*Journal of Sustainability Science and Management*
*Special Issue Number 5, 2018 : 39-46*
*ISSN: 1823-8556*
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# MICROBIAL PHOSPHOLIPID FATTY ACID DISTRIBUTION ASSOCIATED WITH PIG FATTY TISSUE BURIED IN OIL PALM PLANTATION SOIL

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**Abstract:** Estimation of postmortem interval (PMI) is one of the challenges faced in forensic investigations. Such difficulty is caused by the influence of environmental factors towards decomposition process. Thus, it is crucial to conduct this study to investigate the concentration of phospholipid fatty acids in associated carrion soils as one of the forensic markers. A simulated burial experiment was carried out in controlled laboratory. The fatty flesh of a commercial pig *(Sus scrofa)* was buried in an oil palm plantation soil, mimicking a burial in a shallow grave. The fatty flesh was allowed to decompose for 365 days of burial period. The associated soil was collected at each designated sampling point, representing each decomposition stage. The associated soil was analysed in an attempt to identify the concentration of phospholipids fatty acids in the soil. The saturated fatty acid (SAFA) was found to have the highest concentration, i.e. 312.94 mg/g soil dry weight. Furthermore, the associated soils demonstrated a significant different ($p<0.05$) in the concentration of phospholipids fatty acids (PLFAs) between decomposition days. The saturated PLFAs of palmitic acid ($C_{16:0}$) demonstrated the highest concentration, followed by stearic acid ($C_{18:0}$) acids. These PLFA components also exhibited a significant different ($p\leq0.05$) in concentration between decomposition days. The concentration of microbial PLFAs in burial environments may indicate the potential of microbial PLFAs to be developed as a useful tool to estimate post-mortem interval

KEYWORDS: Total lipid extracts, tropical climate, fatty acids, burial biomarkers, postmortem interval

## Introduction

Decomposition is a natural process that will undergo when organism dies. During this process, tissue of the dead organisms is degraded through a series of the chemical and biological degradation which initially may not be visible to naked eyes as it started at the cellular level (Goff, 2009). The decomposition process occurred rapidly, and mainly depending on the environmental factors included soil chemical contents, temperature and moisture (Forbes *et al.,* 2005a; Forbes *et al.,* 2005b). The rate of decomposition of cadaver that buried in humid, fine-textured soil found to be slower. This occurrence may due to the exchange rate of oxygen with carbon dioxide, $CO_2$, was insufficient to meet aerobic microbial demand (Carter, 2005).

It has been recognized the taxonomic group, ecological biomarker, food sources and forth of microbial, phytoplankton and bivalves can be identified throughout signature fatty acids (Piotrowska & Mrozik, 2003; Bachok *et al.,* 2006; Ngosong *et al.,* 2012). Besides, the phospholipids fatty acids (PLFAs) are also an indicator to structure the microbial community in soil. Either they are compared between the individual PLFA components, or the whole PLFA pattern through multivariate statistical technique (Frostegård *et al.,* 2011). The branched chain of fatty acids commonly found in the constituents of gram-positive bacteria (*Bacillus* spp., *Clostridium* spp., and *Actinomyces* spp.). Furthermore, higher molecular weight of fatty acids ($> C_{20}$) indicate the presence of eukaryote communities (Zelles, 1999; Kaur *et al.,* 2005).

The saturated fatty acids were dominant in organisms. In addition, palmitic and stearic acids were frequent components of cellular fatty acids in bacteria (Zelles, 1999). The palmitic acid was initiate as an isotopic marker

of methanotrophs (Crossman *et al.,* 2005). The polyunsaturated fatty acids including $C_{18:2}$ and $C_{18:3}$ were indication of the incorporation of fungi with microorganism in response to the introduction of decomposing fatty tissue into the soil environment (Zelles, 1997). Thus, the soil microbial PLFAs have the potential to be used to estimate post-mortem interval (PMI) of a buried cadaver, subsequently, to locate clandestine graves (Vass, 2001; Carter & Tibbett, 2003; Tibbett *et al,* 2004). A number of studies have proven successful in actual casework, however, most of them are in the early stages of development (Vass *et al.,* 2002).

In order to enhance our understanding in the relationship between cadaver decomposition and types of soil, a controlled laboratory simulated burial experiment, mimicking a burial in a shallow grave, was performed. This experiment shall provide indications on the potential of lipids recovered from the associated soil to be developed as a tool to determine the post-mortem interval (PMI). Furthermore, this experiment may also provide baseline data on the soil lipid distribution of cadaver buried under tropical climate, as previous studies were mainly conducted in temperate countries (Forbes *et al.,* 2002). The reason of using lipids instead of other compound classes such as proteins and carbohydrates was due to its longer period retains in soil compared to other compounds.

