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Patterns of Mortuary Practice Associated with Ethnoreligious Genocides of the Silk Roads: The Role of Bacterial Necrobiome in the Decay of Frozen Wrapped Remains

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Abstract

Frozen body parts are likely to introduce a high moisture level to the wrapped microenvironment; however, the effect of these depositional circumstances on decomposition is not readily understood. The present study is the fourth in a sequence of taphonomic reconstructions focusing on the impact of microbial necrobiome in the decay of detached body parts. Using *Sus scrofa* (domestic pig) body parts, this study investigates the role of bacterial necrobiome in the decay of frozen wrapped remains in two temperature settings. The research methods included the Total Viable Count of bacteria, Gram staining, biomass loss and gross morphological changes. It was hypothesised that ambient temperature would not impact the biomass loss of the remains. The Total Viable Count showed a higher microbial load in a high-temperature setting, indicating that freezing does not necessarily kill the bacterial necrobiome. Gram stain analyses demonstrated that mostly Gram-negative bacteria were associated with higher temperature levels, suggesting a potential involvement in decay. Statistical analyses of body part mass loss yielded significant results, confirming that temperature is the influential variable in the decay of frozen remains. Morphological changes exhibited differential decomposition patterns specific to the tested microenvironments. The first systematic attempt to understand frozen-wrapped microenvironments forms a basis for further research focusing on the taphonomic reconstruction of mass graves in regions with continental climates.

Keywords: *Sus scrofa*, Bacterial necrobiome, Assyrian genocide, Frozen-wrapped body parts, Conflict archaeology

Introduction

The Arrhenius equation asserts that decay is driven by the speed of chemical reactions: with each 10ºC rise in temperature, microbial activity is doubled (Carter and Tibbett 2006). A threshold of 0ºC minimum and 40ºC maximum applies to many microbial decomposers that grow optimally between 15ºC and 40ºC (Carter *et al.* 2017; Pechal *et al.* 2013; Lauber *et al.* 2014). Freezing, on the other hand, promotes differential decay and the preservation of soft tissue due to temperature levels that are uncongenial to the growth of most microbes (Artamanov 1965; Rudenko 1970). Micozzi (1986), for instance, found that freezing may not change the sequence of decomposition stages when compared to bodies that were killed but not frozen, but it may contribute towards faster disarticulation rates. Leygonie *et al.*(2012) explain that although freezing temperatures preserve soft tissue through a

process of sublimation and reduced microbial activity, the body may have been accessible to insects before freezing and might have been frozen in different stages of decomposition. After thawing, hence succession may differ from that of fresh corpses, due to altered bacterial action and the delayed release of volatiles/odour.

Fresh bodies also mostly decompose anaerobically from the inside out (Micozzi 1986). However, Micozzi (1996) reported the decomposition of previously frozen remains from the outside in, preserving the internal organs. The author explained that while temperatures below 12°C may slow down bacterial development, freezing stops the growth of bacteria and tissue is preserved by influencing cell division time. Schäfer and Kaufmann (1999) further note that ice crystal formation damages bacterial cells that shrink or freeze internally during freezing. Soft tissue fibre contains water that, with freezing, creates tissue compartments, impacting tissue degradation by altering the distribution and content of body moisture. Freezing hence damages the connections that hold cells together and once thawed, these result in weakened intercellular junctions with soft and liquefied tissues.

Stokes *et al.* (2009) observed that approximately 80% of the water held by cells during freezing is pulled into occupying interstices and develops crystals when the temperature reaches -5°C, with cellular cytoplasm freezing between -10º and -15°C. It is, hence, uncertain to what extent freezing damages tissue in a way that impacts the decomposition pattern. It is unclear whether the reduction of water from cells would result in a decrease in diffusion and material transfer among cells, which retards chemical reactions. Consequently, it is not readily known how long a body part can be frozen and at what temperature before enteric bacteria is retarded. In fact, Londahl and Nilaaon (1993) argue that it is uncertain whether microbes can resume growth at all once the tissue they occupy is thawed. This is because thawing generally takes longer than freezing, during which period microbes are exposed to a range of temperatures that may or may not promote their growth.

