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PAPER

PATHOLOGY/BIOLOGY

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Volatile Organic Compound Profiling from Postmortem Microbes using Gas Chromatography–Mass Spectrometry*,†

ABSTRACT: Volatile organic compounds (VOCs) are by-products of cadaveric decomposition and are responsible for the odor associated with decomposing remains. The direct link between VOC production and individual postmortem microbes has not been well characterized experimentally. The purpose of this study was to profile VOCs released from three postmortem bacterial isolates (*Bacillus subtilis, Ignatzschineria indica, I. ureiclastica)* using solid-phase microextraction arrow (SPME Arrow) and gas chromatography–mass spectrometry (GC-MS). Species were inoculated in headspace vials on Standard Nutrient Agar and monitored over 5 days at 24°C. Each species exhibited a different VOC profile that included common decomposition VOCs. VOCs exhibited upward or downward temporal trends over time. *Ignatzschineria indica* produced a large amount of dimethyldisulfide. Other compounds of interest included alcohols, aldehydes, aromatics, and ketones. This provides foundational data to link decomposition odor with specific postmortem microbes to improve understanding of underlying mechanisms for decomposition VOC production.

KEYWORDS: forensic science, forensic chemistry, forensic taphonomy, postmortem microbiology, cadaver decomposition, decomposition odor, solid-phase microextraction arrow

After death, the complex process of the decomposition of soft tissues produces a variety of chemicals as macromolecules become broken down into smaller chemical compounds. The major by-products of this process that are responsible for decomposition odor produced from a cadaver are categorized as volatile organic compounds (VOCs) (1). Volatile organic compounds are the compounds present in odors; they are characterized by appreciable vapor pressures that contribute to their preference for existing in a gaseous phase. Decomposition VOCs have been widely investigated in the field of forensic taphonomy to improve applications that involve locating human remains using biological detectors such as cadaver-detection canines (2). It is also possible that improving our understanding of decomposition VOCs could lead to the production of portable sensors to be used in combination with scent detection canine approaches or in challenging mass disaster scenarios that require large-scale multidisciplinary efforts.

The study of decomposition odor is still a developing field that has been under investigation since 2004 (3). Early studies indicated that decomposition VOCs were produced as part of a temporal, dynamic process (3). In recent years, the nature of the VOC profile has been better understood due to an increased focus on generating a more consistent chemical profile for decomposing remains (4–7). Various conditions have been studied, including variations in environments (8), studies in different geographical regions (5,9–11), and seasonal differences (6,12). Different sample collection matrices have been studied (e.g., whole cadaver headspace vs. soil) (9), as well as collection technique comparison and validation of analytical methods (7,13).

Some studies have proposed possible underlying mechanisms responsible for VOC production (14,15); however, these processes are still not well understood and are largely based on reference to literature sources from areas peripheral to forensic taphonomy. To date, no primary studies have identified the specific relationship between postmortem microbes and their role in the emission of volatiles. It is well known that microorganisms play an important role in the decomposition process (16,17). It is highly likely that microorganisms play a dominant role in VOC production through their interactions with cadaveric nutrients and release of volatile by-products from their own metabolism (14,15). A key study identified that the decomposition VOC profile differs significantly between cool and warm seasons, with a major reduction in the abundance of VOCs in winter seasons (12). Correlation analysis suggested that there is an underlying variable responsible for this

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seasonal difference in cooler temperatures that is currently not being measured in decomposition odor studies (12). It was proposed that microbial activity could be such an underlying variable, since it is not usually measured in decomposition odor research (12). More recently, Pascual et al. (2017) concluded that the abundance of several postmortem VOCs is not stochastic; it is significantly correlated with bacterial taxa. It is clear that identifying the functional activity of microorganisms and their responsibility in releasing VOCs will improve the understanding of the source of chemical components in decomposition odor. The aim of the current study was to profile and monitor the volatile organic compounds released from microbial isolates known to be associated with decomposing remains in an attempt to understand the source of chemical components responsible for decomposition odor. Each species was monitored longitudinally using solid-phase microextraction arrow (SPME Arrow) and gas chromatography–mass spectrometry (GC-MS). SPME, first introduced in 1990 by Pawliszyn (18,19), can be used as a simple headspace extraction technique due to its ability to extract volatile compounds with short extraction times and operational simplicity while avoiding the use of organic solvents (20,21). The recently developed SPME Arrow was used in this study to increase sensitivity due to higher phase loading and to increase mechanical robustness (22).

The three bacterial species chosen for this initial study were *Bacillus subtilis*, *Ignatzschineria indica*, and *I. ureiclastica*. *B. subtilis* is a rod-shaped, Gram-positive species, is a facultative anaerobe, and is from the phylum Firmicutes (23). It is widely distributed throughout nature. On decomposing remains, *the genus Bacillus* is typically observed in the later stages of the decomposition process (24). Several strains of *B. subtilis* have also been isolated from *Hermetia illucens* larvae (25). The *Ignatzschineria* species are rod-shaped, aerobic, Gram-negative, non-spore-forming bacteria from phylum Proteobacteria (26), and they are associated with the early stages of decomposition, necrotic tissues, as well as carrion insect larvae (26–28). The three species were chosen as a first set of species for profiling due to their relevance, availability, and lower biosafety level than other postmortem microbes. One valuable resource available in the domain of microbial VOCs is the mVOC 2.0 online database assembled by Lemfack et al. (29) (originally assembled in 2003 (30)), which provides a searchable database for approximately 2000 compounds from 1000 species. While data are available on numerous VOCs from *B. subtilis*, the microbial VOCs released by *Ignatzschineria* species are currently undocumented to our knowledge.

