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Effect of insect exclusion and microbial perturbation on piglet mass loss and total body score

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ABSTRACT

Recent conceptual and empirical developments in decomposition research have highlighted the intricate dynamics within necrobiome communities and the roles of various decay drivers. Yet the interactions between these factors and their regulatory mechanisms remain relatively unexplored. A comprehensive understanding of this facet of decomposition science is important, given its broad applicability across ecological and forensic disciplines, and current lack of research which investigates the inter-dependencies between two critical components of the necrobiome (the microbiome and insect activity), and the consequences of this interdependency on mass loss and total body score. Here we investigated the relationships among these key aspects of the decay process. We experimentally manipulated these variables by physically excluding insects and chemically perturbing the external microbiome of piglet (*Sus scrofa*) carcasses and quantified the effects on mass loss and total body score, as well as insect pre-appearance interval and colonisation. We found that piglets in the insect excluded and microbially perturbed treatment groups exhibited a significant delay in reaching 50 + % of mass loss compared with control piglets with insect access and intact microbiome. However, only remains with insects excluded displayed a significantly slower rate of total mass loss throughout the majority of the experiment *and* remained a significantly higher mass at the endpoint of 11,000 accumulative degree hours. Additionally, all insect excluded and microbially perturbed treatment groups displayed significantly lower total body scores compared to control piglets at corresponding time points. We also observed a significant delay in insect pre-appearance interval and colonisation for piglets with perturbed microbiomes compared to control piglets. Our findings demonstrate the significance of interacting components of the necrobiome, and the power of manipulative experiments in revealing causal relationships between biota and decomposition rates. These considerations are paramount for developing accurate post-mortem interval estimations and a comprehensive understanding of ecological processes during decomposition.

1. Introduction

The decomposition of vertebrate remains involves intricate biological, chemical, and physical processes that break down complex organic compounds while recycling nutrients and energy back into ecosystems [1–4]. Variations in decay rate and patterns across different ecosystems can be explained by considering the "necrobiome." The necrobiome represents the community of organisms and their interactions with the necromass, each other as well as their surrounding habitat and ecosystem; it encompasses the biological and ecological processes of bacteria, insects, vertebrates, and their interactions with abiotic factors, soil, and the environment [1]. The necrobiome concept therefore allows for the testing of hypotheses about how these biotas interact, and the implications of these interactions for decomposition. Yet due to decomposition's inherent variability, applying a universally standardised method for quantifying the process is challenging [5]. Typically, as remains progress through decomposition, they lose biomass, characterised by mass loss, and undergo both qualitative and quantitative changes [6,7]. Various models have attempted to categorise decomposition, typically through a qualitative approach such as decay stages and total body score (TBS) [7–9].

Historically, decomposition has been divided into several stages that

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can be distinguished from one another by taphonomic markers [10]. There is no universally recognised quantitative model for decomposition. Given that decomposition is a continuous process, it should be viewed as a continuum. Therefore, using categorical stages of decomposition to predict PMI does not offer the refined approach that such a complex process warrants [11].

TBS is a method introduced by [9], for quantifying the physical condition of remains. This method involves assigning decomposition scores to three regions of the body—head/neck, torso, and limbs. Scores range from 1 to 13 for the head/neck, 1–12 for the trunk, and 1–10 for the limbs. The scores are determined based on the stage of decomposition (fresh, early decomposition/bloat, advanced decomposition, and skeletonization) and the presence of observable morphological changes associated with decomposition stages. These changes include skin discoloration, the presence or absence of bloat, mummification, adipocere formation, moisture, bone exposure, and bone condition. The scoring system is used in conjunction with a formula to provide an estimation to the post-mortem interval PMI [12]. Research has found the TBS method of PMI estimations to be reliable [13], yet the original statistical model was flawed; this has been developed further into a statistically sound predictive formula developed by [14], which could then be used to retrospectively calculate the accumulative degree days (ADD) and subsequently, the PMI.

Accounting for the variability in decomposition could be achieved, in part, by quantifying the known drivers of variability, which can be facilitated by consideration of the necrobiome [1,15,16], as the process can vary due to internal and external factors such as age, body mass, microbial activity, environmental exposure, burial conditions, and humidity levels [3,17–23]. While many researchers consider temperature and moisture [24–27] as the primary abiotic variables affecting decomposition rate, additional biotic factors are also clearly important. The activity of insects and scavenging vertebrates, microbiome of the individual, clothing, burial, soil chemistry, trauma or injury, body mass, sunlight exposure, submersion, exposure to chemicals, and medications have all been shown to impact the rate of decomposition to varying degrees [6,28–39]. The complexity of these sources of variation in decomposition mean that a robust experimental approach with suitable controls and replication is critical to advancing forensic science, ecological research, and other fields where PMI estimation and knowledge of ecosystem dynamics are of significance.

