (day 14), three samples in category E released between 0.79mg and 0.99mg of fluorescein, in comparison to category C, which released fluorescein in lower amounts, between 0.63mg and 0.96mg (Figure 1). After the experiment, the results revealed a significant decrease in the amount of chemical

released. The levels were lower than at the beginning of the experiment. For category C, the first two samples released 0.05mg, while the third sample yielded a higher amount at 0.60mg. Three samples incubated at 30°C (category E) yielded between 0.05mg and 0.06mg of fluorescein.

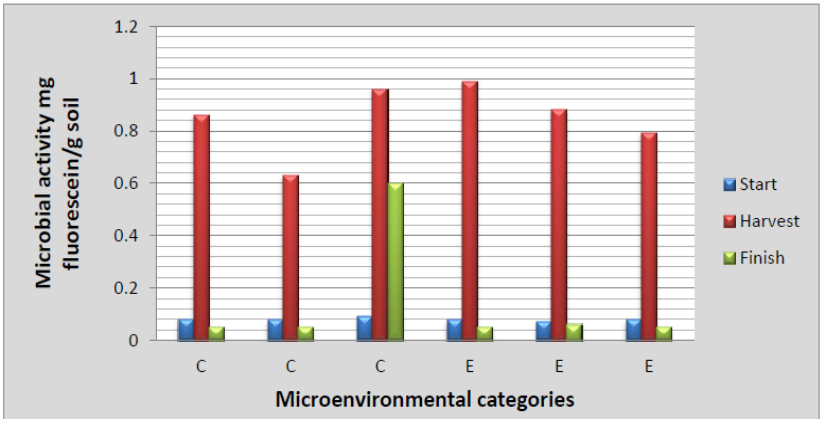

Figure 1: Metabolic activity of soil microbes during the decay

Total Viable Count and Gram stain

There was some difference in the number of microbes grown on MacConkey agar during the harvest period, with more microbial growth on the R2A plate at the end of the trial. More colonies grew on agar incubated at 30°C (category E) than at 20°C (category C). TVC of the anaerobic colonies was higher in numbers during the sample interval than at the end of the experiment. The examination of MacConkey agar plates showed small, purple, and brown, slightly raised colonies that were round (e.g. Figure 2). R2A plates had the most colony counts, but not much difference in appearance was noticed, as they were mainly small, white, and round in shape.

Gram staining of bacteria cultured anaerobically showed predominantly Gram-negative bacteria associated with the decomposition of buried frozen remains. This was more prominent on the soft tissue of remains in category E (Figure 3). Bacteria were rod-shaped and evident in large numbers. A combination of fungi and Gram-negative bacteria was observed in the anaerobic samples incubated at 20°C (category C). However, it was difficult to establish whether Gram-positive or Gram-negative colonies were predominant in the main samples due to the density of numbers. Fungi tend to thrive in lower temperatures, which may explain why only a few appeared on Gram stain in anaerobic conditions of category E. A similar pattern was shown with the anaerobic and the main samples in this category, exhibiting predominantly Gram-negative bacteria.

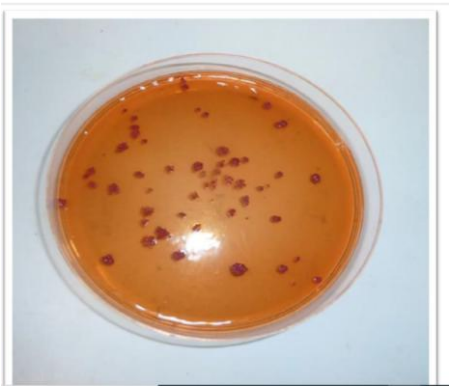

Figure 2: Typical MacConkey plate associated with buried previously frozen tissue, category C

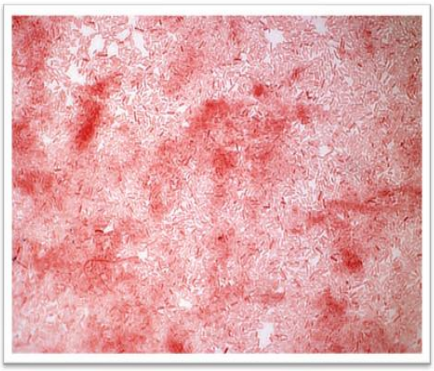

Figure 3: Gram-negative bacteria recovered at the end of the experiment in anaerobic conditions, category E

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Body part mass loss

One-way ANOVA yielded a significant difference in the effect of temperature on the decay of analogue feet at the $p < 0.05$ level [$F(3, 12) = 8.23$, $p = 0.003$]. However, this was not the case with animal heads [$F(3, 12) = 1.40$, $p = 0.291$]. Hence, the null hypothesis, stating there will be no difference in decomposition rates between the two temperature settings, is rejected, and the alternative hypothesis is accepted. Category E feet samples lost the most biomass (62.35%), followed by the head samples of the same category (30.7%). The largest difference in biomass was between animal feet in category E (Figure 4) and category C (21.75%).