This study is part of an ongoing project that will contribute to a larger database of information on VOCs associated with postmortem microorganisms. This database would be different from preexisting databases, as it would be focused specifically on postmortem microorganisms, be collected under conditions that represent forensic taphonomic scenarios, and have consistent methodological controls in place for all entries (e.g., length of time collected, comparison to background VOCs from growth medium). Controlled primary data on postmortem microorganisms, collected in the manner as described, do not currently exist. Such information can then be used to improve applications that rely on the detection of decomposition odor.

Materials and Methods

Sampling of Bacterial Headspace

The three bacteria of interest, *B. subtilis*, *I. indica*, and *I. ureiclastica,* were previously isolated from a pig (*Sus scrofa domesticus*) carcass used for decomposition studies at the university's taphonomic research facility (Honolulu, HI) (24). These bacterial species were removed from −80°C storage and inoculated onto a growth medium (4 mL) of Standard Nutrient Agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) in sterilized 20-mL headspace vials (Restek Corporation, Bellefonte, PA). Each species was cultured in triplicate to produce three biological replicates of each. For each bacterial species, three replicate vials were filled with 4 mL of Standard Nutrient Agar (HiMedia Laboratories Pvt. Ltd.) that were verified to be sterile and were not inoculated with bacteria. These acted as a control to which the bacterial isolates could later be compared. The headspace vials were sealed, and SPME Arrow sampling was performed through each vial septum. SPME Arrow sampling parameters were previously optimized in another study (22) and are described here in brief. A carbon wide range/polydimethylsiloxane (CWR/PDMS) stationary phase was used on the SPME fiber (Restek Corporation). Fiber preconditioning time was 30 min at 270°C. An exposure time of 5 min was performed. The fiber was desorbed in splitless mode in the GC inlet for 3 min at 250°C. After the sampling procedure was complete, each headspace vial was opened inside a sterilized laminar flow cabinet in order to allow the purging of the headspace for 5 min to prevent carryover of volatiles built up from previous days in the next 24-h monitoring period. The vials were resealed and incubated at 24°C until the following day when sampling was repeated. The headspace of each culture was analyzed and resampled every day for a five-day period. A five-day period was chosen to ensure that bacteria completed at least one full growth phase cycle during the time trial under normal laboratory conditions at 24°C; bacteria were in exponential and stationary growth phases at this time.

Gas Chromatography–Mass Spectrometry (GC-MS) Method

The analysis was conducted on a Focus GC coupled with a Dual Stage Quadrupole II (DSQ II) Mass Selective Detector (MSD) (Thermo Scientific, Bellefonte, PA). All SPME Arrow injections were performed using a Thermo Ultra Inlet Conversion Kit (Restek Corporation) which was equipped with a splitless liner (Restek Corporation, 2.0 × 8.0 × 105 mm). A 1.1 mm Merlin Microseal was also used in the inlet conversion configuration (Merlin Instrument Company, Newark, DE).

Analyte separation was accomplished using an Rxi-624Sil MS capillary column (Restek Corporation, 30 m × 0.25 mm ID × 1.4 μm film thickness) using ultra-high-purity helium as the carrier gas at a constant flow rate of 1.0 mL/min (Airgas, Radnor, PA). The GC oven program started at 35°C where it was held for 5 min, followed by a temperature increase of 5°C/min up to 240 °C, which was maintained for 5 min. The MS transfer line and ion source were set to 250 and 200°C, respectively.

Data Processing and Statistical Analysis

Data acquisition was performed initially using the default detection settings for the ICIS algorithm. The settings for data processing of all samples were finally set according to the following parameters. The settings for ICIS included using an area noise factor of 5, peak noise factor of 20, baseline window of 100, minimum peak width of 3, multiplet resolution of 10, area tail extension of 5, and an area scan window of 0. The noise method was repetitive and peak width was not constrained. MS bunching was used with peak dependent correlation set each at

$n = 3$ for the left region bunch, right region bunch, as well as the peak spectrum bunch. Peaks were smoothed using a 7-point Gaussian smoothing function. Library screening for tentative compound identification was made using the National Institute of Standards and Technology (NIST) 2014 Mass Spectral Library with a forward and reverse match factor threshold of 700. Compounds that met this threshold were input into the MS component table, and retention windows were adjusted manually when necessary. For each peak, the MS quantitation peak and MS confirming peaks were adjusted manually to ensure accurate and consistent integration across batch data. Where the default detection algorithm did not identify appropriate quantitation or confirming peaks, smoothing and MS detection settings were manually adjusted for the peak of interest. Chemical standards were also obtained (GC grade, HPLC grade, or pure) from Restek Corporation (Bellefonte, PA) or Sigma-Aldrich (St. Louis, MO). These solutions are described in further detail in previous optimization work (22). When possible, compound identifications were supported through the injection of chemical standards. Standards included 2-ethyl-1-hexanol, 1-propanol, 2-propanol, 2-butanone, cyclohexane, 2-methylfuran, 2-methylpentane, 3-methylpentane, 2,4-dimethylheptane, 2-methylhexane, naphthalene, 1,2,3-trimethylbenzene, benzene, toluene, *m*-xylene, *p*-xylene, *o*-xylene, heptanal, hexanal, dimethyl trisulfide, and C_6-C_{17} saturated alkanes.