Insects are integral to the decomposition process [40] and have held a significant place in forensic literature for quite some time [29,41,42]. Insects quickly locate animal remains shortly after exposure and colonise them in a predictable successional order [43–45]. Primary colonisers, typically blow flies (Diptera: Calliphoridae), are attracted to remains through their specialised olfactory senses, and their larvae utilise remains as food source [3,6]. Primary colonisers are followed by other arthropod species, which are also attracted to the remains as a food source, or to predate on other larvae colonising the remains [46]. The exposure of remains to insects play a pivotal part in driving the remains through the decomposition process, as insects are important consumers of the soft tissues of carrion and remove biomass as decomposition progresses [4,13,47,48].

Throughout the decomposition process, decaying remains emit volatile organic compounds (VOCs) that act as scent signals attracting insects and scavengers [49]. These VOCs result from the breakdown of cells via autolysis and the uncontrolled growth of microorganisms, influenced by factors like temperature and treatments applied to the remains before and after death, as well as insect colonisation [32,50]. Despite this connection, there's been limited experimental manipulation of a subject's microbiome to understand its impact on decomposition rates, with significant interest in this topic emerging only in the last decade [50,51]. The breakdown of cells, rapid microbial growth, and the release of VOCs collectively serve as initial attractants for primary insect colonisers to remains [56]. Disruptions to these attractants could potentially delay insect arrivals at decomposing remains, although this hasn't been explicitly tested. Such delays might manifest as a shift in the timing of insect arrival (known as the pre-appearance interval or PAI) [52], with colonisation and subsequent oviposition also being delayed. Additionally, insects have the potential to introduce diverse microorganisms to the remains [49,51]. The breakdown of complex organic molecules is well known to be facilitated by microbial decomposers, but what happens when the community of these micro-decomposers is disturbed? Understanding this potential for variability in decomposition is essential for measuring, comprehending, and predicting decomposition rates under a diverse range of circumstances [1,53]. This knowledge is particularly valuable for determining the time since death, otherwise known as PMI, which is crucial across a wide range of medicolegal applications [20,54,55]. These disturbances may change the trajectory and rate of decomposition and is an important consideration for forensic scientists when estimating the PMI.

Here we experimentally manipulated the microbiome and insects associated with piglet carcasses to determine their relative effects on decomposition. We researched the effects of microbial perturbation and insect exclusion on the decomposition of vertebrate remains, through measures of mass loss and total body score, to investigate individual and combined effects on the rate of decay.

We had two primary questions that we wanted to answer with our study design:

1. Does microbial perturbation, insect exclusion, or their interaction have a significant effect on decomposition?
2. Does microbial perturbation affect insect PAI and oviposition?

We hypothesised that microbial perturbation would delay PAI, oviposition, and decay rate compared to the control. We also expected insect exclusion to slow decay, with the double exclusion group showing the slowest decay rate of all.

2. Methodology

2.1. Study design

We used a randomised block design of 20 piglets grouped into five blocks of four piglets across a five-hectare study area (5 blocks × 4 treatments) in each year (40 piglets total). Blocks were separated by approximately 200 m, and piglets within blocks separated by approximately 20 m (Fig. S1**)**. The experiment was repeated over the summer and autumn season (February, March) in 2022 (for 28 days starting 23-Feb-2022) and 2023 (for 22 days starting 11-Feb-2023) in rural Victoria, Australia (143°48'13"E, 37°36'31"S). During these times, temperature ranged from approximately 2 – 45 degrees Celsius (C) in both years, with an average temperature between 15 and 25 degrees C during experiment 1, and 14–20.5 degree C during experiment 2. The stillborn piglets (*Sus scrufa*) were sourced locally in a fresh state from a local piggery and confirmed to have similar time of death. Each piglet weighed approximately 1.27 kg (± 0.35 s.d.).