Morphological appearance of body parts

Animal heads appeared to decay faster than animal feet. Most category C head samples were in the Early disintegration stage, and most category E head samples in the Active decay stage. Internal decomposition in most category E head samples corresponded with Advanced decay, showing sagging of the skin and bone detachment and did not align with the outer appearance (Figure 5). Control animal heads in both categories exhibited different decay patterns by appearing deflated (Figure 6). Many category C and E foot samples, including the two control samples, were classified into the Early disintegration stage. No insect/fly infestation was found at or near the remains.

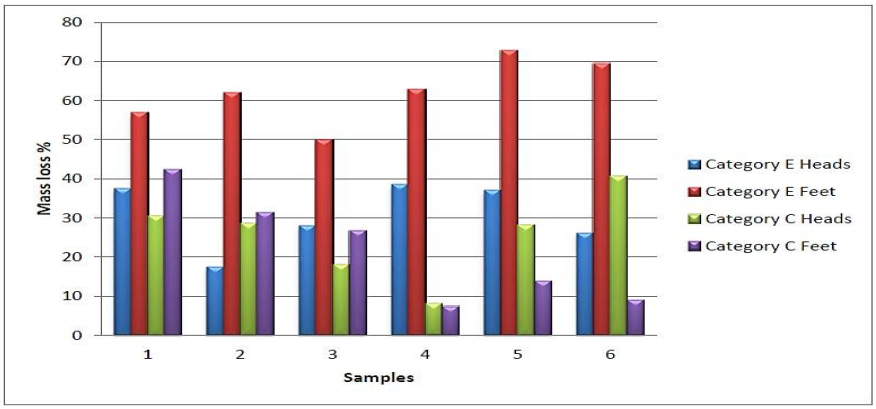

Figure 4: Summary of mass loss for buried body parts

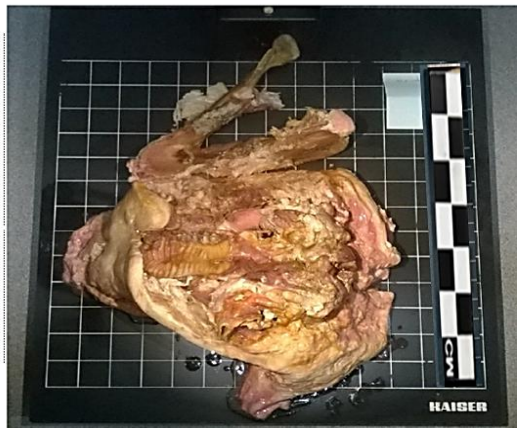

Figure 5: *Sus scrofa* head decay at the end of experiment, category E

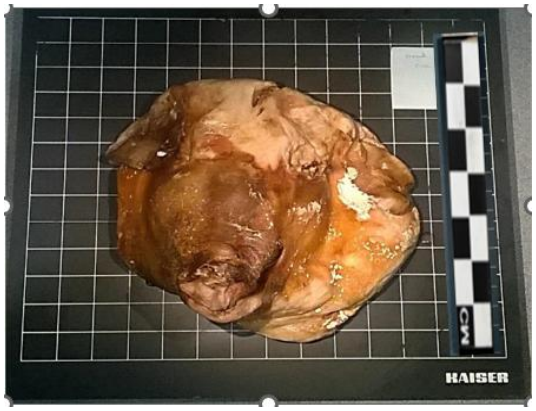

Figure 6: Deflation of the animal head at the end of the experiment, category E control

Discussion

The study investigated the impact of ambient temperature on the decay of frozen buried remains by analysing the physiochemical and microbiological properties of compost, the microbial components of pork tissue, and measuring and categorising body part decay. It hypothesised that ambient temperature will not impact the decay rates of body parts. Because certain statistical analyses yielded significant results, the null hypothesis is accepted and the alternative rejected. There was a significant difference in the soil pH levels according to the temperature. The results are in line with Carter and Tibett (2008) and Rodriguez and Bass (1985) who showed the presence of corpses impacts the soil pH, initially increasing alkalinity, then decreasing the pH. In comparison with fresh buried remains from previous studies (e.g. Cater *et al*. 2010), however, there was no radical difference. Compost containing frozen remains of category

E (30°C) was slightly alkaline during the four weeks, reverting at the end of the experiment.