The summary data were exported from Chromeleon as *.csv files for processing in Microsoft Excel 2013. Nondetected values were converted to zero in excel, and the data were organized in a matrix and imported into Prism 7 (Graphpad Software, La Jolla, CA) for univariate analysis and the Unscrambler X version 10.5 (CAMO Software, Oslo, Norway) for multivariate analysis. For univariate analysis, a Student's *t*-test was performed between the triplicate samples of bacteria and control agar from the longitudinal series to determine whether their trends were significantly different over the course of the trial (i.e., when comparing control agar data series to microbe data series for each compound). Longitudinal data were also plotted in Prism 7 to visualize whether trends differed due to consumption of VOCs from the media or production of VOCs by the bacterial isolate. For multivariate analysis, data were unit vector normalized, mean-centered, and scaled to standard deviation. These data were then subject to principle component analysis (PCA) in order to demonstrate the differences between the blank agar sample in comparison to the bacteria samples on each of the days of analysis. This further highlighted the changes in VOCs during the growth phases of each of the bacteria.

Results and Discussion

Compounds Identified in B. subtilis*,* I. indica*, and* I. ureiclastica

In total, 35 compounds were detected by the GC-MS analysis, as listed with chemical and processing information in Table 1. Compounds were classified into one of three categories (Table 2). Examples of the types of trends within each of these categories are shown in Fig. 1. Detailed figures of all compound trends can be found in the supplementary information files (Figures S1–S9). Compounds that were referred to as "produced" had a significantly higher abundance ($p < 0.05$, Student's *t*-test) over time in the bacteria samples compared to the control samples. Compounds that were classified as "consumed" VOCs had a significantly lower abundance ($p < 0.05$, Student's *t*-test) over time in the bacteria samples compared to the control samples. VOCs categorized as "not significant" were those that were detected from the samples using GC-MS but showed no significant difference ($p > 0.05$, Student's *t*-test) in abundance between the controls and the bacteria samples. The categorization of compounds was necessary in order to observe and fully understand the volatiles being produced during the bacteria growth cycles. Preliminary results monitoring trends in *B. subtilis* VOC profile showed that some compounds exhibited significant trends over the five-day period, while others were not statistically significant (22).

It is essential to distinguish the VOCs produced from those that decrease in abundance for individual species, as there is a wide range of species that are present in a microbial community on a decomposing cadaver (16). These different species will interact with one another, and the dynamic nature of VOCs is likely no exception to this. An initial review on cadaveric VOCs and their potential microbial sources from 2011 focused on VOCs which are produced by microorganisms (14). This information is very important in considering possible bacterial species that may have produced them. However, to understand complex longitudinal trends on decomposing remains, it is important to consider the nonlinear nature of microbial VOC production. While isolates were investigated in this study, this is not the case for the complex microbial community on a decomposing cadaver where many species will interact with the remains and one another. For example, on decomposing remains, one species of microorganism may undergo metabolism that converts an amino acid to an aldehyde and then to an alcohol, and another species may convert that same alcohol back into an aldehyde or another product such as a methylketone (31). One species can release a compound while another is undergoing metabolism to break it down; thus, the produced and consumed VOCs are equally important in this study to record for further understanding of microbial interactions in the future when combining isolates. While the specific mechanisms of VOC decrease for each compound/species is still not known for the "consumed" category, these VOCs were in many cases drastically reduced in the bacterial samples compared to the agar controls, so it is hypothesized that some form of interaction between the bacteria and the medium caused this decrease. Compounds that decreased in the agar controls and bacterial samples simultaneously (i.e., categorized as not significant) were considered to be undergoing chemical reactions in the vial environment (e.g., oxidation or formation with hydroxyl radicals in the air to other compounds) and therefore were not considered to be of importance for that species.

Bacillus subtilis

In *B. subtilis*, five compounds were found to be produced (Table 1), while 18 compounds were detected at higher abundance in agar control vials. The compounds 1-butanol, 2-pentanone, and 3-methyl-2-pentanone were found to increase continuously over the monitored period; the compounds 1-methylcyclohexene, 1,3-pentadiene, and acetic acid butyl ester increased substantially prior to decreasing in abundance toward the later days in the monitored period. It is possible for 1-butanol to be formed through microbial carbohydrate metabolism (32). Ketones, methylketones, alkenes, and acetates can be produced through different fatty acid precursors (31). Of the compounds in the produced category, only 1-butanol and acetic acid methyl ester were previously identified in *B. subtilis* (33). Lee et al. (33) also identified dimethyldisulfide and 2-butanone as being produced by *B. subtilis*, whereas in the current study,

TABLE 1—*Analytical parameters for compounds of interest using gas chromatography–mass spectrometry.*