Each experimental block included four piglets exposed to a different combination of insect and microbial treatment intended to isolate the effects of variation in insects and microbes, and their interaction, on decomposition. These treatments were: (i) a negative control with no condition/treatment affecting insects or microbes; (ii) insect exclusion but microbes undisturbed (iii) external microbe perturbation but insects present; and (iv) both insect exclusion and external microbe perturbation. Insect and microbial treatments, and their combinations, were established as follows (Images of the contraptions can be observed in Fig. S2.):

i. ***Negative control:*** For those in the control group, this involved unwashed piglets placed into a tub, with holes cut in each side and the bottom of the tub, to allow for ventilation and insect access.

ii. ***Insect exclusion:*** For those in the insect exclusion treatment, insect mesh (18 × 16 stands per inch) was glue-gunned over the holes and gaffer taped on the edges to ensure complete coverage of any potential entrances.
iii. ***Microbial perturbation*:** For those in the microbially perturbed group, these were washed in chlorhexidine and left to soak in this solution in a large, covered plastic tub overnight. Prior to placement in the field, piglets had additional chlorhexidine injected into their orifices. Chlorhexidine was chosen for this experiment due to its global availability, safety, affordability, and effectiveness as a broad-spectrum bactericide and fungicide [56, 57].
iv. ***Insect exclusion*** + ***Microbial perturbation:*** involved both ii and iii above.

Those not washed were also stored in a separate covered plastic tub, to prevent premature colonisation from any insects. All piglets were placed in 4 degree Crefrigeration overnight until placement in the field the following morning at approximately 0900 hours after warming to ambient temperature.

2.2. Measurement of mass loss and TBS

Cooling racks were locally sourced and placed under each piglet with rope attached to all 4 corners of the rack and threaded through a hole drilled in roof of the tub. A metal ring was attached to the end of the ropes to allow for easy weighing of the piglets. Handheld weight scales were calibrated for accuracy prior to use in the study.

Prior to placement in the field, each piglet was individually weighed, along with the metal wire rack they were placed on, inside the plastic tubs. The piglets were sampled daily at approximately mid-day when insects would be at their most active. Measurement parameters collected each day included mass loss, TBS, and insect data.

2.2.1. Mass loss data

Each piglet was weighed using a handheld luggage weight device connected to the metal ring and ropes of the racks underneath the piglets. A piglet was gently lifted for a few seconds to record mass as to not disturb the insect activity on the remains. Mass loss was recorded to two decimal places and converted to percent mass loss to standardise between piglets.

2.2.2. Total body score

The TBS of each piglet was transcribed on an Excel spreadsheet by noting in real-time the state of decomposition (Tab. S1**)** and capturing this data on a paper document, using standardised guidelines from [9]. In the analysis, the TBS, initially ranging from 3 to 35, was treated as a continuous response variable. To enhance interpretability, the TBS scale was rescaled to range from 0 to 32 by subtracting 3. This adjustment was made to logically quantify the state of no decomposition, represented by [9] "fresh," as zero. Setting the starting value at zero simplifies the conversion between decomposition scales, facilitating straightforward comparisons and providing a meaningful origin for plots involving these variables.

2.3. Insect data

To determine insect pre- appearance interval, each piglet was monitored for 6 hours after placement in the field and any insects arriving at the piglets were recorded. After the initial placement of the piglets on the first day, the piglets were then sampled daily at midday. Initial sampling included noting the time of colonisation of remains by insects via the first appearance of eggs or first instar larvae in the absence of eggs. Subsequent sampling included collecting immature insect species from the piglets, to rear through to adulthood for identification. Larvae were collected with forceps and placed into 20 mL sampling containers, while eggs were collecting using a wet paint brush. Adult insects were identified in the field, while those which were unidentifiable were caught using insect nets and identified under a Nikon SMZ 745 dissection microscope, with reference to [50].

Immature insect samples were reared on beef liver inside an insect rearing shed on the property. Small plastic containers were prepared with 2 cm of sand, a plastic plate, wet paper towel and beef liver (2.5 g/larvae). The larvae and eggs were transferred to the beef liver and covered with damp paper towel to prevent desiccation. The container was double meshed to protect against parasitic insects and closed with a lid that had a 3 cm × 3 cm cut out to provide ventilation. The paper towel was sprayed with water every day to keep the liver moist until the larvae pupated. Once matured and emerged from their puparium, insects were placed in a –20 degree C freezer for approximately 20 minutes (until death) and then identified under a Nikon SMZ 745 dissection microscope with reference to [50] for species identification.

2.4. Temperature data

Four Thermochron iButton devices were programmed using a One-wire viewer on an Analogue Devices platform to collect temperature data every hour; three of which were placed in differing locations across the property and one of which was placed inside a tub with a piglet to compare temperatures between the inside and outside of the tubs to ensure there were no significant differences. One of the external data loggers were then selected to be the primary temperature logger for data analysis.