## Materials and Methods

### *Experimental Design*

A stimulated laboratory experiment was performed for 365 days of burial periods with a total of 16 sampling days started from September 2014. The soils for this burial study were collected from a palm oil plantation in Terengganu (102.877789° E; 5.127961° N). Approximately, 10 g of fatty flesh of commercial pig, Sus scrofa was buried in a 10 cm (H) x 2 cm (D) vial and allowed to decompose to a particular burial interval under aerobic condition (Figure 1). Each of the burial intervals was replicated three times. The ambient condition at 27 °C of the soils was closely monitored for every week with the observation of soil pH. The associated soils were then collected at the 16 designated sampling days, corresponding to the stages of decomposition process in loamy soils.

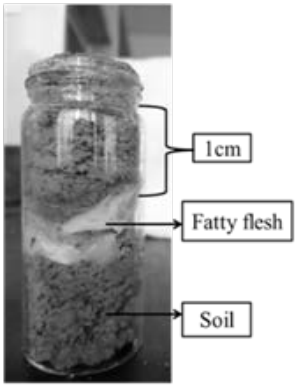



Figure 1: The burial of fatty flesh of commercial pig, (*Sus scrofa*) in oil palm plantation soil under aerobic condition.

### *Total Lipids Extracts (TLEs)*

Modified Bligh-Dyer extraction method was used to extract lipids from the associated soils. Approximately 2 g of the freeze-dried soil was placed into a culture tube. Three millilitre of dichloromethane DCM/methanol was added, spiked with 100 µl internal standard $C_{19}$ alkane, sonicated (15 min) and centrifuged (5 min, ~3000 rpm). The supernatant was transferred

into a clean vial. The process was repeated three times with 2 ml of DCM/ methanol (2:1/ v/v). Then, the soil was treated with 3 ml of modification Bligh Dyer solvent, sonicated for 15 min and centrifuged for 5 min (~ 3000 rpm). The supernatant was then transferred into the same vial. The extraction was repeated with 2 ml of Bligh Dyer solvent. The 2 ml of each buffered water and chloroform were added to the supernatant to break the organic phase. The mixture was then centrifuged for 1 min (~3000 rpm). The obtained organic layer was then transferred into another clean vial. The solvent was evaporated under a gentle flow of nitrogen. The obtained TLE was stored in a freezer (< 20 °C) prior to further analysis.

### *Microbial Phospholipids Fatty Acids (PLFAs) Extractions*

The obtained TLEs were dissolved in DCM/ acetone (9:1, v/v) and applied to a column comprised of a glass Pasteur pipette plugged with pre-extracted cotton wool and filled with solvent extracted silica gel for further separation. The total lipids extracted were further separated using clean Pasteur pipette as solid phase extraction (SPE) column into neutral and acidic fractions. The extracts were eluted with the solvent of 8 ml dichloromethane: Isopropanol (2:1 v/v) corresponding to the neutral fraction. The neutral fraction underwent separation using column chromatography technique. The solvent system of dichloromethane: methanol (1:1 v/v, 4 ml) was applied to yield alcohol fractions. Then, were dissolved in a 5 ml methanolic solution of potassium hydroxide and heated (120 °C, 1 h). Ten millilitre distilled water was added, acidified to pH 3 with 6.0 M hydrochloric acid (HCl) solution and extracted with aliquots of 10 ml chloroform for three times. The phospholipid extracted was methylated with 1 ml hydrochloric-methanol (10% v/v). The extracts were stored dry at 4 °C after removing solvent under nitrogen blown. Dried sample aliquots (<1 mg), were trimethylsilylated by adding 30 µl N,O-bis(trimethylsily)trifluoroacetamide (BSTFA) containing 1 % trimethylchlorosilane (TMCS) and heating (1 h, 60 °C). After cooling, excess BSTFA was evaporated under a gentle stream of nitrogen gas at 40 °C. The samples were re-dissolved in solvent of 50-100 µl depending upon nature of sample and prior to analysis. The concentrations of PLFAs were determined via comparison with an internal standard, n-nonadecane ($C_{19}$). Data were analysed using ANOVA (2-ways) with IBM SPSS statistical software for Windows, Version 20.0. Armonk, NY: IBM Corp.

