



**CHARACTERISATION, ANTIBACTERIAL ACTIVITY AND MODE OF
ACTION OF CLAY AGAINST FOOD-BORNE
Salmonella Typhimurium AND *Staphylococcus aureus***

By

NUR NAQIYAH BINTI AZMI

**Thesis Submitted to School of Graduate Studies, Universiti Putra
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Doctor of Philosophy

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August 2024

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Institute : Halal Products Research

In halal logistics, the discovery of clay with effective antibacterial activity is needed to combat bacterial contamination on surfaces, to ensure *halalan toyyiban* aspects of the products. Clays have unique properties such as fine particle size ($<2\mu\text{m}$), high cation exchange capacity, mineralogical and geochemical composition, generating antibacterial properties. The abundance of clay materials from local deposits have remained largely unstudied for their antibacterial potential, focusing on the removal of food-borne pathogens. This study was undertaken to discover the antibacterial activity of locally sourced clay soils against representative food-borne bacteria, which were gram-negative *Salmonella* Typhimurium ATCC 14028 and gram-positive *Staphylococcus aureus* ATCC 13565, followed by characterisation and the study of antibacterial mode of action. A total of 13 soils were sampled from

Selangor, and their physicochemical analysis such as moisture content, soil particle size, pH and cation-exchange capacity (CEC) were determined. Antibacterial screening was performed on 13 soils using disc diffusion, which revealed that Munchong and Carey soils significantly inhibit *S. Typhimurium* (11.17 ± 0.76 mm) and *S. aureus* (7.90 ± 0.17 mm). Further treatment (untreated and treated) with these 2 soils were carried out to evaluate their difference in pH, CEC, metal content and further antibacterial analysis. Treated preparation caused significant contrasting effect for Carey and Munchong in pH, and the increased in CEC values of both samples were in line with the increased clay fraction. Soluble metal analysis revealed significantly higher concentration of treated Carey in comparison to untreated for all metals tested, except Pb. For bacterial viability testing, treated Carey clay leachate and suspension were the most effective with complete killing of *S. Typhimurium*, while *S. aureus* viability is reduced (2 to 3 log) after 6 h of exposure. The minimum inhibitory concentration (MIC) and minimum bactericidal activity (MBC) were 125 mg/mL and 250 mg/mL, respectively, for leachate and suspension, for both of the tested bacteria. Time-kill experiments revealed bactericidal effects of treated Carey on *S. Typhimurium* within 1 h exposure to both leachate or suspension at 4x MIC. Meanwhile, *S. aureus* was completely killed after 8 h and 24 h of treatment with treated Carey suspension and leachate, respectively, at 4x MIC. XRD analysis confirmed the presence of pyrite and magnetite, while EDX analysis revealed the presence of Al, Fe and S, consistent with the presence of pyrite. Correlation analysis indicated that the high concentration of metals, especially Al, Cu, Fe, Ni and Zn could be responsible in contributing to antibacterial activity against *S. Typhimurium*,

while Ag was found to have strong positive correlation with log reduction of *S. aureus*. Results of transmission electron microscopy (TEM) showed abnormalities, including disrupted cell walls following exposure to Carey clay, while analysis of membrane integrity also highlighted leakage of proteins, cellular constituents and changes in membrane potential in treated cells with the generation of reactive oxygen species (ROS) that likely promotes bacterial toxicity. In conclusion, treated Carey clay showed effective antibacterial activity, thus supports the potential application of treated Carey clay as surface sanitising agent in halal logistics.

Keywords: Antibacterial clay; antibacterial mode of action; gram-positive bacteria; gram-negative bacteria; metal analysis

SDG: GOAL 9: Industry, Innovation and Infrastructure

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia
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**PENCIRIAN, AKTIVITI ANTIBAKTERIA DAN CARA TINDAKAN TANAH
LIAT TERHADAP *Salmonella* Typhimurium DAN *Staphylococcus aureus*
BAWAAN MAKANAN**

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Dalam halal logistik, penemuan tanah liat dengan aktiviti antibakteria yang berkesan diperlukan untuk memerangi pencemaran bakteria pada permukaan, bagi memastikan aspek halalan toyyiban produk. Tanah liat mempunyai sifat unik seperti saiz partikel halus ($<2\mu\text{m}$), keupayaan pertukaran kation yang tinggi, komposisi mineralogi dan geokimia, yang menghasilkan sifat antibakteria. Kekayaan bahan tanah liat dari simpanan tempatan masih banyak yang belum dikaji untuk potensi antibakteria mereka, dengan tumpuan kepada penghapusan patogen bawaan makanan. Kajian ini dijalankan untuk mengetahui aktiviti antibakteria tanah liat yang diperoleh secara tempatan terhadap wakil bakteria bawaan makanan, iaitu *Salmonella* Typhimurium ATCC 14028 yang merupakan gram-negatif dan *Staphylococcus*

aureus ATCC 13565 yang merupakan gram-positif, diikuti dengan pencirian dan kajian mod tindakan antibakteria. Sebanyak 13 sampel tanah telah diambil dari Selangor, dan analisis fizikokimia mereka seperti kandungan kelembapan, saiz partikel tanah, pH dan keupayaan pertukaran kation (CEC) telah ditentukan. Skrin antibakteria telah dilakukan ke atas 13 jenis tanah menggunakan kaedah difusi cakera, yang menunjukkan bahawa tanah Munchong dan Carey secara signifikan menghalang *S. Typhimurium* (11.17 ± 0.76 mm) dan *S. aureus* (7.90 ± 0.17 mm). Rawatan lanjut (tidak dirawat dan dirawat) dengan 2 jenis tanah ini telah dijalankan untuk menilai perbezaan pH, CEC, kandungan logam dan analisis antibakteria lanjut. Persediaan yang dirawat menyebabkan kesan berbeza yang ketara bagi Carey dan Munchong dalam pH, dan peningkatan nilai CEC bagi kedua-dua sampel adalah selaras dengan peningkatan pecahan tanah liat. Analisis logam larut menunjukkan kepekatan Carey yang dirawat adalah jauh lebih tinggi berbanding yang tidak dirawat untuk semua logam yang diuji, kecuali Pb. Untuk ujian kebolehlangsungan bakteria, larut lesap dan ampaian tanah liat Carey yang dirawat adalah yang paling berkesan dengan pembunuhan lengkap *S. Typhimurium*, manakala kebolehlangsungan *S. aureus* berkurangan (2 hingga 3 log) selepas 6 jam pendedahan. Konsentrasi perencatan minimum (MIC) dan aktiviti bakterisida minimum (MBC) masing-masing adalah 125 mg/mL dan 250 mg/mL, untuk larut lesap dan ampaian, bagi kedua-dua bakteria yang diuji. Eksperimen bunuh masa mendedahkan kesan bakterisida Carey yang dirawat terhadap *S. Typhimurium* dalam masa 1 jam pendedahan kepada kedua-dua lakuan atau ampaian pada 4x MIC. Sementara itu, *S. aureus* telah dibunuh sepenuhnya selepas 8 jam dan 24 jam rawatan dengan ampaian

Carey yang dirawat dan larut lesap, masing-masing, pada 4x MIC. Analisis XRD mengesahkan kehadiran pirit dan magnetit, manakala analisis EDX mendedahkan kehadiran Al, Fe dan S, yang konsisten dengan kehadiran pirit. Analisis korelasi menunjukkan bahawa kepekatan tinggi logam, terutamanya Al, Cu, Fe, Ni dan Zn mungkin bertanggungjawab dalam menyumbang kepada aktiviti antibakteria terhadap *S. Typhimurium*, manakala Ag didapati mempunyai korelasi positif yang kuat dengan pengurangan log *S. aureus*. Keputusan mikroskop elektron transmisi (TEM) menunjukkan keabnormalan, termasuk dinding sel yang terganggu selepas terdedah kepada tanah liat Carey, manakala analisis integriti membran juga menonjolkan kebocoran protein, komponen selular dan perubahan dalam potensi membran dalam sel-sel yang dirawat dengan penghasilan spesies oksigen reaktif (ROS) yang mungkin meningkatkan ketoksikan bakteria. Kesimpulannya, tanah liat Carey yang dirawat menunjukkan aktiviti antibakteria yang berkesan, oleh itu menyokong potensi penggunaan tanah liat Carey yang dirawat sebagai agen pembasmi kuman permukaan dalam logistik halal.

Kata kunci: Tanah liat antibakteria; mod tindakan antibakteria; bakteria gram-positif; bakteria gram-negatif; analisis logam

SDG: MATLAMAT 9: Industri, Inovasi dan Infrastruktur

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different concentrations



LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrometer
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BET	Brunauer, Emmett and Teller
CEC	Cation-exchange capacity
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DiBAC ₄ (3)	bis-(1,3-dibutylbarbituric acid) trimethine oxonol
DNA	Deoxyribonucleic acid
EDX	Energy dispersive X-ray spectroscopy
FIC	Fractional inhibitory concentration
FL	Factor loading
g	gram
h	hour
H ₂ O ₂	Hydrogen peroxide
HCl	Hydrochloric acid
HNO ₃	Nitric acid
ICP-OES	Inductively coupled-optical emission spectroscopy
JAKIM	<i>Jabatan Kemajuan Islam Malaysia</i>
MBC	Minimum bactericidal concentration
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
min	minute

mL	millilitre
mm	millimetre
MS	Malaysian Standard
NA	Nutrient agar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient broth
NBT	Nitroblue Tetrazolium
nm	nanometer
OD	Optical density
PBS	Phosphate buffered saline
PC	Principle component
PCA	Principle component analysis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SEM	Scanning electron microscopy
spp.	Species
TEM	Transmission electron microscopy
UPM	Universiti Putra Malaysia
USDA	United State Department of Agriculture
XRD	X-ray diffraction
°C	Degree Celsius
%	Percentage
µm	micrometer
x <i>g</i>	Unit gravity

CHAPTER 1

INTRODUCTION

1.1 Background

In the food industry, bacteria are the main culprit of food contamination, with *Salmonella* and *Staphylococcus aureus* reported as the major contributors of food-borne illnesses globally (Balaban & Rasooly, 2000; Besser, 2018). Infections by the gram-negative *Salmonella* can cause a variety of health problem, such as diarrheal disease and typhoid fever (Popa & Popa, 2021). *Staphylococcus aureus*, a gram-positive bacterium, release enterotoxins that can lead to food poisoning when food contaminated with bacteria is consumed (Balaban & Rasooly, 2000). In food production, contamination with *Salmonella* Typhimurium and *S. aureus* can arise during food handling and processing, as well as transfer between other foods, surfaces and utensils in the kitchen (Kadariya et al., 2014; Moore et al., 2007). Contamination of bacteria can also occur during storage and transportation (Karanth et al., 2023; Sperber, 2009; Zeng et al., 2021). The dissemination and potential cross-contamination of these pathogens can be restricted through effective cleaning strategies to ensure purity of food products from farm to fork.

Halal food products require compliance with halalan toyyiban standards. The toyyiban aspect refers to clean, hygiene, safety, and wholesomeness of the halal product. This concept is essential in halal supply chain management, where the integrity of halal food products have to be protected along the supply

chain from any cross-contamination that can turn the product into becoming non-halal. Any contact with *najs al-mughallazah* (severe impurities) must be washed and ritually cleansed (*sertu*) as required by Shariah law prior. *Sertu* or Islamic cleansing procedure requires washing the affected areas seven times with clean water, one of which is a portion of clay with water (Department of Standards Malaysia, 2014). Malaysia Standard of MS 2400: 2019 Part 1 (Transportation), Part 2 (Warehousing), and Part 3 (Retailing) were established to provide guidelines to the manufacturers in maintaining the halal integrity in halal logistics.

The specific mention of clay as the cleansing agent in *sertu* procedure indicates the importance of this material in the purification of filth-contaminated surface. In the traditional practise of *sertu*, any type of soil can be used for this cleaning procedure as long as the soil is free from *najs* and impurities. The unique properties of clay includes the fine particle size and high adsorption capacity, which had significant contribution in removing impurities on surfaces in comparison to regular soil which has coarser texture (Bhattacharyya & Gupta, 2008). The fine particle size of clay creates a smooth slurry when mixed in water, which ease its application on surfaces during the *sertu* process (Hashim et al., 2013). In contrast, regular soil often contains varying particle sizes (sand, silt, organic material), which could create an inconsistent slurry texture and cause damage and scratches on the surface. In modern technology, clay-based products have been developed as the cleansing agent for *sertu* (Kassim et al., 2014). More recently, the *sertu* cleansing procedure with soil has been suggested as an approach to nullify the germs and illnesses

that may be caused by the contaminants present in *najs mughallazah*. (Ahmad & Shariff, 2016). As bacterial contamination may occur in any stage along the food supply chain, the risk of exposure to pathogens in logistics may also occur, of which the integrity of halal food products could be compromised.

Emerging science has showed that clays possess antibacterial effectiveness against a broad spectrum of human pathogens that include antibiotic resistant properties bacteria. Clays are the fine-grained fraction of natural rock or soil material that is a combination of one or more clay minerals and contains traces of metal oxides and organic matter which have been used as natural remedies since prehistory. Aside from its healing and soothing properties, clay has been investigated for its antibacterial activities, exemplified by the successful application of French green clay in the treatment of Buruli ulcer, a necrotizing cutaneous infection caused by *Mycobacterium ulcerans* (Williams et al., 2004) and the antibacterial efficacy of clay leachates against *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Otto et al., 2014). The ability of of clay in the form of leachate and suspension in causing substantial reductions in biofilm population against both Gram-negative and Gram-positive bacteria was also recently reported (Cafilisch et al., 2018). Many of the studies on antibacterial clays have focused on medical applications such as wound care and elimination of human pathogens and antibiotic-resistance bacteria (Haydel et al., 2008; Williams, 2017). This suggests the ability of certain clay minerals in causing significant effect on reducing microbial populations. These studies have stimulated interest in the identification of clays from other localities that possess antibacterial activity and could be utilised as new

antibacterial agents to enhance the effectiveness of *sertu* procedure. This concept is illustrated in Figure 1.1.

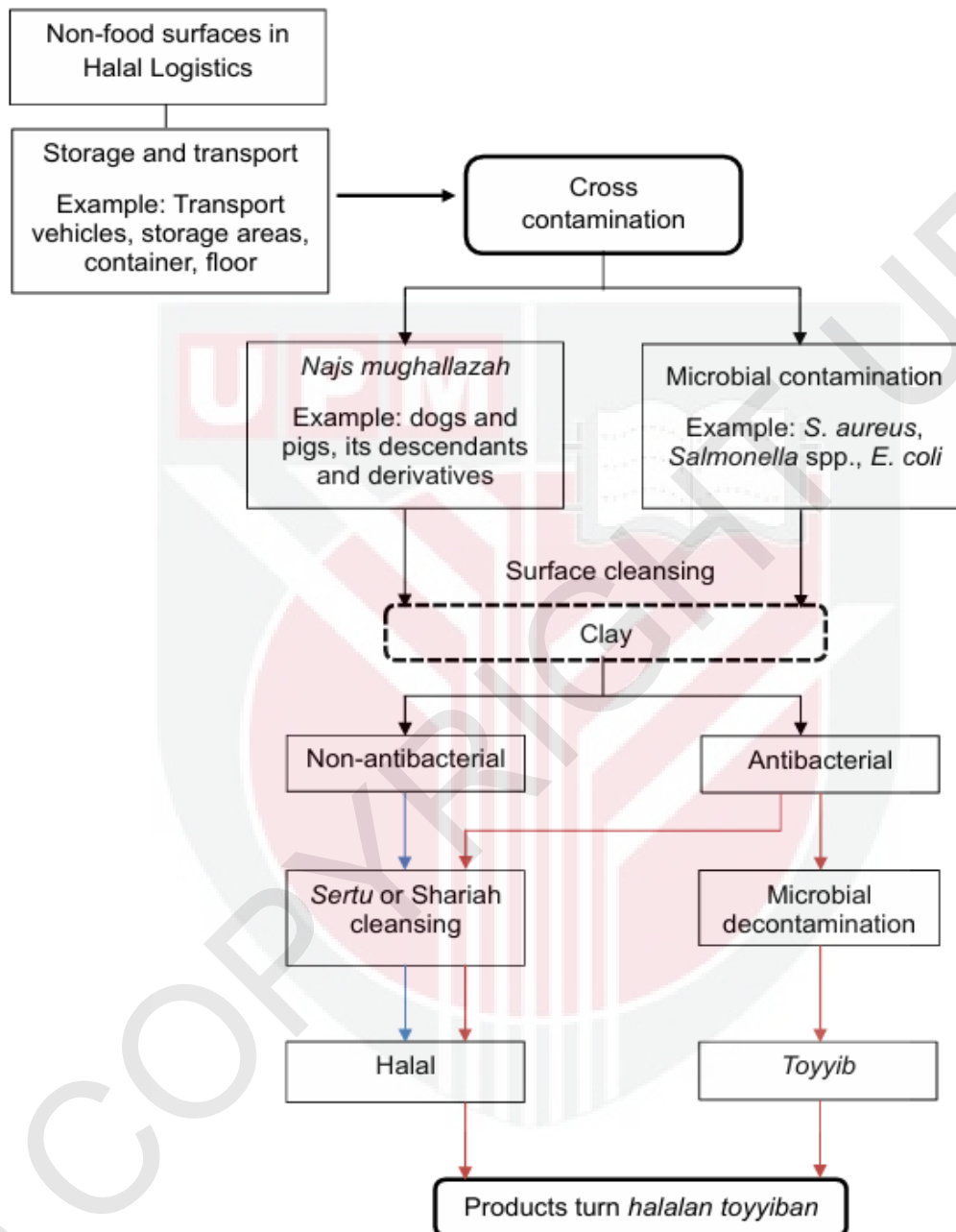


Figure 1.1: Importance of using antibacterial clay in halal logistics.

The blue arrow indicates the flow of surface cleansing that can turn contaminated product into halal when using non-antibacterial clay; the red arrow demonstrates the use of clay with antibacterial activity that can ensure removal of microbes on surfaces, which can turn products into becoming halalan toyyiban. Notes: The specific mention of “*turab*” in *sertu* which refers to soil (or clay) was stated in a Sahih Muslim hadith narrated by Abu Hurairah RA (Ahmad Ayoup@Ayub, 2017; Jabatan Kemajuan Islam Malaysia, 2013).

1.2 Problem Statement

In halal logistics, risks of cross-contamination may occur due to mishandling of the food products in between the various stages from storage and logistics activities have been widely reported (Supian, 2018). *Sertu* or Islamic cleansing procedure in halal logistics requires clay and water as the cleansing agent for purification of contaminated non-food surfaces such as transport, trucks, storage areas and floor from *mughallazah* filths. It has been discovered that clays possess antibacterial properties with significant application in medicine, which were shown effective against broad-spectrum of human pathogens (Williams, 2017). In halal logistics, it is important to assure integrity of food by effective removal of hazards such as bacteria. Clay-based products with antibacterial properties have remained largely unstudied for their application in halal logistic, focusing on the removal of food-borne pathogens. There is a gap in knowledge in antibacterial effectiveness of clays in elimination of pathogenic bacteria. The discovery of antibacterial clay would ensure *toyyib* or hygiene aspect of *sertu* procedure, hence would contribute to both *halalan toyyiban* aspect of food products. Therefore, there is a need to explore how *sertu* principle can direct scientific studies with regards to microbial decontamination and how it would relate to food integrity in halal concept.

The abundance of clay minerals from local deposits have remained largely unstudied for their applications and antibacterial benefits. Although it has been established that clay minerals have antibacterial functionality, the selection of clay to be used is of paramount importance as only certain types exhibit antibacterial activity. Therefore, assessment and characterisation of clay with

antibacterial properties needed to be established to further guide the understanding on the discovery of locally-sourced antibacterial clay deposits. Moreover, the antibacterial activity of clays is reported as somewhat variable, since no natural clay minerals are exactly the same due to differences in mineralogical and geochemical composition.

In order to validate its potential ability to be used as antibacterial agent particularly for halal logistics application, dose efficacy of clay minerals against foodborne pathogens needs to be defined to ensure total elimination of the bacteria. There is a gap in knowledge in antibacterial mode of action of Malaysian clays against foodborne pathogens, in the context of ability of the hydrated clay to generate a low pH environment, through mineral dissolution and sustaining metal release, and oxidative stress production. Thus, this work was carried out to study the antibacterial potential of clays from local deposits and characterise geochemical and mineralogical properties associated as well as defining the possible antibacterial mode of action. The significance of this study is the knowledge contribution of the type of clays with effective antibacterial activity and their important properties. The outcome from this study will provide a theoretical framework for the development of antibacterial products from natural clays for their potential application as cleaning and sanitising agents to be used in non-food surfaces in Halal logistics.

1.3 Objectives

A study was undertaken to discover the antibacterial activity of the locally sourced clay soils against representative food-borne bacteria, followed by characterisation and the study of antibacterial mode of actions. The specific objectives are as follows:

1. To determine physicochemical properties of 13 soil samples collected from Selangor and to screen their antibacterial activity.
2. To compare the effect of different preparation methods (untreated leachate, treated leachate, untreated suspension, and treated suspension) of Carey and Munchong samples on antibacterial effectiveness against gram-negative (*Salmonella Typhimurium*) and gram-positive bacteria (*Staphylococcus aureus*).
3. To characterise the mineralogical, elemental, morphological and surface properties of treated Carey clay and factors influencing the antibacterial activity.
4. To elucidate the antibacterial mode of action of treated Carey clay by morphological changes, cell membrane integrity, changes in membrane potential and production of reactive oxygen species (ROS).

General research flow on objectives 1 to 4 is summarized in Figure 1.2.

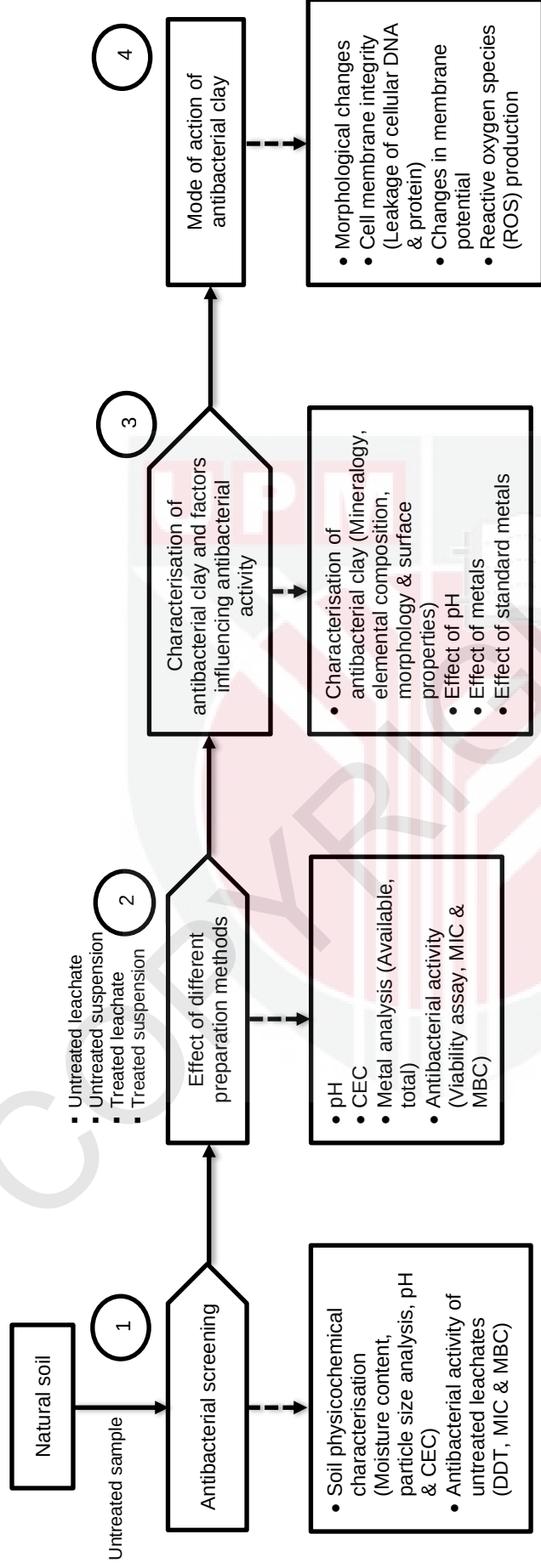


Figure 1.2: Research flow diagram representing objectives 1 to 4.

CHAPTER 2

LITERATURE REVIEW

2.1 Clay and clay minerals

Clays are fine-grained fraction of natural rock or soil material that is a combination of different minerals and other minerals such as quartz, carbonates, metal oxides and organic matter. It is one of the major components of soil, apart from sand and silt, which differ from each other based on their grain sizes. The unique properties of clay are due to its fine particle size ($< 2 \mu\text{m}$), which attributed to having high surface area therefore an excellent adsorbent material (Bhattacharyya & Gupta, 2008). Clays are composed of different minerals, which are formed by weathering of rocks and soils, forming a more stable and finer mineral particles. Structurally, clay minerals are phyllosilicates containing metal ions. The presence of extra charge, created by isomorphous substitution provide clay minerals cation exchange ability. Cation-exchange capacity is another important feature of the clay minerals, which is due to the net negative on the surface of clay hence allows exchange of cations to the surrounding environment.

There are several types of clay minerals, and the characteristics depends on their chemical make-up, layered structure and size. The different classification of clay minerals is shown in Figure 2.1.

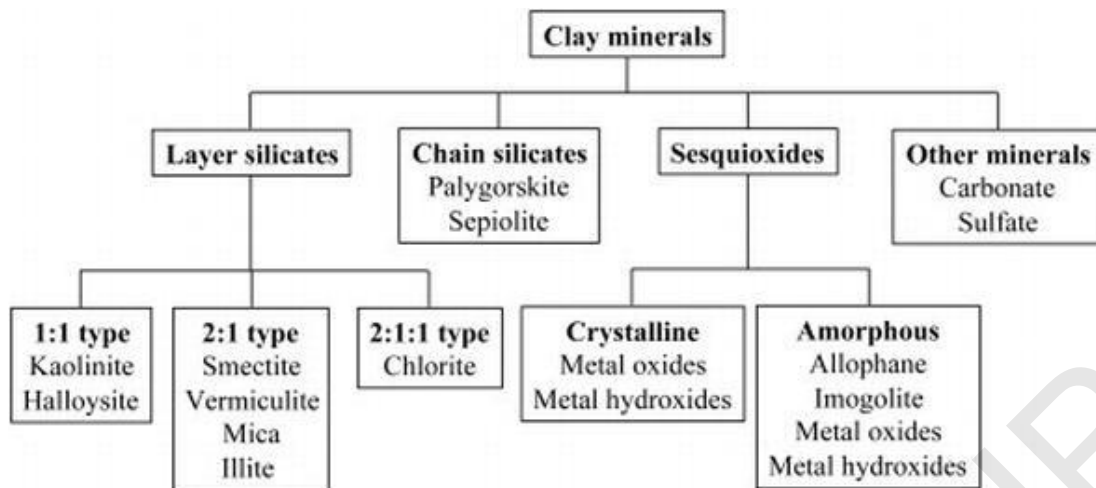


Figure 2.1: Different types of clay and their classification
(Source: Kumari & Mohan, 2021).

In term of structure, the arrangement of silicates in tetrahedral and octahedral sheets is crucial in determining the type of clay mineral. Kaolinite is an example of 1:1 clay mineral, of which the layers consist of one tetrahedral sheet and one octahedral sheet strongly held together by hydrogen bonding (Figure 2.2). On the other hand, smectite is a group of 2:1 clay mineral, which has layers of an octahedral sheet in between 2 tetrahedral sheets, weakly held together by van der Waals forces.

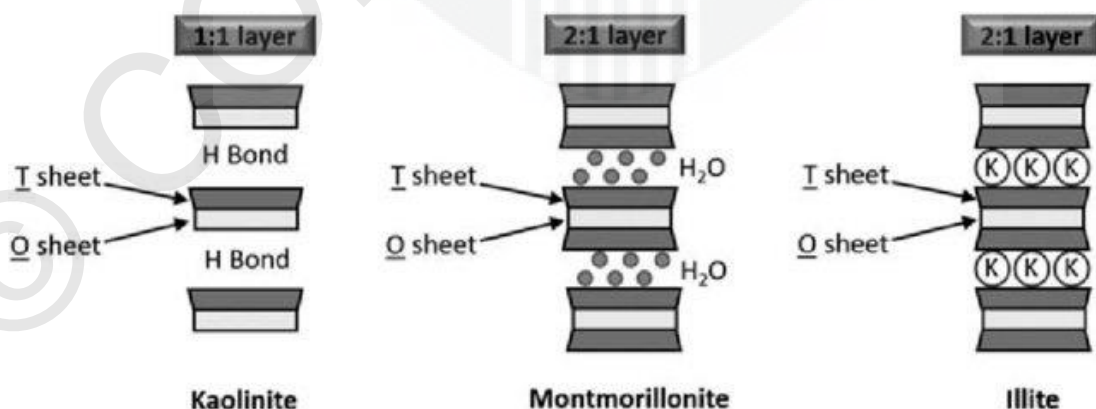


Figure 2.2: Structures of kaolinite (1:1 layer), montmorillonite and illite (2:1 layer)
(Source: Zhang, 2016).

2.2 Importance of clay

2.2.1 Traditional use

Clay has been employed as a natural remedy since prehistory. When moistened with water, the mixture forms mud-like consistency. Applications of clay include in geophagy (Nunn, 2002), pelotherapy (Carretero et al., 2013) and wound care (Williams & Hillier, 2014). Geophagy, which is the direct consumption of earth materials containing clay minerals for soothing effect on gastrointestinal lining and detoxifying effect for treatment of diarrhoea, as well as for supplement of essential micronutrients (Ngozi, 2008; Njiru et al., 2011). Alternatively, clays have also been used in topical application such as geotherapy, healing of wound and skin inflammation due to its excellent adsorption and absorption properties (Carretero, 2002). Clay minerals such as kaolinite and smectite offer various medical benefits such as treatment for diarrhoea and gastrointestinal ailments due to their ability to adsorb lipids, proteins, bacteria and viruses, (Williams & Haydel, 2010).

Clay has also been used for *tayammum*, which is an Islamic practise of dry ablution in which a person conducts ritual cleaning or ablution (*wudhu*) with clean soil or dust, usually sand or clay when water is unavailable (Afifi, 2022; Sunardi et al., 2017). *Tayammum* powder products can widely be found on the market, designed to be in line with Islamic guidelines for cleanliness and purification which offer convenience where access to clean soil or dust may not be practical. Although not specified in Islamic law, the *tayammum* powder is often made from purified clay. Mineralogical analysis on 12 local commercial

tayammum clay products found 4 types of clay minerals which were predominant; palygorskite, kaolinite, phengite, and zeolite, with the presence of amorphous content with low porosity which could contribute to effectiveness in cleansing properties during *tayammum* (Rosli et al., 2024).

2.2.2 The use of clay in *sertu* or Islamic cleansing

Hygiene, sanitation and food safety are necessary in the preparation of halal food (Che Man & Sazili, 2010). In preparation, every unit operation of processing, storage and transportation, halal foods should be physically separated from non-halal foods. Halal can become non-halal (haram) when there is contamination with *mughallazah* filth. Devices, utensils, and machines used previously or in contact with *najs al-mughallazah* (Table 2.1) must be washed and ritually cleansed as required by Shariah law prior halal processing. *Sertu* or Islamic cleansing procedure requires washing the affected areas seven times with clean water, one of which is a portion of clay with water (Department of Standards Malaysia 2019). The requirement for *sertu* is based on the hadith recorded by Imam Muslim in his Sahih, meaning: "From Abu Hurayrah RA, that the Prophet Muhammad SAW said: "When the former one of you is licked by a dog, so to purify it should be washed seven times, the first wash with soil" (Yusof & Subri, 2022). Based on the guidelines by JAKIM, clay or any type of soils with no contaminating impurities can be used in the *sertu* procedure to cleanse *mughallazah* filths, without any restriction on the concentration of clay used (Jabatan Kemajuan Islam Malaysia, 2013).

Table 2.1: Types of najs

Types of najs	Explanation
<i>Mughallazah</i> (severe najs)	Dogs and pigs, including any liquid and objects discharged from their orifices, descendants, and derivatives.
<i>Mukhaffafah</i> (light najs)	The only najs in this category is urine from a baby boy 2 years of age or below who has consumed no food except his mother's milk.
<i>Mutawassitah</i> (medium najs)	Not falling under severe or light najs: for example, vomit, pus, blood, alcoholic drinks (khamr), carrion, liquid and objects discharged from the orifices.

(Source: Department of Standards Malaysia, 2019a)

2.2.2.1 Halal logistics and the importance of *sertu*

Halal industry is a lucrative market with huge opportunities due to proliferating demand both locally and globally, hence the industry must understand and comply with the religious and quality requirement of halal. Halal logistics play an important role in protecting the quality and conditions of products, where halal integrity is maintained by physical separation of halal food products from the non-halal. Logistics include activities such as transportation, warehousing and material handling. Halal logistics operations involve various elements such as transport, trucks and containers, warehouse, surrounding areas, as well as infrastructure such as floors and loading bays (Yaacob et al., 2023). During the process of halal products delivery, risks of cross-contamination may occur due to mishandling of the food products in between the various stages from storage and logistics activities (Mohamed, Shamsudin & Rezai (2013). Other

issues that could cause cross-contamination of halal products include contaminated transport and containers, non-segregation between halal and non-halal products, mixed use of tools, lack of specialised assets and facilities in handling halal products, and the workers attitude in complying to procedures as stated in halal logistics guidelines (Omar & Jaafar, 2011; Riaz & Chaudry, 2004; Talib et al., 2010; Zulfakar et al., 2014). In particular, contamination with *mughallazah* impurities and non-segregation between halal and non-halal food products during transportation involving trucks (Sinar Harian, 2023; Sinar Harian, 2020) and cargo containers (Berita Harian, 2017) were also reported in Malaysia. These reports indicate the high tendency of cross-contamination in storage and transports hence the integrity of halal products could be compromised.

In the case of cross-contamination, a halal product can turn non-halal when physical contact occurs during transportation, or contaminated container. In accordance with standards for halal logistic, *sertu* procedure must be performed to purify areas or surfaces previously contaminated with impurity originated from swine and dogs using soil or clay and water as cleansing agent. The Malaysian Standards have established the procedure for *sertu*, as described in MS1500:2019 - General requirement for halal food, and MS2400:2019 Halal Supply Chain Management System (Part 1 Transportation, Part 2 Warehousing and Part 3 Retailing) (Department of Standards Malaysia, 2019a, 2019b, 2019c, 2019d)

In Halal logistics, the primary goal of *sertu* is to maintain the integrity of Halal products, ensuring they remain uncontaminated from impurities (specifically *najis mughallazah*) that would render them non-Halal. According to guidelines by Jabatan Kemajuan Islam Malaysia (2013), steps involved in the *sertu* process include initial cleaning with water or other cleaning agents to ensure no physical residue remains, mixture of clay in water with a ratio of approximately 1 part clay to 6 parts water, followed by final washing with clean water to ensure that any remaining impurity is completely removed, along with the clay residue. In industrial settings, modern equipment like high-pressure water jets may be used to ensure thorough cleaning. The cleaning is then followed by cleaning of the surfaces using detergent followed by sanitisation using sanitising agents. The personnel handling the *sertu* process should be trained in proper procedures to ensure compliance with Halal standards. The table below (Table 2.2) summarises other key requirements of clay to be used in *sertu* to be used in Halal food industry in general.

Table 2.2: Summary of key requirements for clay in *sertu* in Halal food industry

Requirement	Details	References
Types of clay	Any soil (Natural earth, kaolin clay, bentonite clay, loam soil)	Jabatan Kemajuan Islam Malaysia (2013)
Particle size	50-200 microns (fine clay that mixes well with water). Small particle sizes of clay can fascilitate absorption of impurities through their large surface area and avoid clogging problem during cleaning process	Kassim et al. (2014)
Concentration (in water)	- No specific concentration 0.6% - 2.5%	Department of Standards Malaysia (2019)
pH	Neutral to slightly alkaline (6.5 to 8.5) to avoid rust problem in the machinery or equipment.	Jabatan Kemajuan Islam Malaysia (2013)
Cleanliness/ purity	Slightly acidic to neutral range (pH 4.85 – 6.71) were suggested to be in the safe pH range Must be free from any impurities (<i>najis</i>), harmful chemicals, or pollutants	Kassim et al. (2014)
Moisture content	In dried condition when stored, to avoid contamination or spoilage	Department of Standards Malaysia (2019); Jabatan Kemajuan Islam Malaysia (2013)
Sourcing	Sourced from clean, uncontaminated areas (pollution-free, safe for Halal use)	Hashim et al. (2013)
Documentation	Must adhere to Halal certification standards and may require documentation for auditing purposes	Jabatan Kemajuan Islam Malaysia (2013)

2.2.2.2 Halalan Toyyiban aspect of *sertu*

Apart from halal, the Islamic religion also places strong emphasis on cleanliness (toyyiban). In the context of Halal food, halalan toyyiban refers to clean, hygiene, safety and wholesomeness of the Halal products (Omar & Jaafar, 2011). The toyyiban aspect emphasize on the safety of food products, therefore it is important to assure integrity of food by effective removal of hazards such as bacteria along the food supply chain. Ahmad and Shariff (2016) suggested that *sertu* cleansing or purification procedure with soil as an approach to nullify the germs and illnesses that may be caused by the contaminants present in najas mughallazah. Numerous pathogens that can be found in pork including *Salmonella* spp., *E. coli*, *Campylobacter* spp., *P. aeruginosa* and *S. aureus* which could contribute to foodborne diseases (Baer et al., 2013; Bantawa et al., 2018), while bacteria isolated from dogs' saliva include *Salmonella*, *Yersinia enterocolitica*, *Campylobacter*, *Staphylococcus* spp. and methicillin- resistance *Staphylococcus aureus* (MRSA) (Ghasemzadeh & Namazi, 2015; Tóth et al., 2022).

Since the purpose of *sertu* is to cleanse and removes filth, the principle of this ritual can be further explored to direct scientific studies with regard to microbial decontamination. Condensate generated in refrigeration units can contain bacteria and be disseminated via ventilation systems throughout food-processing plants, making it an unintentional source of contamination during storage (Karanth et al., 2023). A study by Rajkowski et al. (1998) on pig-carrying trailers found contamination of *Salmonella* and *E. coli* on the trailer floors and bedding materials, and the results of washing and sanitizing

demonstrated a significant decreased of the levels of both bacteria. Hence, it is critical to maintain and ensure cleanliness in all storage spaces, as unclean surfaces and places can contribute significantly to cross-contamination, of which the integrity of halal food products could be compromised. This suggests the need to study the potential of clay as decontamination agent for the use in halal logistics. However, the specific and details on the type and condition of the soils for *sertu* are currently still lacking. The general guidelines suggested by Jabatan Kemajuan Islam Malaysia (2013) are as follows: clays sourced from pottery making, taken from at least four meters below ground level to be used for *sertu* procedure, with the concentration of soil and water mixture within 0.6% to 2.5%. As of today, many of *sertu* products are commercially available in the form of powder, liquid suspension and detergent, with kaolin listed as the clay material. According to a recent literature review by Yusof and Subri (2022), the research on this area is still scanty hence further study on this topic is needed to establish new knowledge.