At the conclusion of the experiment, accumulated degree hours (ADH) were calculated by summating the temperature for each hour of the experiment for use as temporal data. The dataset was then merged in an excel spreadsheet, under a column which then specified whether the data was from experiment 1 (2022) or 2 (2023).

2.5. Data analysis

We had two primary questions that we wanted to answer with this study design:

1. Does microbial perturbation or insect exclusion have a significant effect on decomposition?

   To address this question, we analysed the variance homogeneity of the data (Bartlett test) prior to any other statistical testing, after which we employed a Welch's Analysis of Variance (ANOVA) alongside ad-hoc Dunn's test with Bonferroni adjustment, and ANOVA with ad hoc Tukey's HSD test to assess the significant impact of insect and microbial treatments on the final TBS and mass loss values, respectively. The time at which piglets were 50 % decomposed, as measured by mass loss and TBS, was estimated using a 4-parameter sigmoidal model, and t-tests were used to statistically compare between treatments. In the analysis, the TBS, ranging from 0 to 32, was treated as a continuous response variable. All analyses were conducted using the R base package version 2023.12.0 + 369 [58], and DRC package version 3.0–1 [59] while plots were created using the ggplot2 package [60].
2. Does microbial perturbation affect insect PAI and oviposition?

To answer this question, we used a Chi-squared analysis on the raw data. Raw data included the timing of pre-appearance interval and ovi/larvi-posiiton data.

To compare dipteran insects across treatments, we counted daily occurrences of species within each treatment (Tab. S2**)**, therefore providing us with data of occurrence ranging from 0 to 5, where 0 is occurrence at no piglets, and 5 being occurrences at all 5 piglets.

3. Results

3.1. Mass loss

We analysed the variance homogeneity of the data with Bartlett's test and accepted the null hypothesis. By the time (in ADH) that a treatment group (insect excluded and/or microbially perturbed group) reached at least 50 % of mass loss (Fig. 1), we observed a significantly slower rate of decay when compared to the control group ($p \leq 0.01$, $df = 3$, F value = 5.48). After this point, remains in the microbially perturbed treatment groups had their mass loss trajectory align with those in the control group and displayed no significant difference in mass loss by the end of the experiment (ADH = 11,000) (Fig. 1). Contrastingly, we found a significant reduction in mass loss ($p \leq 0.005$) for all insect exclusion treatments compared to the control by the end of the experiment.

3.2. Total body score

We analysed the variance homogeneity of the data with Bartlett's test and rejected the null hypothesis. Throughout the experiment, we observed a gradual incline in TBS scoring over the four different treatment groups (Fig. 2). To note, those in the control group experienced the most rapid increase in TBS over the decomposition period, with all piglets reaching a final score of 33 by the end of the experiment. Those in the microbial perturbed group experienced the second most rapid increase in TBS over time, with those in the insect exclusion groups experiencing a similar increase in TBS throughout the experiment. Statistical analysis found all treatment groups to be significantly different when compared to the control group at 50 % of decay ($p \leq 0.01$, $df = 3$, F value = 31.20) (Fig. 2). Piglets in the insect exclusion groups were also found to have a significantly different TBS to the control group by the end of the experiment (ADH = 11,000) ($p \leq 0.001$).

3.3. Insect colonisation patterns and behaviour

During our experiments, we observed initial pre-appearance interval within the first few minutes of piglet placement in the field for all control piglets. Contrastingly, we did not observe any insect presence on any of the piglets which had been washed and injected with chlorhexidine during the first six observation hours of the experiments. A similar pattern was observed for the colonisation data, whereby all control piglets had been colonised by insects 12 h later, at most. However, the piglets with a perturbed microbiome did not experience colonisation until 24 h for 30 % of the piglets, and longer (72 h) for the remainder. The average colonisation time for piglets in the microbial disturbed groups was 41 h.

The researchers did not observe any eggs or larvae in plastic containers excluded to insects, or on the insect mesh, indicating the success of the insect exclusionary method.

Although there was a high diversity (>10) of dipteran insect species noted at the remains, there were only five dipteran insect species which colonised the remains (Fig. 3): *Calliphora stygia, Calliphora augur, Chrysomya rufifacies, Chrysomya varipes and Lucilia sericata.* We observed common species colonising both control and microbial perturbed piglets: *C. stygia, C. augur, C. rufifacies,* and *C. varipes*, along with similar patterns of succession. However, *L. sericata* only colonised the control piglets. In contrast, *C. rufifacies* were observed more frequently on microbially perturbed piglets.