### *Gas Chromatography Mass Spectrometry Analysis*

Samples were analysed using gas chromatography with mass spectrometry (GC-MS) with BPX 5 nonpolar capillary column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness). Helium was used as a carrier gas. The temperature program after injection was set at 50 °C, the oven temperature was raised to 250 °C at a rate 10 °C $min^{-1}$ and held for 5 min., then increased to 300 °C at the same rate and finally held constant for 10 min. Peaks were identified by comparing their retention times with those of authentic standards that bought from Supelco Inc. of United State (2016).

## Results and Discussions

A number of microbial PLFAs was recovered from the associated soils, which varied in their concentrations. The recovered microbial PLFAs were ranged between $C_{12:0}$ and $C_{24:0}$, together with their monounsaturated analogous and polyunsaturated components, i.e. $C_{18:3}$, $C_{18:2}$, $C_{20:3}$, $C_{20:4}$, and $C_{20:5}$. The concentrations of PLFAs showed a significant difference ($p$<0.05) between decomposition days. These fatty acids were divided into three groups i.e. saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The findings showed that the saturated fatty acids were the most abundant during decomposition days with the total 312.94 mg/g soil dry weight, followed by polyunsaturated fatty acids with total concentration of 74.62 mg/g soil dry weight and monounsaturated fatty acid (48.94 mg/g) (Table 1).

Table 1: Total concentration of saturated (ΣSAFA), monounsaturated (ΣMUFA) and polyunsaturated (ΣPUFA) fatty acids extracted from the associated soils.

| | Oil palm plantation soil (mg/g soil dry wt.) |
|---|---|
| ΣSAFA | 312.94 ±72.09 |
| ΣMUFA | 48.94 ± 38.2 |
| ΣPUFA | 74.62 ± 35.1 |

The microbial PLFA component of palmitic acid ($C_{16:0}$) exhibited the highest concentration, followed by stearic acid ($C_{18:0}$). The other components of microbial PLFAs were recovered with substantial concentrations. The higher and lower of these fatty acids would be useful to indicate the stages of decomposition as suggested by previous study (Notter & Stuart, 2012). It has been recognised that the human activity and soil characteristics may cause the changes in the microbial diversity, qualitatively and quantitatively (Faoro *et al.,* 2010). Therefore, the concentrations of palmitic and stearic acids shall be an anthropogenic indicator as their concentrations could be different daily over the decomposition process, depending on their soil environments.

In this study, a rapid declining in the PLFA concentrations was observable between day 0 and 7 of burial interval, corresponding to the stages of initial decay and putrefaction, respectively. After these days, a sharp inclining was observed between day 7 and 30 of decomposition interval, corresponding to the stages of black putrefaction and butyric fermentation. The PLFA concentrations reached maximum after 120 days of burial period. After this point, the PLFA concentration eventually decreased towards completion of the 365 days of burial experiment.

The saturated fatty acids, including palmitic and stearic acids, were commonly found in soil at a number of decomposition stages. The palmitic acids exhibited highest concentration followed by stearic acid. (Forbes *et al.,* 2002; Forbes *et al.,* 2003; Notter *et al.,* 2008). The long chain of saturated fatty acids were found in higher concentrations than that of unsaturated fatty acids. It has been recognised that the even numbered of saturated fatty acids recovered from the associated soils indicated the presence of gram-negative bacteria (Zelles, 1999).

Table 2: Phospholipids fatty acids compositions (mg/g soil dry wt.) of oil palm plantation soil for a burial period of 365 days. Values represents mean ±SD of three samples. *The values included the other significant fatty acids concentration not written in table.