Using *Sus scrofa* (domestic pig) body parts, the present study investigates the effect of wrapping on the decomposition of frozen remains in two temperature settings. This is accomplished by a combination of taphonomic and microbiological methods, including Gram staining and Total Viable Count of microbes, and by recording the body mass loss and gross morphological changes of soft tissue. It is hypothesised that ambient temperature will not affect the decay rates of animal body parts.

Disarticulated human remains often form part of mass slaughter associated with wars (Schmitt 2002). The findings of the present contribution, hence, may serve as a base for studies focusing on clandestine disposals in continental climates, an example being the Assyrian genocide perpetrated by Ottoman forces and Kurdish tribes in eastern Turkey during World War I.

Indigenous to Upper Mesopotamia, the Assyrians expanded their dominance over the Middle East from 900 to 600 BC, encompassing regions that currently form part of modern-day Turkey (Kirby 2018). Assyrians embraced Christianity in the early centuries AD but set themselves apart from Rome in 410 when they established the Church of the East (ibid.) Within the Ottoman Empire they did not wield disproportionate control over commerce and lacked significant populations in neighbouring adversarial nations in contrast to Armenians (Gingeras 2016). Most resided in secluded, mountainous regions, a strategic choice to evade state authority (Gaunt 2020). While this isolation shielded them from military conscription and taxation, it also fortified internal divisions and hindered the formation of a unified identity comparable to the Armenian national movement (ibid.). Their core region of settlement was the hilly rural areas of eastern Anatolia: Urfa, Harput, Adiyaman and Tur Abdin, where they engaged in agricultural and craft activities (Gaunt 2020). Prior to the nineteenth century, religious minorities rather than ethnic groups were incorporated within the legal millet of the Ottoman Empire (Suny 2015). Assyrian Christian denominations recognised within this framework included Syriac Orthodox (Jacobites), Church of the East (Nestorians), and Chaldean Catholic Church. Midyat in the Diyarbekir province was the only town in the Ottoman Empire with an Assyrian majority (Gaunt 2015).

At the start of WWI, competition for land and the identification of populations based on their religious beliefs triggered the orchestrated elimination of several

ethnoreligious groups by Ottoman officials (Gaunt *et al.* 2017). In 1915, *Sayfo*, also known as the Assyrian genocide, saw the slaughter and mass killing across Anatolia. By 1918, this once ethnic minority, with an estimated 500,000 inhabitants, was reduced by half (Travis 2017). Sadly, the deliberate and systematic destruction aimed to eradicate not only biological but also cultural aspects of the history, destroying their tangible and intangible heritage. The Assyrians are the ancient people of the Silk Roads who revolutionised ancient warfare with war chariots and iron weapons as far as 1250 BC (Roy 2021). The long-distance commercial network between Assur in Assyria and Kanesh in Turkey's Anatolia spanned over 1,000 km from circa 2000 to 1750 BC, comprising an extensive series of merchant colonies and trading stations (Blasweiler 2019). This elaborate network facilitated the trade of textiles and tin from the Assyrian merchants in exchange for silver, gold, and copper, marking one of the earliest known long-distance trading enterprises (Kristiansen *et al.* 2018). To support the trade, merchants relied on inscribed clay tablets as the primary means of communication. They created them by hand and enveloped in clay sheets bearing the names of the sender and addressee, representing an early form of information exchange spanning 4,000 years (Michel 2020). Assyrians are still not recognised as an ethnic minority despite Turkey's compliance with the peace Treaty of Lausanne in 1923. Some 26,000 remain in the country, with a small number maintaining a presence in eastern Anatolia, having no right to education in their native language.

The Assyrian massacres spread across the rugged Anatolian landscape, with an average rainfall of 560 mm and temperatures ranging from -43°C in the winter to 38°C in the summer (World Data 2024). This includes the Hakkari region, characterised by its mountainous terrain, with peaks soaring to heights of 4,000 meters and separated by rugged gorges. While taphonomic research into the early decomposition of frozen, wrapped remains may not directly assist in the recovery and identification of victims of genocide from over a hundred years ago, it could serve as a foundation for studies investigating the advanced stages of decay in mass graves in continental climates.