	Compound	CAS #	Chemical formula	Molecular weight (amu)	Retention time (min)	MS quantitation peak	MS confirming peak 1	MS confirming peak 2
1	1,3-Pentadiene	504-60-9	$C_{12}H_{16}N_4O_5$	68.119	12.632	81	96	67
2	Hexane*	110-54-3	$C_5H_{10}O$	86.178	5.420	57	56	71
3	2-Methylfuran*	534-22-5	$C_{12}H_{16}N_4O_5$	82.102	6.070	82	81	53
4	Butanal	123-72-8	C_4H_8O	72.107	6.450	72	57	
5	2-Butanone*	78-93-3	$C_{14}H_{24}O$	72.107	6.703	72	43	57
6	Trichloromethane	67-66-3	$CHCl_3$	119.369	7.486	83	85	87
7	Butanal, 3-methyl-	590-86-3	$C_5H_{10}O$	86.134	8.992	58	71	86
8	Butanal, 2-methyl-	96-17-3	$C_5H_{10}O$	86.134	9.294	57	58	86
9	1-Butanol	71-36-3	$C_4H_{10}O$	74.123	12.15	80	53	
10	2-Pentanone	107-87-9	$C_{12}H_{26}$	86.134	10.517	43	86	71
11	Methane, bromodichloro-	75-27-4	$CHBrCl_2$	163.823	11.294	83	85	129
12	1,3-Diazine	289-95-2	$C_{12}H_{16}N_4O_5$	80.09	10.087	56	41	43
13	Disulfide, dimethyl	624-92-0	$C_2H_6S_2$	94.19	12.381	94	79	45
14	1-Methylcyclohexene	591-49-1	$C_{12}H_{16}N_4O_5$	96.173	3.280	67	68	53
15	2-Methylbutanenitrile	18936-17-9	C_5H_9N	83.134	12.79	55	54	
16	Toluene*	108-88-3	C_7H_8	92.141	13.105	91	92	65
17	2-Pentanone, 3-methyl-	209.282-1	$C_{12}H_{16}N_4O_5$	100.161	13.280	57	100	72
18	Pyrrole	109-97-7	$C_{12}H_{26}$	67.091	14.990	67	41	40
19	Acetic acid, butyl ester	123-86-4	$C_{12}H_{26}$	116.16	15.289	43	56	57
20	Pyrazine, methyl-	109-08-0	$C_{12}H_{26}$	94.117	15.870	94	67	40
21	Ethylbenzene*	100-41-4	$C_{12}H_{26}$	106.168	17.004	91	106	65
22	Furfural	98-01-1	$C_5H_4O_2$	96.085	17.421	96	95	67
23	Bicyclo[4.2.0]octa-1,3,5-triene	694-87-1	C_8H_8	104.152	18.480	104	103	78
24	2-Heptanone	110-43-0	$C_{12}H_{16}N_4O_5$	114.188	18.913	58	71	114
25	Pyrazine, 2,5-dimethyl-	123-32-0	$C_{19}H_{34}$	108.144	22.530	121	56	94
26	Decane*	124-18-5	$C_{10}H_{22}$	142.286	21.432	57	43	41
27	Oxime-, methoxy-phenyl-		$C_9H_{14}O$	241.29	21.694	133	151	73
28	Benzaldehyde	100-52-7	C_7H_6O	106.124	22.088	105	106	77
29	Pyrazine, 2-ethyl-5-methyl-	13360-64-0	C_6H_8N2	122.171	19.286	108	42	81
30	Limonene	138-86-3	$C_{19}H_{34}$	136.238	22.969	68	93	136
31	2-Acetylthiazole	24295-03-2	$C_{12}H_{26}$	127.161	23.915	127	99	112
32	Phenol	108-95-2	$C_{12}H_{26}$	94.113	24.824	94	66	40
33	Dodecane	112-40-3	$C_{12}H_{26}$	170.34	28.078	57	43	71
34	Decanal	112-31-2	$C_{12}H_{26}$	156.269	29.498	57	70	82
35	Butylated hydroxytoluene	128-37-0	$C_{12}H_{26}$	220.356	37.804	205	220	57

Compounds verified by standard injection are marked with an asterisk*.

these compounds were identified in the category of not significant. This could potentially be from differences in methodology between the two studies, such as instrumental detection limits, growth medium, and differences in the SPME fiber-phase selectivity. Lee et al. (33) also extracted samples at 50°C, which would have been unrealistic for a decomposition odor study as *B. subtilis* would not likely be exposed to these high temperatures. However, it is also possible that the differences arose from the fact that isolates in the study herein were monitored longitudinally in comparison with longitudinal fluctuations in blank agar. While dimethyldisulfide and 2-butanone were detected in these isolates, they did not differ from the detection of their respective blank agar controls and therefore were not included in the "produced" category.

Notably, the most abundant VOC produced by this species was 1-butanol. This compound is a commonly detected decomposition VOC in numerous studies performed under different conditions (6,9–11,34–,37) as well as in decomposing blood (38). 1-butanol is typically detected in the later stages of decomposition from active decay onward (11,37). It is hypothesized that this product becomes triggered during the transition from bloat to active decay due to the introduction of an increasingly aerobic environment after the cadaver ruptures from gas accumulation (39). This increase in 1-butanol may coincide with the proliferation of *B. subtilis* on the cadaver. This is supported due to the fact that *Bacillus* is a facultative anaerobe (23), is commonly found in the gastrointestinal tract of mammals (40), and is observed in later decomposition (24,41). It is likely that the introduction of oxygen after the initiation of active decay could be causing *B. subtilis* to shift metabolic pathways, subsequently releasing 1-butanol as the population of *B. subtilis* increases.

Ignatzschineria indica

The two *Ignatzschineria* species investigated in this study were first described in 2011 after isolation from the gastrointestinal tract of flesh flies (26). There is still little known about these species, and therefore, understanding their volatile profile will contribute to fundamental understanding of their metabolic pathways and interaction with a nutrient source. They are not currently included in the mVOC 2.0 database (29). The bacterial isolates used in this study originated from swabs of decomposing pig carcasses, and thus, it is highly possible that they originate from insects appearing at the decomposing remains.