| PALM OIL /DAY | 0 | 1 | 3 | 5 | 7 | 10 | 15 | 20 | 30 | 40 | 50 | 60 | 90 | 120 | 240 | 365 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SAFA | | | | | | | | | | | | | | | | |
| C14:0 | 3.36 ±0.23 | 0.00 ±0.00 | 0.00 ±0.00 | 0.21 ±0.37 | 0.42 ±0.16 | 0.31 ±0.56 | 0.21 ±0.73 | 0.10 ±0.32 | 0.00 ±0.00 | 0.01 ±0.41 | 0.03 ±0.33 | 0.04 ±0.58 | 0.29 ±0.76 | 0.6 ±0.21 | 0.6 ±0.51 | 0.60 ±0.58 |
| C16:0 | 7.71 ± 0.43 | 6.98 ±0.57 | 6.24 ±0.24 | 5.51 ±0.82 | 4.78 ±0.21 | 3.99 ±0.29 | 3.21 ±0.41 | 2.43 ±0.34 | 1.65 ±0.32 | 3.76 ±0.26 | 5.88 ±1.17 | 7.99 ±0.48 | 6.68 ±0.96 | 8.03 ±0.28 | 7.51 ±0.51 | 7.00 ±0.33 |
| C17:0 | 2.44 ±0.24 | 2.02 ±0.31 | 1.6 ±0.42 | 1.18 ±0.46 | 0.76 ±0.23 | 1.4 ±0.17 | 2.05 ±0.21 | 2.69 ±0.35 | 3.34 ±0.33 | 2.67 ±0.59 | 2.00 ±1.05 | 1.33 ±0.54 | 1.4 ±1.07 | 0.49 ±0.32 | 0.49 ±0.44 | 0.49 ±0.41 |
| C18:0 | 5.09 ±0.32 | 4.61 ±0.39 | 4.13 ±0.58 | 3.66 ±0.55 | 3.18 ±0.29 | 2.72 ±0.2 | 2.26 ±0.29 | 1.79 ±0.38 | 1.33 ±0.34 | 3.14 ±1.16 | 4.95 ±0.85 | 6.76 ±0.5 | 2.88 ±1.33 | 1.34 ±0.4 | 1.34 ±0.47 | 1.34 ±0.36 |
| C22:0 | 2.13 ±0.33 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 1.95 ±0.38 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.65 ±0.54 | 0.65 ±1.45 | 0.65 ±0.34 |
| C24:0 | 1.66 ±0.24 | 1.66 ±0.24 | 0.86 ±0.18 | 0.05 ±0.88 | 0.03 ±0.72 | 1.28 ±0.4 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.97 ±0.58 | 0.92 ±0.41 | 0.87 ±0.45 |
| ΣSAFA* | 42.45 ±3.32 | 28.59 ±3.04 | 23.68 ±3.96 | 19.83 ±5.63 | 22.07 ±4.18 | 15.16 ±1.70 | 14.49 ±4.46 | 12.23 ±3.32 | 15.00 ±3.47 | 14.25 ±5.45 | 17.2 ±4.91 | 20.3 ±4.21 | 17.91 ±9.20 | 19.49 ±3.90 | 16.96 ±7.07 | 13.33 ±4.27 |

Table 2 continued

| PALM OIL /DAY | 0 | 1 | 3 | 5 | 7 | 10 | 15 | 20 | 30 | 40 | 50 | 60 | 90 | 120 | 240 | 365 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MUFA | | | | | | | | | | | | | | | | |
| C16:1ω9 | 1.85 ±0.5 | 1.57 ±0.26 | 1.29 ±0.33 | 1.01 ±0.41 | 0.73 ±0.2 | 0.74 ±0.16 | 0.75 ±0.75 | 0.76 ±0.33 | 0.77 ±0.31 | 0.74 ±0.77 | 0.71 ±0.26 | 0.68 ±0.18 | 0.83 ±0.91 | 0.74 ±0.27 | 0.74 ±0.47 | 0.74 ±0.46 |
| C17:1ω10 | 2.46 ±0.19 | 1.95 ±0.29 | 1.44 ±0.39 | 0.92 ±0.85 | 0.41 ±0.22 | 0.43 ±0.63 | 0.46 ±0.3 | 0.48 ±0.99 | 0.51 ±0.32 | 0.5 ±0.87 | 0.48 ±0.68 | 0.47 ±0.65 | 0.21 ±1.02 | 0.39 ±0.3 | 0.39 ±0.26 | 0.39 ±0.95 |
| C18:1ω9 | 0.00 ±0.00 | 0.13 ±0.37 | 0.26 ±0.55 | 0.39 ±0.53 | 0.53 ±0.28 | 0.39 ±0.2 | 0.26 ±0.71 | 0.13 ±0.4 | 0.00 ±0.00 | 0.25 ±0.38 | 0.49 ±0.12 | 0.74 ±0.51 | 1.29 ±0.43 | 5.19 ±0.39 | 2.9 ±0.76 | 0.60 ±0.48 |
| ΣMUFA* | 6.37 ±1.55 | 3.65 ±1.33 | 2.99 ±1.27 | 2.42 ±3.26 | 2.74 ±1.40 | 1.88 ±1.48 | 1.81 ±2.39 | 1.72 ±2.76 | 1.64 ±0.93 | 1.78 ±3.52 | 1.88 ±1.72 | 2.02 ±3.24 | 2.71 ±3.87 | 7.46 ±1.92 | 5.11 ±2.87 | 2.76 ±4.69 |
| PUFA | | | | | | | | | | | | | | | | |
| C18:2ω6 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 1.33 ±0.25 | 1.27 ±0.18 | 1.21 ±0.29 | 1.14 ±0.36 | 1.08 ±0.34 | 1.56 ±0.27 | 2.04 ±0.26 | 2.52 ±0.71 | 0.32 ±1.17 | 2.17 ±0.35 | 1.48 ±0.49 | 0.80 ±0.29 |
| C20:3ω8 | 0.00 ±0.00 | 0.46 ±0.45 | 0.92 ±0.7 | 1.38 ±0.62 | 1.84 ±0.54 | 1.38 ±0.23 | 0.92 ±0.16 | 0.46 ±0.4 | 0.00 ±0.00 | 0.46 ±0.58 | 0.31 ±0.17 | 0.15 ±0.47 | 0.00 ±0.00 | 5.63 ±0.48 | 4.81 ±0.45 | 4.00 ±0.31 |
| ΣPUFA* | 1.81 ±0.55 | 0.76 ±1.30 | 1.51 ±2.01 | 2.27 ±1.80 | 7.87 ±1.93 | 4.94 ±1.30 | 3.93 ±2.02 | 3.34 ±2.69 | 2.74 ±1.65 | 3.73 ±2.82 | 3.67 ±1.10 | 3.59 ±3.04 | 5.64 ±3.99 | 12.03 ±3.50 | 9.60 ±3.64 | 7.19 ±1.76 |