The pH levels of the remains incubated at 20°C (category C) were also mildly alkaline during the first three weeks, but had changed to acidic by the end of the experiment. With reference to the use of soil pH as a potential indicator of body disposal, the figures indicate that the soil pH around buried frozen remains would not differ much from that associated with body parts buried fresh.

Prior to the experimentation, the compost microbial composition further indicated similar metabolic activity between samples. During the decay, however, the statistical analysis showed the variation between the two temperature levels. This supported the findings of Stokes *et al.* (2009), who suggested that the soil chemistry of buried remains will not be impacted by freezing but that the burial microenvironment will impact soil microbial activity. The study of Londahl and Nilaaon (1993) claimed that it cannot be certain if microbes can resume growth after freezing. The present contribution showed growth in bacterial metabolic activity associated with higher temperatures, implying that freezing in certain conditions does not necessarily kill microbes.

Gram-negative bacteria were also mostly detected on the remains incubated at 30°C in anaerobic conditions. Fungi that prefer cooler temperatures were found on samples incubated at 20°C alongside Gram-negative bacteria. A similar pattern was observed from Gram staining of the main samples, with mostly Gram-negative bacteria in Category C (20°C) and a combination of Gram-positive and Gram-negative in Category E (30°C). Because Gram staining that was conducted prior to the burial of samples showed predominantly Gram-positive microbes, it can be cautiously deduced that the Gram-negative bacteria were mainly involved in the decay of body parts that were possibly body necrobiomes. Because Gram-negative bacteria were also predominant in control anaerobic conditions, it is assumed that facultative anaerobic microbes were involved in the decay process. This is because strict anaerobic Gram-negative bacteria are relatively sparse in these microenvironments (Mhete *et al.* 2020). The results are in contrast to other studies, such as those by Zugibe and Costello (1993), which suggested predominantly aerobic decomposition. The authors, however, used rats as animal analogues and supported their findings with a forensic case ('The Iceman Murder') where the subject was frozen between 18 and 36 months.

TVC also indicated a slight increase in microbial numbers on meat pieces exposed to a higher temperature. The control sample in category C (20°C) that was not in touch with compost exemplified the highest TVC and is assumed to be decomposed by bacteria originating from the body. However, there was not much difference between the microbial counts of the main samples and the control in category E (30°C) to draw more conclusive findings.

There was a statistically significant difference in the biomass loss of frozen, buried feet only. The soles of the feet have harder and thicker skin layers than other parts of the body (Grouios 2004), which could cause slower decomposition, especially with reference to insect succession. Thawed skin, on the other hand, may be easier to decompose. Further, the difference in decay rates between feet and heads could be due to weight and higher surface area to volume ratio. This was in line with the findings of Zugibe and Costello (1993), who suggested that the head of a previously frozen subject decomposed more rapidly in comparison to the rest of the body because of its small size.

The complex and differential decomposition of body parts was further exemplified by their gross morphological changes. Animal head control samples, for instance, exhibited a deflated appearance, which was not the case with the main samples. Differential decay was further evident in category E main head samples, where the decay inside the animal head did not match the decomposition stage of the soft tissue. The decomposition pattern aligns with Micozzi's (1986) findings, which showed the decay to be more prominent internally than externally.

Conclusion

In summary, the tested data showed trends of how frozen organic material reacts to the burial setting in the early stages of decomposition and vice versa. Most findings revealed that

temperature is the main variable in the decay, implying that freezing conditions do not necessarily kill the bacterial necrobiome. Compost pH indicated soil as a potential forensic tool, demonstrating that frozen decaying remains affect alkalinity or acidity levels in higher temperature settings. The metabolic activity of compost bacteria further showed that prior freezing may not necessarily affect the growth and activity of microbes and slow down the decay. These findings were supported by the results from the TVC and Gram stain, which pointed out that the body necrobiome driven by ambient temperature was mostly involved in the decay. Taphonomic results demonstrated a differential decomposition between animal heads and feet and a decomposition pattern specific to the tested microenvironment, indicating a difference in decay pattern within a single body element. The results hence demonstrated that specific microenvironments have a key role in understanding the decay of frozen detached and/or dismembered body parts. This is significant as it can reveal the exposure to previous freezing conditions before burial. The study provides a foundation for future trials focusing on the advanced decay stages in field conditions, essential for understanding the postmortem history of mass burials in regions with a continental climate.

Competing Interests

The authors have no competing interests to declare.

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