Although commercial *sertu* clays products have been placed in the market, their action to eliminate pathogenic bacteria has not been extensively investigated. To date, published data revealed that the adsorption ability of clay minerals (bentonite, kaolin and talcum) onto bacteria isolated from swab culture of canine saliva was influenced by variation of the gram-positive and gram-negative bacterial surface. The use of clay as functional ingredient in liquid detergent has been widely reported, using bentonite clay in combination with vegetable oils in the product formulation (Pratama et al., 2020; Wati et al., 2019). Previous study on the development of clay liquid detergent found

kaolin-based formulation showed highest sensory liking score, with highest surface activity (Angkatavanich et al., 2009).

The potential use clay as antibacterial agent has been reported with the recent development of bentonite clay soap with antibacterial activity against bacteria isolated from saliva of dog reported (Munawiroh et al., 2021). In addition, a study by Das and Tadikonda (2020) does not only suggest the potential use bentonite clay paste as a hand sanitizer, but also proposed decontamination of viruses on medical gears by dispersing bentonite slurry on the affected areas followed by washing with water, which seems similar to *sertu* cleaning procedure. Although clay-based products with antibacterial properties have remained largely unstudied for their application in halal logistic, these studies have suggested the promising application of clay as surface sanitizer.

2.3 Clay as antibacterial agent

Aside from its healing and soothing properties, clay has been investigated for its antibacterial activities, exemplified by the successful application of French green clay in the treatment of Buruli ulcer, a necrotizing cutaneous infection caused by *Mycobacterium ulcerans* (Williams et al., 2004). This discovery is supported by Haydel, Remenih and Williams (2008b), who found that certain clay minerals, when hydrated, have the ability to kill broad spectrum of human pathogens including antibiotic resistant strain. It was the first evaluation of the effectiveness of in vitro antibacterial activity against a broad spectrum of human pathogens that include antibiotic resistant *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, *Pseudomonas aeruginosa* and

Staphylococcus aureus. In addition, the antibacterial efficacy of clay leachates against gram-negative *Escherichia coli* and gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) has also been reported (Cunningham et al., 2010). The most effective antibacterial clay, identified as OMT blue clay was shown to completely eliminate both gram-positive and gram-negative bacteria within 24 h (Morrison et al., 2016). More recently, the OMT blue clay and its aqueous leachate were active when tested against planktonic and biofilm states of several pathogenic bacteria associated with wound infection (Caflisch et al., 2018). Apart from these, deactivation or killing of fish pathogens (Jung et al., 2016) has been reported. These studies have stimulated interest in the identification of clays from other localities that possess antibacterial activity and could be utilised as new antibacterial agents.

2.4 Foodborne disease and incidence

Foodborne illness is one of the major global concerns, affecting millions of people worldwide. 9.4 million cases of foodborne illness are estimated every year in the United States, with 55,961 hospitalizations and 1,351 deaths (Scallan et al., 2011). Meanwhile, the European Union (EU) reported that foodborne outbreak had caused 45,874 cases of illness, 3,892 hospitalisations and 17 deaths in 2015 (Bintsis, 2017). It occurs when a food is contaminated with pathogen and ingested, followed by multiplication of the pathogen in the human host. Foodborne disease can also occur when the pathogen in a food product produces a toxin, which is then ingested by the host. Therefore, the general classification for foodborne illness are foodborne infection and foodborne intoxication. An incubation period is usually involved

for the former, hence time for the symptoms to occur is much longer than that of the latter (Bintsis, 2017). Examples of bacterial foodborne illness includes Salmonellosis, *Campylobacter enteritis*, *Escherichia coli* (*E. coli*) O157:H7 infection, Staphylococcal food poisoning and botulism. These illnesses cause a variety of symptoms, ranging from mild gastroenteritis, flu-like conditions, renal failure and paralysis which leads to death.

2.5 Foodborne pathogens

Food may act as a vehicle for the transmission of bacterial infection or intoxication of humans. Sources of foodborne pathogens can be from poultry, seafood, dairy product, fruit and vegetables. In the food industry, bacteria are the main culprit of food deterioration and *Salmonella* and *Staphylococcus aureus* are major contributors of food-borne illnesses globally (Balaban & Rasooly, 2000; Besser, 2018). Contamination with *Salmonella* Typhimurium and *S. aureus* can arise during food handling and processing, as well as transfer between other foods, surfaces and utensils in the kitchen (Kadariya et al., 2014; Moore et al., 2007). In particular, studies have found a high degree of cross-contamination of various pathogens including *S. Typhimurium*, a gram-negative enterobacterial pathogen and *S. aureus*, a gram-positive species also associated with skin infections due to inadequate cleaning and disinfection of cutting boards (Di Ciccio et al., 2015; Redmond & Griffith, 2003).

2.5.1 *Salmonella* Typhimurium

Salmonella spp. is gram-negative bacteria which is closely related to the genus *Escherichia*, facultative anaerobic, catalase-positive, oxidase-negative, rod-shaped and is non-spore forming. *Salmonella* has been reported to be the major cause of food-borne illnesses globally (Argüello et al., 2018). Infections caused by *Salmonella* of various species and serovars of the genus continue to be threats, and is still a growing public health concern in both the developed and developing worlds (Majowicz et al., 2010; Marathe et al., 2012). *S. Typhimurium* can be spread by food handlers through poor practise of hygiene and sanitation, and when people eat raw or undercooked foods (Bintsis, 2017). Infections by *S. Typhimurium* can cause gastroenteritis, developing diarrhea, fever, vomiting and abdominal cramps. The main sources of human salmonellosis outbreak include poultry meat, eggs and pork (Pires et al., 2014) and the pathogen has virulence factors which it uses to colonise and persist within the host.

2.5.2 *Staphylococcus aureus*

Staphylococcus aureus, is a gram-positive coccal, facultative anaerobic, catalase-positive, coagulase-positive, and non-spore forming bacteria. Some of the strains can release enterotoxins, a highly heat-stable protein toxin that can lead to food poisoning when food contaminated with the bacteria is consumed (Balaban & Rasooly, 2000). Staphylococcal food poisoning (SFP) is a foodborne disease which is common worldwide, with estimated total cases of 241,000 each year in the United States (Scallan et al., 2011). Cooking can

kill the bacteria, but heat-stable enterotoxins are not destroyed which can still cause illness. The symptoms of SFP includes nausea, vomiting, diarrhea and stomach cramps. The source of the contamination is usually food handler, through either direct or indirect contamination of the bacteria on food processing instrument, utensils or storage containers (Stewart, 2017).

2.6 Soils in Malaysia

Malaysia has abundance of clay and clayey materials. The majority of clay has been used in construction sector as well in ceramics industry. The east coast of Peninsular Malaysia has three main rivers that flow into the South China Sea, which are Kelantan, Terengganu and Pahang Rivers. Clay minerals in these three major rivers mainly consist of kaolinite (72%-75%), illite (13%-20%), chlorite (7%-10%) and minor smectite (<1%) (Kee et al., 2015).

The classification of soils in Peninsular Malaysia is based on the United State Department of Agriculture (USDA) soil taxonomy system (Department of Agriculture Peninsular Malaysia, 1993). In Peninsular Malaysia, soils are characterised into 3 main groups; sedentary soils, alluvium soils and miscellaneous soils based on distinct differences in parent materials, type of formations and geomorphology. It is estimated that there are about 400 soil groups or series in Malaysia, however only 100 have been profiled (Paramananthan, 2000; Teh et al., 2017). Some of the examples of soil series were shown in Figure 2.3. In classification of soil, the defined control section in Malaysia is from 25 cm below the mineral soil surface to 100 cm depth. In general, soil can be classified into two main groups, which are organic and

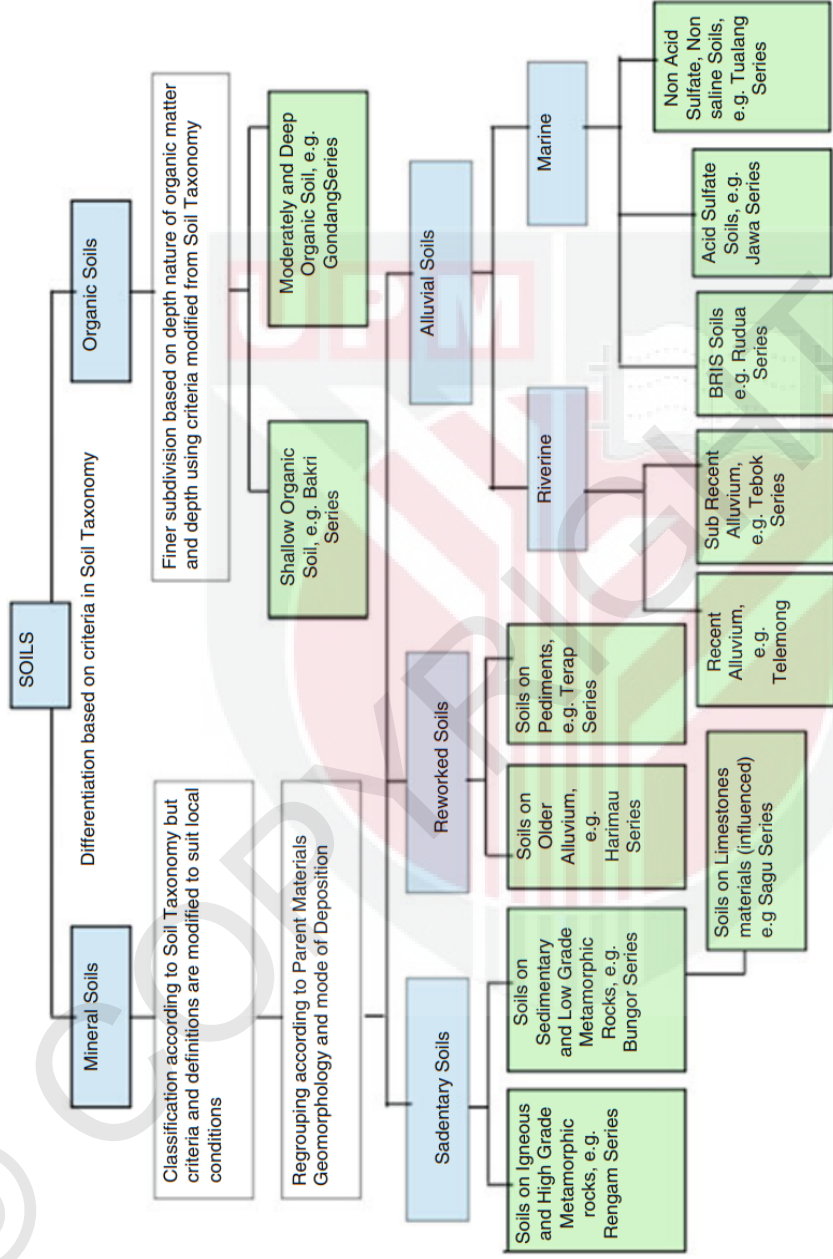


Figure 2.3: Subdivision of soils in Peninsular Malaysia
(Source: Khalid, Ali, and Rahman 2015).

mineral soil. The later type was chosen for this study due to the significant mineral content which is directly related to the clay percentage in the soil. Based on the Department of Agriculture Peninsular Malaysia (1993), soil groups with high clay content have been identified to be from metamorphic and sedimentary rocks, as well as alluvium soil groups. The variety of mineralogical content in the mineral soil include iron-oxides, gibbsite, mica, quartz, kaolinite, montmorillonite group, illite and many more minerals (Paramananthan, 2000).

2.7 Screening of soils with antibacterial activity

Clay is formed in a variety of geologic settings, but only a small proportion of clay minerals have proven to be antibacterial (Londono et al., 2017; Morrison et al., 2013; Morrison et al., 2016; Williams et al., 2008, 2011). Most of the known antibacterial clays are of hydrothermal origin and contain mixed illite-smectite (expandable clay) minerals, such as the French Green clay and Oregon Mineral Technologies (OMT) clays (Morrison et al., 2016; Morrison, Underwood, & Williams, 2013; Williams et al., 2008, 2011). However, not all antibacterial clays are from hydrothermal environments. Londono and Williams (2016) found an antibacterial lacustrine clay from the Colombian Amazon (AMZ), which reduced viability of both gram-negative *E. coli* and gram-positive *Bacillus subtilis* by 4-6 order of magnitude. The antibacterial clays from other regions were also reported, namely Ghanaian Sogakope clay (Borquaye et al., 2016), Jordanian soils (Ibbini et al., 2018), Canadian Kisameet clay (Behroozian et al., 2016) and Germany SAP clay (Zarate-Reyes et al., 2018).

Although there were few studies on local antibacterial clays, none of them were reported in detail. The majority of antibacterial clays reported from other countries are from hydrothermally altered volcanics, where volcanic fluid produce mineral that contain reduced metals (Williams, 2017). In Malaysia, clay-rich soils are derived from erosion of metamorphic and sedimentary rocks, and marine alluvium deposits (Paramanathan, 2000). Since there is no active volcano in Peninsular Malaysia, the search for clay with antibacterial properties should be looked at location of which clay deposited shares similar characteristics of natural antibacterial clays from previous studies. Some of reported antibacterial clay were from the groups of montmorillonite, goethite, kaolinite and pyrite minerals (Morrison et al., 2017; Otto & Haydel, 2013a; Williams, 2017). In addition, aluminium, iron and other metal elements have been established as contributing agent in antibacterial clays (Williams et al., 2008; Williams, 2017).

Among the 12 soil orders in the USDA soil classification system (USDA, 1987), it has been identified that 2 soil orders, namely oxisols and ultisols to be the types that should be screened for their potential antibacterial activity. Oxisols are common in tropical region with a lot of rainfall, and are characterised by the presence of oxides of iron and aluminium in the clay fraction of the soils. Meanwhile, ultisols are clay-rich and acidic type of soil. Ultisols were documented to have moderate CEC values ($< 24 \text{ cmol}(+)/\text{kg}$) according to the classification of Hazelton and Murphy (2007). Therefore, screening of soil series can be narrowed down by targeting the oxisols and ultisols soils.

2.7.1 Physicochemical properties of soils

Although clays have fundamental structural and chemical characteristics in common, each clay mineral has its own unique properties that determine how it will associate with other species. Soils with high clay materials should be sought after, as the amount of fine particles could contribute to antibacterial activity. A study by Haydel, Remenih and Williams (2008b) revealed that the clay minerals with fine particle size ($<0.2\ \mu\text{m}$) showed bactericidal effect when tested against *E. coli*, while the coarser fraction showed no antibacterial effect. This indicates that the smaller size fractions, which consists of clay minerals have an influence on antibacterial activity.

To classify the texture of the soils obtained from sampling, analysis of soil particle was undertaken. In soil science, the most common methods to determine soil texture are pipette and hydrometer methods (Gee & Baulder, 1986), where the particle sizes are quantified using sieve analysis. Although time consuming, pipette method showed more accurate result than hydrometer method, of which the latter was reported to over estimate the total sand fraction resulting in inaccurate texture classification (Beretta et al., 2014; Elfaki et al., 2016). A soil texture may affect its other properties hence the pipette method was used in this study for a reliable and accurate result. Result from the analysis, in which the percentage of each soil fractions, namely sand, silt and clay will determine the soil texture by referring to the soil texture triangle (USDA, 1987) (Figure 2.4). Kassim et al. (2014) suggested the best locally-sourced soil sample to be used for cleaning purposes to be of clayey type with 53.95% clay content, which was determined using pipette method.



Figure 2.4: USDA soil texture triangle
(Source: USDA, 1987).

Cunningham et al. (2010) and Williams (2017) reported the importance of pH as a crucial parameter in preliminary assessment of antibacterial activity, as an acidic environment of hydrated clay minerals contributes to antibacterial efficacy by increasing availability and toxicity of metal ions. This could also be supported by findings by Ibbini et al. (2018), who reported a moderate positive correlation of clay-pH with antibacterial activity against the growth of multi-drug resistant *Klebsiella pneumonia* and *Candida albicans*. In addition, determination of cation-exchange capacity (CEC) were undertaken to characterise the soil surface properties, as previously done by Londono and Williams (2016). In relation to the content of metal ions, CEC value of a soil would suggest the degree of exchangeability of the cations into the surrounding medium (Morrison et al., 2013). Therefore, soils with great CEC values may suggest their potential antibacterial activity. The information on soil

clay content, pH, and CEC of the soil series in Malaysia can be referred to data compilation from Paramananthan (2000), will serve as a reference in screening and selection of soil series with potential antibacterial activity.

In addition, other possible physicochemical parameters likely to be attributed to clay antibacterial activity will be assessed, such as colour and moisture content. Soil colour can be analysed using Munsell colour chart, where the redness index of the soil could indicate the presence of Fe oxides such as haematite or goethite (Barron & Torrent, 1986). While analysis on soil moisture content may not contribute directly to soil antibacterial activity, but is necessary to be included in the database should further investigation be carried out in the future. Previous studies by Ibbini et al. (2018) and Mpuchane et al. (2010) which reported antibacterial soil with physicochemical details could contribute to profiling types of soils with potential antibacterial activity. Understanding the geological environments that produce antibacterial minerals is important for identifying other promising reserves and for evaluating the geochemical diversity within specific deposits that coincide with bactericidal activity.

2.7.2 Preparation of clay leachate and suspension

Antibacterial activity of clay samples can be determined by the standard bacterial viability testing using agar plate. Earlier studies introduced clay mineral samples directly with media (broth) containing bacterial inoculum, as to create texture similar to clay poultice for the treatment of Buruli ulcer (Haydel et al., 2008; Williams et al., 2004, 2008). Cunningham et al. (2010) was the first to investigate the antibacterial activity of clays in the form of leachates and

suspension, which aimed to assess the effect of metals alone in the leachate, and to compare and elucidate the role of clay with the suspension. Cations adsorbed onto the clay surface can be exchanged upon hydration, releasing soluble metals that have the ability to kill pathogenic bacteria (Williams, 2017).

Clay leachates have been prepared with a variety of modifications, depending on the type of clays. Leachate preparation requires removing the insoluble minerals by centrifugation, collecting the supernatant liquid for further testing. Since there were no prior studies that investigate the antibacterial activity of locally sourced clays in detailed manner, the preparation of clay leachates was referred to past studies, as summarised in Table 2.3.

Cunningham prepared clay mineral leachates of 50 mg/mL, which was bactericidal against *E. coli*. Many studies which use mineral-rich clays employed similar clay concentrations while other clays were require higher clay concentrations (> 100 mg/mL) in preparing the leachates. The increased concentration of metallic oxides which are present in the clay minerals were reported to contribute to the antibacterial effectiveness, as increased in frequency of contact between the bacteria and the elements were necessary for the antibacterial effect (Sawai et al., 2000). Therefore, a higher concentration of clays in the leachate would increase the antibacterial effectiveness.

Table 2.3: Comparison of leachate preparation methods with different clays

Sample	Leachate preparation method	Reference
CB clay mineral mixtures	– 50 mg/mL of autoclaved clay in sterile deionised water – Shaking for 18 – 24 h – Centrifugation (31,000 x g) for 3 h – Sterilisation by passing through 0.22 µm filter	Otto et al., 2010
African clays	– 200 mg/mL of autoclaved clay in sterile deionised water – Filtered through sterile filter paper for particulate matter removal – Filtrate collected and antibacterial activity evaluated against test bacteria	Mpuchane et al., 2010
OMT clay	– 50 mg/mL of autoclaved clay in deionised water – Ultrasonication – Shaking for 24 h – Centrifugation (20,000 rpm) for 1 h	Williams et al., 2011
AMZ clay	– 50 mg/mL of autoclaved clay in deionised water – Shaking for 24 h – Centrifugation (26,892 x g for 1 h – Sterilisation of decanted leachate by autoclave (20 min, 120°C)	Londono & Williams, 2016
Ghanaian clays	– 50 mg/mL of autoclaved clay in sterile deionised water – Continuous shaking for 6 h – Centrifugation (4,000 rpm) for 30 min at 4°C	Borquaye, Ocansey & Semanya, 2016
OMT Blue clay	– 200 mg/mL of autoclaved clay in deionised water – Overnight shaking – Centrifugation (9,000 rpm) for 1 h at room temperature	Cafilisch et al., 2018

Other important variables involved in the preparation of clay leachates include addition of sonication treatment and shaking time. Sonication treatment uses ultrasound energy to physically dissociate the soil aggregates (Edwards & Bremner, 1967; Elliott & Cambardella, 1991) which resulted in decreased sand

and silt content and increased clay content (Roscoe et al., 2000; Yang et al., 2009). The shaking of the clay-water mixture promotes cation exchange from the negatively-charged clay surface to the surrounding aqueous environment, releasing the critical antibacterial component from mineral interlayers. In the study of antibacterial clay, many of the studies performed shaking of the clay-water mixture for 18-24 h (Morrison et al., 2016; Williams et al., 2011), purposefully mimicking the overnight application of a clay poultice applied to the skin for healing of infected wounds (Williams, 2017).

No previous study has investigated the comparison of different preparation methods towards antibacterial effectiveness. Therefore, to optimize leachate preparation methods that would effectively enhance the antibacterial activity of clay soils, different clay leachate preparation methods were adopted based on clay concentration, sonication and the duration of shaking in a hydrated state. This approach would provide a baseline data for further studies aimed at screening natural clay soils for antibacterial activity and assessing their potential to be used as natural antibacterial agent.

Suspension is simply prepared as the clay minerals suspended in deionized water, after which the mixture was allowed to equilibrate overnight. There were not much variations in the preparation methods from different studies, except for the different concentrations of clay used (Morrison et al., 2017; Otto & Haydel, 2013a).

2.7.3 Antibacterial test

Disc diffusion method can be used to screen clay soils with antibacterial activity. In comparison to the time-consuming nature of plate counting, this test has many advantages such as simplicity to perform, low cost, has the ability to test large numbers of microorganisms and antimicrobial agents, and the ease to interpret results provided (Balouiri et al., 2016). Due to its convenience, this method was used for antibacterial screening to assess the large number of samples tested in this study. This method is based on the Kirby-Bauer disc diffusion method of antibacterial testing (Bauer et al., 1966) with modifications, where filter paper discs soaked with test compound were placed on the surface of bacteria-inoculated agar plate. The diameter of inhibition zones is measured after overnight incubation, where larger zones of bacterial inhibition would mean that the bacteria are more susceptible to the antibacterial agent. Previous study has reported the use of this test as a rapid qualitative method for screening samples that showed antibacterial potential (Morrison et al., 2017), with leachates of clay were used in the screening assay (Borquaye et al., 2016; Morrison et al., 2017). Alternatively, a study by Mpuchane et al. (2010) employed clay extracts in the form of filtrates. However, the latter method has disadvantage as pipetting the filtrates would be difficult for the mixture with high clay concentration. At this stage, suspension is not preferred as the sediments might interfere transfer of sample during pipetting.

Disc diffusion test is only appropriate as a preliminary test and could not tell the exact concentration of extract needed to achieve a therapeutic result (Klančnik et al., 2010). In addition, although the interpretation of the diameter of inhibition zone has been established for antibiotics, but there has been no standard for clay soils. Hence, the results were compared to each other, with sample showing the largest diameter of bacterial inhibition selected for further analysis.

Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays are quantitative susceptibility testing method performed to evaluate lethality of an antimicrobial to the test bacteria (Davidson et al., 2005), which are necessary to be determined for the clay soils showing antibacterial activity. According to CLSI guidelines, MIC was defined as the lowest concentration of an antimicrobial agent that inhibits observable bacterial growth over 24 hours, while MBC defined as the minimum concentration that produces $\geq 99.9\%$ reduction of the initial bacterial population (CLSI, 1999). MIC and MBC can be determined by agar or broth dilution methods. Klančnik et al. (2010) found the later was the most accurate and faster way of assessing the antibacterial effect. Therefore, the determination of MIC and MBC in this study were conducted using broth microdilution method following the standard protocol as described by CLSI (CLSI, 2015). In this test, two-fold serial dilutions were performed in a 96-well microtiter plate, which involve variations in the concentration of antibacterial sample across the column.

Samples showing growth inhibition were further evaluated for their effect on bacterial viability following the standard antibacterial susceptibility testing (CLSI, 2015) with modifications following procedures described by Williams et al. (2011) and Morrison et al. (2013). This antibacterial assay assesses the effectiveness of natural clays in killing or inhibiting the growth of bacteria by comparing the orders of magnitude in the reduction or increase of treated bacterial population as compared to the untreated ones. Plate counting method is commonly used by many antibacterial clay studies to enumerate number of viable bacteria by formation of visible colonies on agar plates after overnight incubation of clay samples with bacteria. Additionally, Balouiri et al. (2016) recommended time-kill test, which provide information of an antibacterial agent on the nature of the inhibitory effect (bactericidal or bacteriostatic) (time-dependent or concentration-dependent) and the cell damage inflicted to the test microorganism. Dosage efficacy obtained from MIC and MBC tests were selected as concentration of samples to be used against test bacteria.

2.8 Separation of clay fraction from soil

Most of the previous studies of antibacterial clay does not report the procedure of clay processing in great detail. From the earliest discovery of antibacterial French green clays, Argiletz and Argicur, were reported to be supplied from French companies (Williams et al., 2004). Similarly, clay samples CsAg02 and CsAr02 minerals and CB clay mixtures used by the prominent researchers in the study of antibacterial clays were obtained from commercial suppliers, have been subjected to factory processing, which included drying and grinding

before use (Cunningham et al., 2010; Haydel et al., 2008; Otto et al., 2010; Otto & Haydel, 2013a).

The USA's Oregon Mineral Technologist (OMT) clays sourced from hydrothermal mineral deposits which are mineral-rich were simply air-dried, grind and passed through a 250 μm sieve before testing to prevent oxidation that could influence antibacterial activity of the clays (Morrison et al., 2013). Sieving serves as the easiest procedure to collect and rapidly analyse for the potential antibacterial activity of the soil samples. However, larger grains sand and silt, having particle size of 50-500 μm and 2-50 μm , respectively which was broken down by grinding may present in large amount in the sieved soil sample as a result of excessive grinding (Nakata et al., 2001). Aggregation of the clay particles and inconsistent distribution of the clay fraction would negatively affect their potential antibacterial activity.

Depending on the type of soils, especially the ground soil and clay sample may require pre-treatments to remove undesirable substances, such as organic matter. Particle-size fractionation is often undertaken to ensure optimal yield of the fine-sized clay mineral fraction ($<2\ \mu\text{m}$) through either sedimentation or centrifugation methods. The AMZ clay, obtained from fluvial environments was separated into clay-size fraction using standard centrifugation methods (Londono & Williams, 2016). The centrifugation method uses centrifugal forces to separate fine clay particles from coarser materials and impurities that are present in the soils (Jackson, 1979; Moore & Reynolds, 1989). However, this procedure would be time-consuming as only

small amount of soils can be used at a time as the centrifuge available in the laboratory has rotor that can only hold 50 mL tubes, yielding only less than 10% per run (Ibbini et al. 2018). Due to the large number soil samples in this study, this method is not convenient.

Sedimentation method segregate the solid particles from a liquid into size fractions based on their density. Based on the basic principle of sedimentation called “Stokes Law”, fine soil particles in water suspension tend to sink to the bottom as they are denser than water, settling at a velocity due to their higher density higher density than water, settling with a velocity proportionate to their size. Aliquots of clay suspension can be extracted after a muddy solution has been scattered and allowed to settle. Due to the high organic matter in our soil samples (Paramananthan, 2000), sedimentation procedure was undertaken to recover the clay fraction, thus yielded the treated clay. This method is commonly used in soil science in the particle size analysis as well as preparation of sample for X-ray diffraction analysis. The sedimentation method requires pre-treatment with hydrogen peroxide for organic matter removal and Calgon solution as a dispersing agent. However, the chemical treatment can have an effect on the dissolution of mineral constituents (Jensen et al., 2017), hence washing of samples with distilled water numerous times can minimise the effect.

2.9 Characterisation of antibacterial clay

Since there is limited general knowledge on the Malaysian clay soils with antibacterial properties, the comprehensive characterisation of the

antibacterial clay soils in terms of its mineralogical, elemental, morphological and surface properties is necessary. This provides a framework for a better understanding of the basis for the unique properties of Malaysian antibacterial clay including its antibacterial action and the geochemical variables that may influence this activity.

2.9.1 Mineralogical and elemental composition of antibacterial clays

Clay minerals have different chemical and structures which is based on the arrangement of the species in the groups and classification of minerals are based on many criterias including layer type, net layer charge, composition and type of the interlayer material (Al Ani & Sarapaa, 2008). X-ray diffraction (XRD) is an analytical technique that has been used to study clay mineralogy. It was previously reported that most antibacterial clays are composed of minerals containing reduced metals such as ferruginous illite-smectite, and other reduced Fe-bearing phases such pyrite, magnetite and maghemite (Williams, 2017). In particular, the antibacterial OMT clay was found to contain extremely high Fe, suggesting that the antibacterial effectiveness was largely contributed by the mineral-bearing Fe and other transition metals. (Morrison et al., 2013; Svensson et al., 2017). Although commonly present in abundance, feldspars, micas and quartz were identified as non-clay phases and were reported to be non-antibacterial (Haydel et al., 2008). Hence, the contribution of these non-clay minerals towards antibacterial activity of clay can be ignored in this study.

Analysis of elemental composition was carried out in order to support the results given by XRD using scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX). SEM-EDX validate the elemental identification of the sample, of which the minerals were previously identified from XRD analysis, as well as providing quantitative compositional information. Additionally, active ingredients in antibacterial clays are usually performed using modern analytical techniques, such as inductively coupled-optical emission spectroscopy (ICP-OES) to detect known toxic elements. Based on past studies, metals associated with antibacterial activity of clays are Fe, Al, Mg, Cu, Co, Zn, and Ni as shown in Table 2.4. It was previously established that antibacterial metal ions, such Ag, Cu, Fe and Al, were commonly selected as active ingredients due to their strong inhibitory and bactericidal effects (Berger et al., 1976; Domek et al., 1984; Gordon et al., 1994; Nies, 1999). In addition, the high concentration of trace elements such as Pb, Mn and As were also reported to cause toxicity to bacteria (Nies, 1999; Williams et al., 2004, 2008). Thus, in this study, 10 metals were chosen for quantification using ICP-OES which are Al, Ag, As, Cu, Fe, Mg, Mn, Ni, Pb and Zn by preparing their standard solutions for calibration. Leachates and suspensions samples were prepared and their elemental composition were quantified in the form of available metals. To relate these metals with the antibacterial activity, measurements were made for the different MIC values (1x MIC, 2x MIC and 4x MIC). For total metal content, the metals were extracted using the Aqua Regia method, a standard method in soil science, where the clay samples were digested with HCl and HNO₃. The total metal concentration was determined as a reference value.

Table 2.4: Summary of antibacterial component in clay minerals

Type of clay/Source	Component	Findings	Test bacteria	References
Oregon clay, USA	Fe	Release of pyrite caused production of hydroxyl radicals	<i>E. coli</i> , <i>S. aureus</i> , <i>P. aeruginosa</i> , and <i>S. Typhimurium</i> as well as the elimination of antibiotic resistance in extended-spectrum beta-lactamase (ESBL) <i>E. coli</i> and methicillin-resistant <i>S. aureus</i> (MRSA)	Cunningham et al., 2010; Morrison, Misra and Williams, 2016; Williams et al., 2011
OMT clay, USA	Fe	Rapid uptake of Fe impairs cell metabolism, produces ROS	<i>E. coli</i> (ATCC 25922) and <i>Staphylococcus epidermidis</i> (ATCC 14990)	Morrison et al., 2013
CB mixture clay	Fe (II), Cu (II), Co (II), Zn (II) and Ni (II).	Toxicity associated with released metal ions	<i>E. coli</i> O157:H7	Otto et al., 2014
Colombian Amazon clay, Colombia	Al	Elevated levels in the membrane leads to bacterial toxicity	<i>E. coli</i> (ATCC 25922)	Londono, Hartnett, and Williams, 2017
SAP clay, Germany	Mg	Layered mineral Mg induced oxidative stress, resulting in cell damage via lipid peroxidation (LP)	<i>E. coli</i> (ATCC 25922)	Zarate-Reyes et al., 2017

2.9.2 Morphology and surface properties

Morphological assessment using scanning electron microscopy (SEM) can also be utilised as one of the methods in clay minerals identification (Al Ani & Sarapaa, 2008). The different clay minerals exhibit different characterisation in term of its morphological structures. Kaolinite can be identified by the platy, pseudo-hexagonal particles and vermicular stacks. Other examples are halloysite and illite, which generally shows tubular and fibrous shape, respectively. Due to the variations of morphologies exhibited by the clay minerals, the SEM image can therefore serve as complementary assessment to confirm the mineralogical results. In addition, the mineral texture and crystal sizes can be compared to the standard reference, as reported by Williams and Haydel (2010) who suggests the smaller crystal sizes of the French clays in comparison to illite and smectite may play a role in contributing to the antibacterial mode of actions of the clays.

Surface properties include analysis of specific surface area, pore size and pore volume of the antibacterial clay following the Brunauer, Emmett and Teller (BET) method (Dogan et al., 2006) which was based on N₂ adsorption to the exterior mineral surfaces using a surface area and porosity size analyser. In this study, the surface properties were analysed to determine the possibility of physical interaction of clays with bacteria, by comparing their sizes. A high surface area may reflect the roughness of the clay surface, which is one of the possible indication of physical interactions of clay and bacteria (Londono & Williams, 2016). According to Haydel, Remenih, and Williams (2008a), surface attraction between clay minerals and bacteria is the result of physical

bactericidal where uptake of essential nutrients is hampered, resulting in either disruption of cell envelopes or efflux of metabolites. The measurement of CEC of clays is important in indicating the amounts of cations that can be exchanged by the clay mineral interlayer spaces into the surrounding medium. The CEC of samples were determined by the leaching method (Page, 1982) which is a standard method in soil science. Clay minerals with high CEC have been targeted in the creation of synthetic antibacterial materials (Bagchi et al., 2013; Jiang et al., 2016). The high CEC of clay allows free exchange of metal ions from the surface of the particles into the surrounding medium (Otto et al., 2016). Ion-exchange is critical in contributing to complete killing of *E. coli* and MRSA by CB09 leachate after supplementation of metal ions along with pH adjustment, which was previously revealed to be non-antibacterial. Hence, analysis on surface properties and CEC were investigated to better understand the attributes related to antibacterial activity of clays.

2.10 Factors influencing antibacterial activity of clay

2.10.1 Effect of pH

pH is one of the factors affecting bacterial viability (Nolte, 1982). *In vitro* antibacterial activities are associated with low pH environment. At low pH, clay-water mixture generates reactive oxygen species (ROS) and hydrogen peroxide, H_2O_2 and have high oxygen-reduction potential (Otto et al., 2014). In a low pH environment, abundant of protons saturate metal binding sites in the solution, maximising the concentration of soluble metal ions. Metal ions become more bioavailable and possibly more toxic in a low pH solution (Wang

et al., 2009). In soil science, many of elements in the soil are soluble at $\text{pH} < 5$ including Mg, Fe, Mn, Cu and Zn (Hazelton & Murphy, 2007). The high concentration of Al in the OMT leachate was contributed by the low pH environment (Williams et al., 2011), as Al is soluble in extreme pH conditions ($\text{pH} < 4$), but precipitates at circum-neutral fluids (Wesolowski, 1992).

2.10.2 Effect of metals

Although pure clay minerals have unique properties that make them a potential candidate as an antibacterial agent, no antibacterial activity was reported for kaolinite and smectite when tested (Londono & Williams, 2016; Parolo et al., 2011). Otto, Kilbourne, and Haydel (2016) reported that ion-exchanged kaolinite (Fe, Co, Cu and Zn) showed significant antibacterial effectiveness against MRSA as compared with natural kaolinite. Outcome of the study is in agreement with a previous research by Haydel, Remenih and Williams (2008) who found loss of antibacterial activity of clay minerals with natural exchangeable cations removed. This suggests critical role of the metal ions as the active components in the complex antibacterial mode of action.

Certain metal ions are essential for functioning of cells, for example maintaining structure of cell membrane, and participation in cellular processes such as electron transfer and catalysis. Essential metals are lethal when present in excess while non-essential metals are poisonous, have antimicrobial activity at low concentrations (Godoy-Gallardo et al., 2021). A literature by Lemire, Harrison and Turner (2013) stated that certain metals disrupt biofilms formation, have synergistic bactericidal activity, selectively

inhibit pathways & kill multidrug-resistant bacteria. Metals including Ag, Cu, Mn, Ni, Pb and Zn were reported to contribute to antibacterial activity (Godoy-Gallardo et al., 2021; Kawakami et al., 2008; Otto & Haydel, 2013b; Si et al., 2017). Toxicity was found to be affected by a combination of cations rather than a lone metal species (Cunningham et al., 2010). A study on metal ions composition of different clay mixture samples by Otto and Haydel (2013) revealed the significance of the concentration and speciation of exchangeable metal ions, which are responsible for antibacterial activity. Morrison, Misra and Williams (2016) found that the antibacterial activity of Oregon Blue Clay is due to synergistic activities of Fe^{2+} , Fe^{3+} and Al^{3+} which produce greater toxicity to bacteria at lower concentrations than individual metals.

Since the metals may work in concert in contributing to toxicity, synergistic test can be performed by employing fractional inhibitory concentration (FIC) assay to analyse combination of metals in antibacterial activity (Hoseinzadeh et al., 2017). By using checkerboard method, the combination of 2 metals were tested to determine the antimicrobial interaction, by measuring the optical density of the metal mixture solutions incubated with tested bacterial strains at 600 nm using microplate reader. The corresponding FIC values was evaluated from the as: 0.5 = synergy, 0.5–4.0 = indifference, >4.0 = antagonism (Leber, 2016).

In this study, the metals combination were selected based on the work of Otto and Haydel (2013), who reported the critical role of Zn, Ni, Cu, and Fe. These metals were supplemented into the non-antibacterial CB09-L leachate, and

with pH adjusted, were found to be enhancing the antibacterial activity, suggesting the synergistic effect of these metals. In addition, since Al is part of clay structure and the combination of Al and Fe was reported to contribute to antibacterial activity by Morrison, Misra and Williams (2016) as previously mentioned, the metal is also included in this assay. Hence, the different combinations of these 5 metals were tested for their synergistic antibacterial effect.

2.11 Antibacterial mode of action

While study on antibacterial clays has gained attention, their antibacterial mode of actions has only recently been investigated. Both clay minerals and mineral leachates have been reported to display antibacterial activities due to toxicity associated directly with released metal ions (Cunningham et al., 2010; Morrison et al., 2013; Otto & Haydel, 2013a). However, none of the reported antibacterial clays share the same mineral and metal composition as natural samples are highly heterogenous and exhibit varying antibacterial effectiveness (Morrison et al., 2022), therefore it is possible that their antibacterial mode of action could be different. Hence, the underlying mode of action of antibacterial action remained unclear.

Williams et al. (2004) who first reported the discovery antibacterial clay, suggests that clay minerals work more than just an adsorbent of toxins and viruses, as previously suggested by Carretero (2002). It has been considered that clays may deprive bacteria of nutrients by competing with ions for exchange sites on clay surfaces, or that the clay mineral assemblage contains

toxins that may cause dysfunction of the regulatory systems of the bacteria (Williams et al., 2011). Even without the presence of clay, the aqueous leachate solution was able to cause complete killing of bacteria, as previously reported (Morrison et al., 2013; Otto & Haydel, 2013a). This suggests that the antibacterial mode of action is influenced on soluble metal ions released leached out from clay minerals upon hydration, rather than electrostatic interactions or physical destruction caused by clays.