4. Discussion

Our experiment investigated the combined and relative effects of insects and microbes on the decomposition process. While previous research has demonstrated the individual effects of factors such as insects [61] and substances influencing insect behaviour [62,63] on decomposition rates, there is a notable absence of research exploring the inter-relationships among multiple drivers of decay [64]. This study

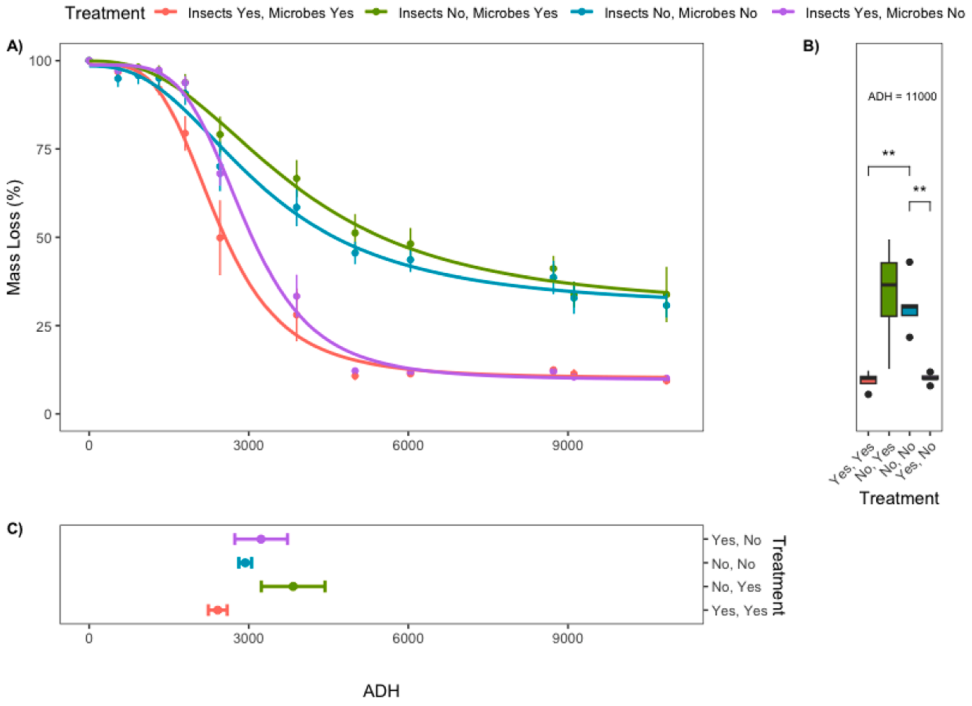

Fig. 1. Panel A: Change in percentage of mass loss of the four treatment groups over time (ADH) fit with a 4-parameter sigmoidal curve: control (insects yes, microbes yes), insect exclusion (insects no, microbes yes), microbe perturbation (insects yes, microbes no), and insect and microbe exclusion (insects no, microbes no). Panel B: Mean mass loss at 11,000 ADH with Tukey's HSD test of comparisons of means with 95 % family-wise confidence level on significance levels between treatment groups. Panel C: ADH estimations at 50 % mass loss (from 4-parameter sigmoidal fit) with 95 % confidence intervals (CI) of the four treatment groups. Datapoints that do not have over-lapping CI are significantly different from one another.