Figure 2 shows the trend of palmitic and stearic acids within decomposition days in associated soil. In this study, the finding showed during day 0 until day 15 there was rapid decreasing of palmitic and stearic acids corresponding to the initial and black putrefaction stages, respectively. During day 15 or at black putrefaction stage was reached minimum concentration of both acids. The black putrefaction stage is known as active decay stage which undergoes rapid decomposition due to the higher concentration of fatty acids released during hydrolysis process (Takatori *et al.,* 1986; Zelles *et al.,* 1994; Dent *et al.,* 2004). Then the concentration of both acids increased until day 30 of decomposition corresponding to the black fermentation stage. The stearic acid continued decreasing in concentration while palmitic acid increased and reached maximum at day 120 and then decreased until completion. These fluctuations of fatty acids concentration during decomposition process could be supported by Notter and Stuart (2012), suggested that the late stage of adipocere formation also can be identified by high and low concentrations of palmitic and stearic acids. The indifferent trend of palmitic acid ($C_{16:0}$) toward the end of completion is probably due to the higher abundant of microbial community in these soil samples.

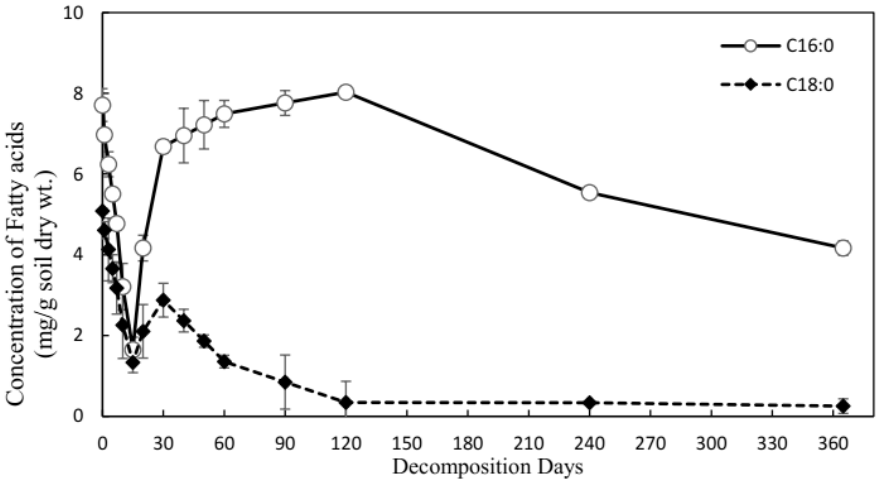



Figure 2: The mean concentration ±SD of palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids in oil palm plantation soil