In general, antimicrobials cause disruption to bacterial cells by affecting synthesis of cell wall, inhibition of protein and nucleic acid synthesis, damaging membrane structure and function, and interfering enzymes involved in microbial metabolic pathways (Williams & Haydel, 2010). Lemire, Harrison and Turner (2013) presented possible mode of action of metal toxicity, which include protein dysfunction, production of ROS and depletion of antioxidants, membrane impairment, interference of nutrient assimilation and genotoxicity. In particular, the antibacterial mechanisms of metals Ag, Zn, Cu and Fe may include cell membrane disruption, intracellular protein and DNA disruption and the induction of metal-mediated ROS (Godoy-Gallardo et al., 2021).

Since the early study on antibacterial clay, it was suggested that the generation of reactive oxygen species (ROS) could cause oxidative damage to bacterial DNA, proteins and lipids (Haydel et al., 2008; Morrison et al., 2013; Williams, 2017). ROS is surface controlled and dependent on clay structural composition and the biological activity of clays strongly relies on the suspension composition (Kibanova et al., 2009). Antibacterial clay mineral assemblages

may contain reduced Fe minerals that drive production of reactive oxygen species (ROS) and can cause damage to cell membranes and intracellular proteins (Morrison et al., 2016). Iron occurs primarily in two oxidation states: oxidised Fe^{3+} (ferric iron) and reduced Fe^{2+} (ferrous iron). Even though bacteria may take Fe^{3+} from the outside, they soon convert it to the more soluble Fe^{2+} . However, Fe^{2+} accelerates ROS generation, resulting in a substantial quantity of hydroxyl radicals via the Fenton and Haber-Weiss reactions. Elevated levels of ROS can lead to DNA damage, lipid peroxidation, protein oxidation and eventual cell death (Ercal et al., 2001; Imlay et al., 1988).

Bioimaging analysis using TEM showed evidence of membrane damage of *E. coli* cells after exposure to OMT clay leachates, with appearance of black precipitates identified as metals that has slowly penetrated into the cell interior after 24 h of incubation (Williams et al., 2011). Visual evidence for the bactericidal effect of OMT clay suspension against *E. coli* and *S. epidermidis* was reported by Morrison et al. (2013), documenting that the membrane permeability of the bacteria may have been compromised. Morrison et al. (2016) studied reaction of clay suspension with *E. coli* in detailed using synchrotron scanning transmission X-ray microscopy (STXM) and found that Fe^{2+} adhered at the cell membrane and produces ROS that may damage membranes. Meanwhile, Al may cause toxicity by competing with Ca and Mg for phosphate binding which may damage the cellular membranes.

Bactericidal activity of an antibacterial agent can compromise bacterial integrity, as alteration of membrane can lead to bacterial death, while

bacteriostatic activity cause bacterial division interruption as a result of inhibition of protein production (Nemeth et al., 2015). The damage of intracellular proteins and DNA was reported by Morrison et al. (2016), with findings that most damage to cell membrane proteins was induced by aqueous Al^{3+} , while Fe^{2+} induced the greatest damage to DNA. Studies on metal toxicity have suggested that intracellular proteins are the evident targets for binding, with the presence of many amino acid-mediated binding sites, after which causing oxidation of the susceptible amino acids, which impairs protein function, reduces protein stability, and marks the protein for destruction (Lemire & Turner, 2016; Lemire et al., 2013). In addition, the similar chemical properties of certain metals and the essential ones may cause the bacterial cells to take up other metals instead, which can cause disorder in the metabolic pathways. Cell metabolism can be irreversibly damage due to the inability of the cells to reduce the metal intake inside the cells (Chitambar, 2016). Lemire et al. (2013) also reported the indirect damage of bacterial DNA that could be caused by many metals, such as the constant uptake of Fe into cytoplasm that contributes to the increased amount of intracellular Fenton chemistry, thereby raising ROS levels and speeds up DNA damage by metal-induced oxygen radicals.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection and on-site soil assessment

Sampling was conducted after selecting few criterias that are known through literature review to contribute to antibacterial activity of soils. Clay content, pH and metal contents (Al and Fe) were found to be the pre-defining criterias, of which these parameters were important in deciding the sampling locations and the soil series that were selected in this study. Therefore, this approach is pre-defined sampling. Based on the outlined criteria, soil series with potential antibacterial properties were selected for further analysis. In this study, soil samples were collected from Selangor, as this state has the second highest number of clayey soils, which is 15 out of 92 (16.30%) (Table 3.1), while the other factor is easy accessibility to the sampling locations.

Soil series represents a group of soils with similar physical and chemical properties such as texture, structure, horizon arrangement, and mineral content. Data for soil series profile were referred from Paramanathan (2000) and the excerpt on soil series information can be referred in Appendix B1.

Table 3.1: Distribution of soil series with high clay content in Malaysia based on state

State	No. of soil series found
Perak	17
Selangor	15
Kelantan	8
Pahang	7
Johor	7
Sabah	7
Negeri Sembilan	5
Sarawak	5
Melaka	4
Terengganu	4
Wilayah Persekutuan	4
Penang	4
Kedah	3
Perlis	2
Total	92

(Source: Paramanathan, 2000)

Potential sites for clays in Selangor, Malaysia were allocated based on data compiled by Paramanathan (2000), of which 10 soil series having key characteristics at specified control depth around 25 – 50 cm were identified (Table 3.2). It was noted that the data on free iron content was not available for some of the soil series, therefore other criterias such as pH and soil textural class were taken into consideration.

Table 3.2: Profile of target samples based on soil series in Selangor, Malaysia

Soil Series ¹	Depth (cm)	% Clay	pH	Textural class	Free iron (%)	Sampling Location
Batu Anam	10-49	55	4.50	Clay	0.55	UPM Serdang
Bernam	14-40	63	4.50	Clay	1.05	Sabak Bernam
	40-60	58	4.60	Clay	1.90	
Malacca	10-110	50	4.60	Stony clay	18.40	UPM Serdang
Munchong	20-81	74	4.96	Clay	0.05	UPM Serdang
Musang	4-27	48	4.20	Clay	-	UPM Serdang
	27-56	52	4.50	Clay	-	
Jerangau	25-57	51	4.50	Clay	-	Puchong
Parit Botak	23-74	53	3.10	Jarosite mottled clay	-	Carey Island
Prang	22-78	55	4.40	Clay	11.97	Putrajaya-Sepang
Sedu	6-32	56	3.50	Clay	-	Selangor
	32-60	54	3.40	Clay	-	
Serkat	22-42	ND	4.75	Clay	-	Sg. Sedu, Banting

¹Soil series are typically named after the geographic location where the type was first identified. For example, the Serkat soil series is named after Serkat, Pontian, Johor where it was first recognized. Serkat soil series are expected to be extensive in Johor, Selangor, Perak, Pahang and Terengganu (Paramanathan, 2000).

Upon site surveying, 13 samples were collected, and the sampling locations are shown in Figure 3.1 which covers areas around the state of Selangor, which were UPM, Sepang, Banting, Carey Island, Pulau Indah and Sabak.

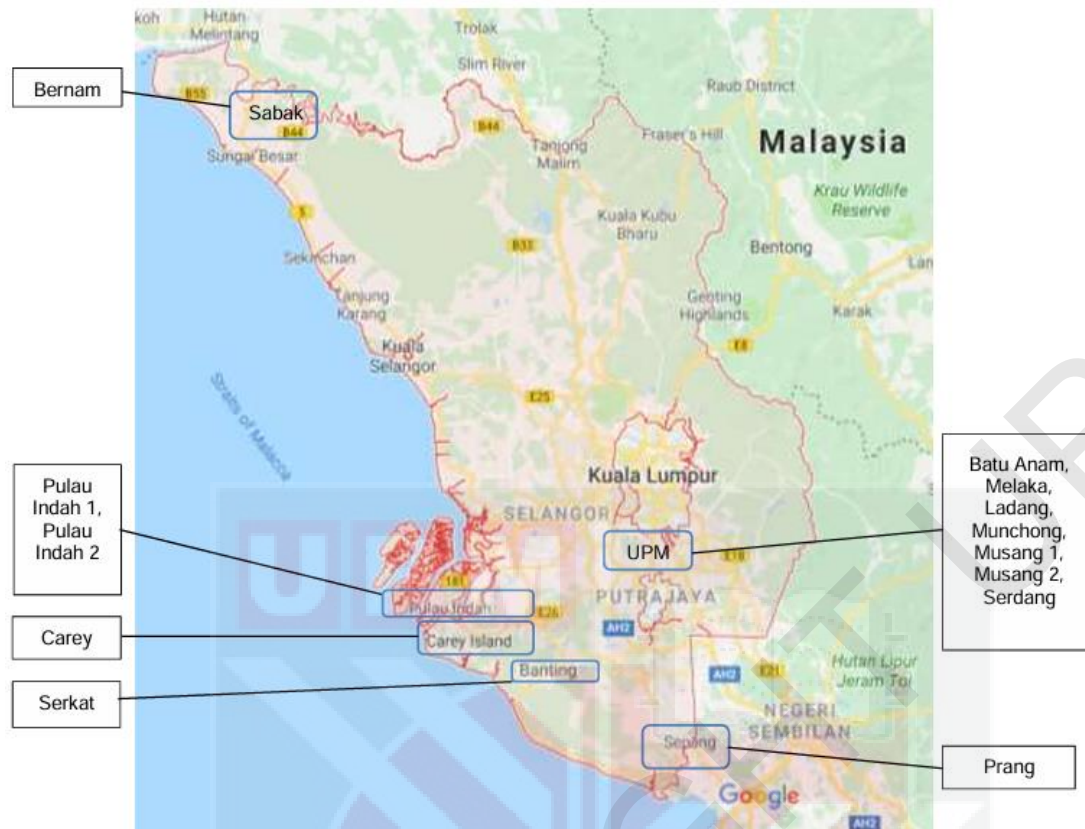


Figure 3.1: Map of soil sampling in Selangor, Malaysia

The overall sampling methodology was summarised in Figure 3.2. Soil samples were collected at depth 25 - 50 cm from surface using hand auger (Appendix B2). The samples (> 3 kg) were then put in labelled plastic bags and brought back to laboratory for further analysis. Sampling details such as coordinates and elevation of the sampling points were recorded using handheld GPS receiver Garmin GPSmap 60CSx (Garmin Ltd., USA). On-site assessments including type of soil, friability and other characteristics were analysed based on guidelines on soil description (Department of Agriculture Malaysia, 2015). Colour of soil was determined in moist condition using soil Munsell chart (Appendix B3) by recording the hue, value and chroma.

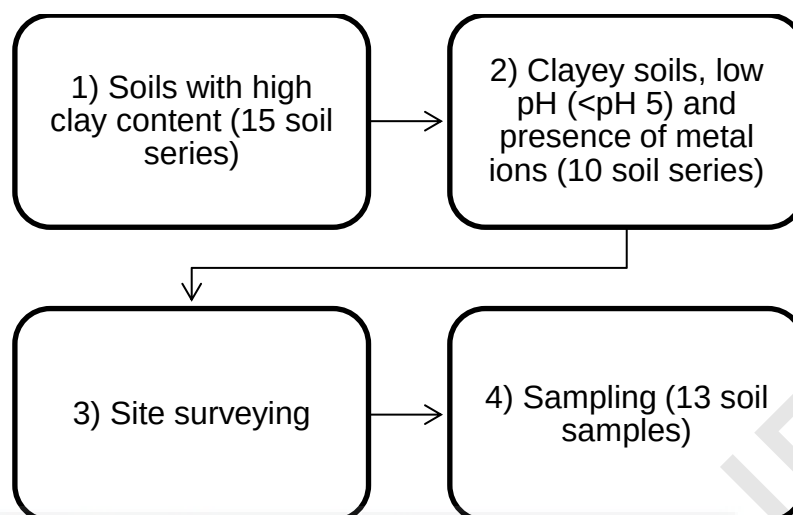


Figure 3.2: Soil sampling methodology

3.2 Sample processing and soil physicochemical analysis

The collected fresh samples were air-dried (30 - 35°C) for 1 – 4 weeks depending on the moisture content of the soil samples. The dried soil samples were then grounded using pestle and mortar, and passed through a 2 mm mesh-sieve to remove non-soil components such as fine roots and gravel. All the sieved soil samples were stored in clean container for further analysis. In this study, the duration of storage was approximately 6 months.

3.2.1 Determination of moisture content

Soil moisture content was determined by oven-drying of samples (105°C, 24 h). 10 g of fresh soil sample was weighted on a pre-weighed crucible. After 24 hours, crucibles containing samples were cooled in a dessicator and the weight was recorded when consistant value was achieved. Percentage moisture content was determined on dry weight basis, calculated as below:

$$\text{Soil moisture content (\%)} = \frac{(\text{initial weight (g)} - \text{oven dried weight (g)})}{\text{oven dried weight (g)}} \times 100$$

3.2.2 Particle size analysis

Particle size analysis was done to determine the percentage of sand, silt and clay in each sample. The particle size distribution for the soils was determined by the pipette method (Gee & Baulder, 1986). Twenty grams of dried soil was weighed into 1000 mL beaker. 100 mL of H₂O₂ (30%) was added and left overnight. The beaker containing soil and H₂O₂ mixture was heated (60 to 90°C), and further quantities of H₂O₂ were added until there was no more frothing. Few drops of alcohol were added in case of bubbling resulted from the heated mixture. This was followed by the addition of 100 mL of 0.2 N HCl and distilled water until the volume reached 400 mL. The mixture was allowed to cool down before washing thrice with 400 mL of distilled water. 50 mL of Calgon solution was added, followed by stirring of the mixture for 5 min using mechanical shaker. The mixture was passed through the 50 µm sieve to collect the sand fraction, transferred into a previously weighed porcelain dish, oven dried (105°C, 24 h), then allowed to cool down in a dessicator before weighing. Particles that passed through the sieve was collected and transferred into a 1000 mL cylinder, with distilled water added until the volume reached 1000 mL. Cylinders containing fine particles were placed into a water bath at the temperature 23°C. The solutions were then thoroughly mixed for 1 min using a plunger. The suspensions were allowed to settle for 7 h, after which, an aliquot was pipetted out and transferred into a previously weighed porcelain

dish. The dishes containing clay particles were oven-dried (105°C, 24 h), then allowed to cool down in a dessicator before weighing.

The determination of the percentages of sand, silt and clay were calculated using the equations below:

$$\% \text{ Sand} = \text{weight of oven dried sand particles (g)} \times \frac{100}{\text{weight of the soil (g)}}$$

$$\% \text{ Clay} = \left(\frac{\text{weight of oven dried clay particles (g)} \times 1000}{\text{volume pipetted}} - C \right) \times \frac{100}{\text{weight of the soil (g)}}$$

$$\% \text{ Silt} = 100 - (\% \text{ sand} + \% \text{ clay})$$

where C = weight of the Calgon in the solution (g)

3.2.3 pH analysis

pH meter was calibrated using buffer solutions (pH 4.00 and pH 7.00) every time before being used for analysis to ensure accuracy of pH measurements. pH of soils was measured with distilled water in the ratio of 1:2.5 (Benton, 2001). After reciprocal shaking of soil-water mixture (30 min, 150 rpm), the suspension is allowed to settled for 1 hour, after which the pH was measured using Mettler Toledo Model Sevengo Pro-SG78 (Mettler Toledo International, USA) probe meter. pH was determined in triplicates in order to improve the variation.

3.2.4 Cation-exchange capacity (CEC)

Cation-exchange capacity (CEC) refers to the capacity of soils or clays to adsorb and exchange cations. The CECs of samples were determined by the leaching method (Page, 1982). Ten grams of soil sample was weighed and placed in leaching tubes. The exchange sites of soil samples were saturated with 100 mL ammonium ions (1 M NH_4OAc , pH 7). Excess free ammonium ions (NH_4^+) in the soils were removed with alcohol, and subsequently leached again with 100 mL of 0.1 N K_2SO_4 . The CEC value was calculated from the concentration of displaced NH_4^+ in K_2SO_4 leachate, which was analyzed using Perkin Elmer AAnalyst 4000 Atomic Absorption Spectrometer (AAS). The concentration of displaced NH_4^+ measured by the AAS is directly related to the total number of exchangeable cations that were present on the soil's cation exchange sites. The CEC is expressed in centimoles of charge per kilogram of soil ($\text{cmol}(+)/\text{kg}$) and calculated using the following formula:

$$\text{CEC (cmol}(+)/\text{kg)} = \frac{\text{Concentration of displaced } \text{NH}_4^+ \text{ in solution (meq/L)} \times \text{Volume of leachate (L)}}{\text{Weight of soil (kg)}}$$

Where:

Concentration of displaced NH_4^+ is obtained from the AAS analysis.

Volume of leachate is the total amount of solution collected after leaching.

Weight of soil is the mass of the soil sample used in the experiment.

3.3 Preliminary screening of untreated soil leachates with antibacterial activity

Antibacterial screening of untreated soil leachates was conducted as illustrated in Figure 3.3. In this section, leachates of the untreated (sieved soil) samples were prepared by using 3 different methods, which are P1, P2 and P3, varying in the concentration of soils, addition of sonication treatment and shaking hour. The leachates were screened for antibacterial activity using disc diffusion test, followed by determination of MIC and MBC, and tested for pH.

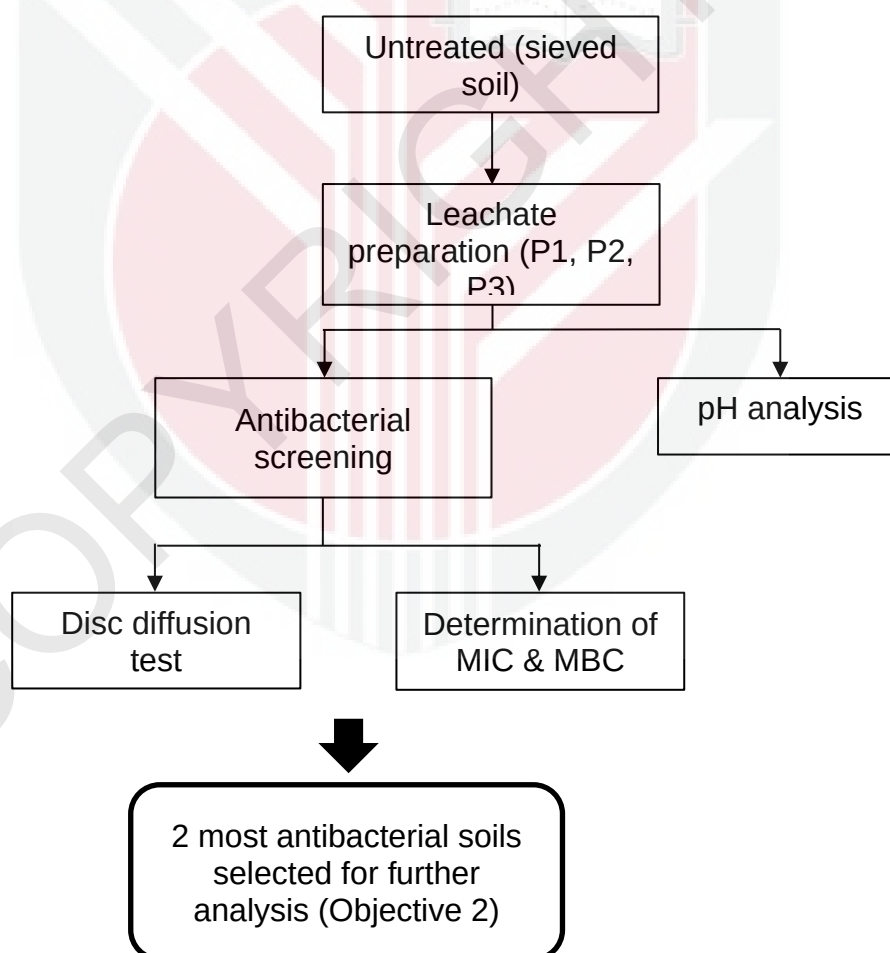


Figure 3.3: Research flow for section 3.3

3.3.1 Preparation of untreated soil leachates and pH determination

Soil sample was passed through a 53 μm mesh-sieve to collect the finest fraction. The sieved soil is henceforth referred to as untreated sample. Samples were sterilised in an autoclave at 121°C for 20 min prior to experimental use.

Soil leachates were prepared from untreated samples with appropriate modifications and grouped accordingly: Preparation 1 (P1): The leachate was initially prepared following methods developed by Morrison et al. (2013) with minor modifications to the shaking time and centrifugation setting. Soil suspensions (100 mg/mL) were prepared in sterile deionised water. The soil and water were mixed using an orbital shaker at 150 rpm for 1 h. Subsequently, the hydrated soil suspensions were centrifuged in an Eppendorf 5810 R using a fixed-angle rotor (10,000 rpm, 25°C, 15 min) to separate insoluble and soluble fractions. The aqueous supernatant (leachate) was recovered for antibacterial testing. Preparation 2 (P2): The soil suspension (500 mg/mL) was prepared in sterile deionised water, subjected to sonication for 1 min following Morrison et al. (2016), then shaken at 150 rpm for 1 h. The hydrated soil suspensions were centrifuged as in P1 and the supernatant leachate retained. Preparation 3 (P3): The soil suspension was prepared at 500 mg/mL, subjected to sonication (1 min), then shaken at 150 rpm for 6 h, centrifuged at 4,000 rpm, 4°C, 30 min (Borquaye et al., 2016) and the supernatant leachate recovered for antibacterial testing.

pH readings for leachates P1, P2 and P3 were determined using a probe meter (Sevengo Pro-SG78, Mettler Toledo International, USA) in triplicates to improve the accuracy of measurements.

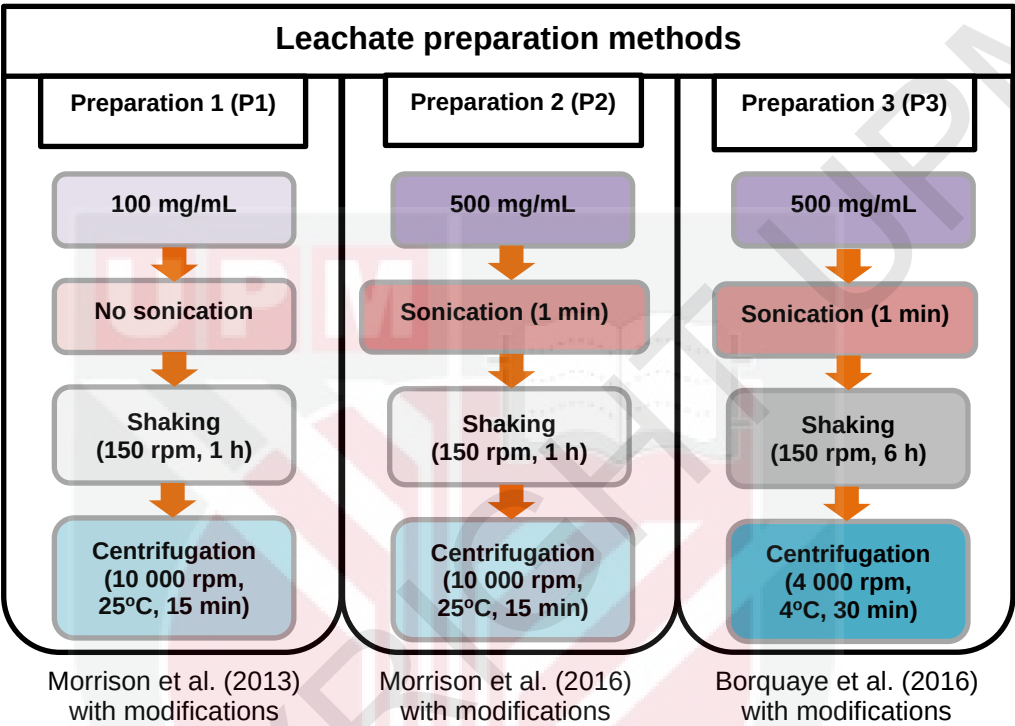


Figure 3.4: Leachate preparation methods

3.3.2 Antibacterial testing

3.3.2.1 Bacterial strains and growth conditions

Salmonella Typhimurium ATCC 14028, isolated from chicken tissue, and *Staphylococcus aureus* ATCC 13565, an enterotoxin-producing isolate (Casman et al., 1963) from a food poisoning outbreak, were used as test organisms. Both bacterial strains were grown on nutrient agar or in nutrient broth at 37°C for 24 h.

3.3.2.2 Disc diffusion assay

Antibacterial activities of untreated soil leachates were measured by using a standard disc diffusion method (Bauer et al., 1966). A single bacterial colony was inoculated into 10 mL of nutrient broth and cultured for 24 h at 37°C to achieve an inoculum concentration of $\sim 10^8$ CFU/mL. An aliquot of 0.1 mL of the inoculum was spread on Mueller-Hinton agar (MHA; Oxoid, England). Sterile paper discs (6 mm in diameter) were immersed into 20 μ L of soil leachate for 15 min and then placed onto the inoculated MHA agar plate. Plates were then incubated at 37°C for 24 h. Bacterial growth inhibition was determined by measuring the diameter of zones of inhibition. Sterile water was used as negative control, while ampicillin (10 μ g) and ciprofloxacin (5 μ g; Fisher Scientific, UK) served as positive controls for *S. Typhimurium* and *S. aureus*, respectively. Testing was performed in triplicate.

3.3.2.3 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined leachates of all 13 soil samples, which was prepared using P1, P2 and P3 methods, as described in Section 3.3.1. The MIC value was defined as the lowest concentration of antimicrobial agent that was able to inhibit the visible growth of bacteria while MBC was defined as the concentration of antimicrobial agent required to completely eliminate viable bacteria from samples. The determination of MIC and MBC were conducted using broth microdilution following the method described by CLSI (2015). Two-fold serial dilutions were performed in a 96-well microtiter plate

for MIC determination. Briefly, bacterial cultures grown in Mueller-Hinton broth (MHB; Oxoid, England) were adjusted to achieve an inoculum concentration of 10^6 CFU/mL. 100 μ L of MHB was applied to each well, then from column 1 until column 10, 100 μ L of the inoculum was added. Untreated soil leachates with initial concentration of 100 mg/mL (P1) and 500 mg/mL (P2 and P3) were diluted in two-fold increments in the respective medium and mixed with the inoculum, whereby column 1 contained the highest leachate concentration (50 mg/mL for P1, 250 mg/mL for P2 and P3) while column 10 had the lowest (0.098 mg/mL for P1, 0.12 mg/mL for P2 and P3). Column 11 served as a positive control for all samples (only medium and inoculum, and no antibacterial agent), whereas column 12 served as negative control (only medium, no inoculum and no antibacterial agent). The microtiter plates were incubated at 37°C for 24 h. The MIC value was determined by the lowest concentration of the soil leachate in that showed no turbidity after incubation. To determine MBC, spot plating was performed by pipetting 10 μ L aliquots from columns which showed no visible bacterial growth on the MHA plate, before further incubation of the microtiter plates at 37°C for 24 h. From the spot plating result, the lowest concentration of leachate where no visible growth was observed on the agar plate was considered as MBC. Experiments were performed in triplicate.

3.4 Effect of different preparations and forms on antibacterial effectiveness of untreated and treated samples

Sample preparation for section 3.4 was conducted as illustrated in Figure 3.5. Samples were prepared in the form of untreated and treated, through sieving

and sedimentation procedure, respectively. These samples were then prepared in the form of leachate and suspension. In this section, Carey and Munchong were processed into untreated and treated before being prepared in the form of leachate and suspension. Therefore, a total of 8 samples were prepared; untreated Carey leachate, untreated Carey suspension, treated Carey leachate, treated Carey suspension, untreated Munchong leachate, untreated Munchong suspension, treated Munchong leachate and treated Munchong suspension.

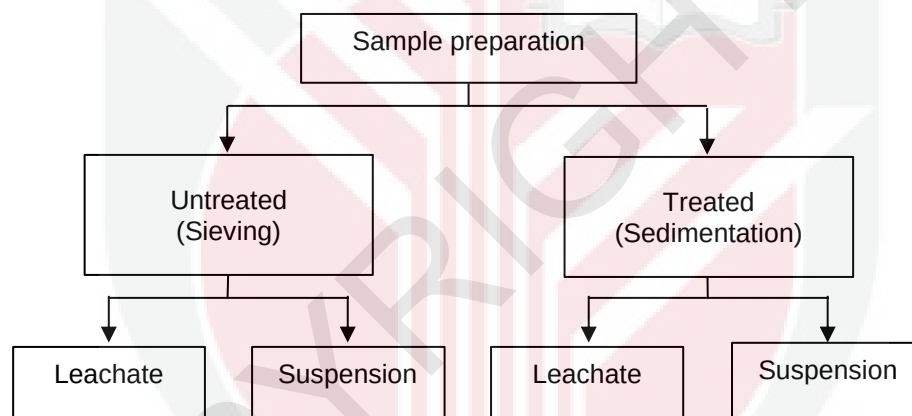


Figure 3.5: Sample preparation for section 3.4.

3.4.1 Preparation of untreated and treated samples

Untreated and treated samples were prepared as illustrated in Figure 3.6.

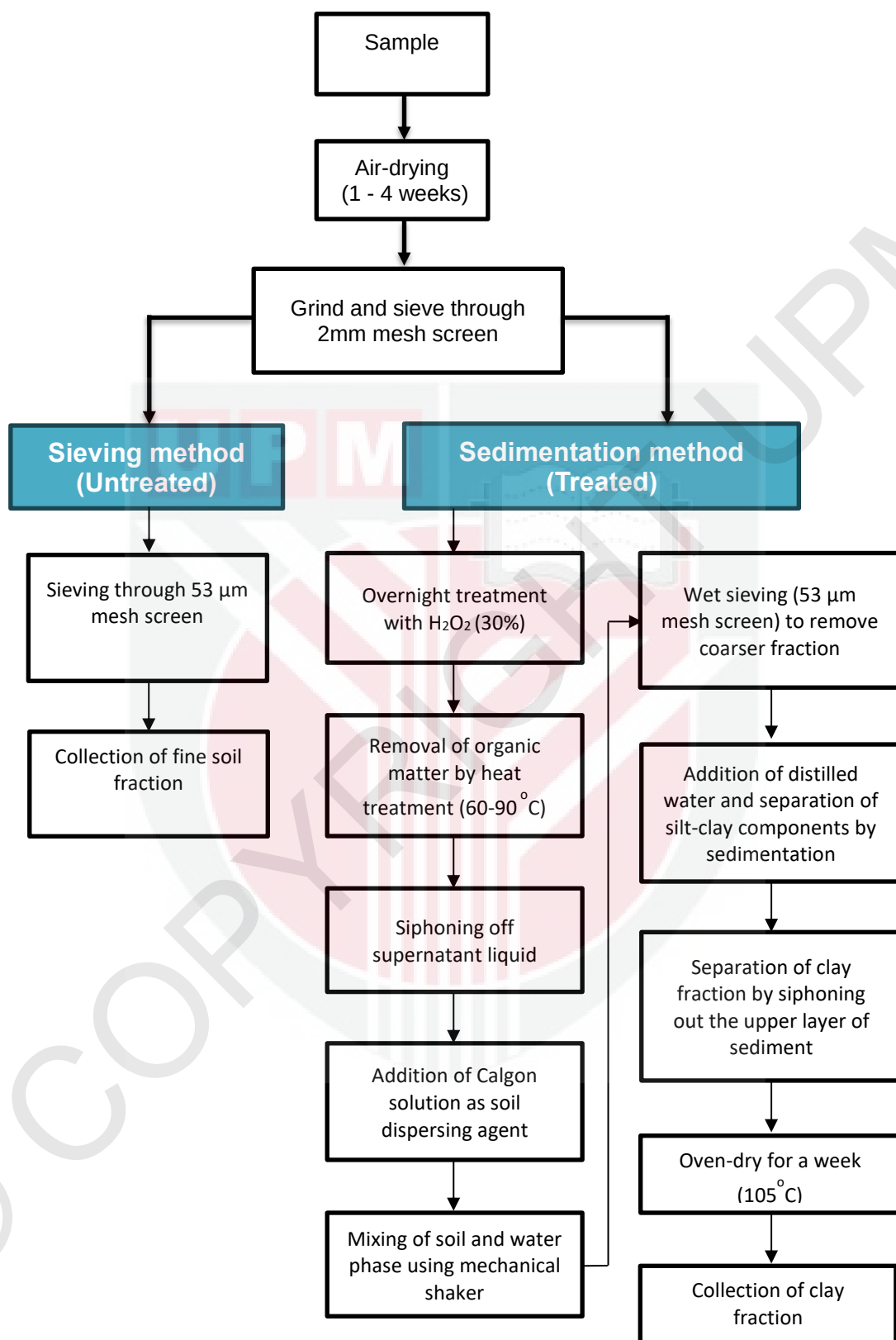


Figure 3.6: Sample preparation using untreated and treated method

3.4.1.1 Untreated samples

Soil samples were further processed by passing through a 53 μm mesh sieve. This sieved soil is henceforth referred to as the untreated sample. Samples were autoclaved (121°C, 20 min) to sterilize prior to experimental use.

3.4.1.2 Treated samples

Soil samples were further processed to separate the clay fraction from the bulk soil. The procedure involves chemical treatment and henceforth, the resulting clay fractions are referred to as treated samples, in order to differentiate them from the untreated soil samples. The clay separation by sedimentation was performed using the method developed by Gee and Baulder (1986), with minor modifications to the weight of samples and the volume of H_2O_2 used. 300 g of soil samples (250 μm), exposed to 150 mL H_2O_2 (30%) overnight, were then heated (90°C) for approximately 30 min to remove organic matter. The marine alluvial soils which are Bernam, Carey, Serkat, Pulau Indah 1 and Pulau Indah 2 require additional time (1 h) to completely remove their organic matter. The rapid reaction with the marine alluvial soils was associated with the presence of high organic matter in these soil samples, therefore temperature was adjusted to 80°C to control and stabilise the reaction. Distilled water was added to a 1000 mL beaker containing the treated soil mixture until the volume reached 1000 mL and left overnight. The resultant soil-water phase was formed whereby soil particles settled at the bottom while the upper layer of liquid consists of distilled water and excess H_2O_2 . The liquid was siphoned off to collect the soil particles. Calgon solution (pH 8.3) was added as a dispersing

agent and the solution was stirred using a mechanical shaker (5 min) to combine both soil and water phases together. The suspension was wet-sieved using a 53 μm mesh screen to collect the finer clay-silt, while removing the coarser sand fraction. Distilled water was added to the beaker containing the silt and clay fraction and left to settle to separate the silt and clay components by sedimentation. The upper layer, containing the clay fraction, was collected by siphoning the solution into a fresh beaker and the clay was oven-dried at 105°C to remove water. The dried fine clay was collected, gently ground in a mortar and pestle, passed through a 53 μm mesh sieve and autoclaved (121°C, 20 min) to sterilize prior to experimental use.

3.4.2 Preparation of leachate and suspension

3.4.2.1 Leachate

Leachates of untreated and treated samples were prepared using P3 method which was previously described in Section 3.3.1.

3.4.2.2 Suspension

A suspension was prepared by referring to Morrison et al. (2017), of which the sterile sample was added in sterile deionized water (500 mg/mL). The resulting suspension was employed in subsequent experiments.

3.4.3 Determination of pH

pH meter was calibrated using buffer solutions (pH 4.00 and pH 7.00) every time before being used for analysis to ensure accuracy of pH measurements. The pH of leachate and suspension samples was measured using a Mettler Toledo Model SevenGo Pro-SG78 (Mettler Toledo International, Columbus, USA) probe meter. For suspension samples, particles were dispersed in water by reciprocal shaking (30 min, 150 rpm), after which the suspension is allowed to settle for 1 hour before pH is measured. The pH was determined in triplicate for improved accuracy.

3.4.4 Determination of CEC

The CECs of untreated and treated samples were determined by the leaching method (Page, 1982), as described in Section 3.2.4.

3.4.5 Metal analysis

3.4.5.1 Available metals

The concentration of water-soluble metals silver (Ag), aluminium (Al), Arsenic (As), copper (Cu), ferum (Fe), magnesium (Mg), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) in leachates of untreated and treated samples were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Perkin Elmer, Optima 8300) following method by Otto & Haydel (2013a). For the suspension, samples prepared using method from Section 3.4.2.2 were shaken at 150 rpm for 6 h, centrifuged at 4000 rpm, 4°C, for 30 min and the resultant supernatant was collected for analysis.

3.4.5.2 Total metals

The total metal concentration was determined as a reference value. The metals were extracted using the Aqua Regia method where the samples were digested with HCl and HNO₃. Briefly, 0.5 g of samples were weighed into boiling tube, then 3 mL of HCl and 1 mL HNO₃ were added, after which, the mixture was digested at 110°C until the solution was reduced. After the solution was allowed to cool down at room temperature, 10 mL of 1.2% HNO₃ were added, then heated at 80°C for 30 min. Distilled water was added after the solution cooled down, then the heating process was repeated once again before transferring the solution to 50 mL volumetric flask, after which, distilled water was added until the volume reached 50 mL. The solution was filtered using filter paper before determining the metal concentrations by ICP-OES.

3.4.6 Antibacterial testing

3.4.6.1 Bacterial strains and growth conditions

Salmonella Typhimurium ATCC 14028, isolated from chicken tissue, and *Staphylococcus aureus* ATCC 13565, an enterotoxin-producing isolate (Casman et al., 1963) from a food poisoning outbreak, were used as test organisms.

Both bacterial strains were grown on nutrient agar or in nutrient broth at 37°C for 24 h.

3.4.6.2 Determination of MIC and MBC

The determination of MIC and MBC of leachate and suspension samples prepared in Section 3.4.2 were performed following the method as described in Section 3.3.2.3. Testing was performed in triplicate.

3.4.6.3 Bacterial viability assay

The antibacterial activity of leachate and suspension samples was further tested using methods developed by Williams et al. (2011) and Morrison et al. (2013), with minor modifications to the shaking time. Bacteria were grown in nutrient broth (NB, Oxoid, Hampshire, UK) by incubation for 24 h at 37°C. The overnight culture was diluted into fresh NB and the bacterial growth was monitored by measuring the optical density at 600 nm using spectrophotometer at regular intervals to plot bacterial growth curve. Based on the growth curve, the log phase occurred when the OD₆₀₀ is between 0.2 and 1.4 (Appendix B4).

Samples (leachate and suspension) were mixed with bacteria in log-phase growth containing approximately 10^7 – 10^8 CFU/mL, in a 1:1 ratio by volume, then continuously mixed in an incubator shaker (120 rpm, 37°C, 6 h). After 6 h of exposure, 1 mL of sample was removed, serially diluted in 0.1% peptone water, and 0.1 mL of the mixture was plated onto nutrient agar (NA). Colonies were enumerated after incubation for 24 h at 37°C using a colony counter (Galaxy230 Colony Counter; Rocker Scientific Co. Ltd.; Taiwan). Analysis was

performed in triplicate, and results expressed as log₁₀ CFU/mL. The bacterial colonies were calculated using the equation below:

Colony forming unit (CFU/mL)

$$= \frac{\text{Number of colonies} \times \text{total dilution factor}}{\text{volume of culture plated (mL)}}$$

3.5 Characterisation of treated Carey clay

3.5.1 X-ray diffraction (XRD)

Clay mineralogy of the treated clay samples was studied using XRD-6000 (Shimadzu, Kyoto, Japan) for powder X-ray diffraction using CuK-alpha radiation target, operated at 40 kV and 30 mA. X'Pert HighScore Plus software was used for qualitative phase analysis.

3.5.2 Elemental composition (EDX)

An elemental analysis was carried out in order to support the results given by XRD in a percentage form using FESEM, JSM-7600F (JEOL, Tokyo, Japan).