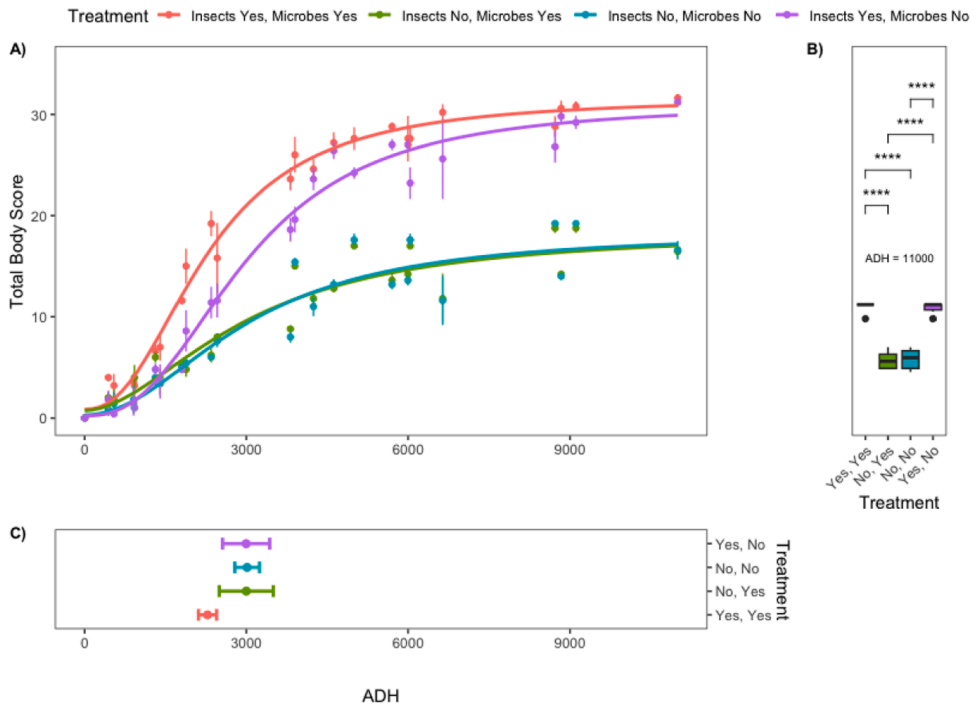

Fig. 2. Panel A: Change in TBS of the four treatment groups over time (ADH) fit with a 4-parameter sigmoidal curve: control (insects yes, microbes yes), insect exclusion (insects no, microbes yes), microbe perturbation (insects yes, microbes no), and insect and microbe exclusion (insects no, microbes no). Panel B: Mean mass loss at 11,000 ADH, with Dunn's test of comparisons of means with 95 % family-wise confidence level on significance levels between the 4 treatment groups. Panel C: Variation of TBS estimations at 50 % of decay (as measured by TBS) with 95 % confidence intervals (CI) of the four treatment groups. Datapoints that do not have over-lapping CI are significantly different from one another.

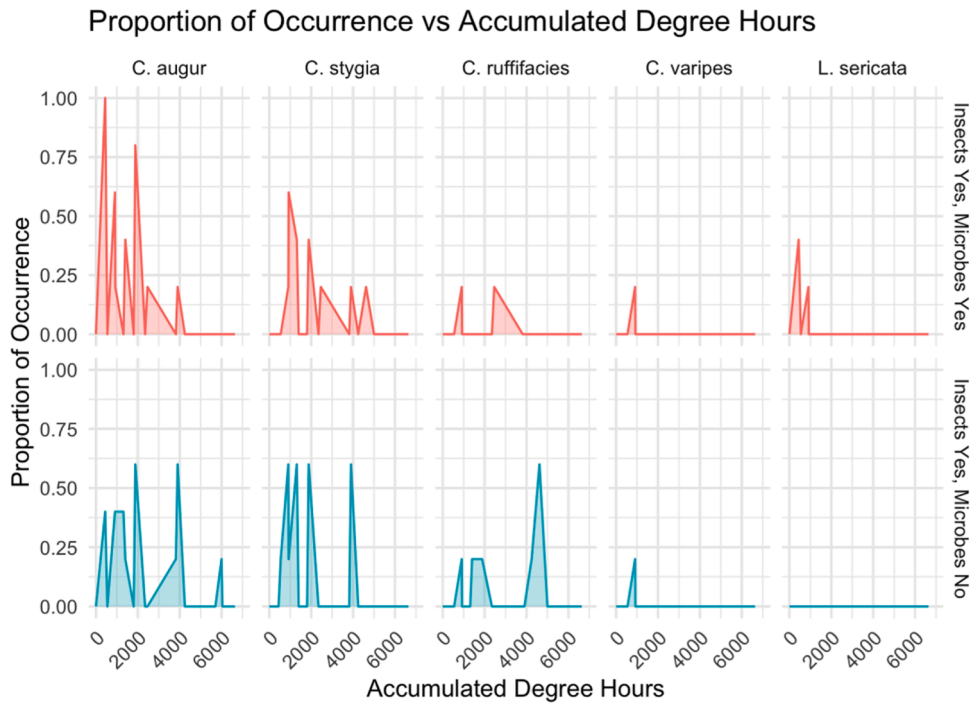

Fig. 3. Dipteran insect species colonisation patterns and proportion of occurrence, where 0.25 = 1 piglet and 1.0 = all five piglets.

addresses that gap by investigating how insects and microbial communities together influence decomposition.

We found that remains with a perturbed microbiome caused a significant delay in the pre-appearance interval (PAI) and colonisation by insects. We also found that remains excluded from insects decayed at a significantly slower rate than remains exposed to insects. Although there was a significant difference in the time (ADH) taken for remains in the microbe disturbed group to reach at least 50 % of decay and TBS increase, there was no significant difference by the end of the experiment. This indicates that microbial effects are limited to the trajectory of decay

but not the end result.