Materials

The domestic pig *Sus scrofa* was used as a human analogue as it is generally accepted to decompose similarly to human remains (Matuszewski *et al.* 2014). A total of 22 pieces of pork belly and 32 pig heads and feet were sourced from John Penny & Sons, a Yorkshire abattoir in Leeds. The pork belly facilitated microbial and soil analyses, while the pig body parts were used for taphonomic trials. Only adult animals aged two years and weighing 30 to 40 kilograms were selected for the study. A dismemberment consisted of five cuts designed to separate the head, front and back limbs, and torso. The animals were slaughtered seven hours before the start of the experiment and maintained in refrigerated conditions at 5°C before being delivered to the University of Bradford Taphonomy lab in double plastic bags. No antibiotics were known to be administered to the animals at any point before slaughter.

Methods

Microbiological analyses

Broad-spectrum enumeration techniques were chosen for the study of microbial presence as a variety of types were assumed to be involved in the decay. The Total Viable Count (TVC) was used to determine the number of non-specific bacterial and fungi colonies on a scale of less than 100 (see Hopkins *et al*. 2000; Weaver 1994). Samples with more than 100 colonies were labelled TMTC (too many to count) and approximated. The population of microorganisms was assessed using Rose Bengal agar (for fungi growth), MacConkey agar (for *E. coli* and other coliform growth purposes), and R2A agar plates (for soil waterborne microflora). The plates were examined at 24 hours for R2A and MacConkey agars and at 48 hours for Rose Bengal agar. Gram staining was employed to identify the prevalence of Gram-negative bacteria (commonly found in the human body) or Gram-positive bacteria (mainly living in the environment) during the decay.

Taphonomic analyses

The taphonomic decomposition model based on studies by Davis and Goff (2000) and Prieto *et al.* (2004) has been modified to reflect the post-mortem changes in the moist microenvironment (Table 1). To determine biomass loss, the weight of each body part was measured using

an electronic scale at the start and end of each experiment.

Table 1: Description of morphological appearances of wrapped 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 in 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. 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 wrapped pork belly

Microenvironment	Category	Harvest	End of trial
Aerobic	A	3+1 control sample	3+1 control sample
Aerobic	D	3+1 control sample	3+1 control sample
Anaerobic	A	--------	3
Anaerobic	D	--------	3

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

Duration	Sample size	Microenvironments/Category	Applied method	Tested variables	Controls
28 days	Aerobic: 16 Anaerobic: 6	A (20^0C) aerobic conditions D (30^0C) aerobic conditions A (20^0C) anaerobic conditions D (30^0C) anaerobic conditions	TVC Gram stain	Ambient temperature	Double wrapping

Table 4: Summary of experiment set ups for wrapped animal body parts

Duration	Sample size	Microenvironments/ Category	Applied method	Tested variables	Controls
28 days	32	A (20.8^0C) D (26.1^0C)	Mass loss Morphological changes of soft tissue	Ambient temperature	Double wrapping

Methods of data analysis

Differences in analogue mass loss were analysed using a one-way ANOVA. When the data failed to pass the normality tests, the Kruskal-Wallis test was employed instead. TVC and Gram staining were deemed qualitative microbiological methods, unsuitable for statistical analysis within the current study design. Their characteristics were utilised to support or contradict the findings of other statistical results.

Research design for pork belly experiments

Two sets of experiments were conducted separately using pork belly and animal body parts. The former was used for Gram staining and TVC, while the latter served for the taphonomic analyses. A total of 22 pork belly samples were utilised, 16 in aerobic and 6 in anaerobic conditions (Table 2; Table 3). Prior to the experiment, the meat pieces (5cm width x 5cm length) were kept in the freezer at -18°C for 24 hours, then wrapped and immediately placed into jam jars (228ml, height 85mm, diameter 63mm with lid/neck of 63mm). The pork belly was incubated at 20°C (category A) and 30°C (category D).

To establish TVC and Gram stain, muscle and skin areas of pork belly were swabbed after they were taken out of the freezer, halfway through the experiment (day 14) and at the end for those decaying in aerobic conditions. A double wrapper was used as a control. The samples were not re-wrapped after harvesting to preserve the body microflora, but new meat pieces were used for the next sampling interval. Pork belly pieces in an anaerobic chamber were not harvested mid-experiment to minimise exposure to oxygen and were swabbed at the start and end of the trial. Control samples were not used either due to the nature of the treatment. Bacterial colonies grown on Petri dishes were observed under a microscope with a 100X objective.