3.5.3 Scanning electron microscopy (SEM)

Clay morphology was observed under field emission scanning electron microscopy (FESEM), JSM-7600F (JEOL, Tokyo, Japan) using 5 kV acceleration voltage.

3.5.4 Surface properties

Specific surface area, pore size and volume of Carey clay was analysed following the Brunauer, Emmett and Teller (BET) method (Dogan et al., 2006) which was based on N₂ adsorption to the exterior mineral surfaces using a surface area and porosity size analyser (Micromeritics Tristar II Plus, Georgia, USA). CEC of clay was determined by the leaching method (Page, 1982) as described in Section 3.2.4.

3.6 Factors influencing antibacterial activity of treated Carey clay

3.6.1 Effect of pH on antibacterial activity

3.6.1.1 pH analysis at different clay concentration

pH was determined for Carey clay in leachate and suspension form for 1x MIC (125 mg/mL), 2x MIC (250 mg/mL) and 4x MIC (500 mg/mL) following method as described in Section 3.4.3.

3.6.1.2 Influence of pH on antibacterial activity

Deionised water was adjusted to pH 2 and pH 4 pH using 0.1 M of hydrochloric acid (HCl, Sigma Aldrich, United States) and 0.1 M of sodium hydroxide (NaOH, Sigma Aldrich, United States). The average pH of of treated Carey clay leachate and suspension at 4x MIC (500 mg/mL), which was previously measured in Section 3.6.1.1, was maintained at pH 2.51 ± 0.05 and pH 2.75 ± 0.04 , respectively. All samples were sterilized by using autoclave (121°C, 20 min) before use. Sample was added to NB containing inoculum of

approximately $10^7 - 10^8$ CFU/mL in a 1:1 ratio by volume, then continuously mixed in an incubator shaker (120 rpm, 37°C, 24 h). At 0 h and 24 h of exposure, 1 mL of sample was serially diluted in 0.1% sterile peptone water, and 0.1 mL of the sample was plated onto nutrient agar (NA). Colonies were enumerated after incubation for 24 h at 37°C using a colony counter (Galaxy230 Colony Counter; Rocker Scientific Co. Ltd.; Taiwan), and results expressed as $\log_{10}$ CFU/mL. Sterile deionised water with pH 7.76 was used as control. Analysis was performed in triplicate, and results expressed as $\log_{10}$ CFU/mL.

3.6.1.3 Stability of antibacterial clay at different pH

The stability of Carey leachate and suspension at different pH were evaluated against test bacteria. pH of clay leachate and suspension at 4x MIC (500 mg/mL) was adjusted to pH 3, pH 5 and pH 7, using 0.1 M of HCl (Sigma Aldrich, United States) and 0.1 M of NaOH (Sigma Aldrich, United States). The samples were then sterilized by using autoclave (121°C, 20 min) before use. Samples were mixed with bacteria in log-phase growth containing approximately 10^7-10^8 CFU/mL, in a 1:1 ratio by volume, then continuously mixed in an incubator shaker (120 rpm, 37°C, 24 h). At 0 h and 24 h of exposure, 1 mL of sample was serially diluted in 0.1% sterile peptone water, and 0.1 mL of the sample was plated onto nutrient agar (NA). Colonies were enumerated after incubation for 24 h at 37°C using a colony counter (Galaxy230 Colony Counter; Rocker Scientific Co. Ltd.; Taiwan), and results expressed as $\log_{10}$ CFU/mL. Sterile deionised water with pH 7.76 was used as

control. Analysis was performed in triplicate, and results expressed as $\log_{10}$ CFU/mL.

3.6.2 Time-kill analysis

Time-kill assay was carried out with the MIC values found previously, according to method described by Caflisch et al. (2018) with modifications to the orbital shaker speed and time intervals of bacteria-sample exposure. Clay samples (suspension and leachate) were adjusted to pH 3 using 0.1 M of HCl (Sigma Aldrich, United States) and 0.1 M of NaOH (Sigma Aldrich, United States). Samples were then diluted with the nutrient broth (NB) medium containing inoculum of approximately $10^7 - 10^8$ CFU/mL to obtain final concentrations of 0x MIC, 1x MIC, 2x MIC, and 4x MIC for each bacterial species. The cultures were continuously mixed in an orbital shaker (120 rpm, 37°C). At different time intervals of exposure (0, 0.5, 1, 2, 4, 6, 8, 10 and 24 h), 1 mL of the sample was removed, serially diluted in 0.1% peptone water and 0.1 mL of the sample was plated onto nutrient agar (NA). Colonies were counted after incubating for 24 h at 37°C, using a colony counter (Galaxy230 Colony Counter; Rocker Scientific Co. Ltd.; Taiwan). Analysis was performed in triplicate, and results expressed as $\log_{10}$ CFU/mL.

3.6.3 Determination of available metals in treated Carey clay leachates and suspension

Clay samples were prepared in different concentrations based on MIC values; 1 MIC (125 mg/mL), 2 MIC (250 mg/mL) and 4 MIC (500 mg/mL). The concentration of water-soluble metals Ag, Al, As, Cu, Fe, Mg, Mn, Ni, Pb and

Zn in leachates of untreated and treated samples was determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Perkin Elmer, Optima 8300).

For the suspension, samples were prepared using method from Section 3.4.2.2 for 1 MIC, 2 MIC and 4 MIC were shaken at 150 rpm for 6 h, centrifuged (4000 rpm, 4°C, 30 min) and the resultant supernatant was collected for ICP analysis.

3.7 Effect of standard metals against *S. Typhimurium* and *S. aureus*

3.7.1 MIC and MBC determination for standard metals against *S. Typhimurium* and *S. aureus*

MIC and MBC determination for individual metal ion was referred to the method by Vaidya et al. (2017). For chemical preparation, single element standard calibration solutions for Ion Coupled Plasma – Atomic Adsorption Spectroscopy (ICP-AAS) of Ag, Al, Cu, Fe, Mg, Mn, Ni, Pb and Zn of 1000 mg/L (Merck, Darmstadt, Germany) were used. Dilution of these metals into respective metal ion concentrations was done using sterile water.

The determination of MIC and MBC were done using broth microdilution following the method by CLSI (2015). A two-fold serial broth microdilution method was performed in a 96-well microtiter plate for MIC determination. Briefly, bacterial cultures in Mueller Hinton broth (MHB; Oxoid, England) were adjusted to achieve an inoculum concentration of 10^6 CFU/mL. 100 μ L of MHB was added into each well. To determine MIC of each metal, 100 μ L of

the test samples were added into the first column, mixed and two-fold diluted the respective medium until column 10, whereby column 1 would have the highest concentration while column 10 would have the lowest concentration. Finally, 100 μ L of the inoculum were added into all column except column 12. Column 11 served as positive control for all samples (only medium and inoculum, and no antibacterial agent) while column 12 served as negative control (only medium, no inoculum and no antibacterial agent). The microtiter plates were incubated at 37°C for 24 h. The MIC value was determined by the lowest concentration of the metal in the column that showed no turbidity after incubation. To determine MBC, spot plating was performed by pipetting 10 μ L aliquots from columns which showed no visible bacterial growth on the MHA plate, before further incubation of the microtiter plates at 37°C for 24 h. From the spot plating result, the lowest concentration of metal where no visible growth was observed on the agar plate was considered as MBC. Experiments were performed in triplicate.

3.7.2 Fractional inhibitory concentration (FIC) determination

The FIC was determined to assess the interaction of two metal combinations in order to determine the potency of two metals in comparison to the individual antibacterial activities of the metal. Determination of FIC was done using synergy checkerboard assay according to Daly et al. (2017). Bacterial cultures in Mueller Hinton broth (MHB; Oxoid, England) were adjusted to achieve an inoculum concentration of 10^6 CFU/mL. 100 μ L of MHB was added to every well of a 96-well microtiter plate. 100 μ L of the test sample (metal A) was added into every well of the the first column, except column 12. Two-fold dilution was

performed by adding 100 µL of the first row of the column (A1 - A11) into the second row (B1 - B11), with the content mixed by reverse pipetting. The procedure was repeated until row G1 – 11, skipping the last row (row H). Next, metal B was added into wells of column 1. Two-fold dilution was performed by adding 100 µL of column 1 into column 2. The content was mixed by reverse pipetting. The procedure was repeated until column 10, skipping columns 11 and 12. Finally, 100 µL of inoculum previously prepared was added to all wells except E12 – H12, which served as negative controls. Wells A12 – D12 containing the inoculum served as positive controls. The microtiter plates were incubated at 37°C for 24 h. The result was assessed by measuring the optical density at 600 nm using multimode microplate reader (Tecan Spark, Switzerland). The least concentration of metal showing growth inhibition of the tested bacterial strain was considered as MIC. Synergy of the two metals was assessed by locating the lowest dilution combination well in which there is no growth.

The FIC values were calculated as;

$$FIC = \left(\frac{\text{MIC of metal A in combination}}{\text{MIC of metal A alone}} \right) + \left(\frac{\text{MIC of metal B in combination}}{\text{MIC of metal B alone}} \right)$$

Where the antimicrobial interaction was evaluated from the FIC values: 0.5 = synergy, 0.5–4.0 = indifference, >4.0 = antagonism.

3.8 Antibacterial mode of action

3.8.1 Transmission electron microscopy (TEM)

Bacteria (approximately $10^7 - 8$ CFU/ml) were incubated in the broth with clay leachate and suspension at the 1, 2 and 4 MIC for 0.5 h for *S. Typhimurium*, and 6 h for *S. aureus* at 37°C. Due to rapid killing of *S. Typhimurium* observed in time-killing analysis (Figure 4.8 and Figure 4.9), the culture was taken after 30 min of exposure to clay samples. Meanwhile, *S. aureus* which showed slower killing rate were taken at 6 h of exposure with clay samples. The untreated cell culture was used as a control. Cells were collected by centrifugation at 5000 rpm for 5 min, the pellet was washed 3 times with 0.1 M PBS (pH 7.0), then fixed with 2.5% glutaraldehyde at 4°C for 6 h. Cells were centrifuged again with fixative removed, then left to clot using animal serum. Clotted sample were thinly diced, then fixed with 2.5% glutaraldehyde at 4°C for 2 h. The samples were washed with 0.1M sodium cacodylate buffer for 10min, repeated for 3 times. The pellets were then postfixed with 1% osmium tetroxide for 2 hours at 4°C, then washed again with 0.1 M sodium cacodylate buffer for 10 min, and repeated for 3 times. A series of acetone were prepared for dehydration. The pellets were dehydrated using 35, 50, 75, 95 and 100% acetone. The samples were infiltrated using a mixture of acetone and resin, embedded into beam capsules, then polymerized in oven at 60°C for 48 h. Ultrathin sections were obtained using an ultramicrotome and then mounted on copper grids. The morphology of the cells was observed by TEM (JEM-2100F, JEOL, Japan).

3.8.2 Leakage of cellular DNA

Leakage of cellular DNA was determined following method by Panicker et al. (2020). The bacteria were grown in NB and incubated at 37°C. The logarithmic growth phase cells of bacteria were treated with clay samples (leachate and suspension) at 1x MIC, 2x MIC and 4x MIC, while the bacteria samples with no treatment was used as a control. The samples were continuously mixed in an orbital shaker (120 rpm, 37°C). For *S. Typhimurium*, samples were incubated for 0, 0.5, 1, 1.5 and 2 h, while for *S. aureus*, incubation was done for 0, 6, 8, 10 and 24 h. An amount of cell cultures was collected at each time interval, and then centrifuged at 10,000 rpm for 10 min. The amounts of cellular constituents released from the cytoplasm was determined by measuring the optical density of the supernatant at 260 nm using multimode microplate reader (Tecan Spark, Switzerland).

3.8.3 Leakage of protein (Bradford assay)

Leakage of cellular protein was determined following method by Siddique et al. (2020). The bacteria were grown in NB and incubated at 37°C. The logarithmic growth phase cells of bacteria were treated with clay samples (leachate and suspension) at 1x MIC, 2x MIC and 4x MIC, while the bacteria samples with no treatment was used as a control. The samples were continuously mixed in an orbital shaker (120 rpm, 37°C). For *S. Typhimurium*, samples were incubated for 0, 0.5, 1, 1.5 and 2 h, while for *S. aureus*, incubation was done for 0, 6, 8, 10 and 24 h. An amount of cell cultures was collected at each time interval, and then centrifuged at 10,000 rpm for 10 min.

The supernatant was added into Bradford reagent, incubated for 15 min, and then the optical density was measured at 595nm using spectrophotometer (Thermo Scientific Genesys 20, USA). A standard graph was developed using bovine serum albumin (BSA), which was prepared in a dilution series of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. A calibration curve of BSA was developed based on the standard absorption reading using spectrophotometer. The bacterial cellular protein leakage was calculated from the calibration curve and expressed as mg/mL.

3.8.4 Changes in membrane potential

The changes in membrane potential of the two bacterial strains were tested with and without treatment (control). In this assay, bis-(1,3-dibutylbarbituric acid) trimethine oxonol DiBAC₄ (3) was used as a membrane potential-sensitive fluorescent probe. For treated samples, the bacteria were incubated with 1 MIC, 2 MIC and 4 MIC of the clay samples in both leachate and suspension forms, continuously mixed in an orbital shaker (120 rpm, 37°C). For *S. Typhimurium*, samples were incubated for 0.5 h, while for *S. aureus*, incubation was done for 6 h. 0.5 mM DiBAC₄ (3) solution was prepared following protocol by Lacombe et al. (2013), and 100 µL of stain was added to 100 µL of treated samples in a black 96-well plate, incubated at room temperature for 30 min. After incubation, fluorescent intensity was measured at the excitation and emission wavelengths of 492 and 515 nm, respectively, using fluorescence mode in the multimode microplate reader (Tecan Spark, Switzerland).

3.8.5 Reactive oxygen species (ROS) detection

The production of superoxide anion radicals from the reaction of clay with bacteria were measured using NBT assay following method by Jung et al. (2015). Clay samples (leachate and suspension) were added to bacterial cells at the concentration of 1x MIC, 2x MIC and 4x MIC in the exponential growth phase, and then continuously incubated in an orbital shaker (120 rpm, 37°C, 15 min). The bacteria sample with no treatment was used as a control. Cells were then collected by centrifugation (10,000 rpm, 10 min). The cell pellet was resuspended in 0.5 ml of 1 mg/ml of NBT reagent for 30 min, and 0.1 M HCl was added afterwards to stop the reaction and then the cells were harvested by centrifugation. To solubilize the reduced NBT, dimethyl sulfoxide was added to the cell pellet. Absorbance of the sample was measured at 575nm using spectrophotometer (Thermo Scientific Genesys 20, USA).

3.9 Statistical analysis

All analytical determinations and measurements were performed at least in triplicate. For antibacterial testings, three distinct sets of samples (bacterial cultures, growth media) were prepared and the analysis were performed independently. The mean was calculated by analysis of variance (ANOVA) using Minitab 18 Software. Significant differences between means were determined by the Tukey test. The level of significance for all statistical analyses was 5%.

Principle component analysis (PCA) was used to define the correlation between variables. Pearson correlation determination was followed taking into consideration that the data were normally distributed. The correlation coefficient (R) was as follows: $R < |0|$ = no correlation, $|0| < R < |0.3|$ = weak correlation, $|0.3| < R < |0.7|$ = moderate correlation and $|0.7| < R < |1.0|$ = strong correlation. The correlation test was conducted using XLSTAT-Pro (2017) statistical software (Addinsoft, Paris, France).



CHAPTER 4

RESULTS AND DISCUSSION

4.1 Selection of soil samples

4.1.1 Sampling detail

The details of 13 soils that have been sampled and on-site assessments, coordinates and soil characteristics are shown in Table 4.1. The 13 soils were the representative of different types of mineral soils. Batu Anam, Munchong, Musang, Prang and Serdang were under sedentary soils group, developed from sedimentary and low-grade metamorphic rocks, while Melaka was reworked soils on pediments. Carey and Pulau Indah are acid sulfate soils, while Bernam and Serkat are non-acid sulfate, and all these 4 types of soils were developed over marine alluvium (Paramanathan, 2000). Out of the 13 soils, 8 (61.54 %) have moist consistency, while another 5 (38.46 %) which were the marine alluvial soils, have wet and sticky consistency.

Musang soils were sampled from different sites to compare the characteristics and potential antibacterial activity of the middle (Musang 1) and bottom slopes (Musang 2). They showed slightly different characteristic, with the mid-sloped sample having more brownish colour than the bottom-sloped soils. Similarly, Pulau Indah soils were collected at different sites since the area was vast. Compared to Pulau Indah 1, Pulau Indah 2 soils had less disturbance from human activities, as the sampling site was far from main road and had stronger

Table 4.1: Soil sampling details

Sample name	Sampling point	Coordinate	Elevation (m) ¹	Surrounding	Characteristics	Soil colour ²
Batu Anam	Kawasan unit sayuran organik (SALM), UPM	N 02° 59' 13.5"	51	Undisturbed soil, surrounded by experimental organic farm.	Soil with small roots (roots <10%), non-friable, moist consistency	Yellowish brown (10 YR 5/8)
		E 101° 42' 26.2" (±3 m)				
Melaka	Ladang Penyelidikan TPU, UPM	N 02° 58' 51.3"	52	Undisturbed soil, surrounded by experimental farm, near papaya plantation.	Soil with medium to small roots (roots <25%), non-friable, moist consistency	Yellowish brown (10 YR 5/6)
		E 101° 42' 43.5" (±3 m)				
Munchong	Ladang Sains Pertanian 10B, Fakulti Pengajian Pendidikan UPM.	N 02° 59' 24.9"	55	Undisturbed soil, surrounded by experimental farm of which the soil has been rotorvated.	Soil with very few roots (roots <5%), non-friable, moist consistency	Strong brown (7.5 YR 5/6)
		E 101° 43' 02.7" (±3 m)				
Bernam	Sabak Bernam, Selangor	N 03° 47' 04.0"	9	Undisturbed soil, drainage area, near Sg. Perak and rice fields irrigation. Surrounded by Nira and Sago palm trees.	Coastal clay, wet and sticky consistency, very few roots (roots <5%)	Dark gray (7.5 YR 4/0)
		E 100° 59' 09.7" (±3 m)				
Ladang	Ladang UPM 15	N 02° 59' 20.6"	73	Undisturbed soil, surrounded by secondary shrub, near vegetation area	Soil from igneous rock, moist consistency, very few roots (<5%)	Brownish (10 YR 4/3)
		E 101° 44' 16.5" (±5 m)				

Table 4.1: Continued

Sample name	Sampling point	Coordinate	Elevation (m) ¹	Surrounding	Characteristics	Soil colour ²
Musang 1	Bukit Ekspo, UPM	N 02° 59' 07.3" E 101° 42' 28.9" (±6 m)	65	6 – 10° middle-sloped, surrounded by natural vegetation. Has some disturbance from human activity (waste disposal). Location is near to MAEPS Serdang.	Soil from igneous rock, stony, presence of mottles, moist consistency, medium to small roots (roots <25%)	Strong brown (7.5 YR 5/8)
Musang 2	Bukit Ekspo, UPM	N 02° 59' 06.7" E 101° 42' 29.0" (±6 m)	65	6 – 10° bottom-sloped, surrounded by natural vegetation. Has some disturbance from human activity (waste disposal). Location is near to MAEPS Serdang.	Soil from igneous rock, stony, presence of mottles, moist consistency, medium to small roots (roots <25%)	Yellowish brown (10 YR 5/6)
Serdang	Hospital UPM	N 02° 58' 48.2" E 101° 42' 44.8" (±3 m)	56	Undulating/rotorvated soil, surrounded by shrub (Zoysia matrala)	Soil with medium to small roots (roots<25%), non-friable, moist consistency	Strong brown (7.5 YR 5/8)
Pulau Indah 1	Pulau Indah	N 02° 57' 02.5" E 101° 21' 22.8" (±7 m)	15	Swamp area with poor drainage, surrounded by mangroves, small trees and shrub. Low to moderate disturbance due to location that is nearer to main road.	Soil with large to medium roots (<25%). Sulfuric marine soil, wet and sticky consistency.	Dark yellowish brown (10 YR 4/3)

Table 4.1: Continued

Sample name	Sampling point	Coordinate	Elevation (m) ¹	Surrounding	Characteristics	Soil colour ²
Pulau Indah 2	Pulau Indah	N 02° 55' 53.7" E 101° 20' 48.3" (±10 m)	21	Swamp area with poor drainage, surrounded by mangroves, small trees and shrub. Low to moderate disturbance.	Soil with large to medium roots (<25%). Sulfuric marine soil, wet and sticky consistency.	Dark yellowish brown (10 YR 4/3)
Carey	Pulau Carey	N 02° 50' 45.4" E 101° 22' 26.8" (±3 m)	11	Near palm oil plantation with moderate to high disturbance.	Soil with very few roots (roots <5%). Acid sulphate soil. Brackish, wet and sticky consistency.	Yellowish red (5 YR 5/8)
Serkat	Sg. Sedu, Banting	N 02° 52' 39.5" E 101° 32' 29.9" (±3 m)	49	Near palm oil plantation, beside highway, moderate disturbance	Soil with very few roots (roots <5%). Sulfuric marine soil, wet and sticky consistency.	Light gray (10 YR 7/1)
Prang	Sepang	N 02° 47' 03.1" E 101° 42' 26.4" (±3 m)	11	Near palm oil plantation, beside highway, moderate disturbance	Soil with medium to small roots (roots <25%). Clay oxic, moist consistency. Has low clay content, high iron content.	Yellowish red (5 YR 4/6)

¹ Elevation refers to vertical distance or height of the sampling location relative to the mean sea level.

² Soil colour inspected on-site and determined using Munsell colour chart based on hue, value and chroma (Appendix B3).

rotten eggs smell, reflecting the higher content of sulfidic materials accumulated in the soil (Paramananthan & Daud, 1986).

Soil colour, inspected using Munsell Chart as part of on-site assessment showed variations, from yellow, red, brown to gray, with hues ranging from 5 to 10 (Table 4.1). The classification of soil colour was based on three attributes which are hue, value and chroma. Batu Anam was read as 10 YR 5/8, of which 10 YR represent the hue (yellow-red scale), 5 was the value (medium lightness) and 8 was the chroma index (high saturation) (Department of Agriculture Malaysia, 2015). Although antibacterial activity of a soil is rather complex to be determined through physical inspection alone, initial assessment through soil colour can be useful indicator of some of the general properties of a soil. Most mineral grains are gray in colour. The natural colour of soil is mostly contributed by the presence of organic matter and iron. Darker colour indicates the abundance in organic matter in a soil, while a range of soil colours, from yellowish-brown to red are dependent on the oxidation state of iron. Majority of the samples had brownish in hue, with different values and chroma indicating the different level and iron oxide formation as part of the soil component. Meanwhile, yellowish red colour of Carey and Prang may indicate a high level of iron and aluminium oxides in the soils, hence could potentially show antibacterial activity (Morrison et al., 2016). Colour analysis of Jordanian soils showed the majority of the collected soils for antibacterial activity screening have the range of 5 YR to 7.5 YR 4/4, which were attributed to the clay minerals and oxides present the soils (Ibbini et al., 2018).

4.1.2 Soil physicochemical analysis

The results of soil physicochemical analysis (moisture content, particle size analysis, pH and cation-exchange capacity) are shown in Table 4.2. Details on soil processing is shown in Appendix A1.

Moisture content determination by gravimetric method revealed the percentage of water in the soil mass of the soil samples. A total of 8 out of 13 samples (61.54%) were found to be moist soils with moisture content in the range of 23.55% to 34.80%, while the remaining 5 samples (38.46%) were wet soils with moisture content of 44.64% to 103.40%.

Particle size distribution describes the relative amounts of sand, silt and clay within soil. Soil properties are largely affected by the percentage of each component. Out of 13 soil samples, 8 (61.54%) have clayey texture with high content of clay fraction, ranging from 45.49% to 78.11%, which are Batu Anam, Bernam, Melaka, Munchong, Serkat, Musang 1, Musang 2 and Ladang. The other 5 soils which are Carey, Pulau Indah 1, Pulau Indah 2, Prang and Serdang were classified as silty clay loam, silt loam, sandy loam, sandy clay loam and sandy clay, respectively, with clay content < 40%.

Table 4.2: Soil physicochemical analysis

Soil Name	pH		Moisture content (%)	Particle size analysis			CEC (cmol(+)/kg) ²
	Fresh	Dried		Clay %	Silt %	Sand % Texture class ¹	
Batu Anam	6.82 ± 0.07 ^a	5.40 ± 0.04 ^b	23.55 ± 0.04	77.60 ± 1.15 ^a	8.51 ± 0.42 ^c	13.96 ± 0.69 ^b Clay	8.64 ± 0.12
Bernam	6.82 ± 0.11 ^a	4.74 ± 0.18 ^b	103.40 ± 0.23	61.89 ± 0.01 ^a	37.32 ± 0.06 ^b	0.79 ± 0.06 ^c Clay	15.83 ± 0.08
Carey	3.02 ± 0.04 ^b	4.01 ± 0.06 ^a	44.64 ± 0.24	34.62 ± 0.37 ^b	50.28 ± 0.15 ^a	15.11 ± 0.52 ^c Silty clay loam	9.67 ± 0.16
Melaka	5.75 ± 0.06 ^a	5.22 ± 0.02 ^b	27.32 ± 1.05	52.40 ± 0.45 ^a	7.56 ± 0.15 ^c	39.96 ± 0.30 ^b Clay	6.89 ± 0.24
Munchong	4.83 ± 0.04 ^a	4.78 ± 0.02 ^a	34.80 ± 0.46	78.11 ± 0.08 ^a	9.03 ± 0.14 ^c	12.85 ± 0.23 ^b Clay	7.11 ± 0.08
Serkat	5.45 ± 0.11 ^a	5.14 ± 0.03 ^b	97.81 ± 3.40	58.08 ± 0.19 ^a	38.53 ± 0.63 ^b	3.25 ± 0.30 ^c Clay	17.44 ± 0.71

Table 4.2: Continued

Soil Name	pH		Moisture content (%)	Particle size analysis			CEC (cmol(+)/kg) ²	
	Fresh	Dried		Clay %	Silt %	Sand %		Texture class ¹
Musang 1	4.15 ± 0.06 ^b	4.69 ± 0.09 ^a	32.54 ± 1.69	56.86 ± 0.72 ^a	13.41 ± 0.02 ^c	29.73 ± 0.71 ^b	Clay	6.61 ± 0.47
Musang 2	5.25 ± 0.05 ^b	5.41 ± 0.01 ^a	33.70 ± 1.03	45.49 ± 0.84 ^a	11.64 ± 0.20 ^c	42.88 ± 0.65 ^b	Clay	7.97 ± 0.68
Pulau Indah 1	6.17 ± 0.02 ^a	4.50 ± 0.02 ^b	61.70 ± 0.93	16.48 ± 0.34 ^b	73.30 ± 1.32 ^a	10.21 ± 1.66 ^c	Silt loam	9.31 ± 3.10
Pulau Indah 2	6.24 ± 0.02 ^a	5.73 ± 0.03 ^b	63.10 ± 0.74	16.79 ± 0.34 ^c	27.01 ± 1.32 ^b	56.20 ± 1.66 ^a	Sandy loam	5.32 ± 0.16
Prang	5.22 ± 0.16 ^a	4.73 ± 0.05 ^b	23.79 ± 2.75	27.18 ± 0.22 ^b	18.54 ± 0.55 ^c	54.28 ± 0.77 ^a	Sandy clay loam	6.68 ± 2.38
Serdang	5.41 ± 0.09 ^a	4.83 ± 0.04 ^b	27.17 ± 0.94	36.05 ± 0.08 ^b	9.96 ± 0.09 ^c	54.00 ± 0.17 ^a	Sandy clay	8.83 ± 0.79

Table 4.2: Continued

Soil Name	pH		Moisture content (%)	Particle size analysis			CEC (cmol(+)/kg) ²
	Fresh	Dried		Clay %	Silt %	Sand %	
Ladang	4.91 ±	4.44 ±	32.50 ± 0.61	59.23 ±	7.26 ±	33.51 ±	11.08 ± 0.90
	0.06 ^a	0.11 ^b		0.13 ^a	0.17 ^c	0.05 ^b	

¹ Texture classification of the soil particle are referenced from the USDA manual (USDA, 1987).

² Low: 6 – 12 cmol(+)/kg; moderate: 12 – 25 cmol(+)/kg (Hazelton & Murphy, 2007).

Pairs of values with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

pH of all soil samples, in both fresh and dried form were all acidic ($< \text{pH } 7$). Carey soils showed lowest pH values (most acidic) which were 3.02 ± 0.04 and 4.01 ± 0.06 for fresh and dried samples, respectively, while the fresh Batu Anam and Bernam samples showed the highest pH values (pH 6.82). Their pH values both decreased in their dried form, which were 5.40 ± 0.04 for Batu Anam and 4.74 ± 0.18 for Bernam, respectively.

The cation-exchange capacity (CEC) values of the 13 soils range from 5.32 to 17.44 $\text{cmol}(+)/\text{kg}$. According to classification by Hazelton and Murphy (2007), only Bernam and Serkat have moderate CEC values (15.83 and 17.44 $\text{cmol}(+)/\text{kg}$, respectively). The rest of the soil samples were categorised as having low CEC values, with the highest was Ladang, followed by Carey, Pulau Indah 1 and Serdang (11.08 ± 0.90 , 9.67 ± 0.16 , 9.31 ± 3.10 and 8.83 ± 0.79 $\text{cmol}(+)/\text{kg}$, respectively).

Moisture content describes wetness of soil samples. The wet soil samples, which are Bernam, Carey, Serkat, Pulau Indah 1 and Pulau Indah 2 were of clayey sulfidic nature, which can be of marine, brackish water or estuarine origin (Paramanathan, 2000). The moisture content of Bernam soils were the highest among other samples (103.40 ± 0.23) which reflects the water-logged condition of the sampling site, of which the location is near to coastal area (see Table 4.1). Seven soils collected within UPM area were all moist samples, with different types of soils reported around the region due to the complex geographical distribution of soils (Shamshuddin & Darus, 1979).

The amount of clay present in the soil is an important characteristic in the selection of soil for antibacterial purposes. The fine particle size of clays (< 200 nm) play crucial role in promoting metal dissolution, while the coarser fraction of soil are not antibacterial (Williams et al., 2008). Clays have a net negative surface charge, allowing the attachment of ions and molecules to the edges or interlayer surface. In hydrated environments, the adsorbed metal cations can be released into the surrounding medium, which is reported to be responsible antibacterial activity (Williams & Haydel, 2010). Hence, the greater amount of the fine clay particles could provide enhanced activity in antibacterial assays by their increased interaction with water, which could lead to mineral dissolution and releasing of exchangeable metals upon hydration (Williams, 2017).

Cunningham et al. (2010) and Williams (2017) reported the importance of pH as a crucial parameter in preliminary assessment of antibacterial activity, as an acidic environment of hydrated clay minerals contributes to antibacterial efficacy by increasing the availability and toxicity of metal ions.

The high CEC of different clay minerals has been targeted in the creation of inorganic antibacterial materials. Synthetic antibacterial clay minerals are created by exchanging known antibacterial ions such as Ag, Cu, Zn and Fe (Ma et al., 2021; Saad et al., 2016; Su et al., 2011; Suzuki et al., 2021) with the native ions of kaolinite and montmorillonite. The gradually release of novel exchanged ions from the synthetic clay provides long-term antibacterial effectiveness.

4.2 Preliminary screening of untreated soil leachates with antibacterial activity

4.2.1 Preparation of untreated soil leachates

The properties of leachates P1, P2 and P3, prepared from soil samples and their corresponding pH values is shown in Table 4.3. All the clay leachate samples showed acidic pH values, with Carey P3 having the lowest value (2.81 ± 0.10). Overall, the different preparation methods had effect on leachate pH except for Batu Anam. Out of the 13 soil samples, Carey, Munchong, Musang 1, Pulau Indah 2, Prang and Ladang leachates prepared using P3 method showed significantly lowered pH values (more acidic) in comparison to P1 and P2 preparation methods.

A preliminary assessment on the antibacterial activity of leachates found that the sample with lowest pH to be the most antibacterial, and the acidity of the clay leachate could potentially be responsible for its antibacterial activity (Borquaye et al., 2016), with more acidic leachates ($< \text{pH } 4$) were reported to show bactericidal activity (Morrison et al., 2017). In this study, many of leachates, regardless of the preparation methods showed $< \text{pH } 7$, hence it was expected that their antibacterial potential could be reflected by the acidity of the leachates.

Table 4.3: pH of soil leachates prepared using methods P1, P2 and P3

Leachate	P1	P2	P3
Soil concentration	100 mg/mL	500 mg/mL	500 mg/mL
Sonication time	0 min	1 min	1 min
Shaking time	1 h	1 h	6 h
Batu Anam	5.23 ± 0.06 ^a	5.32 ± 0.14 ^a	5.13 ± 0.11 ^a
Bernam	5.42 ± 0.19 ^a	5.15 ± 0.04 ^{ab}	5.14 ± 0.02 ^b
Carey	4.50 ± 0.38 ^a	3.50 ± 0.09 ^b	2.81 ± 0.10 ^c
Melaka	5.71 ± 0.10 ^a	4.84 ± 0.03 ^b	4.79 ± 0.02 ^b
Munchong	5.52 ± 0.04 ^a	4.57 ± 0.05 ^b	3.60 ± 0.18 ^c
Serkat	3.48 ± 0.10 ^a	2.95 ± 0.02 ^b	2.98 ± 0.04 ^b
Musang 1	4.43 ± 0.07 ^a	4.18 ± 0.08 ^b	3.70 ± 0.03 ^c
Musang 2	5.71 ± 0.07 ^a	5.42 ± 0.03 ^b	5.29 ± 0.09 ^b
Pulau Indah 1	6.18 ± 0.03 ^a	4.52 ± 0.04 ^b	4.47 ± 0.06 ^b
Pulau Indah 2	6.26 ± 0.04 ^a	5.72 ± 0.03 ^b	5.55 ± 0.06 ^c
Prang	5.22 ± 0.16 ^a	4.73 ± 0.05 ^b	4.14 ± 0.02 ^c
Serdang	5.42 ± 0.09 ^a	5.31 ± 0.03 ^a	4.89 ± 0.05 ^b
Ladang	6.40 ± 0.13 ^a	5.23 ± 0.04 ^b	4.34 ± 0.02 ^c

Pairs of values with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

4.2.2 Disc diffusion test

Table 4.4 and 4.5 show the inhibition zone of soil leachates P1, P2 and P3 against *Salmonella* Typhimurium and *Staphylococcus aureus*, respectively.

Out of 13 soil samples tested, six (46.15%) showed antibacterial activity against both of the test bacteria for both of the test bacteria, which were Batu Anam, Bernam, Carey, Melaka, Munchong and Serkat.

Table 4.4: Inhibition zone of soil leachates against *S. Typhimurium*

Sample	Inhibition zone (mm)		
	P1	P2	P3
Batu Anam	6.13 ± 0.06 ^{Bc}	6.40 ± 0.17 ^{Ba}	8.83 ± 0.14 ^{Ab}
Bernam	6.30 ± 0.17 ^{Bc}	6.83 ± 0.29 ^{Ba}	8.50 ± 0.50 ^{Ab}
Carey	6.67 ± 0.29 ^{Bbc}	6.83 ± 0.58 ^{Ba}	8.08 ± 0.14 ^{Ab}
Melaka	6.09 ± 0.09 ^{Ac}	7.00 ± 1.14 ^{Aa}	7.83 ± 1.04 ^{Ab}
Munchong	7.39 ± 0.41 ^{Ba}	7.00 ± 0.00 ^{Ba}	11.17 ± 0.76 ^{Aa}
Serkat	7.00 ± 0.00 ^{Bab}	7.33 ± 0.29 ^{Ba}	8.78 ± 0.26 ^{Ab}
Musang 1	n.a	n.a	n.a
Musang 2	n.a	n.a	n.a
Pulau Indah 1	n.a	n.a	n.a
Pulau Indah 2	n.a	n.a	n.a
Prang	n.a	n.a	n.a
Serdang	n.a	n.a	n.a
Ladang	n.a	n.a	n.a

n.a : No activity.

Diameter of inhibition zones in mm (including 6 mm disc).

Pairs of values with different uppercase and lowercase superscripts indicate a significant difference ($p < 0.05$) between columns and rows, respectively.

Positive control with ampicillin (10 µg) produced zones of growth inhibition of 30.69 ± 0.96 mm.

Soil leachates P1 and P2 showed little inhibition zone, with diameter between 6.09 to 7.39 mm. Meanwhile, the soil leachates prepared using method P3, with an extended hydration shaking period, showed the greatest antibacterial efficacy against *S. Typhimurium* relative to the P1 and P2 leachate preparation methods. The P3 soil leachates from Munchong proved to be the most effective against *S. Typhimurium* (11.17 ± 0.76 mm) with a significantly increased growth inhibition zone than P1 and P2. P3 soil leachates from all samples except Melaka showed robust antibacterial activity, with the antibacterial efficacy was evidently enhanced with P3 relative to P1 and P2.

The clay leachates also displayed some antibacterial activity against *S. aureus* (Table 4.2), as judged by the diameter of zones of inhibition showed by leachates P1, P2 and P3, which was between 6.30 to 7.90 mm. The Carey soil leachates prepared by method P3 showed significantly improved antibacterial activity compared with methods P1 and P2, with improved efficacy over the other five clays.

Table 4.5: Inhibition zone of soil leachates against *S. aureus*

Sample	Inhibition zone (mm)		
	P1	P2	P3
Batu Anam	6.40 ± 0.17 ^{Aa}	6.40 ± 0.17 ^{Ab}	6.40 ± 0.17 ^{Ab}
Bernam	6.40 ± 0.17 ^{Aa}	6.30 ± 0.17 ^{Ab}	6.30 ± 0.17 ^{Ab}
Carey	6.30 ± 0.17 ^{Ca}	7.17 ± 0.29 ^{Ba}	7.90 ± 0.17 ^{Aa}
Melaka	6.50 ± 0.00 ^{Aa}	6.40 ± 0.17 ^{Ab}	6.30 ± 0.17 ^{Ab}
Munchong	6.33 ± 0.12 ^{Aa}	6.33 ± 0.12 ^{Ab}	6.33 ± 0.12 ^{Ab}
Serkat	6.40 ± 0.17 ^{Aa}	6.70 ± 0.17 ^{Aab}	6.30 ± 0.17 ^{Ab}
Musang 1	n.a	n.a	n.a
Musang 2	n.a	n.a	n.a
Pulau Indah 1	n.a	n.a	n.a
Pulau Indah 2	n.a	n.a	n.a
Prang	n.a	n.a	n.a
Serdang	n.a	n.a	n.a
Ladang	n.a	n.a	n.a

n.a : No activity.

Diameter of inhibition zones in mm (including 6 mm disc).

Pairs of values with different uppercase and lowercase superscripts indicate a significant difference ($p < 0.05$) between columns and rows, respectively.

Positive control with ciprofloxacin (5 µg) produced zones of growth inhibition of 26.35 ± 0.67 mm.