Ignatzschineria indica produced fifteen different compounds (Table 2), which was the largest number of detected volatiles in this study. Six compounds were detected in higher abundances in the agar control vials. Dimethyldisulfide increased significantly in abundance throughout the study. Its abundance was substantially greater than all other VOCs produced by the other microbial species studied. At the peak of its production, the abundance of dimethyldisulfide was approximately two orders of magnitude greater relative to all other decomposition VOCs produced in the study. It is possible that this species of bacteria is

TABLE 2—*Compounds of interest from* Bacillus subtilis*,* Ignatzschineria indica*, and* I. ureiclastica *over a five-day period.*

Produced VOCs	Consumed VOCs	Not significant VOCs
Bacillus subtilis		
1-Butanol	1,3-Diazine	2-Butanone
1-Methylcyclohexene	2-Acetylthiazole	2-Heptanone
1,3-Pentadiene	2-Methylbutanenitrile	2-Methylfuran
2-Pentanone	Bicyclo[4.2.0]octa-1,2,5-triene	Decane
2-Pentanone, 3-methyl-	Butanal	Disulfide, dimethyl
Acetic acid, butyl ester	Butanal, 2-methyl -	Dodecane
	Butanal, 3-methyl-	Ethylbenzene
	Butylated hydroxytoluene	Hexane
	Decanal	Methane, bromodichloro-
	Furfural	Toluene
	Limonene	Trichloromethane
	Oxime-, methoxy-phenyl-	
	Phenol	
	Pyrazine, 2-ethyl-5-methyl-	
	Pyrazine, 2,5-dimethyl-	
	Pyrazine, methyl-	
	Pyrrole	
Ignatzschineria indica		
1,3-Diazine	1-Methylcyclohexene	1-Butanol
1,3-Pentadiene	Butanal	2-Acetylthiazole
2-Butanone	Butanal, 2-methyl-	2-Methylfuran
2-Heptanone	Butanal, 3-methyl-	2-Pentanone, 3-methyl-
2-Methylbutanenitrile	Decanal	Acetic acid, butyl ester
2-Pentanone	Limonene	Benzaldehyde
Disulfide, dimethyl		Bicyclo[4.2.0]octa-1,3,5-triene
Ethylbenzene		Butylated hydroxytoluene
Hexane		Decane
Methane, bromodichloro-		Dodecane
Oxime-, methoxy-phenyl-		Furfural
Phenol		Pyrrole
Pyrazine, 2-ethyl-5-methyl-		Toluene
Pyrazine, 2,5-dimethyl-		Trichloromethane
Pyrazine, methyl-		
Ignatzschineria ureiclastica		
2-Pentanone, 3-methyl-	1-Butanol	1,3-Pentadiene
Phenol	1-Methoxycyclohexene	2-Butanone
	1,3-Diazine	2-Heptanone
	2-Acetylthiazole	2-Methylfuran
	2-Methylbutanenitrile	2-Pentanone
	Bicyclo[4.2.0]octa-1,3,5-triene	Acetic acid, butyl ester
	Butanal	Benzaldehyde
	Butanal, 2-methyl-	Decanal
	Butanal, 3-methyl-	Decane
	Butylated hydroxytoluene	Disulfide, dimethyl
	Furfural	Dodecane
	Limonene	Ethylbenzene
	Oxime-, methoxy-phenyl-	Hexane
	Pyrazine, 2-ethyl-5-methyl-	Methane, bromodichloro-
	Pyrazine, 2,5-dimethyl-	Toluene
	Pyrazine, methyl-	Trichloromethane
	Pyrrole	

Examples of the trends for each category are shown in Fig. 1.

responsible for producing the large amounts of dimethyldisulfide detected in the early stages of decomposition. Dimethyldisulfide is commonly detected at high abundance in all decomposition odor studies (2).

Ignatzschineria ureiclastica

In *I. ureiclastica,* only two compounds were produced, seventeen were consumed, and the other sixteen were not significant (Table 2). The two compounds that were produced were phenol and 3-methyl-2-pentanone. However, along with phenol, increasing amounts of benzaldehyde were produced during each day of the study. Pascual et al. (2017) also observed an increase in phenol associated with bacterial family Xanthomonadaceae, which may have been represented by genus *Ignatzschineria*. Microbes are known to produce different metabolites, many of which are VOCs and products of various metabolic processes such as fermentation, amino acid metabolism, and sulfur reduction (42). All three of these compounds (phenol, 3-methyl-2-pentanone, benzaldehyde) may be products of such processes. In a study done on *Ignatzschineria* isolated from flesh flies, *I. ureiclastica* was found to be positive for the phenylalanine deaminase reaction (26). Several microbial metabolic pathways leading to the formation of benzaldehyde have been postulated, and it has been suggested that it is a product accumulated from the deamination of phenylalanine (43). Phenol, which was significant for both *Ignatzschineria* species, is an antiseptic chemical that can inhibit the growth of other microorganisms, meaning that it may be