Research design for body part experiments

A total of 32 body parts were utilised: six animal heads and six feet, and two control heads and two control feet per category (Table 4; Table 5). The sample size included one repeated trial. After the delivery, body parts were weighed, photographed and deposited in the freezer at -18°C for 24 hours with close attention paid to avoid insect succession. Skin and muscle tissues from selected animal heads and feet were swabbed after being taken out of the freezer and at the end of the experiment for the comparison of TVC and Gram stain with pork belly samples. Body parts were wrapped in heavy-duty plastic waste bags, sealed with gaffer tape and left to decompose individually in fabric storage boxes (33 x 38 x 33 cm) undisturbed for the duration of the experiment.

Body parts in category A decayed at room temperature, averaging 20.8°C, and those in category D at an average temperature of 26.1°C. One of each body element was left to decompose in transparent bin liners with Tinytag® Gemini PT100 loggers left inside to monitor the ambient temperature hourly. The control group consisted of body parts double-wrapped in two plastic bags, with the inner bag placed on top of the bottom one and both bags double-sealed. At the end of the trial, all animal heads and feet were again observed for any signs of insect succession, photographed, weighed, cleaned and categorised into decomposition stages.

Table 5: Breakdown of sample size for wrapped body parts

Category	Sample	Sample size
A	Head	6
A	Foot	6
A control	Head	2
A control	Foot	2
D	Head	6
D	Foot	6
D control	Head	2
D control	Foot	2

Results

1. Qualitative and quantitative assessments prior to wrapping

Microbiological analyses

Predominantly Gram-negative bacteria were observed, with the muscle areas exhibiting a comparable number of colonies (average 3.7 x 105) to those found on the skin of the tested belly slices (average 3 x 105).

Taphonomic analyses

Examination of the body parts revealed no signs of fly or insect infestation. All remains were categorised in the "Fresh" stage of decomposition. The average weight of animal heads was 5.01 kg, and the average weight of animal feet was 0.91 kg.

2. Micro-environmental monitoring during the decay

Total Viable Count and Gram stain

TVC of microorganisms in anaerobic conditions (category D, incubated at 30°C) increased consistently throughout the experiment, indicating ongoing decay. In category A (incubated at 20°C), the microbial numbers at the end of the trial were even higher. In aerobic conditions, there was no clear trend in the TVC of microorganisms in either category. However, the high number of colonies may have affected the accuracy of the plate counts. Most bacterial growth on Rose Bengal appeared flat, round white, or pale yellow. The colonies that grew on MacConkey were mainly small, round, raised, and dark purple (Figure 1).

Gram staining of the samples in category D (incubated at 30°C) demonstrated predominantly Gram-negative bacteria in large numbers (Figure 2), with a combination of bacilli and cocci-shaped colonies. Mostly bacilli-shaped colonies were further evident in a control sample of the same category. Gram staining of the samples in category A (incubated at 20°C) indicated possible Gram-positive cocci, diplococci, and streptococci colonies involved in the wrapped remains decomposition (Figure 3). The control sample of the same category showed mainly Gram-negative rods in large numbers. The samples left to decompose in anaerobic conditions had a combination of Gram-positive and Gram-negative bacteria in both categories.

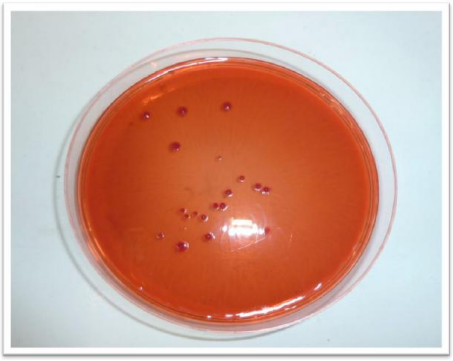

Figure 1: Typical MacConkey agar plate from frozen wrapped pork belly muscle, Category D