This highlights the dynamic nature of decomposition, where early microbial activity alters the trajectory of decay, but over time, other factors, such as insect access, mitigate these effects, leading to similar end results across groups. Understanding this distinction is pivotal in decomposition science, as it emphasises the complexity of interpreting decomposition timelines and the importance of comprehensive analysis that considers both microbial influences and subsequent ecological processes, such as insect colonisation. We also observed different species of dipteran insects across the insect exposed groups, as well as rate of occupancy. Below we discuss our key findings of how insect access and microbial perturbation to remains affects the rate of decomposition in carrion.

4.1. Insect exposure and microbial perturbation effect on mass loss and total body score

Significant differences in mass loss and TBS were observed among treatment groups during decomposition. Our findings reinforce that insects play a crucial role in driving the decomposition process [48,65]. The delay in PAI and colonisation is likely due to the altered VOC production and potentially compounded by any repellent effects of chlorhexidine [66].

Mass loss and TBS are valuable metrics in estimating the PMI, particularly when used together. However, determining mass loss can be challenging in forensic cases where initial mass is unknown, and research on human cadaver decomposition remains limited [67]. In addition, there will be situations where access to decomposition remains are limited or excluded to insects, and our research has demonstrated that these cases decompose at a significantly slower rate than those exposed to insects. Our experiment has demonstrated that piglets with a disturbed microbiome exhibit a significantly slower rate of mass loss and increase of TBS, when compared to the control group by the 50 % mark in decay rate. While this was chosen as an optimal comparison point, it is essential to consider decay rate differences beyond this point. Therefore, unless remains have been discovered at or near the end of the decomposition endpoint, the PMI is even more uncertain. In addition, treatment groups exposed to insects showed a more rapid increase in TBS scores and mass loss than the insect exclusion groups, reflecting the accelerated decomposition associated with insect consumption of remains [1,68].

4.2. Microbial perturbation effect on insect colonisation and behaviour

Our study demonstrated how insects significantly influenced the rate of decomposition, while also highlighting the impact of microbial perturbation of remains. This interaction was apparent early in the decomposition process, particularly in attracting insects to the remains. Perturbing the external microbial environment on piglets significantly delayed the PAI and insect colonisation when compared to the control group. These results demonstrate the significance of microbial communities after death, and potential VOCs released from their unchecked proliferation, and the role they play in the attraction of insect to remains. This attraction initiates accelerated decomposition, with insect larvae consuming the remains' biomass as a transient resource for their life cycles [1]. One must also consider the ability of insects to introduce bacteria and influence the VOC production which further complicates the decomposition process, highlighting the intricate interplay between microbial communities and insect activity. Insects can act as vectors for microbial dispersion, introducing new species that can outcompete or coexist with the existing microbial flora [69], thus altering the VOC emissions [70] and subsequent insect behaviour. This interaction is dynamic; as insects modify the microbial environment, the VOC profile shifts, potentially attracting or repelling other insect species, creating a feedback loop that continuously shapes the decomposition process [69,70].

The addition of chlorhexidine to remains may have also induced a repellent and/or suppressant effect or masked the chemical cues of decomposition, which insects rely on to locate food resources and breeding grounds [71]. There is a myriad of research which describes repellent effects of drugs, chemicals and toxins to insects, and in turn result in a delayed PAI and oviposition, or mortality of insects which feed on contaminated substrates [72]. One such study by [38] investigated common household items such as gas, mosquito citronella repellent, perfume, bleach, hydrochloric acid and caustic soda, and how they may induce a delay in insect colonisation of a body. They found that some common household products effect fly behaviour and cadaver attraction, and thereby advise forensic examiners to quantify their PMI estimations with the consideration of this possible effect and provide only a minimum PMI.