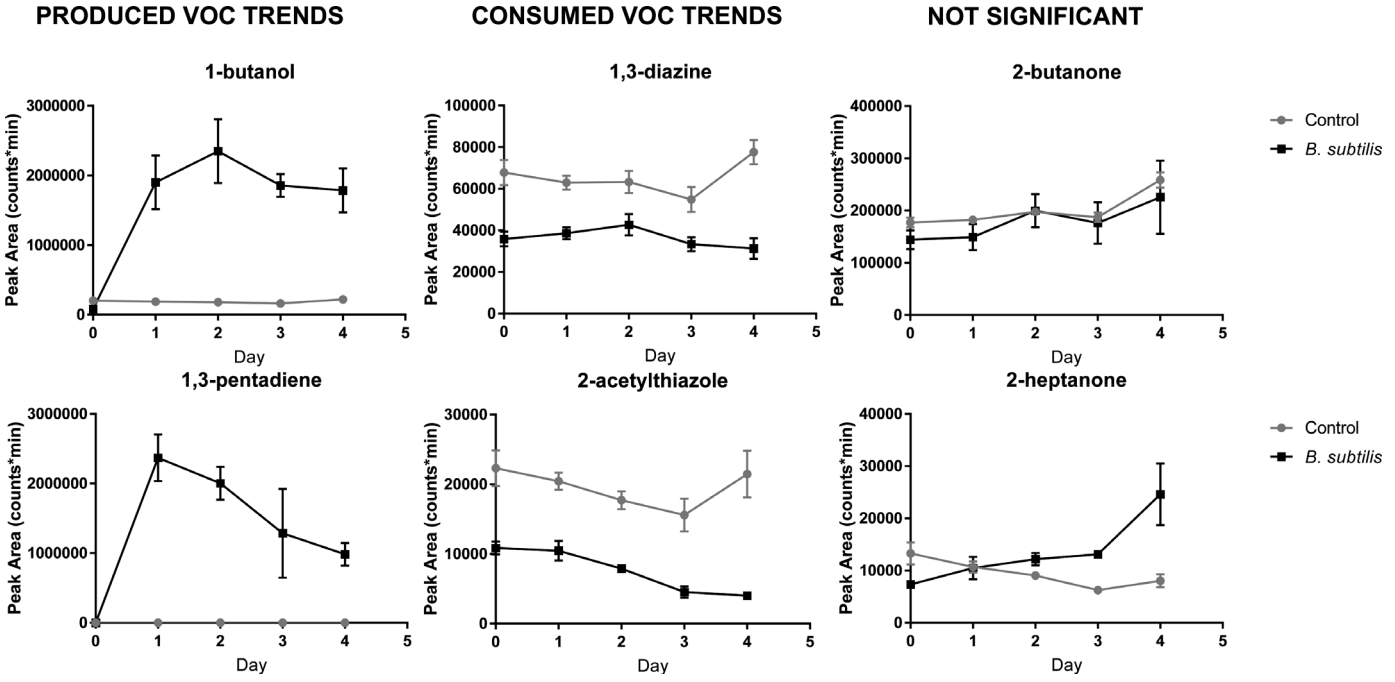

FIG. 1—*Example of the categories of volatile organic compounds (VOCs) detected from* Bacillus subtilis*. "Produced" compounds showed a higher abundance in the bacteria samples than control samples. "Consumed" compounds were higher in the control samples than in bacteria samples. Compounds that were not statistically significant between the two data sets were "not significant." Compound trends for all compounds of interest for* B. subtilis, Ignatzschineria indica*, and* I. ureiclastica *are shown in the Supplementary Material. Error bars represent standard deviation with* n = *3 biological replicates.*

released as a competitive strategy on decomposing remains (14). Benzaldehyde and its derivatives have also been shown to exhibit antimicrobial properties (44).

Phenol may also be related to bacterial motility and communication such as swarming and quorum sensing. Ma et al. (45) observed that phenol, a fly attractant, also triggered swarming motility in *Proteus mirabilis*. I*gnatzschineria* spp., members of the same class (Gammaproteobacteria) as *Proteus mirabilis*, may use phenol similarly because *Ignatzschineria* spp. are known to stimulate insect activity. Two unidentified species of *Ignatzschineria* were observed to stimulate the oviposition of *Hermetia illucens* in a laboratory setting (46). These are important observations to consider because they provide insight into the highly complex postmortem food web where bacteria attract insects to decomposing remains and then are transported by those insects to other decomposing materials. The current data

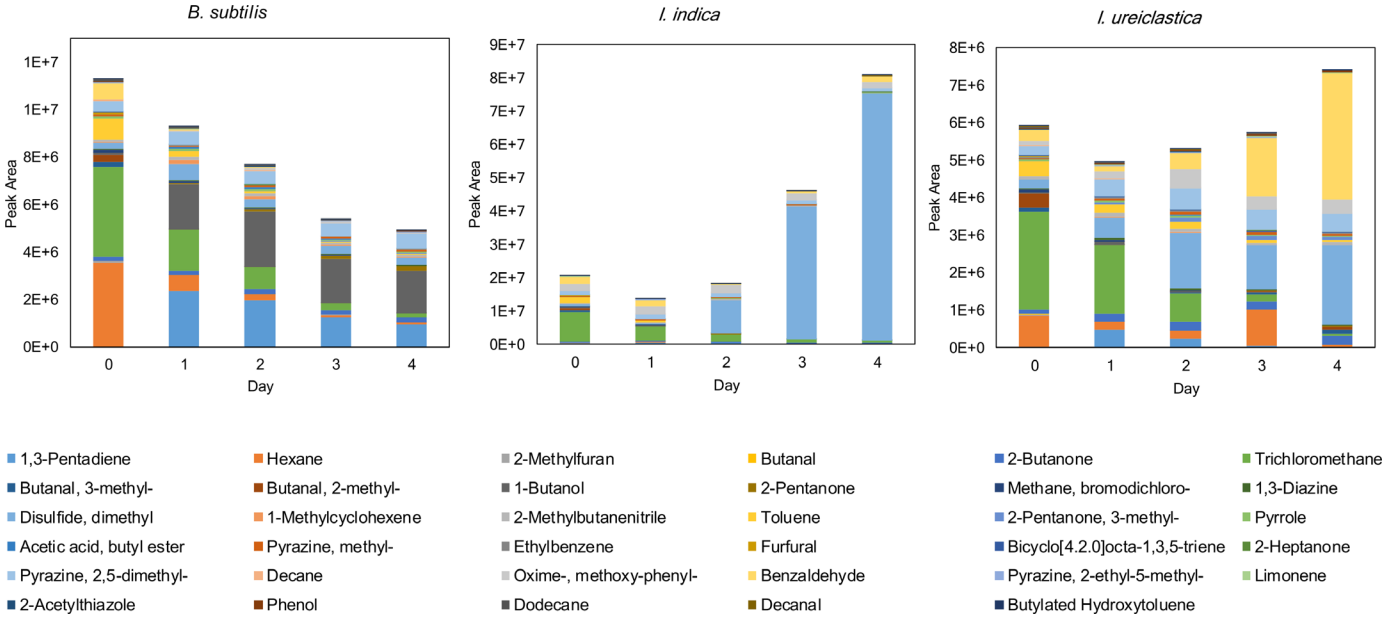

FIG. 2—*Overall volatile organic compound profile detected throughout the longitudinal study for each microbial species:* Bacillus subtilis, Ignatzschineria indica*, and* I. ureiclastica*. Each species exhibited a different pattern of total released VOCs and different major contributors to the profile over time. Bars represent the average production of each VOC from* n = *3 biological replicates.*