Previous studies generally used 50 – 200 mg/mL of clay-water mixture (see Section 2.7.2). Results from preliminary studies indicated that the samples used in this study showed low to no inhibition. This suggests the difference in the antibacterial activity in comparison to the past studies which could be to the mineralogy and geochemical composition. Therefore, different leachate preparation methods were adapted and compared to determine their effect on the pH and bacterial growth inhibition. The disc diffusion testing showed that the acidic condition of the 13 soil leachates was not effective enough to inhibit the bacterial growth by itself. The antibacterial activity of Batu Anam and Bernam clay soils (pH > 5) against *Salmonella* Typhimurium was not significantly different from Carey and Serkat (pH < 3) or Melaka (pH 5) (see Table 4.3). Only the Munchong clay leachate with a pH 3.60 produced a significantly greater zone of inhibition (11.17 ± 0.76 mm) when tested against the gram-negative *Salmonella* Typhimurium. In contrast to the gram-negative bacterial screening, the Carey leachate showed the greatest activity (7.90 ± 0.17 mm) against the gram-positive *S. aureus*. The remaining soil leachates with differing levels of acidity were less effective at inhibiting bacterial growth.

In general, the leachates showed larger zones of inhibition with *Salmonella* Typhimurium relative to *S. aureus*, indicating that gram-negative bacteria are more susceptible, which is in agreement with previous study by Borquaye et al. (2016) who reported larger zone of inhibition of Ghanaian Sogakope clay against *S. typhi* than *S. aureus*, which was 14 mm and 8 mm, respectively. The interpretation of the diameter of the inhibition zone has been established for antibiotics, but there has been no standard for clay soils. Based on the

preliminary results, the leachate samples showed moderate sensitivity (range of 6.09 – 11.17 mm zone). Testing against the positive controls (ampicillin against *S. Typhimurium* and ciprofloxacin against *S. aureus*) indicate that both test bacteria were susceptible (ampicillin >17, ciprofloxacin >21) (CLSI, 2016). The 6 samples showed much smaller diameter of inhibition zones in comparison to past studies (Borquaye et al., 2016; Morrison et al., 2017; Mpuchane et al., 2010) which suggests that locally-sourced soils samples recovered through untreated (sieving) were not effective against the test bacteria. Pure kaolinite leachates were also tested for comparison and the results showed no inhibition when tested against both *S. Typhimurium* and *S. aureus* (Appendix A2).

In comparison to other clay samples, both Munchong and Carey had low CEC values (Table 4.2) and acidic pH (Table 4.3). Soils with acidic pH have been targeted for their potential antibacterial activity, as Williams (2017) stated that pH may assist in assessing antibacterial potential of soils, as more metals are soluble at $\text{pH} < 5$. Although Bernam and Serkat had greater CEC values in comparison to Munchong and Carey (see Table 4.2), that does not reflect their antibacterial potential. Since the clay fraction recovered in this preliminary screening is through sieving, the CEC values could be influenced from the traces amount of organic matter (Tan, 2011) present in the fraction, which have not been removed using this procedure and not possible to be separated by sieving. However, it was revealed that organic matter does not have influence on antibacterial activity (Haydel et al., 2008). Greater CEC values could be obtained with clay recovered through sedimentation method, which might give

better reflection of antibacterial potential of the clay samples since organic matter and coarser soil components are removed, producing only pure, fine clay fraction. The antibacterial effectiveness of clay soils recovered from both sieving and sedimentation will be discussed in Section 4.3.

Munchong has clayey texture, with clay content of 78.11 ± 0.08 % (see Table 4.2). The low CEC value of Munchong indicated that the soils were highly leached, along with the detection of 1:1 clay minerals in the soil (kaolinite, gibbsite and goethite), which showed the sign of extreme weathering (Shamshuddin & Darus, 1979). Munchong soil is derived from iron-rich parent materials, which possibly released into the leachate mixture and could be responsible in causing significant growth inhibition to *S. Typhimurium*. Carey soil is an acid sulfate soil, which is characterised by presence of pyrite, acidic pH and high quantities of Al and Fe, which could either be in the solution or on the exchange complex (Shamshuddin, 2006). Antibacterial activity of Carey could be associated with metals Al and Fe, which were found to cause damage to bacterial cell wall and intracellular proteins through production of hydroxyl radicals (Morrison et al., 2016).

4.2.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC results for all 13 untreated soils leachates prepared using P1, P2 and P3 methods were shown in Table 4.6. All 6 soils previously showing antibacterial activity in disc diffusion test does not show MIC and MBC values, indicating the samples does not show decent antibacterial activity.

The minimum inhibitory concentration (MIC) is used to guide the selection of soil for further analysis. However, none of the soil leachates tested showed activity for MIC and MBC value, which was not consistent with the disc diffusion results (Section 4.2.2). This could be due to the different concentration used in both of the analysis. The initial stock solution of soil leachates was prepared using P1, P2 and P3 methods, which were 100 mg/mL, 500 mg/mL and 500 mg/mL, respectively. The diameter of inhibition zones was rather little even when tested at high concentration (500 mg/mL). MIC MBC assay requires microdilution procedure, where the soil leachates stock solution undergoes a series of 2-fold serial dilutions of the active ingredient are prepared in broth (growth medium). The first column employed soil leachates with the concentration of 50 mg/mL for P1 and 250 mg/mL for P2 and P3. This resulted in lesser concentrations of the active ingredients in the soil leachates in comparison the the initial concentration used in disc diffusion assay, which contributes to the no activity when tested for MIC and MBC.

Table 4.6: MIC and MBC of soil leachates P1, P2 and P3 against *S. Typhimurium* and *S. aureus*

Sample	Leachate preparation	<i>S. Typhimurium</i>		<i>S. aureus</i>	
		MIC	MBC	MIC	MBC
		(mg/mL)	(mg/mL)	(mg/mL)	(mg/mL)
Batu Anam	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Bernam	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Carey	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Melaka	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Munchong	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Serkat	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Musang 1	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Musang 2	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Pulau Indah 1	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a

Table 4.6: Continued

Sample	Leachate preparation	<i>S. Typhimurium</i>		<i>S. aureus</i>	
		MIC	MBC	MIC	MBC
		(mg/mL)	(mg/mL)	(mg/mL)	(mg/mL)
Pulau Indah 2	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Prang	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Serdang	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a
Ladang	P1	n.a	n.a	n.a	n.a
	P2	n.a	n.a	n.a	n.a
	P3	n.a	n.a	n.a	n.a

n.a : No activity

Following this outcome, according to the disc diffusion result (Tables 4.4 and 4.5), Munchong and Carey samples which previously showed the most effective antibacterial activity against *S. Typhimurium* and *S. aureus*, respectively, were selected for further antibacterial testing as described in Section 4.3. The variation found in these initial experiments may indicate that the soil preparation method and assay by disc diffusion have some limitations as means of determining the antibacterial efficacy of different types of soil samples. Sieving served as a simple approach to collect and rapidly prepare the 13 soil samples. The leachates were prepared by the sieving method, in which larger grains of sand and silt, having particle sizes of 50–500 μm and 2–

50 µm respectively, are likely to be retained. It is possible that aggregation of the clay particles and inconsistent distribution of the clay fraction could negatively affect their antibacterial activities.

4.2.4 Correlation test and principal component analysis (PCA)

4.2.4.1 Effect of soil leachates preparation variables with pH and antibacterial activity

The correlation between variables contributing to the *S. Typhimurium* and *S. aureus* growth inhibition is shown in Table 4.7. The results showed that shaking time and growth inhibition zone of *S. Typhimurium* showed a strong positive correlation, with $r = 0.775$ and significant at 99% confidence level ($p < 0.01$). Soil concentration and sonication treatment had a moderate positive correlation with *S. Typhimurium* growth inhibition, while leachate pH and growth inhibition zone display a moderate negative correlation.

Soil concentration, sonication treatment and sonication treatment showed weak positive correlation with *S. aureus* growth inhibition, while leachate pH produced a negative moderate correlation with the inhibition zones of *S. aureus*. This result indicates that the antibacterial activity of each of the soil leachates against *S. aureus* was not significantly affected by any of the leachate preparation variables. The effect of different preparation conditions on the antibacterial activity of soil leachates was particularly apparent with *S. Typhimurium*, with increased length of time of shaking of the soil-water suspension correlating with improved antibacterial effects.

Table 4.7: Correlation between soil leachate preparation variables with the corresponding diameter of *S. Typhimurium* and *S. aureus* inhibition zone and leachate pH of the 6 antibacterial soil samples

Variables	Soil concentration	Sonication treatment	Shaking time	Inhibition Zone (ST)	Inhibition Zone (SA)	pH
Soil concentration	1					
Sonication treatment	1.000**	1				
Shaking time	0.500**	0.500**	1			
Inhibition Zone (ST)	0.471**	0.471**	0.775**	1		
Inhibition Zone (SA)	0.204	0.204	0.135	0.055	1	
pH	-0.366**	-0.366**	-0.298*	-0.404**	-0.523**	1

Data used for this analysis were derived from 6 soil samples which showed antibacterial activity (see Table 4.4 and Table 4.5)

*Correlation is significant at the 0.05 level (two-tailed).

**Correlation is significant at the 0.01 level (two-tailed).

The Pearson correlation matrix showing strength of relationship between variables ($r < |0|$ = no correlation, $|0| < r < |0.3|$ = weak correlation, $|0.3| < r < |0.7|$ = moderate correlation and $|0.7| < r < |1.0|$ = strong correlation).

ST: *S. Typhimurium*.

SA: *S. aureus*.

The Munchong soil leachate prepared by the P3 method was the most effective at inhibiting *S. Typhimurium* growth. In contrast, the leachates produced by any of the three methods tended to be ineffective at restricting *S. aureus* growth; although, increased clay concentration and sonication of the Carey soil did enhance antibacterial efficacy. The difference in correlation values for each of the tested bacterial species do suggest that the clay leachates selectively prevent the growth of gram-negative (*S. Typhimurium*) over gram-positive (*S. aureus*) bacteria. This difference in susceptibility between gram-negative and gram-positive species has been observed with clay mineral leachates previously. Otto et al. (2010) reported a rapid bactericidal activity on the gram-negative *Escherichia coli*, whereas a methicillin-resistant *S. aureus* strain was killed at a slower rate. This is likely to be due to differences in the cell envelope architectures of these bacteria, with the outer membrane of gram-negatives, absent from gram-positives, representing a permeability barrier to toxic leachate components.

It is important to note the differences in the concentration of sample material used in experiments since no clay deposit is precisely the same with respect to its mineralogy and chemistry. Two clay concentrations (100 mg/mL and 500 mg/mL) were assessed following a study which reported that the antibacterial efficacy of African clay filtrates was concentration-dependent (Mpuchane et al., 2010). In contrast, the results show that soil concentration had only a moderate correlation with inhibition of *S. Typhimurium* growth and a weak correlation with *S. aureus*. This highlights the importance of the leachate preparation method in altering the solution chemistry, or alternatively, that the

quantities of specific antibacterial ions can be released by different clay types, rather than simply the abundance of material in a particular volume of water.

Shaking of the soil-water mixture was initially performed for a short time interval (1 h) compared to 18-24 h (Morrison et al., 2016; Williams et al., 2011), the latter purposefully mimicking the overnight application of a clay poultice applied to the skin for healing of infected wounds (Williams, 2017). The shaking of the soil-water mixture promotes soil-water interaction, therefore promotes cation exchange from the negatively-charged clay surface to the surrounding aqueous environment, releasing the critical antibacterial component from mineral interlayers. Beside reduced Fe^{2+} and Al^{3+} (Morrison et al., 2016), Cu^{2+} and Fe^{2+} were identified as the active antibacterial component (Otto & Haydel, 2013a), responsible for damaging bacterial membranes. The results show that increased hydration shaking time from 1 h to 6 h does result in improved release of active substances that are potent at inhibiting the growth of *S. Typhimurium*.

Sonication treatment was employed to promote the dispersion of fine clay particles in water, disrupting weak Van der Waal forces that attract and loosely bind clay minerals together, thereby encourage metal ion diffusion (Morrison et al., 2017). It has been reported that sonication treatment increases the specific surface area of the mineral (Čavajda et al., 2015; Roscoe et al., 2000; Yang et al., 2009). However, this result suggests that sonication treatment alone has no significant influence on the release of antibacterial components from the clays tested here. This could be due to the use of soil instead of clay,

which might need longer time for sonication for improved clay value after dispersion (Edwards & Bremner, 1967).

It was initially anticipated that a more acidic leachate would prove to be superior in antibacterial activity, as metal ions become more bioavailable and potentially more toxic in a low pH solution (Wang et al., 2009). Although it has been suggested that pH as one of the parameters for screening of soils with antibacterial activity, the moderate correlation with antibacterial activity against both test bacteria (see Table 4.7) suggested that the low pH is not the only factor affecting antibacterial activity of the soil leachates. It is possible that antibacterial activity against *S. aureus* requires the presence of soil, whereby the mineral interlayer of clay acts as pH buffer and promotes dissolution of metal elements over time (Williams, 2017). In this study, the removal of the soil may reduce the availability of metal ions in the leachate which are less effective in penetrating the peptidoglycan layer at the bacterial cell surface. Further investigations could compare the viability of *S. aureus* following overnight incubation with soil-water suspensions in addition to leachates in order to determine the importance of clay in antibacterial efficacy.

4.2.4.2 Selection of samples for further analysis

Biplot graph was constructed to visualise sample grouping of P3 soil leachates based on the correlation with inhibition zones of *S. Typhimurium* and *S. aureus* (Figure 4.1). The biplot exhibited two clusters, Munchong P3 and Carey P3. Munchong P3 was positioned closely with *S. Typhimurium* inhibition zone at the right side of the F1 axes, indicating that the antibacterial activity against

S. Typhimurium was enhanced with Munchong P3 soil leachates. Carey P3 which was positioned with the *S. aureus* inhibition zone at the left side of the F1 axes, indicating that Carey leachate with P3 treatments was the best in inhibiting growth of *S. aureus*.

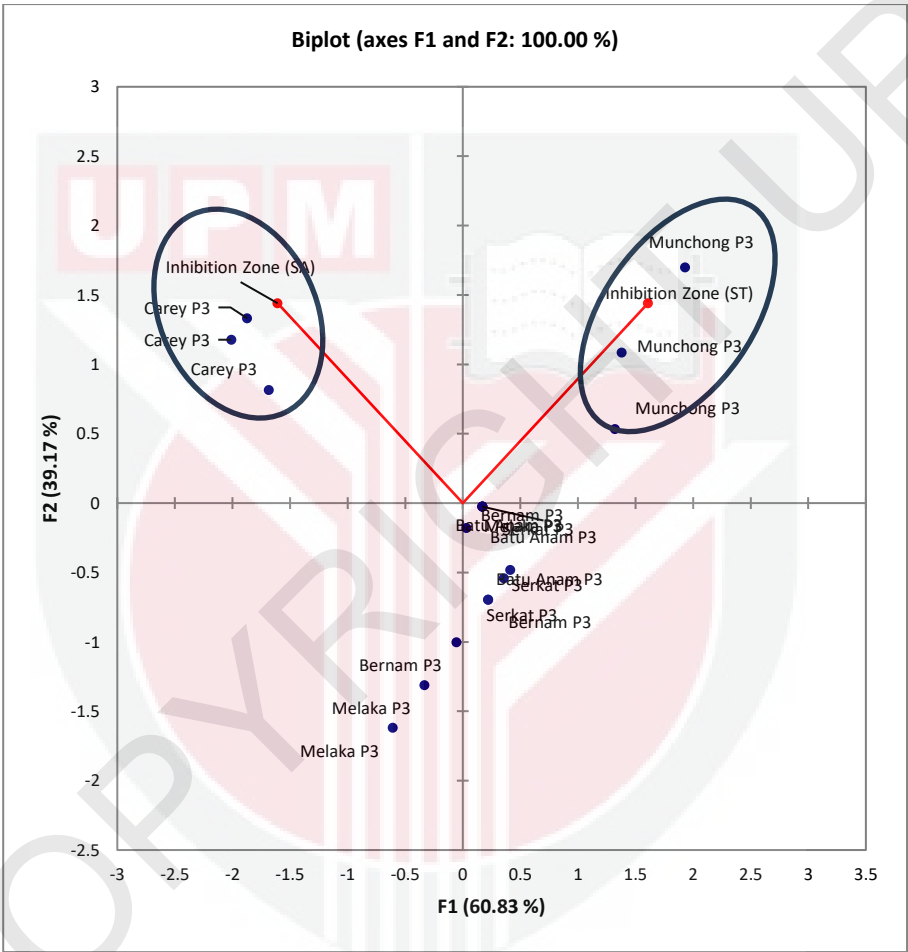


Figure 4.1: Biplot showing grouping of samples with inhibition zones of *S. Typhimurium* (ST) and *S. aureus* (SA).

From these evaluations, Carey and Munchong P3 leachates were exhibited to be the best soil samples to inhibit *S. aureus* and *S. Typhimurium*, respectively. Section 4.3 further discusses on the antibacterial activity of Carey and Munchong soils.

4.3 Antibacterial effect of untreated and treated Carey and Munchong samples

Following result from preliminary screening of soils with antibacterial activity in Section 4.2, Carey and Munchong soils showing the most effective antibacterial activity against *S. aureus* and *S. Typhimurium*, respectively, were investigated for their physical and chemical properties (pH, CEC, metal analysis) and further analysed for their antibacterial activity against *S. Typhimurium* and *S. aureus* by determination of MIC and MBC, followed by bacterial viability assay. The results are discussed in 4.3.1 and 4.3.2.

4.3.1 Soil sample treatment and analysis

Leachates and suspensions were prepared for untreated and treated Carey and Munchong samples as shown in Figure 4.2. The untreated samples, previously used in Section 4.2, were collected by sieving procedure which contains a mixture of sand, silt and clay, while treated samples undergone additional treatments (see Section 3.4.1) to produce pure clay fraction. Both untreated and treated samples were processed into leachate and suspension. Leachates refers to the liquid produced by collecting the supernatant after centrifugation of clay-water mixture, containing dissolved ions and other small soluble compounds leached from the clay. Clay particles are not visible, and the liquid appears clear or coloured, depending on the compounds dissolved in it (Figures 4.2a-d). Suspension consists of clay particles dispersed in water but not dissolved, forming 2 separate phases, which are liquid-solid phase where the particles settle at the bottom over time when left undisturbed (Figures 4.2e-h).

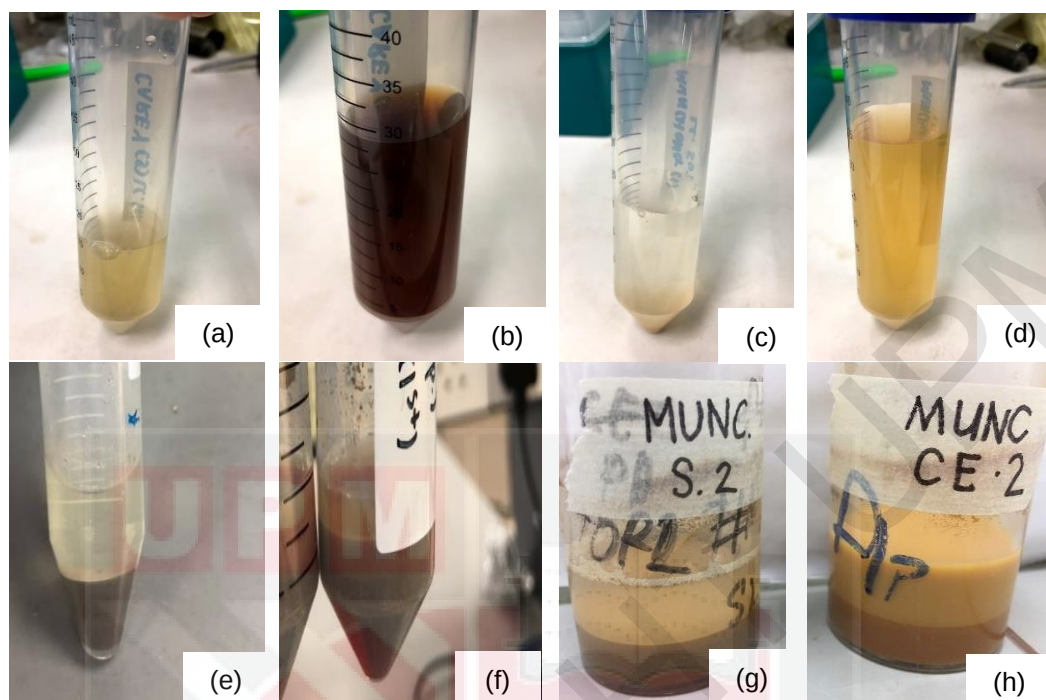


Figure 4.2: Leachates and suspensions prepared from untreated and treated samples a) Untreated Carey leachate; b) Treated Carey leachate; c) Untreated Munchong leachate; d) Treated Munchong leachate; e) Untreated Carey suspension; f) Treated Carey suspension; g) Untreated Munchong suspension; h) Treated Munchong suspension.

4.3.1.1 pH analysis of untreated and treated samples

The pH of the leachate and suspension of both untreated Carey and untreated Munchong samples showed acidic values, which were less than pH 4 (Table 4.8). Similarly, treated Carey had very acidic pH values for its leachate and suspension, ranging from 2.51 ± 0.05 to 2.75 ± 0.04 , respectively. Meanwhile, treated Munchong had less acidic pH ($\text{pH} > 6$). The sedimentation procedure had significant effect on the acidity of the clay sample, of which the treated Carey showed lower pH values in comparison to the untreated Carey. In contrast, pH of treated Munchong samples were higher in comparison to the untreated samples, in both leachate and suspension form.

Table 4.8: pH of untreated and treated samples

Sample	pH	
	Untreated	Treated
Carey Leachate	2.80 ± 0.09 ^a	2.51 ± 0.05 ^b
Carey Suspension	2.84 ± 0.01 ^a	2.75 ± 0.04 ^b
Munchong Leachate	3.66 ± 0.05 ^b	6.19 ± 0.01 ^a
Munchong Suspension	3.48 ± 0.07 ^b	6.10 ± 0.03 ^a

Pairs of values with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

The sedimentation method (treated) involves pre-treatment with hydrogen peroxide for organic matter removal may cause in pH drop (Mikutta et al., 2005) as observed in treated Carey clay samples. Meanwhile, the use of Calgon solution (sodium hexametaphosphate) as a dispersing agent to produce the treated samples, could cause changes of pH in treated samples due to the presence of free phosphate anions which will form bond with free H^+ released from various components of soils including sand, silt and clay minerals. As shown in the result, the slight drop of pH in treated samples for Carey was due to the stability of metal ions Al^{3+} and Fe^{2+} present in high amount in the acid sulfate Carey (Shamshuddin, 2006) than that of Munchong, which is a type of oxisols with high value of exchangeable Al (Paramananthan, 2000; Shamshuddin & Kapok, 2010), of which the high Al^{3+} content cause imbalance of overall charges. The sedimentation treatment caused loss of H^+ from Munchong, which form bond with phosphate anions and washed away during wet sieving procedure and therefore caused the drastic increase in pH of the sample. In addition, the higher pH of treated Munchong could be due to

organic matter, which can contribute to acidity of soils (Zhou et al., 2019), has been removed as a result of the sedimentation procedure.

4.3.1.2 CEC of untreated and treated samples

The CEC values (Table 4.9) showed the untreated Carey sample had low CEC value, while the treated samples yielded clay with higher CEC value. Similar results were obtained for Munchong samples, where the CEC value was much lower for the untreated sample by 3-fold difference in comparison to the treated Munchong. Both treated Carey and Munchong had moderate ranges of CEC values (12–25 cmol(+)/kg) according to the classification of Hazelton and Murphy (2007).

Table 4.9: CEC of untreated and treated samples

Sample	Carey		Munchong	
	Untreated	Treated	Untreated	Treated
CEC (cmol(+)/kg)	9.67 ± 0.16	15.00 ± 0.63	7.11 ± 0.08	21.33 ± 0.43
Classification ^a	Low	Moderate	Low	Moderate

^a Low: 6–12 cmol(+)/kg; moderate: 12–25 cmol(+)/kg (Hazelton & Murphy, 2007).

The result showed that both untreated Carey and Munchong samples possess low CEC values, whereas the CEC values of both treated clays were higher. This fits with a higher distribution of clay in the treated sample, as the larger particles have been removed during the purification procedure for treating the

clay. The increased yield of clays in the treated samples creates a larger surface area due to the finer particle size, which could provide enhanced clay–bacterial contacts in antibacterial assays (Williams, 2017).

4.3.1.3 Metal analysis of untreated and treated samples

Table 4.10 shows metal content in untreated and treated Carey and Munchong leachates. In untreated Carey, higher levels of Mg and Fe were found (56.88 ± 4.65 and 43.45 ± 2.67 mg/L, respectively) followed by Al with a much lower amount (9.92 ± 2.28 mg/L). The other 7 metals (Ag, As, Cu, Mn, Ni, Pb and Zn) were present in a trace amount. In treated Carey, the 3 metals Al, Mg and Fe were present in higher levels than others (92.63 ± 2.18 , 88.38 ± 2.29 and 67.46 ± 3.09 mg/L, respectively). Out of the 10 different metals, nine metals, (the exception being Pb) are present in significantly higher levels in treated samples of Carey in comparison to the untreated samples. Three elements, namely Al, Mg and Fe, are present at much higher concentrations, with 9-fold, 1.6-fold and 1.5-fold increase, respectively, in comparison to untreated Carey.

All metals were present in low amounts in untreated Munchong samples, with Fe, Mg and Al present in higher level in comparison to the other metals (1.80 ± 0.22 , 0.27 ± 0.00 and 0.13 ± 0.06 mg/L, respectively). In treated Munchong, high level Al and Fe

Table 4.10: Soluble metal content in untreated and treated Carey and Munchong leachates

Metal Content (mg/L)	Carey		Munchong	
	Untreated	Treated	Untreated	Treated
Ag	0.002 ± 0.001 ^b	0.005 ± 0.00 ^a	0.002 ± 0.001 ^a	<0.001 ^b
Al	9.92 ± 2.28 ^b	92.63 ± 2.18 ^a	0.13 ± 0.06 ^b	36.45 ± 1.98 ^a
As	0.02 ± 0.01 ^b	0.06 ± 0.01 ^a	0.01 ± 0.00 ^b	0.40 ± 0.03 ^a
Cu	0.002 ± 0.001 ^b	0.02 ± 0.00 ^a	0.002 ± 0.000 ^b	0.01 ± 0.00 ^a
Fe	43.45 ± 2.67 ^b	67.46 ± 3.09 ^a	1.80 ± 0.22 ^a	6.38 ± 4.03 ^a
Mg	56.88 ± 4.65 ^b	88.38 ± 2.29 ^a	0.27 ± 0.00 ^a	0.11 ± 0.03 ^b
Mn	0.12 ± 0.02 ^b	0.21 ± 0.02 ^a	0.01 ± 0.00 ^a	<0.001 ^b
Ni	0.01 ± 0.00 ^b	0.06 ± 0.01 ^a	<0.001 ^a	<0.001 ^a
Pb	0.002 ± 0.001 ^a	0.004 ± 0.001 ^a	0.01 ± 0.01 ^a	0.01 ± 0.01 ^a
Zn	0.05 ± 0.01 ^b	0.14 ± 0.02 ^a	0.004 ± 0.000 ^a	0.003 ± 0.001 ^b

Pairs of values (mg/L) with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

were found, which were 36.45 ± 1.98 and 6.38 ± 4.03 mg/L, respectively). In comparison to untreated sample, only Al, As and Cu showed significantly higher concentration in treated Munchong. Notably, Al varied greatly with a 280-fold difference between treated and untreated Munchong samples. Although the metal analysis of Munchong samples revealed higher concentrations of Al and Fe compared to the other metals, the values are much lower relative to the Carey samples.

Total metal content analysis of Carey and Munchong revealed high Al and Fe content in both treated and untreated samples, with Mg also showing high content in the Carey samples (Table 4.11). In particular, the high Al and Fe content reflect the mineral composition of the samples. Clays are composed of aluminosilicates, which are made of aluminium (Al), silicon (Si) and oxygen (O). Mineral structures of clays such of kaolinite, montmorillonite and illite includes Al in their crystalline lattice (Al Ani & Sarapaa, 2008), contributing to the high aluminium content. Meanwhile, the high Fe indicates the presence of iron oxides or hydroxides in the samples. The treated samples showed significant differences in all metals tested, except for Ag and Cu for Carey; for Munchong samples, the Cu, Fe, Mg and Zn content was not significantly different for both treatments. The total metal concentration was determined as a reference value to reflect the actual amount of metals present in Carey and Munchong clay samples. The metal concentration in soil can vary slightly if sampling is done on different days, even in the same location, due to several factors that influence the distribution and availability of metals in the soil, such as soil moisture, temperature changes, sampling depth and the influence of

Table 4.11: Total metal content in untreated and treated Carey and Munchong solid samples

Metal Content (mg/L)	Carey		Munchong	
	Untreated	Treated	Untreated	Treated
Ag	0.32 ± 0.04 ^a	0.48 ± 0.24 ^a	0.04 ± 0.00 ^b	0.18 ± 0.06 ^a
Al	29450 ± 678 ^b	71660 ± 700 ^a	123620 ± 17740 ^b	213680 ± 6280 ^a
As	2.22 ± 0.54 ^b	3.82 ± 0.26 ^a	14.94 ± 3.70 ^b	25.52 ± 1.28 ^a
Cu	4.04 ± 0.08 ^a	6.64 ± 5.60 ^a	8.20 ± 0.28 ^a	7.96 ± 0.44 ^a
Fe	3800 ± 109 ^b	4924 ± 16 ^a	5382 ± 98 ^a	5502 ± 26 ^a
Mg	261.52 ± 1.12 ^b	353.92 ± 4.64 ^a	13.76 ± 0.64 ^a	23.92 ± 9.60 ^a
Mn	18.76 ± 1.56 ^b	40.88 ± 0.56 ^a	30.90 ± 0.74 ^a	12.76 ± 0.68 ^b
Ni	0.64 ± 0.00 ^b	2.76 ± 0.00 ^a	1.48 ± 0.24 ^b	2.60 ± 0.08 ^a
Pb	3.46 ± 0.02 ^b	7.04 ± 0.60 ^a	1.58 ± 0.26 ^b	3.78 ± 0.42 ^a
Zn	11.60 ± 2.00 ^b	54.02 ± 1.82 ^a	13.62 ± 1.78 ^a	13.10 ± 1.22 ^a

Pairs of values (mg/L) with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups

human activity (Rahman & Zaim, 2015). However, if the environmental conditions and sampling methods remain consistent, the concentrations were generally be similar (Hazelton & Murphy, 2007). Unlike leachates that had metal ions leached out into the solution upon hydration, the total metals were extracted acids (HCl and HNO₃), revealing extremely higher levels of metals in comparison to the soluble metals in leachates (Table 4.10).

4.3.2 Antibacterial activity

4.3.2.1 MIC and MBC

The MIC and MBC of the leachates and suspension of four clay soil samples were determined against *S. Typhimurium* and *S. aureus* and the results are shown in Table 4.12. Treated Carey leachate and suspension displayed antibacterial activity against both the gram-negative and gram-positive bacteria tested, while the other three samples (untreated Carey, untreated Munchong and treated Munchong) do not show any antibacterial activity.

The result for treated Carey leachate and suspension showed that MIC values of *S. Typhimurium* and *S. aureus* were the same, which was 125 mg/mL, while MBC values of both bacteria was 250 mg/mL. MIC and MBC values were the same for both bacterial species, indicating that treated Carey samples had the capacity to inhibit bacterial growth and kill the cells present irrespective of their different cell wall architectures.

Table 4.12: MIC and MBC of leachates and suspensions against *S. Typhimurium* and *S. aureus*

Sample	<i>S. Typhimurium</i>		<i>S. aureus</i>	
	MIC	MBC	MIC	MBC
	(mg/mL)	(mg/mL)	(mg/mL)	(mg/mL)
Leachate				
Untreated Carey	n.a	n.a	n.a	n.a
Treated Carey	125	250	125	250
Untreated Munchong	n.a	n.a	n.a	n.a
Treated Munchong	n.a	n.a	n.a	n.a
Suspension				
Untreated Carey	n.a	n.a	n.a	n.a
Treated Carey	125	250	125	250
Untreated Munchong	n.a	n.a	n.a	n.a
Treated Munchong	n.a	n.a	n.a	n.a

n.a : No activity

There was no previous report for activity of clays against the same *S. Typhimurium* and *S. aureus* strains as used in this study. The closest study that can be compared to is by Londono et al. (2017) who reported the antibacterial Colombian Amazon clay suspended in an isotonic solution (0.85% NaCl), which showed MIC and MBC values of 50 mg/mL and 90 mg/mL, respectively, when tested against gram-negative *E. coli*. Although a direct comparison is impossible, it can be said that the treated Carey showed a considerably high MIC and MBC values. The value of MIC and MBC of treated Carey samples were referred to for further antibacterial viability assay since there were no MIC and MBC value reported for the other 3 samples

(untreated Carey, untreated and treated Munchong) in both of their leachate and suspension form. Hence, a higher concentration of 4x MIC (500 mg/mL) was selected for the viability assay in Section 4.3.2.2.

4.3.2.2 Bacterial viability assay

Exposure of *S. Typhimurium* to treated Carey leachates (500 mg/mL) resulted in complete killing (5.7 log reduction), while for *S. aureus*, viability decreased by 1.82 log (Figure 4.3). The other three leachate samples (untreated Carey, untreated Munchong and treated Munchong) showed no antibacterial activity against either *S. Typhimurium* or *S. aureus*.

The effect of clay suspension samples (500 mg/mL) on *S. Typhimurium* and *S. aureus* viability is shown in Figure 4.4. The treated Carey sample killed *S. Typhimurium* (6.7 log reduction) within 6 h and also resulted in a 4.02 log decrease in *S. aureus* viability. Suspensions of untreated Carey showed 1.82 log decrease in *S. Typhimurium* viability; however, the viability of *S. aureus* was minimally affected. Exposure to untreated Munchong resulted in modest reductions in *S. Typhimurium* and *S. aureus* viability, whereas suspensions of treated Munchong showed no growth inhibition when tested against these species.

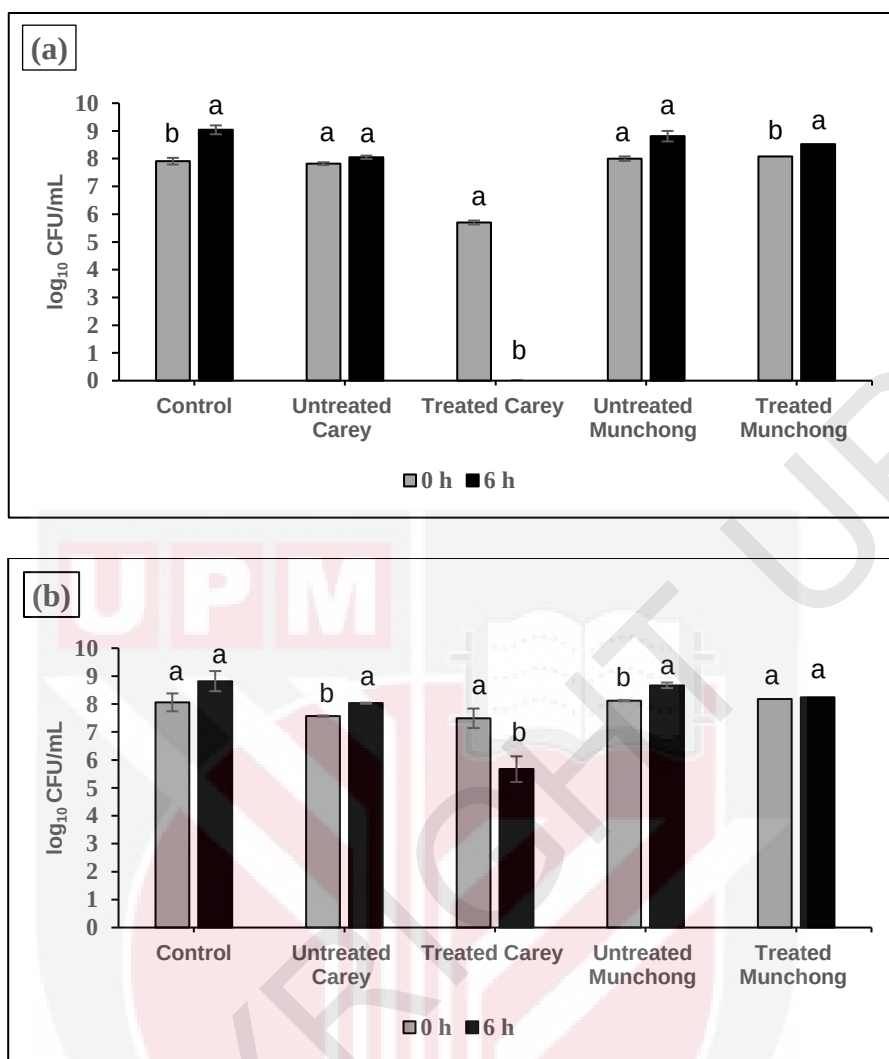


Figure 4.3: Antibacterial activity of untreated and treated clay leachate of Carey and Munchong soils (500 mg/mL) following 6 h of exposure, with sterile deionized water as control: (a) Viability of *S. Typhimurium*; (b) Viability of *S. aureus*. Pairs of values with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

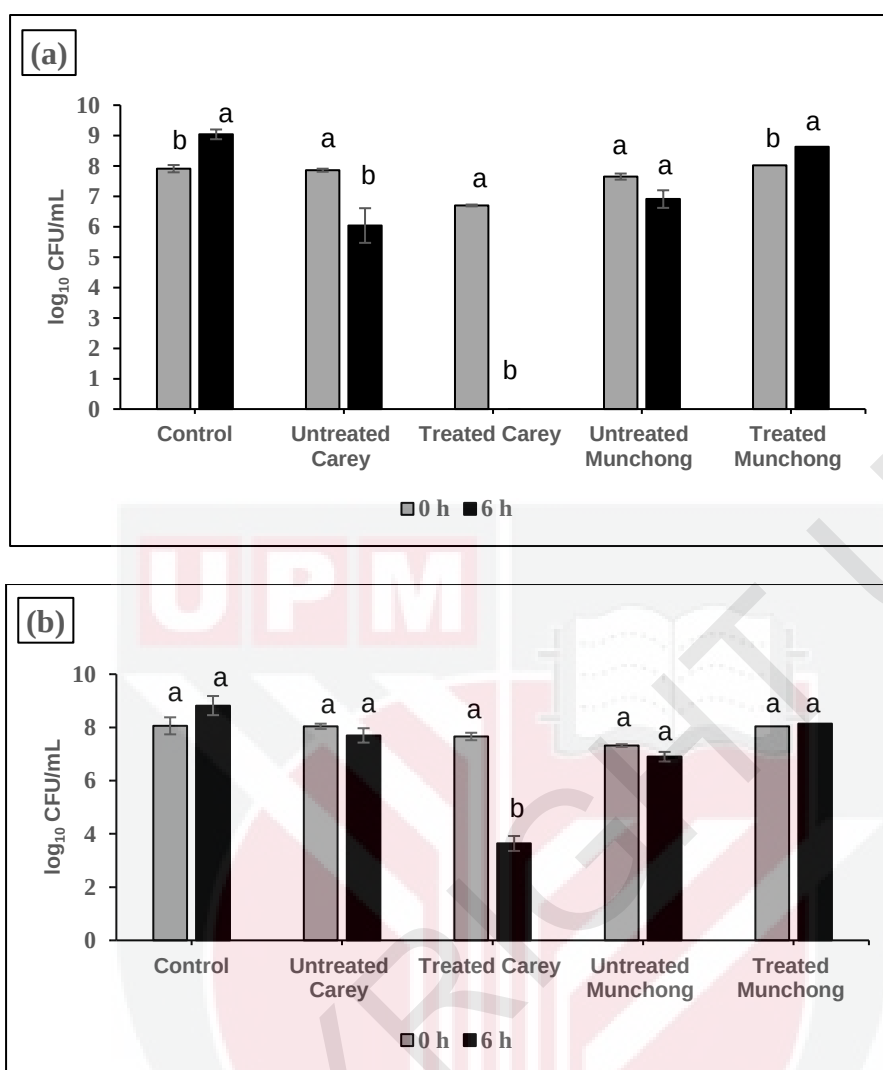


Figure 4.4: Antibacterial activity of untreated and treated clay suspension of Carey and Munchong soils (500 mg/mL) following 6 h of exposure, with sterile deionized water as control: (a) Viability of *S. Typhimurium*; (b) Viability of *S. aureus*. Pairs of values with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

Overall, Carey leachates and suspensions originating from treated soils displayed the greatest antibacterial efficacy against both of the gram-negative and gram-positive bacteria compared to the untreated clay leachates and suspensions. The treated Carey clays (suspension and leachate) proved the most effective at killing *Salmonella Typhimurium*. However, the viability of *S. aureus* was also reduced by the Carey clay suspension and leachate (>3 log

and <2 log, respectively). The untreated Carey and all the Munchong clays showed little or no antibacterial activity against either bacterial species.