We noted variations in dipteran colonisation frequency and insect species between the control and microbially perturbed groups. In the control groups, all piglets were colonised by the same species, *Calliphora augur*, primarily at the initial stage of decomposition which aligns with their status as primary colonisers of remains. *Calliphora augur* was also prevalent across piglets with a perturbed microbiome, suggesting their potential utility as an insect of forensic significance in our study location as it is in other areas of southeastern Australia [73], regardless of microbial conditions over the summer and autumn period. Our experiment also identified *C. stygia* as a common insect in both treatment groups, colonising throughout the decomposition process until 6000 accumulated degree hours (ADH), indicating its forensic significance as well. In addition to these, the control piglets observed colonisation by three other insect species: *C. rufifacies, C. varipes, and L. sericata,* all common necrophagous insects in the rural Victorian region[52]. Except for *L. sericata*, these species also colonised the microbially perturbed piglets, with *C. rufifacies* showing higher colonisation rates in the microbially perturbed remains, possibly due to reduced competition of other species on these remains [74]. The absence of *L. sericata* in the perturbed group might be due to the effect of chlorhexidine and subsequent changes in chemical cues emitted by remains with a disturbed microbiome. Studies suggest that microbial metabolites can influence blow flies' decisions regarding resource attractiveness and utilisation [75]. Furthermore, species richness differences between the groups may be linked to the type of remains, as indicated by previous research [76]. Specifically, carcass type directly influences species richness and abundance of scavenger insects. Additionally, studies have shown that higher diversity does not necessarily lead to greater carrion mass loss, while less diverse fly assemblages are associated with higher mass loss [40].

The absence of *L. sericata* across all piglets in the microbially perturbed group may also have resulted from shifts in insect species colonisation patterns within the treatment group, altering competition dynamics on the remains. For instance, *C. rufifacies*, known as predators of other larvae on remains [77], exhibited higher abundance and prevalence of colonisation in our microbial perturbed piglets, which could have influenced the presence of *L. sericata* on the remains. Our research has demonstrated the importance of taking these variable factors into consideration when analysing insect evidence and patterns of colonisation of remains.

Microbial perturbation of remains can be achieved through a myriad of conditions, both ante and post-mortem. Ante-mortem, the ingestion of drugs can influence the complex ecosystem which is the gut microbiome [78,79]. Additionally, research highlights the potential impact of ante-mortem disease treatments. For instance, one study [7] found that antibiotics and cytotoxic drugs administered before death can influence the decomposition process, altering its rates and patterns [7]. This is an important consideration for forensic scientists as it has the potential to affect the accuracy of PMI estimations. A study by [80] investigated the effects of freezing remains on the community of microbes and found that although the community diversity of microbes were not significant across frozen versus unfrozen remains, frozen remains observed changes in the abundance of specific phyla; this may subsequently effect the

number and type of VOCs produced, and consequently alter the attractiveness of the remains to insects. The effect of chemical and/or toxicological influence on microbes after death is a novel area for research, evident by the lack of literature in this area, despite the evidence which demonstrates the significant impact of drugs and toxins in the peri-mortem sense.

Our research highlights the necessity of discerning which insects hold forensic significance across remains with altered microbial communities and those without. Neglecting the disruption of microbial activity and the subsequent VOC production, which may alter PAI and insect colonisation patterns, can ultimately impact forensic entomological analysis, leading to underestimations of the minimum PMI; a pivotal factor in legal cases [8,29,30,81–83]. In addition, when factors such as TBS and mass loss are used to estimate PMI, insect access to remains must be considered. Our study has shown that insect access significantly affects the rate of decay, and failure to account for this could lead forensic entomologists to provide unreliable PMI estimations. Hence, our findings emphasise the necessity of future research on human cadavers which can integrate microbial dynamics into forensic entomology protocols to ensure more precise and reliable investigative outcomes.

5. Conclusion

Our research addressed a key gap in our understanding of carrion decomposition by examining the dynamic interplay between microbes and insects, two fundamental drivers of decay. The study employed the necrobiome model, a framework that encompasses the intricate interplay between microbes, insects, and even vertebrates during decomposition, highlighting its critical role in understanding post-mortem processes [1]. By manipulating microbial communities and insect access to carrion, we were able to systematically evaluate their individual and combined influences. Our findings demonstrate that both microbes and insects play significant roles in slowing decomposition, with microbial disruption leading to a delay in insect activity. These results have significant implications for forensic investigations, where accurate estimates of time since death (PMI) rely on a comprehensive understanding of the complex interactions between these decay drivers. Beyond the practicalities of forensic science, our work establishes the necrobiome model as a powerful tool for dissecting the roles of different players in carrion decomposition. Incorporating the necrobiome into forensic science becomes increasingly important for accurate PMI estimations and a more holistic understanding of underpinning ecological processes. Our study opens avenues for future research, including exploring the complexities of microbial communities and insect interactions under various environmental conditions.

Compliance with Ethical Standards

Not applicable.

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CRediT authorship contribution statement

Philip S. Barton: Writing – review & editing, Supervision, Methodology, Conceptualization. **Blake M. Dawson:** Writing – review & editing. **Benjamin M. Long:** Writing – review & editing, Visualization, Supervision. **Donna McIntyre:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Donna McIntyre reports financial support was provided by Australian Government Research Training Programme. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2024.112336.

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