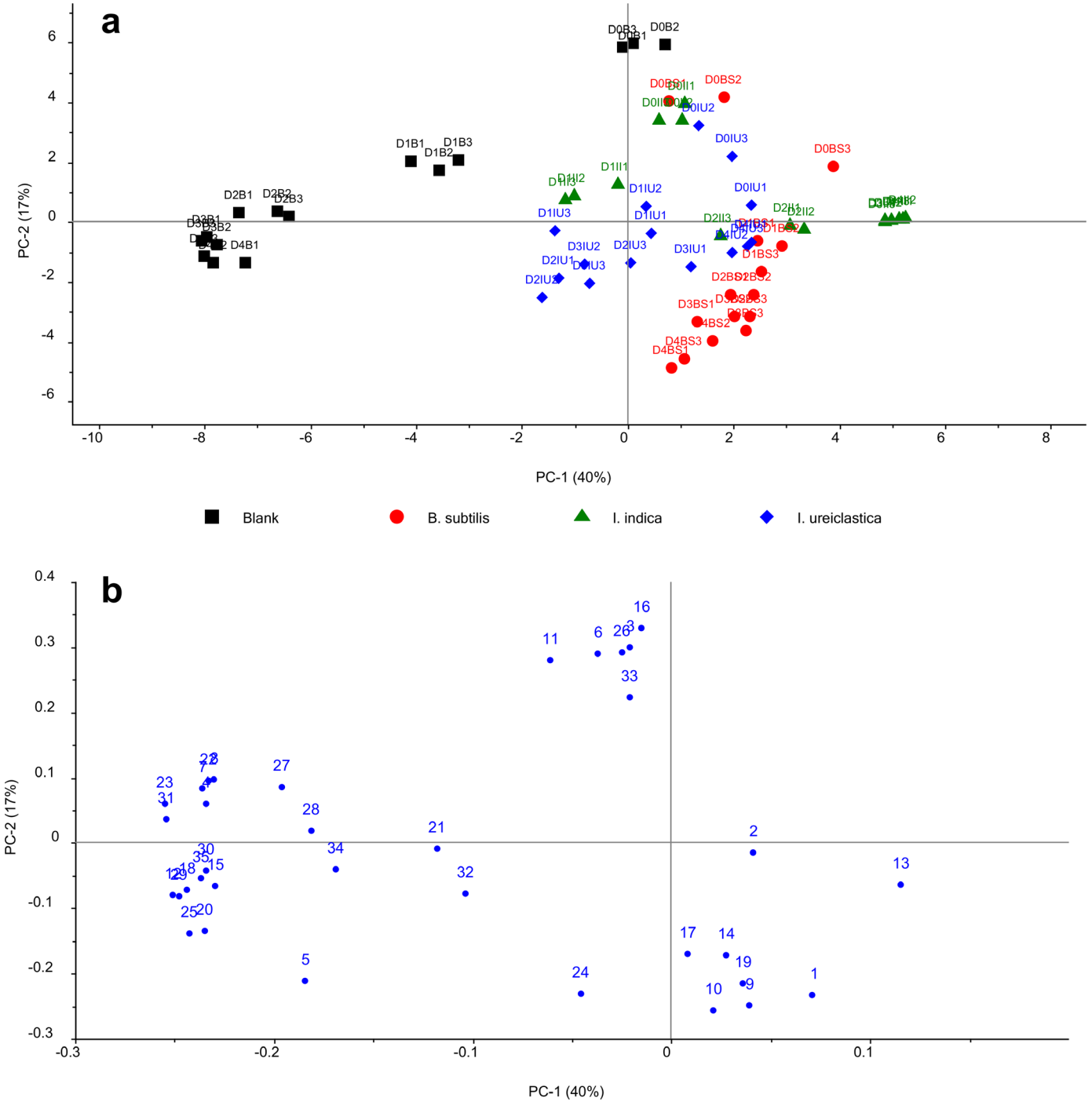

FIG. 3—*Principle component analysis (PCA) for peak area data from gas chromatography–mass spectrometry. (a) Scores plot including* Bacillus subtilis *(BS),* Ignatzschineria indica *(II),* I. ureiclastica *(IU) and blank agar vials (B). Labels represent the day of analysis (e.g., D1 = Day 1), sample type (i.e., B, BS, II, IU), and replicate number (i.e., sample 1, 2, or 3 sampled that day). (b) Correlation loadings plot with numbered labels referring to compounds in Table 1.*

are significant because they represent the first research done on the VOCs of the *Ignatzschineria* bacteria that could link the presence of different microbes found on the body to decomposition odor.