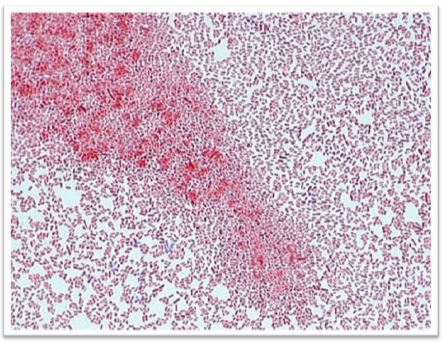

Figure 2: Gram-negative bacteria from the surface of animal tissue, Category D. Scale: 100µm

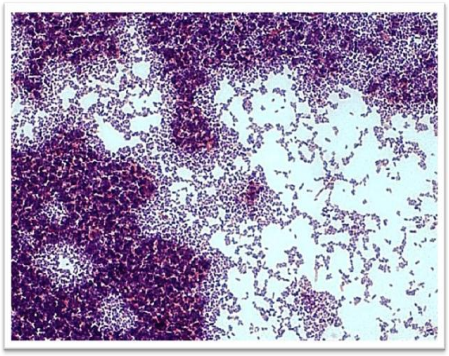

Figure 3: Gram-positive bacteria from the surface of animal tissue, Category A. Scale: 100µm

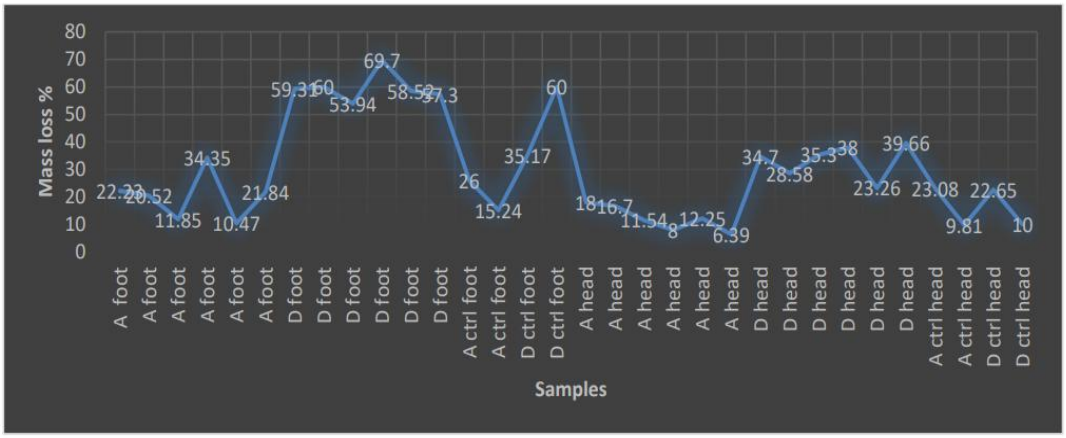

Figure 4: Summary of tissue mass loss of frozen wrapped body parts (%)

Body part mass loss

Kruskal-Wallis test yielded a significant difference in mass loss between animal heads ($H= 10.73$, $p < 0.05$), and One-Way ANOVA showed a significant difference in mass loss of animal feet between the two categories [$F (3, 12) =34.99$, $p < 0.05$]. The null hypothesis, stating that there would be no difference between the two tested groups, was therefore rejected, and the alternative hypothesis was accepted. There was not much difference between the temperature inside the bin liner and the room temperature for category A heads (21.1°C vs 20.8°C) or feet (21.4°C vs 20.8°C). For category D samples, the temperature inside the wrapper was higher by an average of 1.4°C for the heads and 1°C for the animal feet. Category D feet and head samples showed the highest biomass loss (Figure 4). There was minimal variance in tissue mass loss between the main category A samples and their controls, and category D samples and their controls.

Morphological appearance of body parts

A slight variation in gross morphological changes was observed among category D heads (ranging from Early disintegration to Active decay). However, some samples deviated from established decay stages and displayed soft tissue characteristics of submerged remains (Figure 5). Animal feet in the same category demonstrated a more uniform decomposition pattern, with most body parts reaching the Advanced decay stage. More variation in morphological changes was noted in category A heads, where decomposition stages ranged from Early disintegration to Advanced decay. Animal feet decomposed slower than the samples from category D and were categorised into either Putrefaction or Early disintegration stages. Control samples for both body elements in both categories remained at the Putrefaction stage.