The difference in susceptibility between gram-negative and gram-positive bacteria has been observed with clay mineral leachates previously (Cunningham et al., 2010; Haydel et al., 2008; Williams, 2017). Otto et al. (2010) reported bactericidal activity on the gram-negative *Escherichia coli*, whereas a methicillin-resistant *S. aureus* strain was killed at a slower rate. This is likely to be due to differences in the cell-envelope architectures of these bacteria, with the outer membrane of gram-negatives, absent from gram-positives, representing a permeability barrier to toxic components. Caflisch et al. (2018) reported that OMT clay suspensions are more effective in killing multiple strains of representative gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria than clay leachates at the same concentration. While the previous study employed clay at 200 mg/mL in both suspensions and leachates, this study used a higher concentration (500 mg/mL), although the outcome is similar. These findings support a direct antibacterial contribution for clay minerals for clay prepared as suspension in causing antibacterial effect, rather than metal ion toxicity in the solution chemistry being the sole factor, as previously concluded with clay leachates (Otto & Haydel, 2013a).

The sedimentation treatment caused removal of coarser particles which are not antibacterial, including sand, silt and organic matter. The higher CEC values of treated Carey and Munchong in comparison to the untreated samples (see Table 4.9) reflected that the clay distribution is higher. This may

have an influence in the potential antibacterial activity of the treated samples, as the greater amount of fine particle size of clays would create a larger surface area, providing enhanced clay–bacterial contacts in antibacterial assays. Although this explanation fits with the antibacterial properties of the Carey treated clays, it does not account for the reduced efficacy of the Munchong treated clays. Hence, the higher portion of clay in samples is not the sole contributor to their antibacterial properties. This result is consistent with a previous study by Ibbini et al. (2018) who found a weak correlation of clay content and CEC with the antibacterial activity 7 bacterial strains tested, including *S. aureus*, *E. coli* and MRSA.

4.3.3 Correlation between pH and metals on antibacterial activity of untreated and treated leachates against *S. Typhimurium* and *S. aureus*

A principal component analysis (PCA) was performed as an exploratory analysis of the obtained data in order to verify the correlation between the different Carey and Munchong treatments (untreated and treated clay leachates), pH, metals and the associated log reduction of *S. Typhimurium* and *S. aureus* at 6 h exposure (Figure 4.5). A total of 11 variables were examined, which are the individual metal ions (Ag, Al, As, Cu, Fe, Mg, Mn, Ni, Pb, and Zn) and pH, with 4 observations analysed, which are untreated Carey leachate, treated Carey leachate, untreated Munchong leachate and treated Munchong leachate. The first and second main components represent a total explained variance of 89.08%, with PC1 explained 68.52% and PC2 explained 20.56% of the variance, respectively. Of the 11 variables, metal ions Ag, Al, Fe, Mg, Mn, Ni and Zn had strong FL ($FL \geq |0.75|$) that were dominant in this

study. Cu and pH had moderate FL ($|0.500| < FL < |0.749|$) while metals As and Pb had a weak FL ($FL \leq |0.499|$).

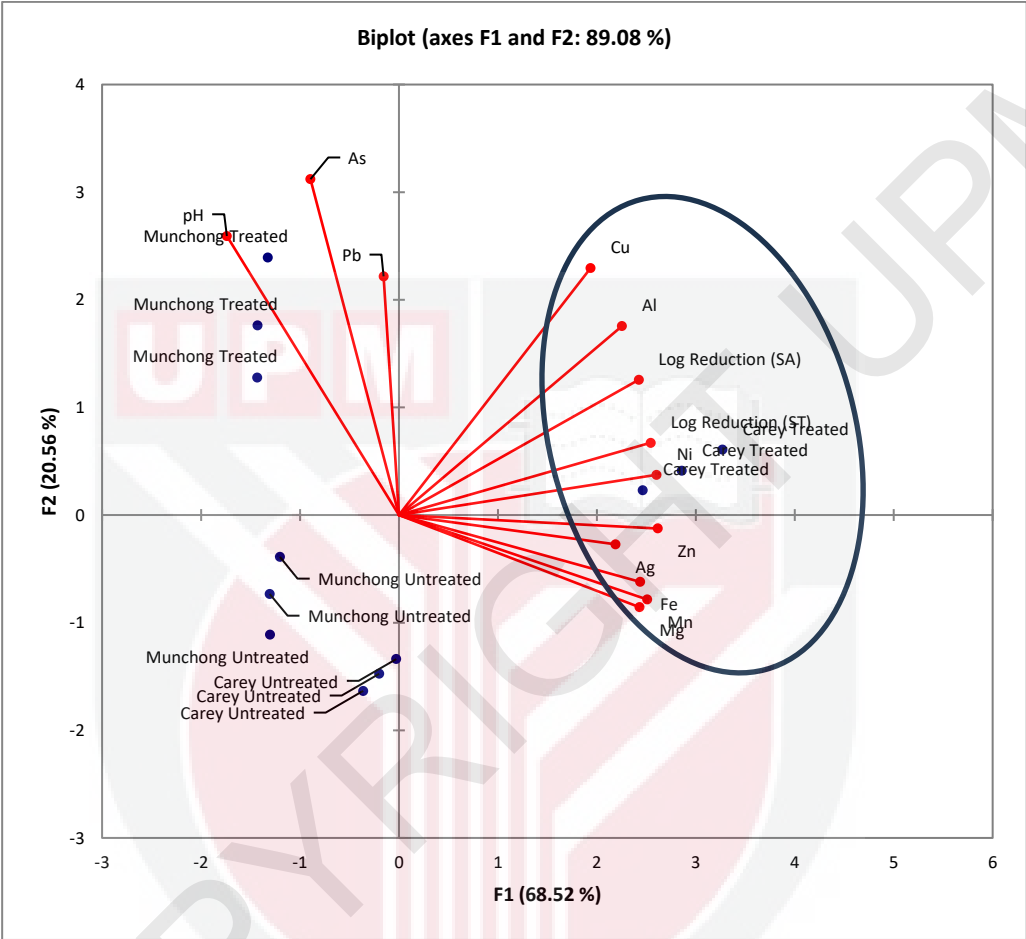


Figure 4.5: Biplot of correlation between available metals and pH on the antibacterial activity of untreated and treated leachates against *S. Typhimurium* (ST) and *S. aureus* (SA).

The biplot displayed one cluster consisting of treated Carey, which was positioned closely with log reduction of both *S. Typhimurium* and *S. aureus*, along with most of the metal ions except As and Pb. Treated Carey, which was plotted nearest to eight variables shows that they are highly correlated to each other. Meanwhile, pH was positioned about 90° angle from log reduction of

both *S. Typhimurium* and *S. aureus*, indicating that pH did not affect inhibition of both of the test bacteria. On the other hand, negative correlation was observed between pH and metals Fe, Mg and Mn as the variables were located in inverse direction to pH, which indicates that a lower pH were correlated with higher Fe, Mg and Mn content. The result from this evaluation indicates that leachates of treated Carey leachate to be the most effective in growth inhibition of *S. Typhimurium* and *S. aureus*, with the antibacterial activity was strongly correlated with the presence of metal ions Ag, Al, Cu, Fe, Mg, Mn, Ni and Zn.

In the effort to further determine the role of metal toxicity, it was discovered that the soluble metal content was higher in treated Carey clay samples compared to the untreated ones. This finding fits with the greater antibacterial activity of treated Carey, as judged by the bacterial log reduction value. These results are similar to previous studies which have shown that metal ion toxicity is responsible for bactericidal activity against *E. coli* and *S. aureus* (MRSA), with toxicity directly associated with the released metal ions (Fe^{2+} , Cu^{2+} and Zn^{2+}) in an acidic environment (Otto et al., 2014; Otto & Haydel, 2013a). Aqueous leachates derived from known antibacterial clays tend to have high levels of Mg, Al and Ca, with some containing exceptionally high concentrations of Fe (Williams, 2017; Zarate-Reyes et al., 2017). Previous work has concluded that Ca was not significant in bacterial cell death (Borrok et al., 2004; Williams et al., 2011), hence analysis of Ca levels was not included in this study. For OMT clay minerals, the dissolution of reduced Fe^{2+} and Al^{3+} was identified as the active antibacterial component, acting in concert to damage bacterial membranes (Morrison et al., 2016). Speciation modelling by

Otto and Haydel (2013) suggests that increasing the soluble metal ions Cu^{2+} and Fe^{2+} are the key to increasing the antibacterial activity of the leachates.

The activity could be influenced by the pH of the sample, as acidic conditions should favour metal ion solubility (Cunningham et al., 2010). As noted previously, pH alone is not the only factor contributing to antibacterial activity, as previously verified in the PCA analysis (Figure 4.5). However, in an acidic environment, abundant protons saturate metal binding sites in the solution, maximising the concentration of soluble metal ions. Metal ions become increasingly more bioavailable and potentially more toxic in a low-pH solution (Wang et al., 2009). It seems likely that the abundance of metal ions in treated Carey clay samples, especially in an acidic environment, contributes to bacterial killing due to metal ion toxicity.

The available metals (Al, As and Cu) were higher in the treated Munchong than the untreated leachate by 280-, 80- and 4.5-fold, respectively (see Table 4.10). The higher soluble metal content could be attributed to the higher clay percentage in the treated sample, as evidenced by their larger CEC values compared to the untreated sample. However, metal analysis revealed much lower Fe, Mg, Mn, Ni, and Zn leachate content compared to both of the Carey leachate samples. This could mean that the metal content in untreated and treated Munchong is below the threshold value that could cause death of bacteria.

Relative to the other three non-antibacterial leachates, the antibacterial treated Carey leachate contains significantly higher levels of available Ag, Al, Cu, Fe, Mg, Mn, Ni and Zn. Treated Carey clay has a greater CEC in comparison to the sieved sample due to increased finer clay fractions in the sample. The elevated quantities of these metals suggest that a greater amount of elements is concentrated in the finer clay fractions, as previously suggested by Williams (2017), and is only released into solution upon hydration. The high total Al and Fe content in the treated Munchong clay did not seem to improve its antibacterial activity. Al is abundant as it provides the structural framework of clay minerals, but may not necessarily be present in a bioactive form. Similarly, Fe may be present in pyrite and hematite within the clay minerals but may not be readily available in a reduced state with its associated higher toxicity. On the other hand, the available metals in leachates, when present above the threshold amount, can cause death of bacteria due to metal toxicity. Williams et al. (2011) reported that the leachate of antibacterial OMT clay contained significantly higher Al, Fe, Cu and Pb content, with the values of 23.9, 51.6, 0.23 and 0.00002 mg/L, respectively. These are among the metals associated with *E. coli* death. Although the metal threshold value was not determined in this study, it can be concluded, based on literatures, that Fe content is likely to make significant contribution to the antibacterial effectiveness of the sample against the test bacteria.

The result from biplot analysis indicates that leachates of treated Carey leachate to be the most effective in growth inhibition of *S. Typhimurium* and *S. aureus*. Therefore, treated Carey samples, including both leachate and

suspension were also selected for further analysis due to the same MIC and MBC value reported with clay suspension and leachate (see Table 4.12).

4.4 Characterisation of treated Carey clay and factors influencing the antibacterial activity

Based from results from Section 4.3, treated Carey was found to be the most effective clay when tested against *S. Typhimurium* and *S. aureus* in both leachate and suspension form. This section includes characterisation of clay (mineralogy, elemental composition, morphology and surface properties) and analyses the factors influencing antibacterial activity of treated Carey clay, which include time-kill analysis (*S. Typhimurium* and *S. aureus*), effect of pH and metals.

4.4.1 Characterisation of treated Carey clay

4.4.1.1 Mineralogy

The clay mineralogy of the treated Carey sample revealed the presence of kaolinite, mica, pyrite and magnetite (Figure 4.6). The detection of pyrite and magnetite in the Carey sample indicates the presence of iron-rich mineral as part of the clay mineralogy.

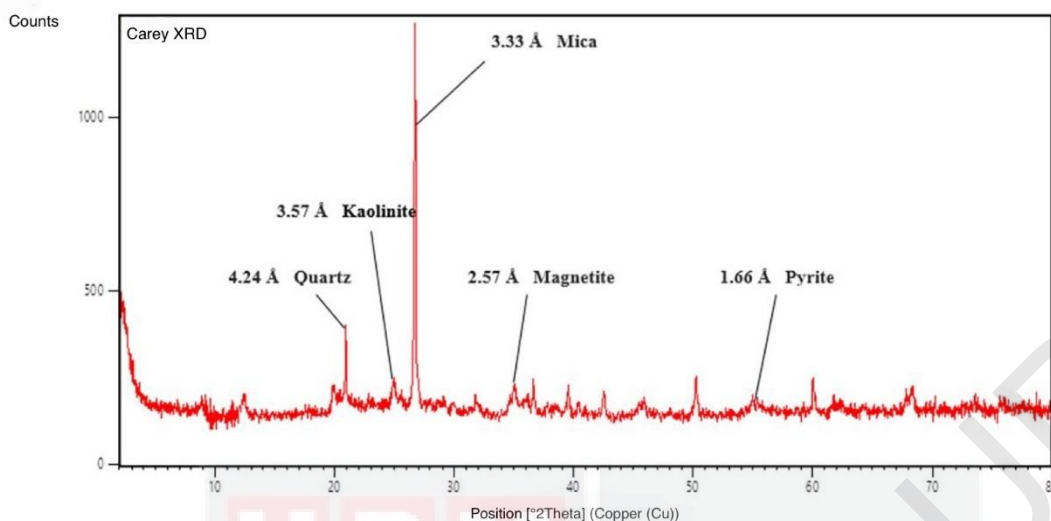


Figure 4.6: XRD (powder X-ray diffraction) graph showing peaks for mineralogy identification of treated Carey clay

Carey is an acid sulfate soil, characterised by the presence of pyrite and high Al and Fe content (Shamshuddin, 2006). XRD analysis confirmed the presence of pyrite, as well as magnetite in the Carey sample. The pyrite and magnetite minerals are sulfur and iron composite minerals, which could potentially contribute as sources of sulfur and iron in the clay leachate and suspension. Pyrite showed reduction in *E. coli*, with antibacterial activity linked to iron leached out from pyrite dissolution (Friedlander et al., 2015). Therefore, sulfur content was not measured in this study.

Kaolinite, mica and quartz were also found in the XRD analysis, which were common constituent of soil. Kaolinite mineral was previously reported inefficient in antibacterial activity (Adusumilli & Haydel, 2016), however Londono et al. (2017) reported Al-rich Colombian Amazon (AMZ) clay, which was dominated by kaolinite, was found to show effective antibacterial activity

in solution (suspension), suggesting Al dissolution from kaolinite to be the cause of membrane damage in bacteria.

4.4.1.2 Elemental composition

Energy dispersive X-ray spectroscopy (EDX) analysis revealed the major elemental composition of the treated Carey clay (Figure 4.7). Quantitative analysis showed that oxygen (O) makes up the majority of the elements (55.94 %), followed by carbon (C) and silicon (Si) (13.50 % and 12.61 %, respectively). Aluminium (Al), iron (Fe) and sulfur (S) are present in moderate amounts (8.31 %, 4.36 % and 1.76 %, respectively) while other elements (Cl, Na, K, Mg) are present in traces quantities.

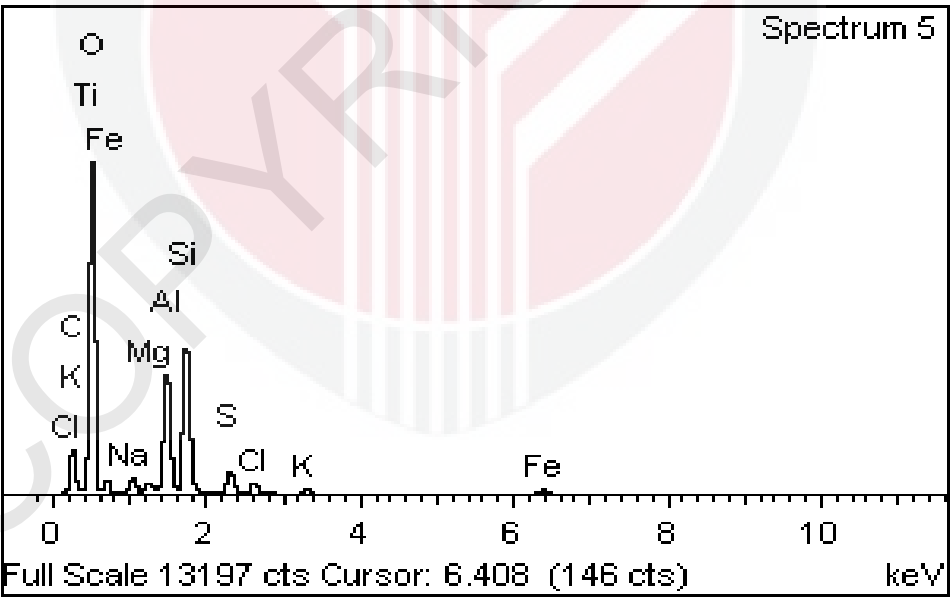


Figure 4.7: Elemental analysis by EDX showing elemental composition of treated Carey clay

Carey clay belongs to the acid sulfate type, which contains high concentrations of metals, including Al and Fe (Shamshuddin, 2006). The additional SEM-EDX

analysis also confirmed the presence of sulfur, one of the elemental components of pyrite mineral (FeS_2) as previously detected in the XRD analysis.

4.4.1.3 Morphology

Treated Carey clay as determined by scanning electron microscopy (SEM) (Figure 4.8) consists of crystals with layered structure. The morphology shows an irregular shape, platy-like and “cornflake” texture with various sizes, which can be identified as kaolinite. The characteristic images seemed to be similar with the results obtained by the kaolinite-rich antibacterial clay although less well-defined, with the reference kaolinite clay reported to showed smooth surfaces with hexagonal shape of kaolinite crystals (Londono & Williams, 2016).

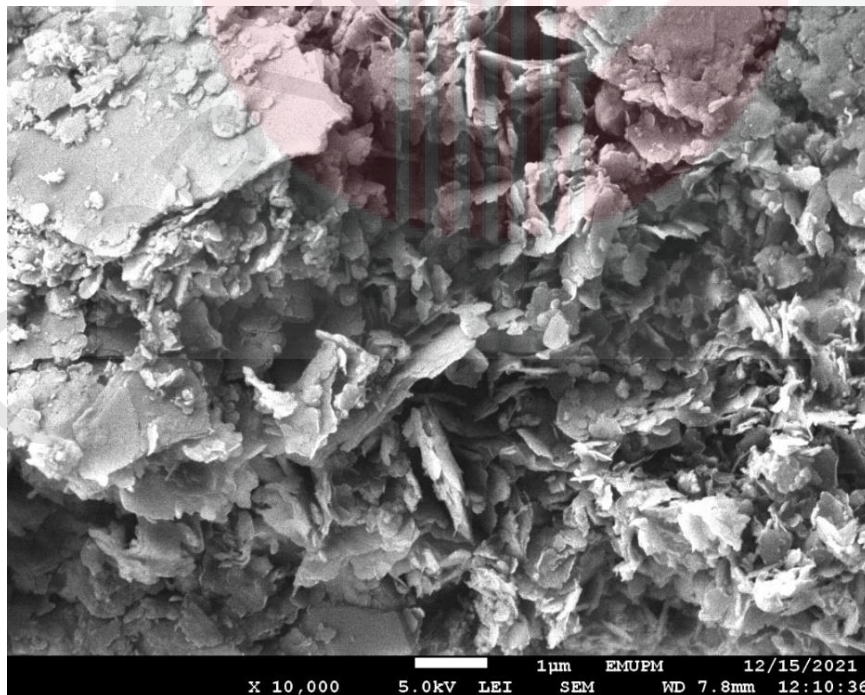


Figure 4.8 : Morphology of treated Carey clay analysed by SEM

4.4.1.4 Surface properties

Surface properties of treated Carey clay are presented in Table 4.13. The results of a Brunauer-Emmett-Teller (BET) of the clay was analysed to have the value of $11.19 \pm 0.07 \text{ m}^2/\text{g}$. Other properties analysed include pore volume and size, which are, respectively. CEC value of treated Carey clay, which was previously presented under Section 4.3.2.1 was also included under this section for further elaboration.

Table 4.13: Surface properties of treated Carey clay

Properties	Value
BET surface area (m^2/g)	11.19 ± 0.07
Pore volume (cm^3/g)	0.0023
Pore size (nm)	0.83
Cation-exchange capacity ($\text{cmol}(+)/\text{kg}$)	15.00 ± 0.63

The cation-exchange capacity (CEC) of clay refers to its ability to exchange cations with the surrounding medium. Significantly, toxic metal ions released in this fashion from clay interlayers can exert bacteriostatic and bactericidal effects (Babich & Stotzky, 1977). In comparison to kaolinite as reference clay, the CEC value of Carey had 5-fold higher value (Philen et al., 1971). The CEC is considered to be in moderate value when compared to the antibacterial Colombian Amazon (AMZ) clay, of which the value is $29.32 \text{ cmol}(+)/\text{kg}$ (Londono & Williams, 2016).

Analysis of surface properties of Carey were undertaken to determine whether adsorption of clays on the surface of bacteria could be the factor leading to

bacterial cell death. The higher surface area indicates the higher adsorption capacity of the clay (Lu et al., 2016). Carey clay presents a much lower BET surface area in comparison to the AMZ clay, which value is 51.23 m²/g (Londono & Williams, 2016). The relatively small surface area of Carey clay might indicate that adsorption is not a factor for bacterial killing. Additionally, the pore size of clay was categorised as micropore (pores less than 2 nm diameter), with over 1000 times much smaller in comparison to the average size of bacterial cell of the test bacteria (Fàbrega & Vila, 2013; Monteiro et al., 2015), suggesting the adhesion of bacterial cells to the pores of Carey clay seemed highly unlikely. Londono and Williams (2016) also reported a weak physical interaction between the AMZ clay and bacteria, as measured by zeta potential which further suggested lack evidence to support bacterial death by adsorption of bacteria to clay surfaces.

4.4.2 Effect of pH on antibacterial activity of treated Carey clay

4.4.2.1 pH analysis of leachates and suspensions at different concentrations

The pH of all samples was measured and all were found to have a highly acidic pH, ranging from pH 2.51 to pH 3.04 (Table 4.14). Higher leachate and suspension concentrations resulted in increasingly more acidic pH in the samples. Leachates exhibited slightly greater acidity than suspensions at the same concentrations. The sample with most acidic pH was leachate 4x MIC (2.51 ± 0.05), while the 1x MIC suspension yielded the least acidic pH (3.04 ± 0.05). The increase in clay concentration in leachates and suspensions decreases their pH value, becoming more acidic. The higher concentration of clay in water could increase the frequency of contact between the large surface area of clay particles with water, which increased the hydration of exchangeable metals and dissolution of minerals. The acidic environment that improves the solubility of metals in the clay mineral interlayer, such as Fe^{3+} , Fe^{2+} and Al^{3+} (Morrison et al., 2016).

Table 4.14: pH analysis of leachates and suspensions at different concentrations

Sample	pH
Leachate 1x MIC	2.74 ± 0.07
Leachate 2x MIC	2.66 ± 0.04
Leachate 4x MIC	2.51 ± 0.05
Suspension 1x MIC	3.04 ± 0.05
Suspension 2x MIC	2.87 ± 0.09
Suspension 4x MIC	2.75 ± 0.04

4.4.2.2 Influence of pH on antibacterial activity

Preliminary assessment was conducted to assess the effects of pH alone on the viability of *S. Typhimurium* and *S. aureus*, as shown in Figure 4.9. A series of deionised water with pH adjusted was prepared: pH 2 (2.04 ± 0.01) and pH 4 (4.06 ± 0.04). For control, pH-unadjusted sterile deionised water (pH 6.32 ± 0.00) was tested for comparison. The results showed that exposure to a lesser acidic condition (pH 4) resulted in increase in viability of *S. Typhimurium* at 6 h in comparison to the initial (0 h), and remained unchanged at 24 h. When exposed to highly acidic condition (deionised water adjusted to pH 2), the viability of *S. Typhimurium* was initially remained unchanged after 6 h of incubation, however after 24 h, viability was increased (Figure 4.9a). In comparison, the treated Carey leachate and suspension, with pH 2.51 and pH 2.75 respectively, resulted in complete killing of *S. Typhimurium* after 6 h of incubation.

The viability of *S. aureus*, showed no difference when exposed to pH 4 (Figure 4.9b) after 24 h of incubation in comparison to 0 h. At lower acidic condition (pH 2), viability of *S. aureus* was reduced to 4.28 log within 6 h of incubation, with no viable bacteria were recovered after 24 h. Comparative viability assays with treated Carey leachate and suspension resulted in a more gradual decline in *S. aureus* viability. The viability was slightly reduced within 6 h of incubation with treated Carey leachate (1.45 log reduction), while greater log reduction was shown with treated Carey suspension (3.95 log reduction). Both of the samples demonstrated complete killing after 24 h of incubation.

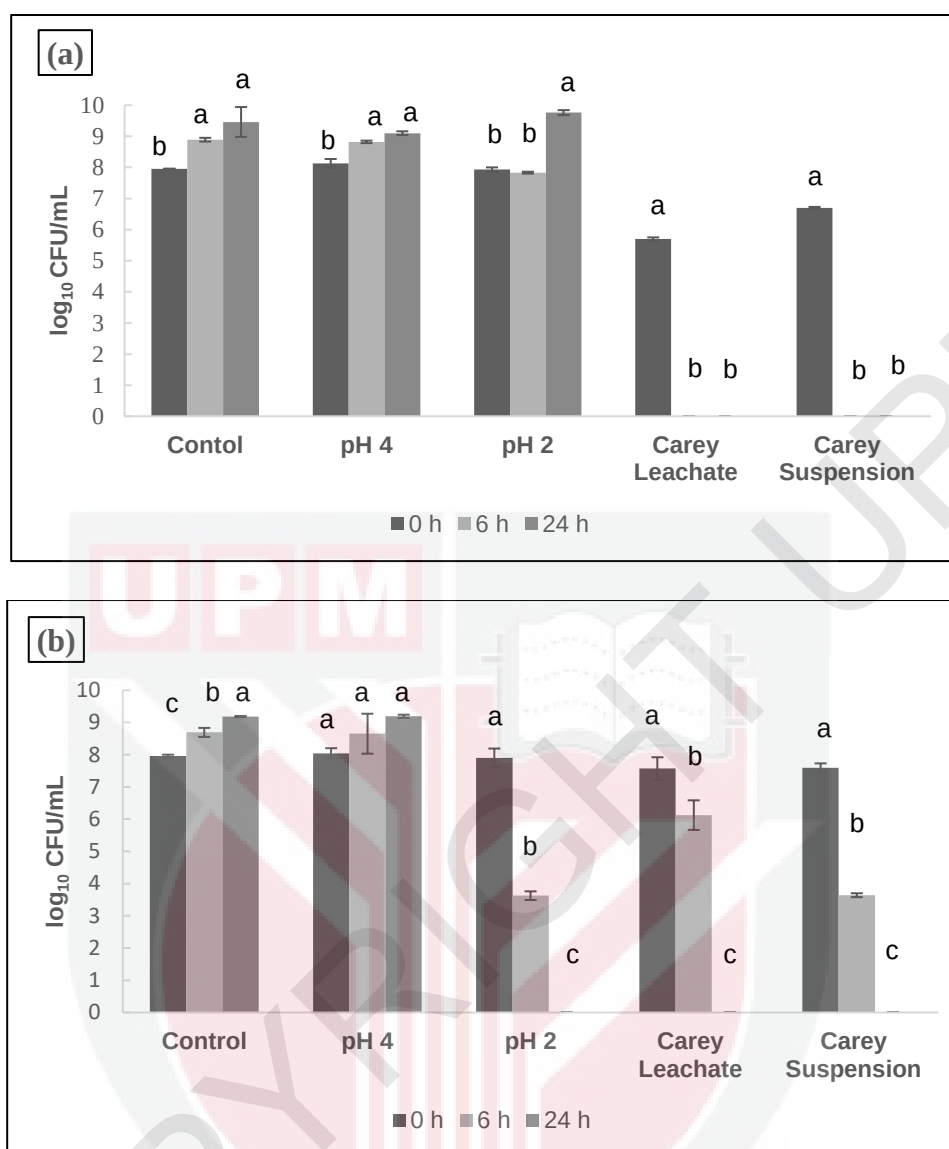


Figure 4.9: Effect of pH on the antibacterial activity against a) *S. Typhimurium* and b) *S. aureus*. A series of deionised water with pH adjusted was prepared: pH 2 (2.04 ± 0.01) and pH 4 (4.06 ± 0.04) and unadjusted (pH 6.32 ± 0.00) as control. Viability of both *S. Typhimurium* and *S. aureus* incubated with low pH solutions have been compared with 500mg/mL (4xMIC) treated Carey clay samples (leachate pH 2.51; suspension pH 2.75). Pairs of values with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

The result suggests that *S. aureus* is more sensitive to pH than *S. Typhimurium*. Gram-positive *S. aureus* demonstrated low tolerance to low-pH conditions in comparison to the gram-negative bacteria, which is in line with

results from previous studies (Behroozian et al., 2020; Cunningham et al., 2010). While both *S. Typhimurium* and *S. aureus* are sensitive to acidic conditions (Jay et al., 2008; Lund et al., 2000), *S. Typhimurium* displays better capacity to survive low-pH stress (pH 3.3) (Foster, 1993). It was demonstrated that the active components of treated Carey clay can be extracted with water. The resultant leachates and suspensions showed pH below 3 (see Table 4.14). Certain exchangeable metal ions are more soluble at low pH, therefore it is hypothesized that the acidic nature of treated Carey clay leachates and suspension may be required for their antibacterial activity. It is concluded that while low pH is a characteristic of treated Carey leachates and suspensions, it was not the only factor contributing to the antibacterial activity against *S. Typhimurium* and *S. aureus*, which is in agreement with a previous study (Behroozian et al., 2020).

4.4.2.3 Stability of antibacterial treated Carey clay at different pH

pH-adjusted samples were tested for its antibacterial activity against both of the test bacteria to determine if adjusting the pH of treated Carey leachates and suspensions could change their activity (Figure 4.10). pH of treated Carey clay at 4x MIC (500 mg/mL) was adjusted to pH 7 (7.04 ± 0.06), pH 5 (5.02 ± 0.02) and pH 3 (3.03 ± 0.03).

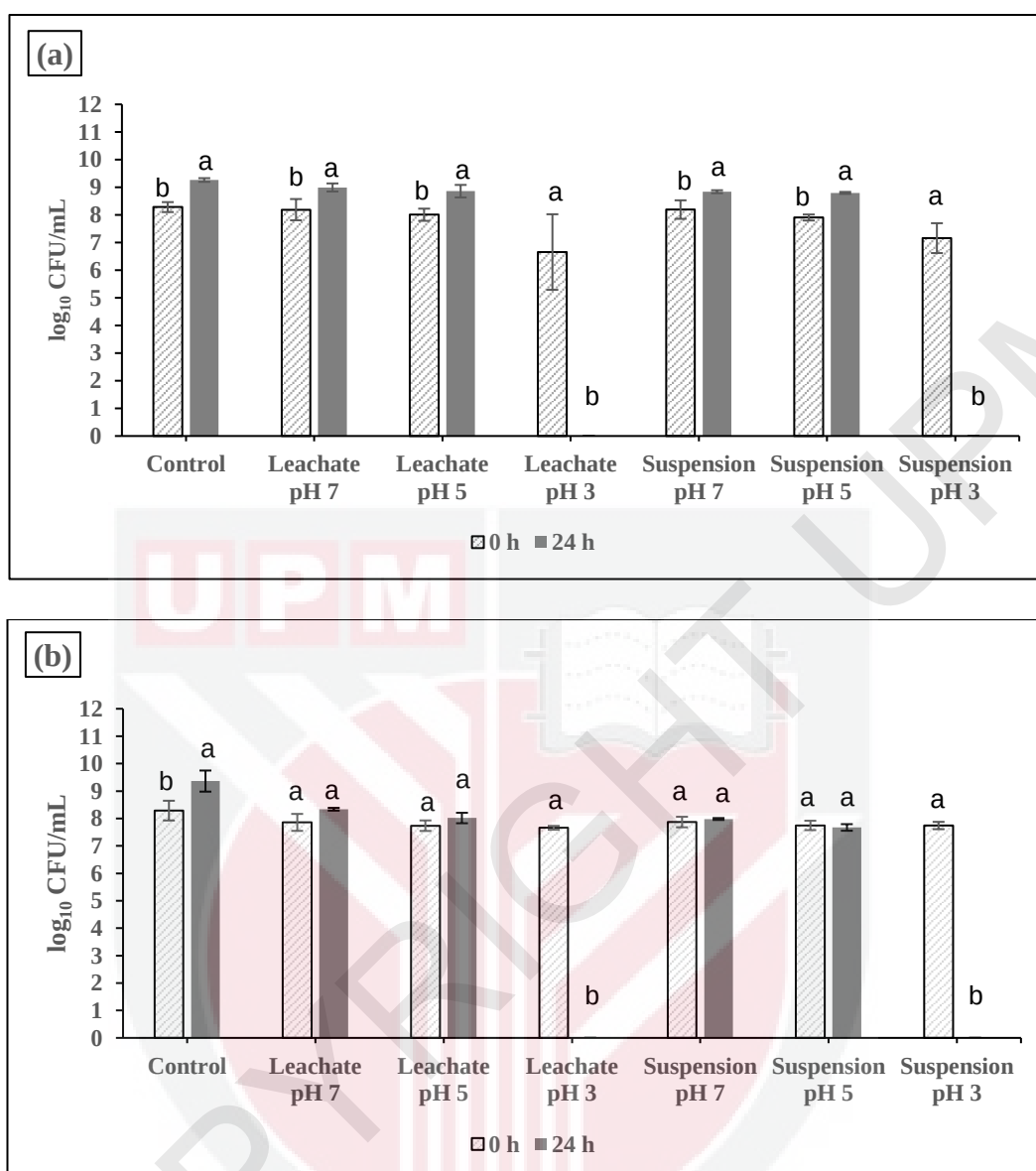


Figure 4.10: Changes of antibacterial activity of Carey at different pH of clay-bacteria mixture before (0 h) and after incubation (24 h) for (a): *S. Typhimurium* and (b): *S. aureus*. pH of treated Carey clay at 500 mg/mL (4x MIC) was adjusted to pH 7 (7.04 ± 0.06), pH 5 (5.02 ± 0.02) and pH 3 (3.03 ± 0.03). Sterile nutrient broth (pH 7.76) as negative control. Pairs of values with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

The result shows that the antibacterial activity of treated Carey clay is pH-dependent. When pH was slightly higher (pH 3) than the original sample pH (pH 2.51 – pH 2.75), bactericidal activity was retained for both leachate and

suspension against *S. Typhimurium* (Figure 4.10a). However, adjusting the pH of both leachate and suspension to a lower acidity to neutral (pH 5 and pH 7) rendered them ineffective. A similar trend was observed for *S. aureus* (Figure 4.10b). This reflected that the slight increase in pH does not affect the antibacterial activity. However, at a higher pH (pH 5 and 7), the samples lose their antibacterial activity due to precipitation of metal ions which reduces their bioavailability and toxicity (Morrison et al., 2016). Relating the pH of clay-bacteria mixture, the consistently acidic environment seemingly important in sustaining the antibacterial activity. This result is consistent with a study by Otto and Haydel (2013), who reported an increase in pH decreases the antibacterial activity (become non-antibacterial) and suggested the combining effect of metal ion availability and a low pH environment that causes complete killing of bacteria.

As observed by Morrison et al. (2016), an increase in pH reduces the concentration of soluble metals Fe^{3+} , Fe^{2+} and Al^{3+} in the clay-water mixtures. Acidic conditions can have detrimental effects on membrane integrity (Guan & Liu, 2020) and this may also help toxic metals gain entry to the cell and exacerbate their effects on intracellular proteins. It seems likely that the dissolution of metal ions in Carey clay under an acidic condition to be causing antibacterial effect due to metal ion toxicity.

4.4.3 Time-kill analysis

4.4.3.1 *S. Typhimurium*

The correlation between treated Carey clay concentration and its bactericidal effect on the test bacteria was evaluated in a time-kill assay. The assay showed that both leachates and suspensions at 2x and 4x MIC display significant bactericidal activity against *S. Typhimurium* (Figure 4.11a and b). At 4x MIC, a >6 log reduction in *S. Typhimurium* was evident after only 30 min with the leachate and after an hour with the suspension. At the 0 h time point, *S. Typhimurium* exposed to both 4x MIC leachates and suspension already showed decreasing in bacterial cell viability, when 1.5 log reduction was observed than the respective controls containing sterile deionised water (0x MIC). Experimental procedures require about 3 min to complete, which implicates the initial decrease in cell viability and demonstrated the rapid killing of *S. Typhimurium* with 4x MIC clay samples. The 2x MIC concentration was also bactericidal but took longer exposure time to eradicate the bacteria, which are 2 h with the leachate and 4 h for suspension.