Profiling Approach to Compare Bacterial Species

The VOCs produced by each bacterial species comprised a very specific metabolic profile as seen in Fig. 2. They produce a characteristic odor based on the individual VOCs that are being produced or consumed, so the coloration of each set of bars on the three figures look unique. Therefore, it is also possible to view the dataset in a profiling approach, considering all components in the total profile in order to compare the different species to one another over time. Multivariate analysis (i.e., PCA) was conducted to decrease the dimensionality of the dataset and facilitate observation of the trending VOCs throughout the 5-day period (Fig. 3*a*). Each of the three bacteria species clustered

separately on the PCA plot (Fig. 3*a*). Although some overlap can be seen, the individual clustering indicates that each species has its own unique VOC profile, based on whether they produced or consumed specific compounds. It can be inferred from this that each species has different microbial metabolic pathways that may be impacted by different bacterial enzymes, their effect on available nutrients from decomposing organic matter, or other factors. While it was originally hypothesized that *B. subtilis* would yield very different results from the two *Ignatzschineria* species, it is interesting that the results obtained for both *Ignatzschineria* species were substantially different from each other as well, perhaps indicating that each of these species plays a unique role during decomposition.

During the first 2 days of monitoring, the VOC profiles of all three species moved in a linear direction on the PCA plot that diverged from the control samples. Samples were similar to controls on Day 0 (D0) and over time became increasingly different from controls toward the final day of monitoring on Day 4 (D4). Despite the change in volatile profile of the control samples, bacterial samples were seen to change in a different manner. This represents a buildup of volatile products and use of volatile compounds from the agar. The PCA loadings plot demonstrates that the separation between points on the PCA scores plot was due to a large number of compounds and not necessarily a few that affected variation in samples alone. However, the compounds located in the lower right quadrant of the PCA loadings plot (i.e., 1,3-pentadiene, hexane, 1-butanol, 2-pentanone, dimethyldisulfide, 1-methylcyclohexene, 3-methyl-2-pentanone, acetic acid butyl ester, 2-heptanone, ethylbenzene, and phenol) were all compounds that were found to exist in the produced category for at least one of the species. Since these loadings affect the direction of these points moving down and toward the bottom right of the plot over time, this highlights the ability to distinguish microbial trends based on key compounds they are producing. These compounds contribute to the ability to differentiate these microbes but also to track their temporal trends.

Using this approach, it was possible to summarize the compounds that fell into the first category of compounds (i.e., produced VOCs) that also contributed to temporal trends and differentiation between bacteria. These compounds may have significance in the future when coupling decomposition VOC monitoring with microbial community monitoring from decomposing remains. *Bacillus subtilis* produced significant amounts of the following compounds that also held a temporal trend for the species over time: 1,3-pentadiene, 1-butanol, 2-pentanone, 1-methylcyclohexene, 3-methyl-2-pentanone, and acetic acid butyl ester. The relevant compounds produced by *I. indica* that were indicative of temporal trends included 1,3-pentadiene, hexane, 2-pentanone, dimethyldisulfide, 2-heptanone, ethylbenzene, and phenol. The significant compounds produced by *I. indica* that appeared to be significant in its temporal profile included 3-methyl-2-pentanone and phenol. As these compounds are common decomposition VOCs, this will provide information about the source of these compounds in future decomposition odor studies.

Source of Nutrients

A major driver of microbial metabolism is the source of nutrients that are available to them. In this study, Standard Nutrient Agar was used as a source of nutrients. The specific nutritional content of Standard Nutrient Agar in comparison with the resources available from decomposing remains is not currently known. The ingredients are listed as peptic digest of lean meat, beef extract, sodium chloride, and agar. It is hypothesized that this is much lower in fatty acids compared to decomposing remains with adipose tissue being stored in abundance. Therefore, it must be stated that the results of bacterial metabolism could be different given these two environments. In addition, the use of a different media (e.g., tryptic soy agar, blood agar) may provide different results than those obtained herein. However, the investigation of microbial isolates apart from decomposing remains in a single growth medium nonetheless provides valuable information that can help to build an improved linkage between decomposition odor and postmortem microbes.

Conclusion

In this study, three postmortem bacterial species were cultured and monitored for their VOC profile over a five-day study. The results in this work indicated that each microbial species produced a different VOC profile that included compounds traditionally reported in decomposition odor studies. This is the first study that performs primary work linking VOCs from microbes specifically collected from decomposing remains. These data will assist in understanding the source of VOCs detected in decomposition odor and potential reasons for their cycling. More studies are needed to understand other postmortem microbes and their volatile emissions. Species–species interactions may also play a role in the dynamics of VOCs released from the postmortem microbial community. Finally, it is important to also consider that the source of nutrients may influence the results obtained and should be investigated in future work. Examining the trends of microbial VOCs will improve understanding of odor associated with decomposition and therefore lead to improved search and recovery efforts.

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Conflict of Interest

Restek Corporation donated chemical standards used in this study through the Restek Academic Support Program. Restek Corporation had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Temporal trends for compounds produced by *Bacillus subtilis* identified using gas chromatography – mass spectrometry.

Figure S2. Temporal trends for compounds consumed by *Bacillus subtilis* identified using gas chromatography – mass spectrometry.

Figure S3. Temporal trends for compounds that were not significant for *Bacillus subtilis* identified using gas chromatography – mass spectrometry.

Figure S4. Temporal trends for compounds produced by *Ignatzschineria indica* identified using gas chromatography – mass spectrometry.

Figure S5. Temporal trends for compounds consumed by *Ignatzschineria indica* identified using gas chromatography – mass spectrometry.

Figure S6. Temporal trends for compounds that were not significant for *Ignatzschineria indica* identified using gas chromatography – mass spectrometry.

Figure S7. Temporal trends for compounds produced by *Ignatzschineria ureiclastica* identified using gas chromatography – mass spectrometry.

Figure S8. Temporal trends for compounds consumed by *Ignatzschineria ureiclastica* identified using gas chromatography – mass spectrometry.

Figure S9. Temporal trends for compounds not significant for *Ignatzschineria ureiclastica* identified using gas chromatography – mass spectrometry.