Figure 5: Sus scrofa head decomposition at the end of the experiment: category A

Discussion

The study investigated the significance of bacterial necrobiome in the decay of frozen wrapped remains by recording the impact of ambient temperature on TVC and Gram stain type, body part mass loss and gross morphological changes. It was hypothesised that ambient temperature would not impact the biomass loss of the remains. Statistical analysis yielded significant differences in mass loss between body parts. The null hypothesis was therefore rejected, and the alternative hypothesis accepted.

Freezing of the samples likely reduced the number of anaerobic microbes. This was evident by the rising colony numbers throughout the experiment in those incubated at 20°C (category A) and, to a lesser extent, on microorganisms incubated at 30°C (category D). Metabolising and replicating DNA in frozen conditions and continuing metabolic activity have been noted in previous studies (e.g. Christner *et al.* 2003). Baust *et al.*(2001) and Baust (2007) further suggested the possibility that certain types of microbes can survive freezing (-17°C), as not all mammalian cells can equally cryopreserve. The study indicated that even if they do preserve, approximately 30-70% would be destroyed within the first 48 hours after thawing. Baust *et al.* (2001) further observed that some microbes can indeed endure deep freezing below –100°C, but the intermediate temperature zones that they need to go through twice during freezing and thawing are the ones that destroy them.

Variability in microbial numbers according to the temperature settings, presumably due to unequal cell cryopreservation, would be consistent with the study by Christner *et al.* (2003) that recorded specific conditions for frozen microbes to repair damaged DNA (Dieser *et al.* 2013).

Gram staining revealed the division of Gram-positive and Gram-negative bacteria, with the latter mostly found at higher temperature levels among the main samples. Control samples (double-wrapped remains) were in line with these results, indicating that reduced oxygen levels were not the main factor in deciding whether Gram-positive or Gram-negative bacteria take the lead in tissue decomposition. The study of Schäfer and Kaufmann (1999) showed that cells can either freeze internally or shrink during freezing. It is, therefore, possible that temperature affected not only microbial numbers but also Gram type in aerobic and anaerobic conditions, causing variability in the decay between microenvironments tested. The lack of fungi colonies in both microenvironments points to freezing having destroyed the microorganisms that existed beforehand or the presence of water crystals in the incubator that did not promote their growth.

Statistical analysis of the biomass loss of both animal body parts yielded significant results. The percentage of biomass loss range, however, was wide: between 6.39% and 39.66% for the main head samples and 10.47% and 69.70% for the main feet samples. The

temperature inside the bin liners was slightly higher than the ambient temperature, approximately a degree Celsius.

Roberts *et al.*(2017) suggested that freezing accelerates decay rates, while Schäfer and Kaufmann (1999) claim to slow it down. The decomposition of frozen wrapped remains in this study showed that temperature influenced decay rates, resulting in differential decomposition even between the same animal elements. Since the remains were frozen for only 24 hours, it is possible that ice crystal formation did not significantly damage bacterial cells (Christner *et al.* 2003). A longer freezing period or lower temperatures could have impacted microbial growth and activity differently. Decomposition patterns between body parts varied. Overall, the bespoke decay model accommodated five out of six decomposition stages (Fresh to Advanced decay) in both categories. The exception was category A head samples that resembled submerged remains, with gross morphological appearance also observed by Micozzi (1986).

Conclusions

In summary, this study demonstrated discernible decay patterns in the response to a frozen-wrapped microenvironment in the initial phases of decomposition. Most findings indicate that temperature is the dominant determinant in decomposition, implying that freezing does not invariably kill the bacterial necrobiome. Subzero temperatures likely reduced the number of anaerobic microbes, slowing the decomposition. This was evidenced by the increasing bacterial numbers at the end of the experiment in both anaerobic conditions. The lack of trend in TVC in aerobic conditions further emphasised the complexity of bacterial decay. Gram staining revealed the presence of both Gram-positive and Gram-negative bacteria, suggesting the potential involvement of the body's necrobiome in decomposing remains at higher temperatures. Taphonomic experiments further demonstrated a complex decay pattern, with most body parts exposed to higher temperatures losing the most biomass, differential decomposition among the same animal elements and soft tissue characteristics of submerged remains. The findings demonstrate that microenvironments have a key role in understanding the decay of individual body parts. 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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