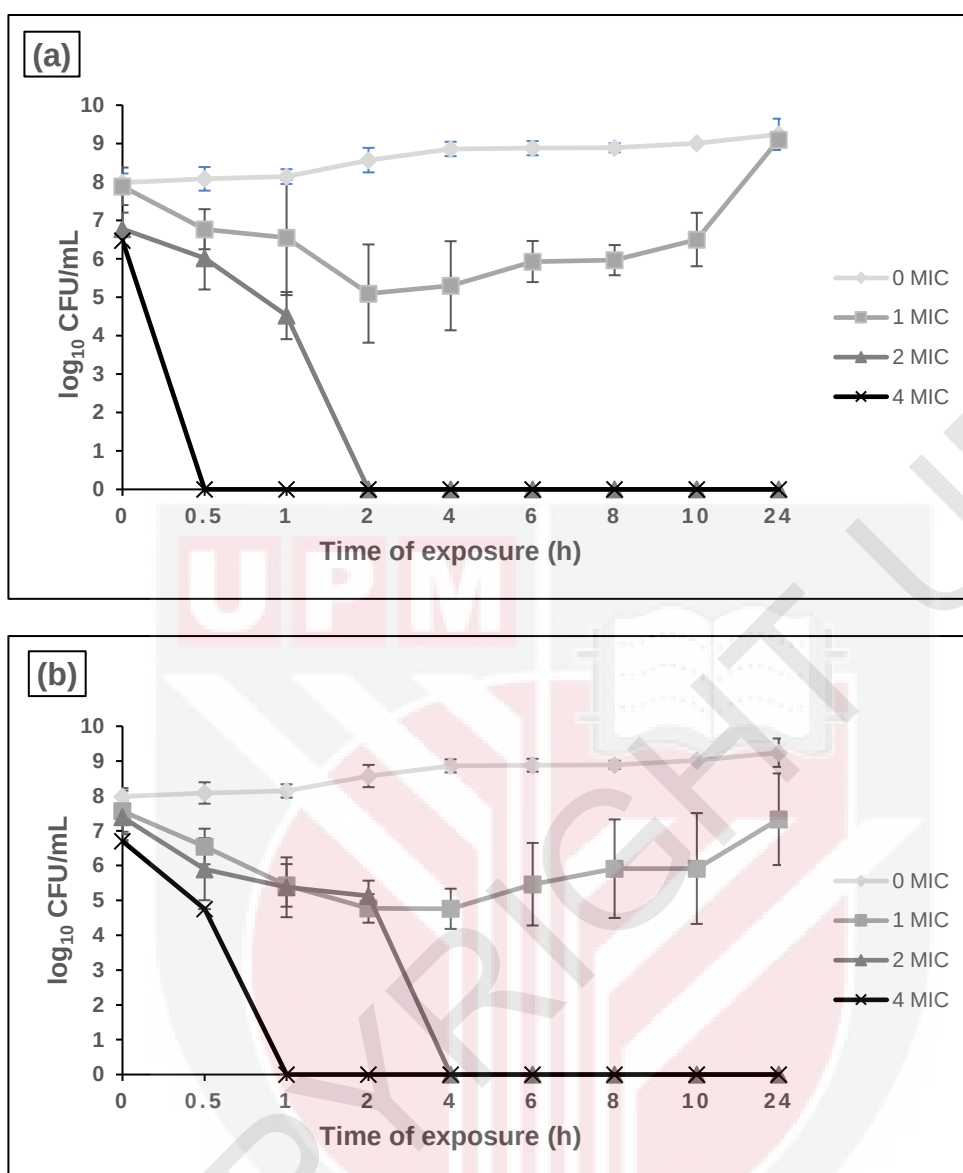


Figure 4.11: Viability of *S. Typhimurium* following 24 h exposure to (a) clay leachate and (b) clay suspension. (1x MIC: 125 mg/mL; 2x MIC: 250 mg/ml; 4x MIC: 500 mg/ml), with sterile deionised water (0x MIC) as control.

4.4.3.2 *S. aureus*

The leachate had considerably less efficacy against *S. aureus*, with bactericidal activity only observed at the end of the 24-hour clay-bacteria incubation period with 2x MIC and 4x MIC (Figure 4.12a). Meanwhile, complete killing of *S. aureus* with the Carey clay suspension required a minimum of 8 hours at 2x MIC and 4x MIC (Figure 4.12b). The results show

that the Carey clay displays robust antibacterial activity as judged by its ability to reduce viability of both *S. Typhimurium* and *S. aureus*. At 2x MIC and 4x MIC, Carey clay in both leachate and suspension forms resulted in complete killing of both bacterial species.

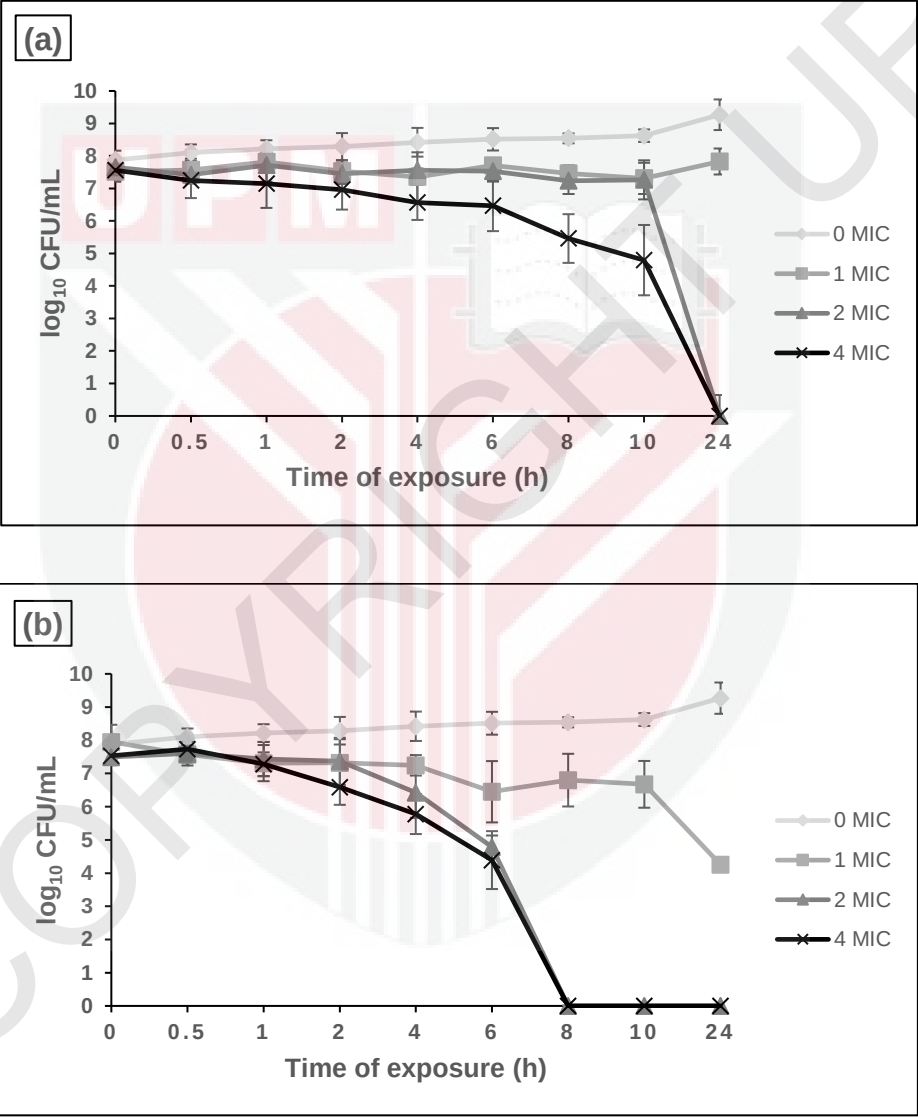


Figure 4.12: Viability of *S. aureus* following 24 h exposure to (a) clay leachate and (b) clay suspension. (1x MIC: 125 mg/mL; 2x MIC: 250 mg/mL; 4x MIC: 500 mg/mL), with sterile deionised water (0x MIC) as control.

The leachates took the longest time to completely kill *S. aureus*, which is at the end of 24 h exposure while suspension was at 8 h for both 2x MIC and 4x MIC. These findings are similar to those of Caflisch et al. (2018), although their antibacterial clay was tested against different *S. aureus* strains. The greater antibacterial efficacy of the Carey suspensions compared to the leachates is consistent with previous studies with different antibacterial clays (Caflisch et al., 2018; Morrison et al., 2016). This enhanced antibacterial activity can be explained by the continuous supply of toxic metals released from fine-sized clay interlayers, perhaps in close proximity to the bacteria to aid metal delivery to the cell surface.

Bacterial killing occurred rapidly in gram-negative *S. Typhimurium*, whereas the gram-positive *S. aureus* took considerably longer to be completely eradicated following exposure to the Carey clay. There was clearly greater potency with both leachates and suspensions against the gram-negative bacteria relative to the gram-positive. To some extent this matches previous work showing preferential bactericidal effects of clay minerals on gram-negative species, while growth of gram-positive bacteria was only moderately inhibited after 24 h of incubation (Haydel et al., 2008). It is possible that the presence of the protective outer peptidoglycan shell found in gram-positives contributes to the delayed toxicity noted with *S. aureus* following treatment with Carey clays. Direct damage to the outer membrane of gram-negatives, may also help make them much more susceptible to killing.

The application of treated Carey clay as surface sanitizers would be possible, with the minimum requirements and standard of efficacy testing (AOAC 960.09, 2013; EN 13697, 2015) which stated that a $\geq 99.9\%$ or 3 log reduction of test microorganism within 5 min is adequate for non-food contact surface sanitizers. The time-kill results showed that a long incubation period is needed for reduction of bacterial viability especially with *S. aureus*. In industrial setting, the effectiveness of clay can be enhanced with the combination of increasing the contact time and temperature setting (Sansebastiano et al., 2007) to ensure effective killing of bacteria.

4.4.4 Effect of metals on antibacterial activity of treated Carey clay

4.4.4.1 Available metals in treated Carey clay leachates

As previous studies linked the presence of certain metals in clay samples to antibacterial activity, the types of soluble metals present in both leachates and suspensions were determined at 3 concentrations (1x MIC, 2x MIC and 4x MIC) as shown in Table 4.15. Higher levels of Ag, Al, As, Cu, Fe, Mg and Zn were found in the 4x MIC leachate sample relative to the 1x MIC and 2x MIC samples. In particular, the 4x MIC leachate contained much higher concentration of Al (92.63 ± 2.18 mg/L), Fe (67.46 ± 2.19 mg/L) and Mg (88.48 ± 2.29 mg/L).

Table 4.15: Soluble metals content in clay leachates in different concentrations

Soluble metals (mg/L)	Leachate concentration		
	1x MIC	2x MIC	4x MIC
Ag	0.001 ± 0.000 ^b	0.002 ± 0.000 ^b	0.005 ± 0.002 ^a
Al	30.53 ± 4.42 ^c	53.22 ± 5.42 ^b	92.63 ± 2.18 ^a
As	0.01 ± 0.01 ^b	0.02 ± 0.01 ^b	0.07 ± 0.01 ^a
Cu	0.003 ± 0.001 ^c	0.007 ± 0.002 ^b	0.02 ± 0.00 ^a
Fe	23.97 ± 0.54 ^c	44.04 ± 0.57 ^b	67.46 ± 2.19 ^a
Mg	51.48 ± 2.79 ^c	79.98 ± 1.20 ^b	88.48 ± 2.29 ^a
Mn	0.08 ± 0.04 ^b	0.13 ± 0.06 ^{ab}	0.21 ± 0.02 ^a
Ni	0.02 ± 0.01 ^b	0.04 ± 0.02 ^{ab}	0.06 ± 0.01 ^a
Pb	0.01 ± 0.00 ^a	0.009 ± 0.002 ^a	0.004 ± 0.001 ^b
Zn	0.04 ± 0.02 ^b	0.08 ± 0.03 ^b	0.14 ± 0.02 ^a

Pairs of values (mg/L) with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

4.4.4.2 Available metals in treated Carey clay suspension

Similarly, the 4x MIC suspension also contained high concentration of these three metals, with Al at 59.48 ± 4.05 mg/L, Fe at 25.64 ± 2.92 mg/L and Mg at 108.34 ± 2.36 mg/L (Table 4.16). While other metals were present in significantly higher amounts in the 4x MIC samples, there was no significant difference in Ag, Cu, and Pb content between the three clay suspension concentrations.

Table 4.16: Soluble metals content in clay suspension in different concentrations

Soluble metals (mg/L)	Suspension concentration		
	1x MIC	2x MIC	4x MIC
Ag	0.28± 0.00 ^a	0.28 ± 0.01 ^a	0.28 ± 0.00 ^a
Al	18.05 ± 2.95 ^c	29.43 ± 1.00 ^b	59.48 ± 4.05 ^a
As	n.d.	n.d.	n.d.
Cu	0.01 ± 0.00 ^a	0.006 ± 0.005 ^a	0.01 ± 0.00 ^a
Fe	9.53 ± 0.22 ^c	18.41 ± 0.32 ^b	25.64 ± 2.92 ^a
Mg	33.97 ± 1.10 ^c	57.53 ± 1.72 ^b	108.34 ± 2.36 ^a
Mn	0.07 ± 0.00 ^c	0.14 ± 0.01 ^b	0.25 ± 0.03 ^a
Ni	0.02 ± 0.00 ^c	0.03 ± 0.00 ^b	0.05 ± 0.01 ^a
Pb	0.004 ± 0.000 ^a	0.002 ± 0.002 ^a	0.004 ± 0.001 ^a
Zn	0.05 ± 0.00 ^c	0.08 ± 0.00 ^b	0.13 ± 0.02 ^a

Pairs of values (mg/L) with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

n.d.: Not determined. As analysis was unable to be carried out due to unavailability of sample, which was the limitation during the Movement Control Order (MCO).

Differences in the metal content between leachate and suspension were noteworthy. For the 4x MIC, Al and Fe in the leachate were elevated over the suspension by 1.6- and 2.6-fold, respectively. In contrast, the suspension contained had 56-fold higher Ag and 13-fold higher Mg than the leachate. Analysis of soluble metals in the suspension form revealed a significantly higher Ag and Mg content, although much lower amounts of Al, Cu and Fe in comparison to its leachate form.

4.4.4.3 Correlation analysis and principal component analysis for the effect of metals concentrations against antibacterial activity of treated Carey clay

The correlation between metal concentrations in treated Carey clay samples (leachate and suspension) and *S. Typhimurium* and *S. aureus* log reduction is shown in Table 4.17. Metals Al, Cu, Fe, Ni and Zn had strong positive correlation with *S. Typhimurium* log reduction ($r > 0.7$), which are significant at 99% confidence level ($p < 0.01$). Metals Mg and Mn showed moderate positive correlation with log reduction of *S. Typhimurium* ($p < 0.05$). This result suggested that the high concentrations of metals especially Al, Cu, Fe, Ni and Zn could be responsible in inhibiting growth of *S. Typhimurium*.

For log reduction of *S. aureus*, only Ag showed strong positive correlation with $r = 0.837$ and are significant at 99% confidence level ($p < 0.01$). Metal Pb had strong negative correlation, while Mn and Zn showed moderate positive correlation against *S. aureus* inhibition. This indicates that most of the metals tested had very little effect on growth inhibition of *S. aureus*, except for Ag. However, it is possible that these metals work in combination to produce antibacterial effect against *S. aureus*.

Table 4.17: Correlation analysis for the effect of metals concentrations against antibacterial activity of treated Carey clay

Variables	Log Reduction (ST)	Log Reduction (SA)	Ag	Al	Cu	Fe	Mg	Mn	Ni	Pb	Zn
Log Reduction (ST)	1										
Log Reduction (SA)	-0.043	1									
Ag	-0.433	0.837**	1								
Al	0.891**	0.007	-0.468	1							
Cu	0.748**	0.330	0.065	0.651**	1						
Fe	0.883**	-0.295	-0.707**	0.930**	0.511*	1					
Mg	0.469*	0.276	-0.154	0.788**	0.359	0.602**	1				
Mn	0.506*	0.544*	0.133	0.721**	0.543*	0.447	0.855**	1			
Ni	0.758**	0.311	-0.186	0.909**	0.662**	0.729**	0.806**	0.900**	1		
Pb	-0.174	-0.775**	-0.718**	-0.049	-0.341	0.139	-0.116	-0.351	-0.171	1	
Zn	0.710**	0.482*	-0.003	0.843**	0.689**	0.623**	0.789**	0.928**	0.980**	-0.331	1

*Correlation is significant at the 0.05 level (two-tailed).

**Correlation is significant at the 0.01 level (two-tailed).

The Pearson correlation matrix showing strength of relationship between variables ($r < |0|$ = no correlation, $|0| < r < |0.3|$ = weak correlation, $|0.3| < r < |0.7|$ = moderate correlation and $|0.7| < r < |1.0|$ = strong correlation).

ST: *S. Typhimurium*.

SA: *S. aureus*.

The biplot in Figure 4.13 exhibited two clusters, which are observations and variables with high correlations with log reduction of *S. Typhimurium*, while the other one is associated with *S. aureus* log reduction. The leachate 4x MIC and suspension 4x MIC was positioned near to *S. Typhimurium* log reduction, indicating that these two observations are highly correlated with bacterial inhibition of *S. Typhimurium*.

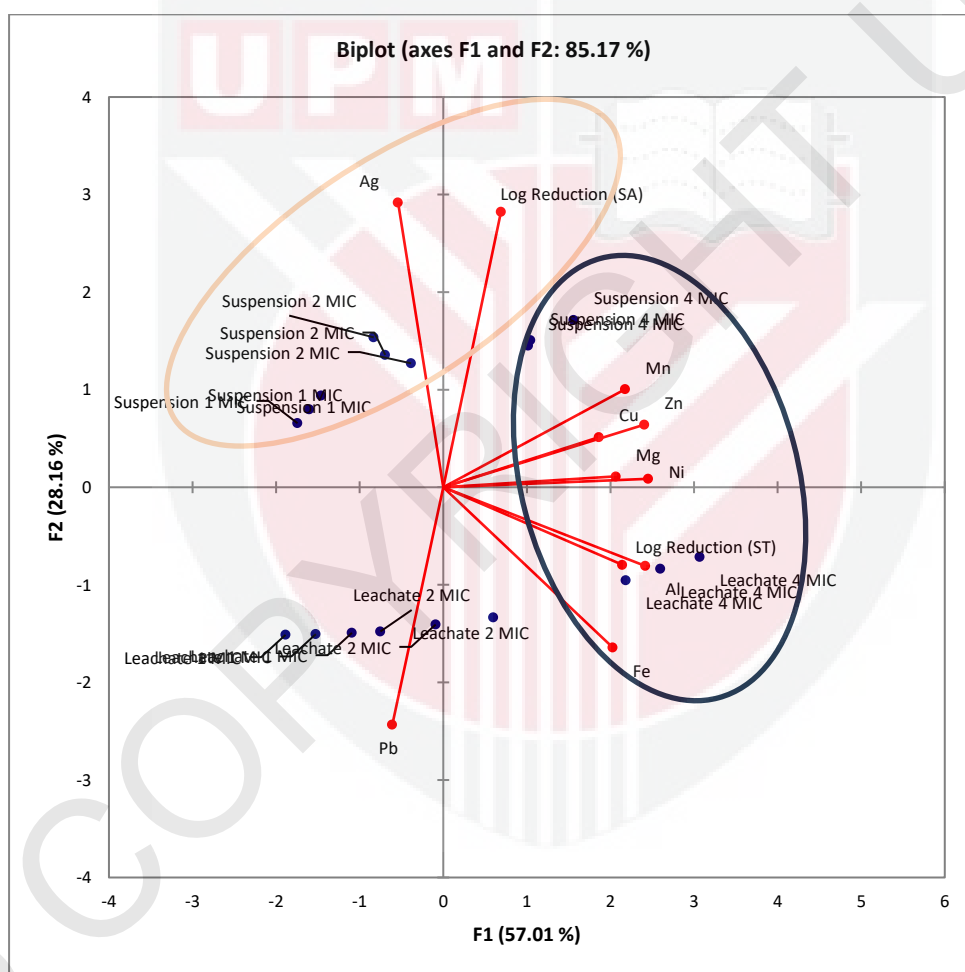


Figure 4.13 : Biplot of correlation between soluble metals concentrations on the antibacterial activity of treated Carey leachates and suspensions against *S. Typhimurium* (ST) and *S. aureus* (SA).

Log reduction of *S. aureus*, which was previously found to have strong positive correlation with Ag (Table 4.17), had all clay suspension observations (1x MIC, 2x MIC and 4x MIC) clustered together. This result indicates that all suspension samples, regardless of the concentrations are effective in inhibiting growth of *S. aureus*.

Carey clay belongs to the acid sulfate type, which contains high concentrations of metals, including Al and Fe (Shamshuddin, 2006). The cation-exchange capacity (CEC) of clay refers to its ability to exchange cations with the surrounding medium. Significantly, toxic metal ions released in this fashion from clay interlayers can exert bacteriostatic and bactericidal effects (Babich & Stotzky, 1977). Although pH is not the sole factor in clay antibacterial efficacy, the presence of an acidic environment clearly aids the solubility of known toxic metals (Cunningham et al., 2010). The metals associated with bacterial toxicity include Al (Londono & Williams, 2016) and Fe (Morrison et al., 2013; Williams et al., 2008, 2011). The combination of high Al and Fe in an acidic environment with the Carey clay seems likely to be the major factors responsible for its antibacterial activities. Carey clay also contains lower quantities of additional metals, including Ag, Mn, Ni, Pb and Zn that could contribute to antibacterial activity (Godoy-Gallardo et al., 2021; Kawakami et al., 2008; Si et al., 2017). Fe, Cu, Al and Zn can induce irreversible damage to bacteria by stimulating ROS production, mismetallation of metalloenzymes, disrupting protein binding to biological molecules or displacing essential ions involved in membrane stabilization (Collins & Stotzky, 1989).

4.4.5 Effect of standard metals against *S. Typhimurium* and *S. aureus*

4.4.5.1 MIC and MBC determination

Antibacterial activity of individual standard metal was tested against both *S. Typhimurium* and *S. aureus*, as shown in Table 4.18. The metals showed MIC values ranging from 15.63 mg/L to 125 mg/L for *S. Typhimurium*, and 7.81 mg/L to 125 mg/L for *S. aureus*. In particular, Ag showed the lowest MIC values, which was 15.63 mg/L and 7.81 mg/L for *S. Typhimurium* and *S. aureus*, respectively. It is also noted many of the metals showed the same MIC and MBC values when tested against both of the bacteria, which are Cu, Fe, Mg, Mn, Pb and Zn.

Table 4.18: Values of MIC and MBC for standard metals against *S. Typhimurium* and *S. aureus*

Metals	<i>S. Typhimurium</i>		<i>S. aureus</i>	
	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)
Ag	15.63	31.25	7.81	15.63
Al	62.5	125	125	250
Cu	62.5	125	62.5	125
Fe	62.5	125	62.5	125
Mg	125	250	125	250
Mn	125	250	125	250
Ni	125	250	62.5	125
Pb	125	250	125	250
Zn	62.5	125	62.5	125

4.4.5.2 Fractional inhibitory concentration (FIC) determination

The fractional inhibitory concentration (FIC) assay revealed certain combination of certain metals can produce different antibacterial effects. The combination of Al-Fe against *S. Typhimurium*, along with Al-Fe and Al-Zn combinations against *S. aureus* resulted in FIC value of less than 0.50, which demonstrated synergistical antibacterial efficacy (Table 4.19). Other metal combinations showed indifference antibacterial effect.

Table 4.19: Fractional inhibitory concentration index for metal ion combinations

Metal combinations	<i>S. Typhimurium</i> ^a	<i>S. aureus</i> ^a
Al-Fe	0.38 ± 0.00	0.38 ± 0.00
Al-Zn	0.63 ± 0.34	0.38 ± 0.00
Cu-Fe	0.50 ± 0.00	0.63 ± 0.34
Cu-Zn	0.75 ± 0.34	0.75 ± 0.00
Fe-Zn	0.50 ± 0.00	0.75 ± 0.00
Ni-Zn	0.75 ± 0.00	0.75 ± 0.00

^a Synergy = <0.5, indifference = 0.5-4.0, antagonism = >4.0

Five metals (Al, Cu, Fe, Ni and Zn) with different combinations were chosen based on the outcome from correlation analysis (see Table 4.17), of which these metals showed strong positive correlation with *S. Typhimurium* log reduction ($r > 0.7$) and are significant at 99% confidence level ($p < 0.01$). Ag was the only metal showing strong positive correlation with log reduction of *S. aureus*, hence its interaction with other metals was not tested in this study. Silver ions (Ag^+) and silver nanoparticles (AgNPs) were reported to demonstrate a broad spectrum of microbicidal activity against bacteria, fungi

and viruses, and also promising to combat antibiotic resistant *S. aureus* (Marambio-Jones & Hoek, 2010; Salah et al., 2021). The strong positive correlation of Ag with *S. aureus* inhibition might be due to the cell walls of the gram-positive bacteria, which have higher charge capacity, thus allowing more cations interaction in comparison to the gram-negative bacteria (Mullen et al., 1989).

As previously determined, individual metals showed a considerably high MIC and MBC values when compared to the values of Carey clay (125 mg/L and 250 mg/L, respectively). On the other hand, the FIC assay demonstrated some metal combinations showed synergistical antibacterial efficacy. The combination of metals can magnify toxicity due to synergistic effects. This is in line with a study by Morrison et al. (2016) who found that the antibacterial activity of Oregon Blue Clay is due to synergistic activities of Fe^{2+} and Al^{3+} which produce greater toxicity to bacteria at lower concentrations than individual metals. Supplementation of a non-antibacterial leachate with combinations of Fe, Co, Cu, Ni and Zn metals ions has been demonstrated to exhibit antibacterial activity against *E. coli* and MRSA (Otto & Haydel, 2013a) working synergistically to induce cell death. This suggested that the available metals in the Carey clay may work together in contributing to the antibacterial activity.

4.5 Antibacterial mode of action of treated Carey clay

To investigate in more detail the antibacterial mode of action of the treated Carey clay against *S. Typhimurium* and *S. aureus*, the treated cells were visualised using transmission electron microscopy (TEM) to look for any abnormality in bacterial morphology, as well as an examination of the impact on cell membrane permeability and membrane integrity. Bacterial toxicity due to the high metal content of treated Carey clay was investigated, alongside an examination of reactive oxygen species (ROS) production.

4.5.1 Morphological changes

To visualise any effects on cell morphology that might account for the observed bactericidal activity, both strains were exposed to treated Carey clay and analysed by TEM. As expected, *S. Typhimurium* grown in nutrient broth (NB), as an untreated control, exhibited a normal cellular morphology (Figure 4.14a, Figure 4.15a). However, there was clear evidence of damaged and lysed cells in samples exposed to the highest clay concentration (4x MIC) after only 30 min incubation with either leachate (Figure 4.14d) or suspension (Figure 4.15d). Some loss of integrity and cohesion of the cell wall was evident at 1x MIC (Figure 4.14b, Figure 4.15b), which was even more pronounced at 2x MIC concentrations (Figure 4.14c, Figure 4.15c). At 2x MIC and 4x MIC, rupture of the cell wall is visibly accompanied by release of cellular constituents. Despite clay suspensions taking longer than leachates to kill cells (see Figure 4.11a and Figure 4.11b), there was greater cell wall disintegration and lysis with the Carey suspension (Figures 4.15b, c, d). TEM images capture 2D projections



Figure 4.14: TEM images of *S. Typhimurium* ATCC 14028 after treatment with clay leachate for 30 min a) control; b) 1x MIC; c) 2x MIC; d) 4x MIC. Arrow showed morphological damage of treated bacteria cells.

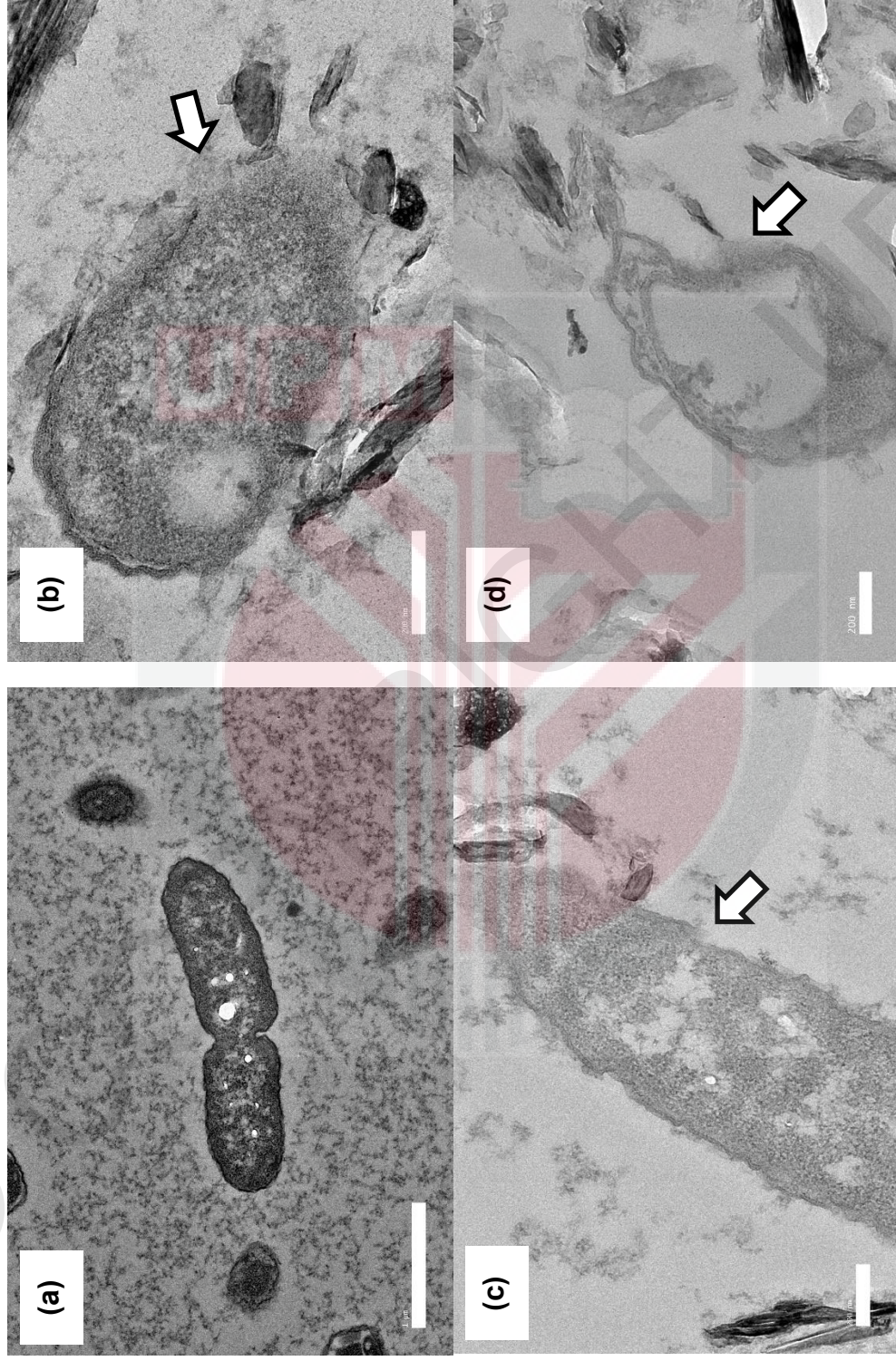


Figure 4.15: TEM images of *S. Typhimurium* ATCC 14028 after treatment with clay suspension for 30 min a) control; b) 1x MIC; c) 2x MIC; d) 4x MIC. Arrow showed morphological damage of treated bacteria cells.

of 3D objects. If the *S. Typhimurium* cell is viewed from a specific angle, it may appear more spherical rather than elongated, as shown in Figure 4.14c.

The untreated *S. aureus* cells showed typical coccal morphology and cell wall structure with the dark line of the cytoplasmic membrane surrounded by an intact peptidoglycan cell wall (Figure 4.16a, Figure 4.17a). At the lowest concentration of the clay leachate (1x MIC) there was little indication of cell wall changes after 6 h of treatment (Figure 4.16b). However, at 2x MIC there were indications of loss of cell wall integrity (Figure 4.16c) and at 4x MIC there was clear evidence of cell lysis in some cells (Figure 4.16d). Consistent with the greater antibacterial efficacy of the suspension against *S. aureus* (Figure 4.17c, d), cell wall deterioration was noted at 1x MIC (Figure 4.17b). At 2x MIC and 4x MIC with the clay suspension the dark stained lines of the membrane disappear when treated, indicating substantial cell wall degradation (Figure 4.17c, Figure 4.17d) and likely cell death as evidenced in the time-kill analysis (see Figure 4.12). In suspension samples, dark, sharp needle-like particles surrounding the cells appeared to be the clay particles, which regularly associate with the membrane-damaged cells (Figures 4.16b-d and Figures 4.17b-d).

The images of the treated bacteria cells revealed significant damage to the cell wall of both *S. Typhimurium* and *S. aureus* and lysis at higher clay concentrations. These findings differ from those of Otto et al. (2010), who found that *E. coli* and MRSA cells remained intact and failed to induce lysis

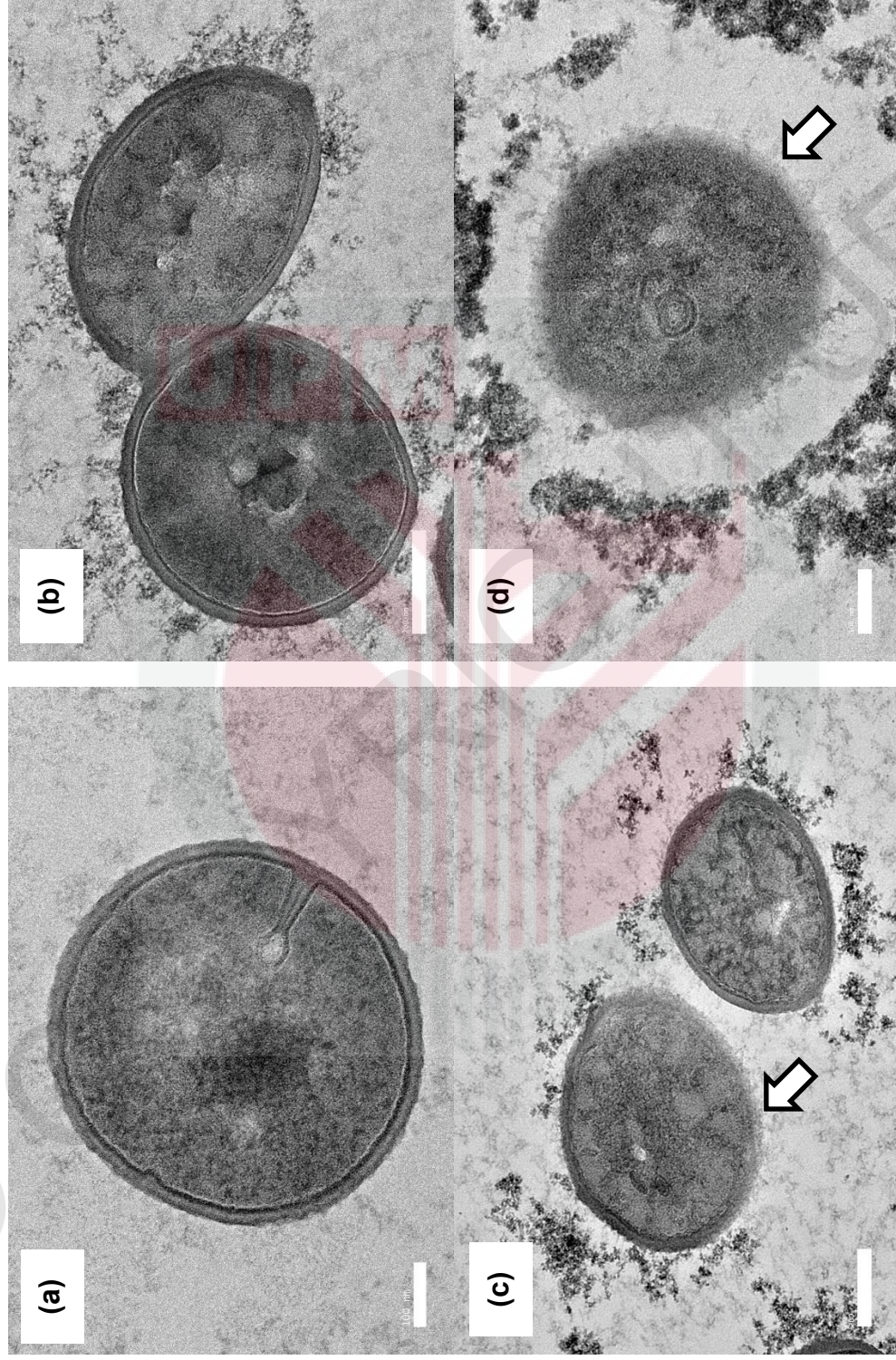


Figure 4.16: TEM images of *S. aureus* ATCC 13565 after treatment with clay leachate for 6 h a) control; b) 1x MIC; c) 2x MIC; d) 4x MIC. Arrow showed morphological damage of treated bacteria cells.

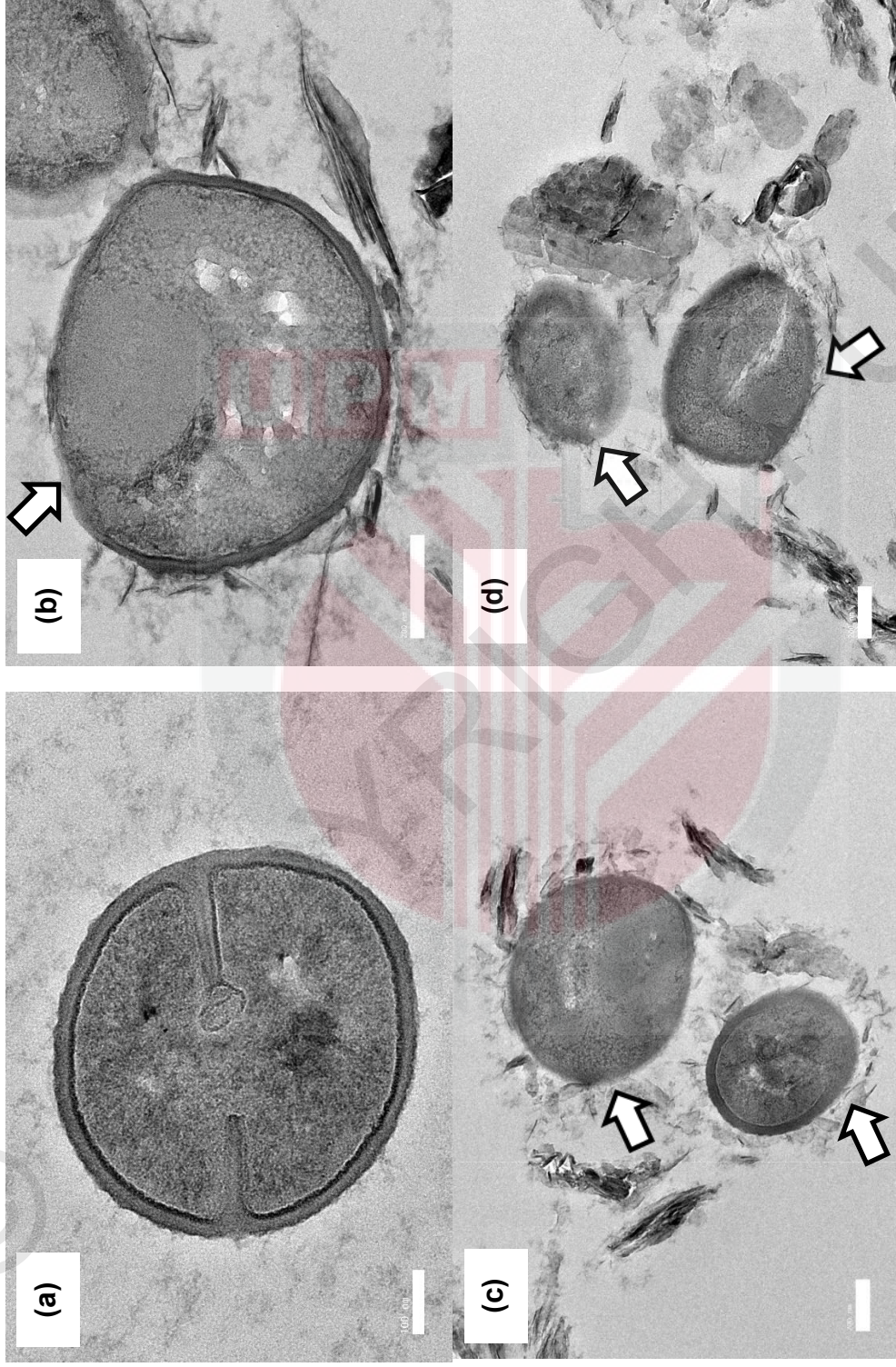


Figure 4.17: TEM images of *S. aureus* ATCC 13565 after treatment with clay suspension for 6 h a) control; b) 1x MIC; c) 2x MIC; d) 4x MIC. Arrow showed morphological damage of treated bacteria cells.

after 24 h incubation with mineral leachates. Morrison et al. (2013) also showed that no lysis occurred with *E. coli* and *S. epidermidis* cells when tested with 100 mg/mL mineral suspension.