The total microbial PLFAs found were contributed by those gram-negative and gram-positive bacteria, and fungi. These PLFAs of $i\mathrm{C}_{14:0}$, $i\mathrm{C}_{15:0}$, $a\mathrm{C}_{15:0}$, $i\mathrm{C}_{16:0}$, $i\mathrm{C}_{17:0}$, $a\mathrm{C}_{17:0}$ were indicators to represent gram -positive bacteria whilst, saturated fatty acid of $\mathrm{C}_{14:0}$ until $\mathrm{C}_{20:0}$, $\mathrm{C}_{16:1}$ and $\mathrm{C}_{18:1}$ for gram -negative bacteria, and higher molecular weight of fatty acids (> $\mathrm{C}_{20}$) and presence of $\mathrm{C}_{18:2\omega6}$ were classified as fungi indicators (Bååth *et al.,* 1980; Federle, 1986; Wilkinson, 1988; Tunlid *et al.,* 1989; Zelles, 1999). The total concentration of microbial PLFAs demonstrated the dominance of gram -negative bacteria at most of the decomposition stages, followed by fungi and gram -positive bacteria (Figure 3). These finding was supported by Zelles (1999), stated the human-associated bacteria *(Bacteroids)* was mostly from the phylum of gram-negative bacteria.

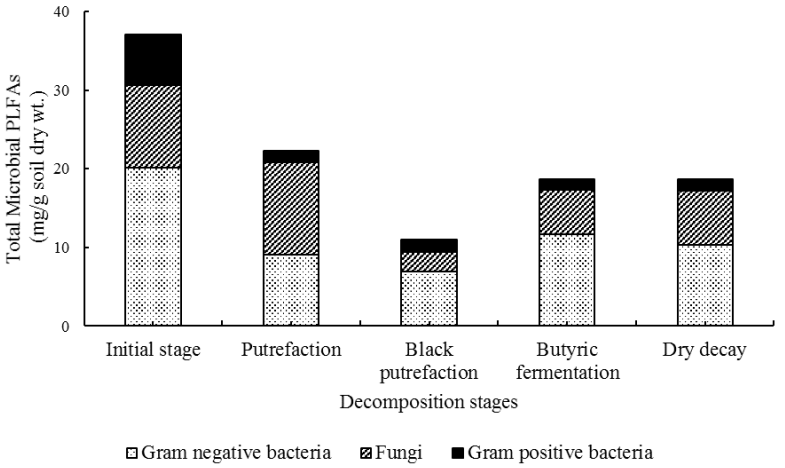



Figure 3: Total microbial PLFA distributions between different stages of decomposition in oil palm plantation soils of 365 days burial period.

## Conclusion

Microbial phospholipids fatty acids were successfully being analysed for associated soil of pig tissues buried in oil palm plantation soils. The general microbial communities (i.e., gram-positive and gram-negative bacteria, and fungi) that involved in the decomposition process were identified via microbial PLFA profiling. The temporal variation in the concentrations of microbial PLFAs recovered from the associated soils may indicate the potential of these PLFAs to be developed as a forensic tool, particularly to locate a clandestine grave. Furthermore, the variation of their concentrations at each decomposition stage may also provide an indicator for post-depositional interval (PDI) that describe the duration of the body being buried in a disposal site/grave. Subsequently, these microbial PLFA distribution may potentially be used to estimate the post-mortem interval (PMI) or time since death of the body. The most abundance microbial PLFA components that recovered from the soils were palmitic ($\mathrm{C}_{16:0}$) and followed by stearic ($\mathrm{C}_{18:0}$) acids, varied in their concentrations over time of decomposition. The other microbial PLFA components were also recovered with considerable concentrations. Furthermore, the dominance of gram-negative bacteria throughout most of the decomposition stages, followed by fungi and gram-positive bacteria may provide a better information on estimating the decomposition state of a cadaver buried under the soil environment of an oil palm plantation. Therefore, it is not impossible to develop microbial PLFAs as decomposition biomarkers for forensic investigations. However, more studies should be conducted to examine PLFAs concentrations at different soil

environments under the tropical climate. As the disposing of a cadaver into ground is extremely environmentally unfriendly, therefore, the response exhibited by the different groups of microorganisms upon the introduction of decomposing body into the soil environmental system can be used to establish a better process of cadaver disposal.

**Acknowledgments**

We would like to thank the Ministry of Higher Education Malaysia for the research grant of Fundamental Research Grant Scheme (Vot 59405). We would also like to acknowledge the Royal Malaysia Police for granting this study with the soil samples of a number of forensic cases and ideas. Thanks to all staff at the Central Laboratory and School of Marine and Environmental Sciences of Universiti Malaysia Terengganu for technical assistance throughout the research.

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