The results suggest that the antibacterial mode of action is different from those in previous studies, although this could possibly be due to the higher clay concentrations applied. Cells do not appear to be killed through physical penetration, as treated Carey clay particles do not enter the membrane-damaged cells. They only surround the cells and attach around the membrane-damaged cells. A previous study found insufficient attraction between clay particles and bacterial surfaces, hence physical interaction of clay and bacteria was considered weak (Londono & Williams, 2016). Haydel et al. (2008) found that K^+ exchange with antibacterial clay mineral resulted in the complete loss of bactericidal activity, suggesting the importance of chemical exchange, rather than surface properties in contributing to the antibacterial properties of the clay minerals.

4.5.2 Cell membrane integrity

4.5.2.1 Leakage of cellular DNA

Loss of membrane integrity was also indicated by measuring the nucleic acid content (OD_{260nm}) released from bacterial cells treated with either leachate or suspension (Figure 4.18). The nucleic acid content of the clay-treated bacterial cells increased significantly over time ($p < 0.05$), whereas the untreated control did not alter ($p > 0.05$). For *S. Typhimurium*, leakage of cellular DNA increased

from a baseline value of 0.14, to 0.25 ± 0.01 and 0.22 ± 0.01 after 0.5 h for clay leachate and suspension, respectively, and remained stable thereafter. A similar pattern of cellular DNA release was apparent with *S. aureus*, which increased from 0.11 to 0.25 ± 0.02 and to 0.22 ± 0.01 for clay leachate and suspension at 6 h, respectively, and remained unchanged until the end of the 24-hour treatment (Figure 4.18b).

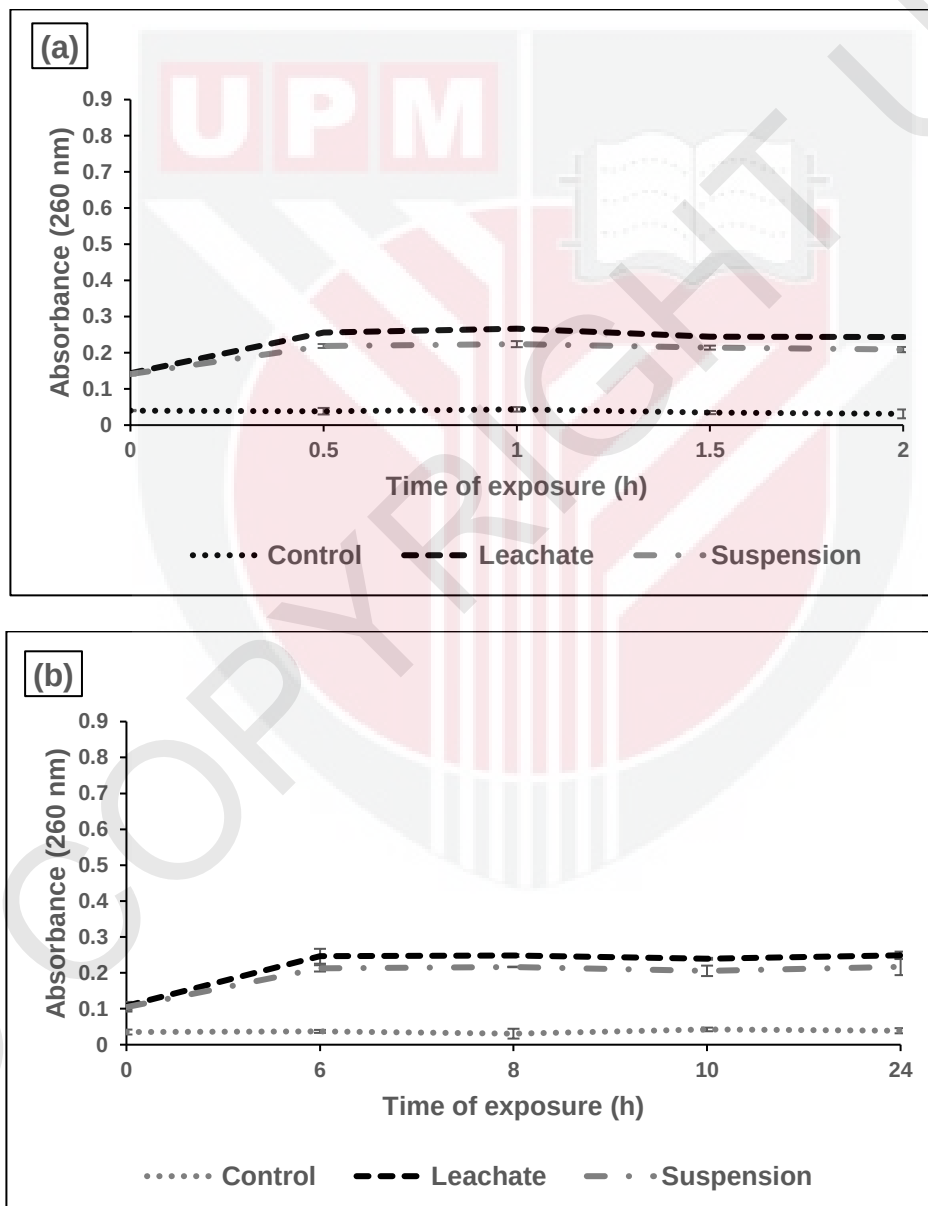


Figure 4.18: Effect of clay leachate and suspension treatment at 4x MIC on cellular DNA leakage from *S. Typhimurium* (a) and *S. aureus* (b) cells.

4.5.2.2 Leakage of protein

Leakage of protein from bacteria following exposure to clay samples at 4x MIC concentration was monitored using the Bradford assay. In order to calculate bacterial protein leakage, a standard BSA curve was developed; $y = 0.4714x + 0.0197$ (Appendix B5). As shown in Figure 4.19, leakage of proteins, consistent with membrane damage and lysis, gradually increased over time. Due to the rapid killing of *S. Typhimurium* by clay samples (see Figure 4.11), protein levels were assessed over a 2-hour period. Similar amounts of protein were released throughout the experiment with both forms of clay, with 0.35 ± 0.02 mg/mL recovered from leachate samples and 0.35 ± 0.04 mg/mL from the suspension after 2 hours (Figure 4.19a). These levels were significantly higher ($p < 0.05$) than the untreated *S. Typhimurium* control, which released 0.24 ± 0.01 mg/mL at this time point.

For *S. aureus*, the leakage of protein by treated samples began to increase significantly in comparison to the control after 6 hours of exposure to the clay samples (Figure 4.19b). By the end of the 24-hour incubation period, *S. aureus* treated with the clay suspension released similar amounts of protein (0.22 ± 0.04 mg/mL) relative to the leachate (0.21 ± 0.04 mg/mL). At the same time point, the control *S. aureus* only resulted in 0.12 ± 0.01 mg/mL protein, which was significantly lower ($p < 0.05$) in comparison to the clay-treated bacterial cells.

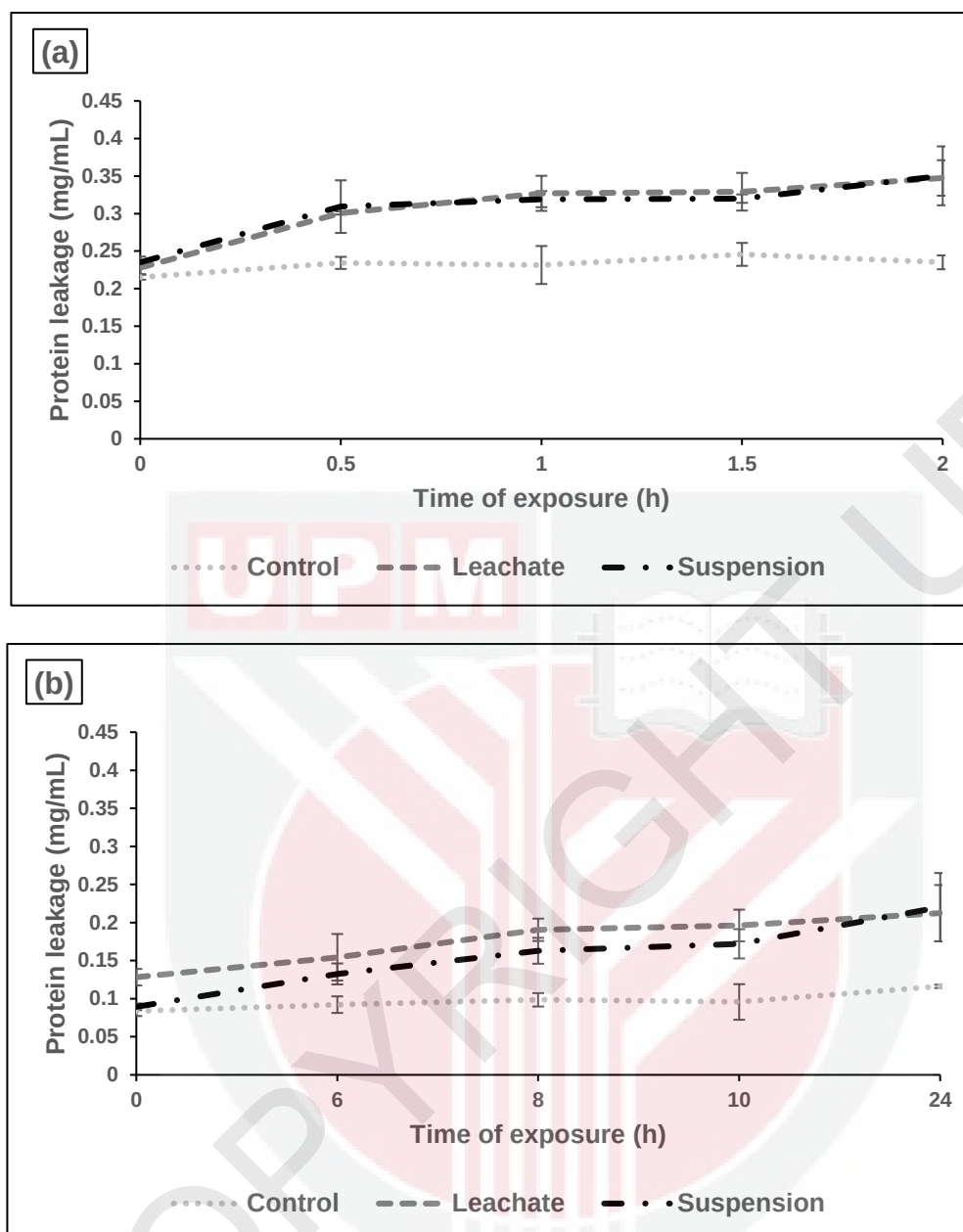


Figure 4.19: Leakage of protein, measured from a) *S. Typhimurium* and b) *S. aureus* suspensions without (control) and after exposure to the clay leachate and suspension at concentration of 4x MIC, measured at different exposure times.

Analysis of cell membrane integrity can provide information on the antibacterial mode of actions, as it is critical for understanding the effect on bacterial growth and survival. Release of cytoplasmic constituents such as DNA and RNA has been reported to be an indicator of cell membrane integrity disruption, which

can be evaluated by measuring absorbance of the bacterial suspension at 260 nm (Bajpai et al., 2013; Li et al., 2019). Exposure of the two test bacteria to the treated Carey clay leachates and suspensions, did result in significant increases in the release of cytoplasmic constituents in comparison to control (untreated cells). Leachates caused greater leakage than clay suspension in both of the test bacteria, which could be related to the presence of metals which are readily available for interaction with bacterial cellular membrane. The release of 260 nm absorbing materials (DNA and RNA) was previously reported for both gram-negative *E. coli* and gram-positive *S. aureus* after incubation with antibacterial Ni and Ag, indicating the ability of the metal ions in causing membrane damage (Li et al., 2019; Panicker et al., 2020).

Similarly, leakage of intracellular proteins can also be used to assess cell membrane damage (Bouyahya et al., 2019; Du et al., 2012; Li et al., 2010). The increased amount of protein leaked after incubation of both test bacteria with both clay leachates and suspensions in comparison to control further indicates that irreversible damage might occur to the cytoplasmic membranes. Siddique et al. (2020) reported the potential of Ag, in the form of nanoparticles (AgNPs) to disrupt cell membrane thus causing leakage content of gram-negative multidrug-resistant *Klebsiella pneumoniae* as detected by the quantification of membrane proteins using Bradford assay. Similar outcomes were reported for both *E. coli* and *S. aureus* after incubation with Zn, with the concentration of protein leakage was higher by *E. coli* than *S. aureus* (Haghniaz et al., 2021). This is similar in comparison to the results in this study, which showed that protein concentration released by gram-negative *S.*

Typhimurium was higher than that of gram-positive *S. aureus* (see Figure 4.19). The differences in the structure and chemical composition of the bacterial cell wall may have resulted in such occurrence. These results indicate the role of metals, released from clay minerals in causing loss of cellular membrane integrity by membrane damage.

The protein leakage result is almost similar for both bacteria, however the time-kill result (see Section 4.4.3) shows a different pattern, where bacterial killing of *S. aureus* took longer time in comparison to rapid killing of *S. Typhimurium*. Protein leakage reflects the damage to the bacterial cell membrane or wall, indicating disruption, but not necessarily immediate death (Silhavy et al., 2010). Time-kill assays measure the actual bacterial viability over time. While *S. aureus* may show signs of membrane disruption early on (leading to protein leakage), it may still take longer for the cells to die completely due to their robust cell wall and different metabolic processes. Gram-positive bacteria like *S. aureus* can activate stronger repair mechanisms and maintain some cellular processes even after partial damage to their membrane (Jordan et al., 2008; Kohanski et al., 2010). This can cause the bacteria to survive for a longer period despite exhibiting protein leakage. The rate of protein leakage may reflect rapid membrane disruption, which can happen similarly in both bacterial types when exposed to antibacterial clay. However, cell death can take longer depending on how vital cellular functions are compromised, and Gram-positive bacteria may withstand membrane damage longer before dying.

4.5.3 Changes in membrane potential

S. Typhimurium and *S. aureus* cell membrane potential was analysed following incubation with clay samples at 30 minutes and 6 hours, respectively (Figure 4.20). Membrane depolarization occurs in membrane-damaged cells, and DiBAC₄(3) was employed as membrane probe, where an increase in fluorescence indicates membrane damage.

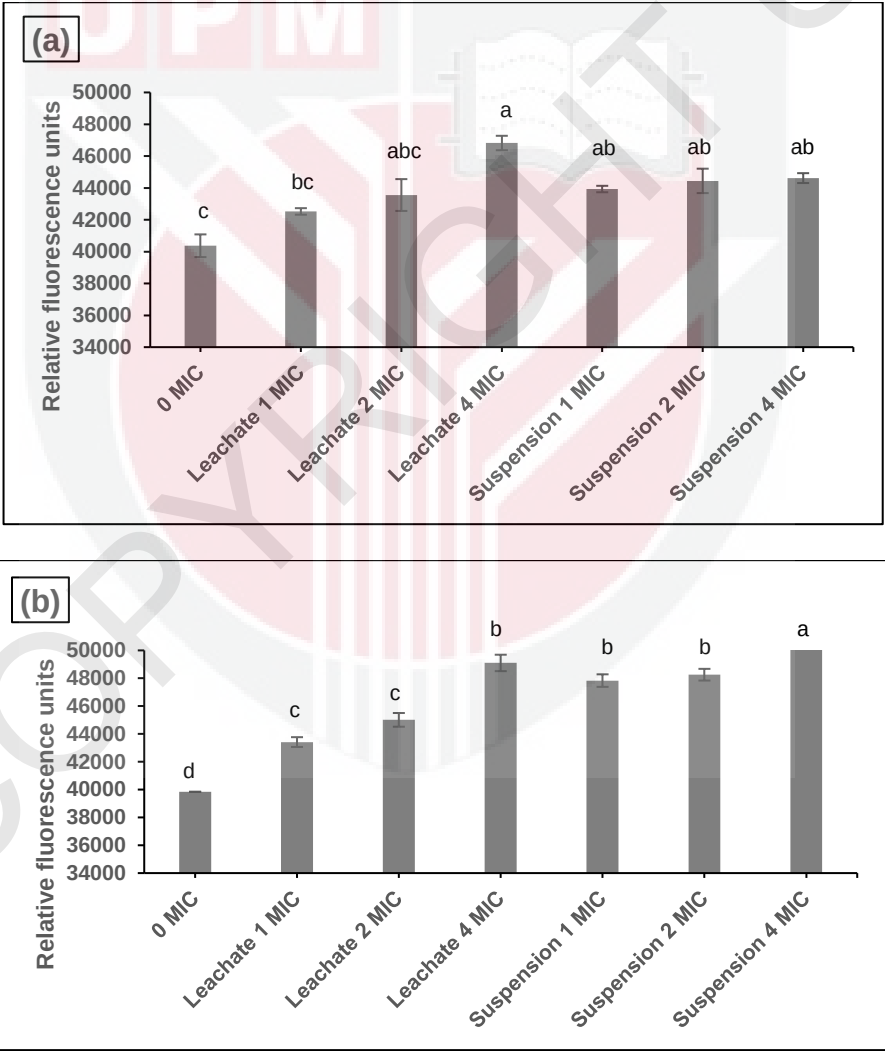


Figure 4.20: Changes in membrane potential for a) *S. Typhimurium* after 30 min of exposure to samples and b) *S. aureus* after 6 hours of exposure to samples, respectively. Pairs of values with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

Relative to the untreated control, *S. Typhimurium* cells exposed to clay leachates showed a gradual increase in DiBAC₄ fluorescence with a peak (46765 ± 247) at the highest concentration (4x MIC) (Figure 4.20a), while suspension-treated cells showed no difference with the increased in clay concentration. The experiment was repeated with *S. aureus* cells exposed to clay samples after a 6-hour incubation period (Figure 20b). Treatment with clay leachates or suspensions seemed to result in increased DiBAC₄ fluorescence with increased clay concentration, consistent with effects on membrane potential. The 4x MIC suspension-treated sample produced the highest fluorescence (52387 ± 1253), with the suspensions showing higher changes in membrane potential relative to the leachates at the same concentration.

DiBAC₄(3) is a membrane probe that allows measurement of an anionic potential response which is usually excluded by healthy cells. Membrane depolarization occurs in membrane-damaged cells, allowing DiBAC₄(3) to enter, bind to intracellular proteins and membrane and produce an increase in fluorescence (Bridges et al., 2020). In this study, clay samples resulted in membrane depolarization of *S. Typhimurium* and *S. aureus* cells. Depolarization of the cell membrane is consistent with structural damage to the bacterial cell membrane, as it is associated with influx of Na⁺ into the cell, causing a charge imbalance due to the reduction in intracellular negative charge (Gries et al., 2013).

4.5.4 Reactive oxygen species (ROS) detection

The NBT assay was used to probe bacterial cells for the generation of oxidative stress arising from exposure to the different clay samples. Higher absorbance values at OD_{575nm} indicate increased ROS production in cell supernatants. As shown in Figure 4.21, the result revealed that overall, both leachate and suspension-treated *S. Typhimurium* showed increased absorbance readings that correlated with increased clay concentration (Figure 4.21a). The nitroblue tetrazolium (NBT) reduction method relies on the blue NBT formazan formation, which is produced by reduction of the membrane permeable, yellow-colored, nitroblue tetrazolium by superoxides (O_2^-). Absorbance was taken for formazan formed using UV spectroscopy in order to quantify the ROS generation by the test bacteria after treatment with clay leachate and suspension with different concentrations. The formation of superoxide leads to hydrogen peroxide (H_2O_2) generation which ultimately produces highly reactive hydroxyl radicals ($\text{OH}^\cdot$).

Substantially increased absorbance OD_{575nm}, corresponding to greater ROS, was evident with the clay suspensions over the leachates, with the highest absorbance value of 1.75 ± 0.03 shown by suspension at 4x MIC. Very similar results were obtained with *S. aureus*, with greatest oxidative stress shown with the clay suspensions 2x MIC and 4x MIC, with absorbance values of 1.20 ± 0.07 and 1.37 ± 0.21 , respectively (Figure 4.21b).

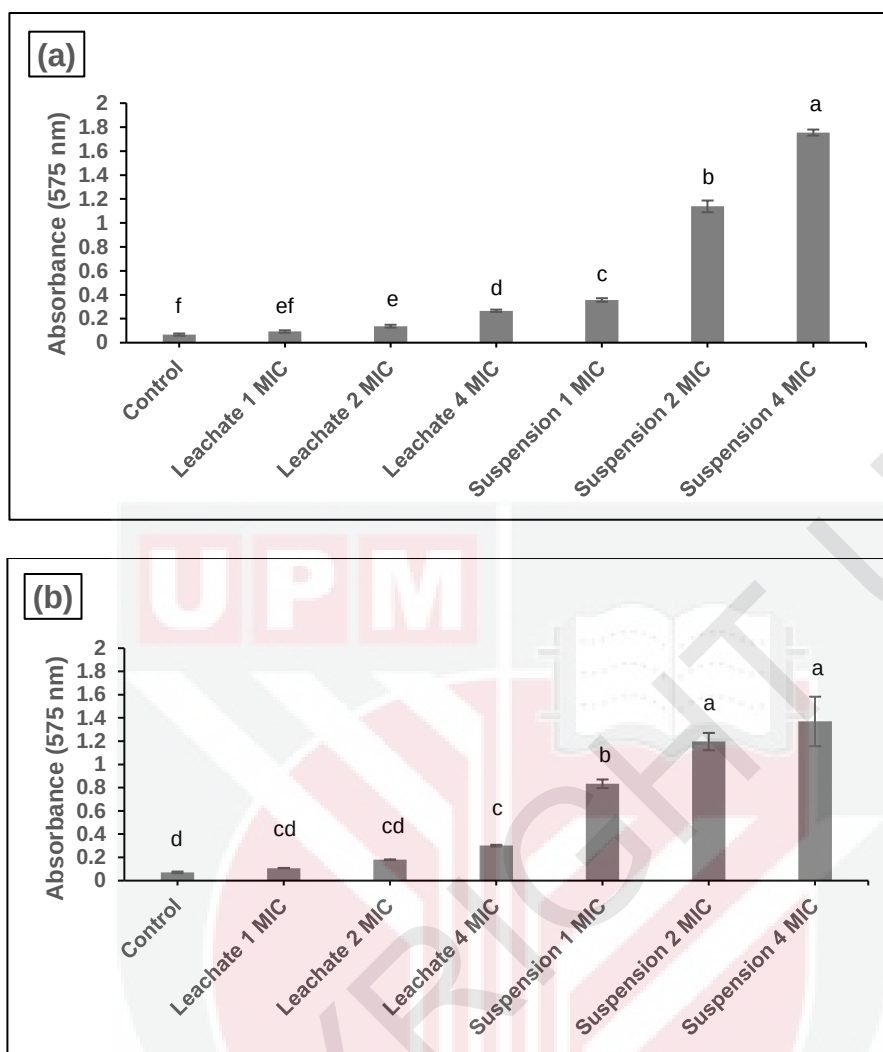


Figure 4.21: NBT assay showing the production of ROS for (a) *S. Typhimurium* and (b) *S. aureus* suspensions without (control) and after exposure to the clay leachates and suspensions at different concentrations. Pairs of values with different lowercase superscripts indicate a significant difference ($p < 0.05$) between groups.

Leachate-treated *S. aureus* did not improve with increasing concentration, however the ROS produced by 4x MIC was significantly higher in comparison to control ($p < 0.05$). Comparison of the absorbance values shown by 4x MIC suspension-treated cells indicated production of ROS in *S. Typhimurium* was greater than *S. aureus*. This is consistent with previous studies that reported greater toxic effect of clay and metal ions on gram-negative than gram-positive bacteria (Fayaz et al., 2010; Jung et al., 2015). The difference could be

attributed to the presence of peptidoglycan layer, which consists of cross-linked polysaccharide chains that causes the rigidity of the gram-positive bacteria (Shockman & Barrett, 1983). On the other hand, the outer membrane and the thin peptidoglycan layer do not form rigid structure in in gram-negative bacteria (Beveridge, 1999).

Membrane-damaged bacteria might be expected to exhibit stress due to increased reactive oxygen species (ROS) production, and these results are consistent with a previous report showing membrane-damaging action by clay in generating oxidative stress from bacteria (Jung et al., 2015). The treated Carey clay suspension caused greater membrane damage than the leachates, with the generation of ROS corresponding to the increasing clay concentration. The ongoing release of toxic metals from clay particles may directly contribute to the increased ROS generation by the clay suspension samples. For instance, Ag has been reported to cause oxidation of cellular proteins in the plasma membrane of the membrane-damaged cells, which is likely to disrupt cell homeostasis (Su et al., 2009).

The exact mode of action for antibacterial clay has not been established due to the difference in geochemistry and mineralogical compositions (Williams, 2017). Clay minerals play an important role in buffering aqueous conditions so that metals remain within solution for a period of time sufficient to cause oxidation reactions which can harm bacteria's defenses. The main antibacterial mode of action of treated Carey clay might be attributed to the release of soluble species that interact with bacterial membranes.

TEM studies provided insight on changes of bacterial ultrastructure, membrane impairment, and leakage of intracellular materials induced upon treatment of *S. Typhimurium* and *S. aureus* with treated Carey clays. The same approach was previously used for analysing the mode of action of metal ions and novel antimicrobial agents (Li et al., 2010; Woo et al., 2008). In this study, TEM images of both leachate and suspension-treated *S. Typhimurium* and *S. aureus* bacterial cells clearly show cell wall degradation and lysis. Lemire et al. (2013) stated that results from morphological studies alone is insufficient to distinguish whether membrane disruption or cell wall detachment is a cause or a consequence of bacterial death, hence other complementary studies on are needed.

Analysis on cell membrane integrity revealed greater leakage of cellular DNA by leachates in comparison to the clay suspension in both *S. Typhimurium* and *S. aureus*. For protein leakage, similar result was observed for *S. Typhimurium*. However, suspension-treated *S. aureus* revealed to cause greater leakage of protein in comparison to the leachates. It was reported that nanoparticles can compromise bacterial cell envelope integrity through the production of ROS, which cause lipid peroxidation (Kibanova et al., 2009). Exposure to toxic levels of metals such as Al and Ag were reported to have severely compromising the cell membrane integrity (Li et al., 2010; Yaganza et al., 2004). In particular, Al^{3+} was reported to have the ability to forms complexes with phosphate ligands which were found both in bacteria and in clay minerals (Macdonald & Martin, 1988). The results of this chemical affinity include alterations of lipid–protein interactions that would lead to membrane damage (Pina & Cervantes,

1996) and destabilising the membrane of gram-negative bacteria by displacement of the structural cations Ca^{2+} and Mg^{2+} , the cations that binds to LPS (Borrok et al., 2005; Hancock, 1984). Londono et al. (2017) stated the possibility of AMZ clay to behave similarly, with outer membrane of *E. coli* found to be compromised, suggesting that the clay may have caused damaged to the phospholipid in the outer membrane of the gram-negative bacteria. Thus, based on the high concentration of Al in treated Carey clay, the metal ions could cause bacterial cell membrane damage by compromising the integrity of the membrane, potentially causing leakage of cytoplasm (Beveridge & Koval, 1981). The results of this study seem to be in agreement, with the result showing loss of membrane integrity, as shown by the leakage of cellular protein and DNA (see Figure 4.17 and Figure 4.18). However, a study by Otto et al. (2010) found bactericidal activity of clay mineral leachates against *E. coli* without occurrence of permeabilization of the bacterial cell membrane, which suggests the difference in the mode of action.

Membrane potential is one of the factors affecting bacterial cell viability. Analysis on membrane potential changes showed that *S. Typhimurium* cells exposed to clay leachates caused a gradual increase in DiBAC₄ fluorescence with a peak at the highest concentration, and the DiBAC₄ fluorescence was greater in comparison to suspension-treated cells. The increased in fluorescence indicates depolarization of the membrane. Cell membrane depolarization also occurred in *K. pneumoniae* cells treated with Ag nanoparticles, as the structural changes of the bacteria cause blocking of the transport channels (Khalandi et al., 2017; Molina-Hernandez et al., 2021).

The treated Carey clay showed presence of pyrite (see Figure 4.5) which has been reported to be antibacterial. When there is water present, dissolution of pyrite occurs which produces ROS, such as hydrogen peroxide and hydroxyl radicals through the Fenton reaction, which degrade nucleic acids in RNA and DNA (Schoonen et al., 2010). In this study, *S. aureus* cells exposed to clay suspensions showed higher changes in membrane potential relative to the leachates at the same concentration. Substantially increased absorbance OD_{575nm}, corresponding to greater ROS, was evident with the clay suspensions over the leachates for both *S. Typhimurium* and *S. aureus*. Bacterial cell walls have strong interactions with metal cations, governing the type and amount of ions that can enter the cytoplasm (Beveridge & Koval, 1981). Previous studies reported metal-induced oxidative stress of certain metals that elevate intracellular ROS, especially Fe²⁺ and Cu²⁺ when present in toxic amounts (Lemire & Turner, 2016). Hydroxyl radicals formed by Fe²⁺ and hydrogen peroxide was reported to cause significant damage to cellular DNA (Park & Imlay, 2003). In addition, the presence of other transition metals in clay minerals can produce hydroxyl radicals through Fenton-like reaction (HaMai et al., 2005). Mn²⁺ and Mn³⁺ was reported to also generate hydroxyl radicals via oxidative stress (Das et al., 2020; Syed et al., 1995), and caused morphological changes in the biofilm structure of *P. aeruginosa* (Jabłońska-Wawrzycka et al., 2021). Morrison (2016) reported that aqueous Al³⁺ produced greater damage to cell membrane proteins than intracellular proteins, whereas Fe²⁺ caused the most damage to DNA. Studies on antibacterial clay revealed the production of H₂O₂ by Oregon blue clay (Morrison et al., 2016) and Columbian lacustrine clay (Londono et al., 2017). The ROS generated by the

presence of various metals in treated Carey could be one of the major mode of action.

Based on the analysis on the antibacterial mode of action, it can preliminarily conclude that the treated Carey clay cause antibacterial activity by affecting integrity of the bacterial cellular membrane, which result in leakage proteins and cellular constituents, alterations of membrane potential and generation of ROS, which lead to bacterial death. The bactericidal activities of treated Carey clay could be associated with ROS-mediated bacterial damage, of which the metal-mediated reactions could contribute to DNA and membrane damage. The synergistic effect of metal ions especially Al and Fe, which were presence in high concentration in the clay could also cause detrimental damage on the bacterial membrane. Overall, these results suggest multi-target mode of action for treated Carey clay against *S. Typhimurium* and *S. aureus*. The fundamental understanding of antibacterial mode of action of treated Carey clay could provide a theoretical framework for the development of antibacterial products from natural clays.

CHAPTER 5

SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

5.1 Summary

Natural clays have recently been proven to possess antibacterial properties. However, only a small proportion of clay minerals have been proven to be antibacterial. Depending on their chemical structure and geochemistry, clay minerals can display potent antibacterial properties against a range of bacterial pathogens. In view of emerging science describing clay as an efficient killing agent against several bacterial pathogens, this study investigates the antibacterial activity of clay from Selangor, Malaysia against food-borne bacterial pathogens in an effort to demonstrate the potential of naturally produced clay as surface antibacterial agent in Halal logistics.

In this study, 13 soils samples that were identified as potentially having antibacterial activity based on pre-defined criterias were collected around Selangor. Many of the soils had clayey texture, while others had clay fraction ranging from 16-36%. All of the soils were all acidic (< pH 7) in both fresh and dried form. The preliminary screening using disc diffusion test showed that only 6 out of 13 soil samples showed antibacterial activity against gram-negative *S. Typhimurium* and gram-positive *S. aureus*. P3 leachate of Munchong produced a significantly greater zone of inhibition (11.17 ± 0.76 mm) when tested against *S. Typhimurium*, while Carey leachate P3 showed the greatest

activity (7.90 ± 0.17 mm) against *S. aureus*. The longer clay suspension shaking times produced a significant increase in antibacterial efficacy against *S. Typhimurium*. Result from this study demonstrate the importance the importance of the leachate preparation method in altering the solution chemistry that would effectively enhance the antibacterial activity of clays.

Munchong and Carey soils, previously showing most antibacterial effectiveness against *S. Typhimurium* and *S. aureus*, respectively, undergone further treatment (untreated and treated) to evaluate their antibacterial activity in the form of leachate and suspension. Treated Carey clay demonstrated effective antibacterial activity against *S. Typhimurium* and *S. aureus* with MIC values of 125 mg/mL and MBC values of 250 mg/mL for leachate and suspension, for both of the test bacteria. Result of bacterial viability assay revealed the treated Carey clays (suspension and leachate) to be the most effective in complete killing *S. Typhimurium*. The viability of *S. aureus* was also reduced by the Carey clay suspension (>3 log) and leachate (<2 log), respectively. The untreated Carey and all the Munchong clays showed little or no antibacterial activity against either bacterial species. Based on correlation analysis, the high contents of available metals Ag, Al, Fe, Mg, Mn, Ni and Zn could be a possible indication that these elements are the most effective factors in antibacterial activity of the treated Carey clay against *S. Typhimurium* and *S. aureus*.

Treated Carey clay was chosen for further analysis due to its effective antibacterial activity. Characterisation of treated Carey clay was carried out to

determine the geochemical variables that may influence its antibacterial activity. EDX analysis revealed the presence of Al, Fe and S, consistent with the presence of pyrite as analysed using XRD. Morphology and surface properties found treated Carey clay with kaolinite. It was found that pH is not the only factor contributing to the antibacterial effectiveness, however it is likely that acidic conditions with treated Carey favours metal ion solubility. Time-kill experiments revealed that the clay showed bactericidal effects on *S. Typhimurium* within 1 h exposure to either leachate or suspension at 4x MIC. Meanwhile, *S. aureus* was completely killed after 8 h of treatment with a treated Carey suspension at the same concentration, whereas the leachate resulted in bactericidal effect after 24 h of exposure. There was clearly greater potency with both leachates and suspensions against the gram-negative bacteria relative to the gram-positive. The result of soluble metals in Carey clay suggested that high levels of metals, with the possibility of the metals working in synergy to cause bacterial killing, especially Al and Fe.

The results from the study of antibacterial mode of action of treated Carey clay revealed that TEM showed abnormalities, including disrupted cell walls following exposure to treated Carey clay. Analysis of membrane integrity also highlighted leakage of proteins and cellular constituents after treatment with the clay. Changes in membrane potential were evident in treated cells and the NBT assay showed generation of ROS that likely promotes bacterial toxicity. The results implicate metal induced toxicity that damages DNA and cellular membranes, as the antibacterial mode of action.

5.2 Conclusion

This study revealed the potentials of soils from Malaysia to be used as an antibacterial agent against selected foodborne pathogens. Treated Carey clay has the potential to be used as broad-spectrum antibacterial agent due to its effectiveness against both gram-positive and gram-negative bacteria. The comparison of antibacterial effectiveness from leachates prepared using different methods would provide a baseline data for further studies aimed at screening natural clay soils for antibacterial activity and assessing their potential to be used as natural antibacterial agent. The results provide a fundamental understanding of the antibacterial mode of action of treated Carey clay. The outcome from present study has put forward a theoretical framework for the clay-based products development for the use as sanitizer on non-food contact surfaces, where sufficient reductions of bacteria (> 3 log) has been especially observed by the clay leachates and suspensions. Although the result showed a rather high metal contents, there is possibility to further this work as an application to non-food contact surfaces such as on containers and floors by developing formulations with lowered concentration of clay in the suspension to assure toxicological safety. In the context of Halal food integrity, the use of clays would be beneficial, not only for Islamic cleansing procedures but for effective removal of bacterial hazards in the Halal logistics.

5.3 Recommendation for future research

This study revealed the potentials of soils from Malaysia to be used as an antibacterial agent against selected foodborne pathogens. The comparison of

antibacterial effectiveness from leachates prepared using different methods suggested the effectiveness of P3 preparation method in enhancing the antibacterial activity against *S. Typhimurium* and *S. aureus*. Therefore, leachates prepared using P3 method could be used for studies aimed at screening natural clay soils for antibacterial activity.

Results from the study on antibacterial mode of action suggested the bactericidal effect of treated Carey clay in causing irreversible damage on DNA and cellular membrane. Therefore, treated Carey clay has the potential to be used as broad-spectrum antibacterial agent due to its effectiveness against both gram-positive and gram-negative bacteria. Further work can be done by testing the antibacterial activity against other food-borne pathogens.

Soils and clays used in this study have been autoclaved to can inactivate any bacteria or contaminants present in the samples. Additional research such as metagenomic sequencing can be pursued, of which any viral genetic material (including phages) in the clay samples can be detected. This would provide more comprehensive screening, which exclude the possibility of phage activity in the study of antibacterial clay.

The acidity of treated Carey clay in solution and the high concentrations of metals should be taken into consideration so it does not cause any adverse effect on human or the environment. To expand the potential use of treated Carey clay as sanitising agent, further study could determine its antibacterial effectiveness with adjusted pH and analysing their corresponding metals

content that could contribute to the antibacterial activity. In addition, further toxicological analysis should be conducted to determine its suitability as a surface sanitising agent. Formulations of *sertu* product by metals-supplemented kaolin clay can be developed and tested on-site to analyse the potential use of this clay as surface sanitising agent for the use in Halal logistics.



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APPENDICES

A. Tables

A1. Soil processing

Sample	Raw	Processed
Batu Anam		
Bernam		
Carey		
Ladang		
Melaka		

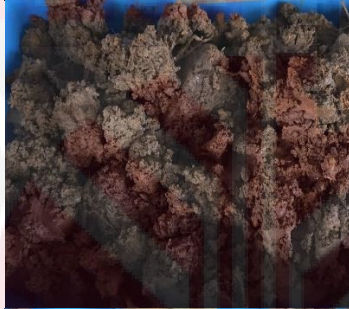
Munchong



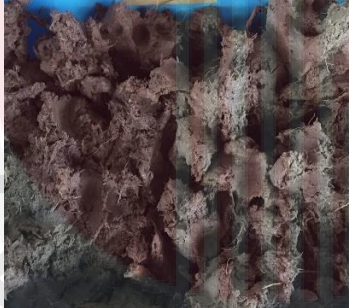
Musang 1



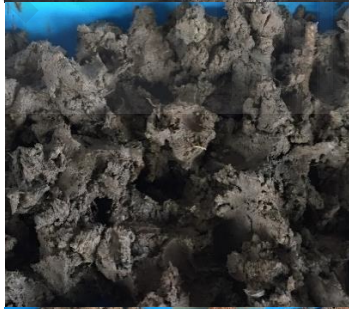
Musang 2



Pulau Indah 1



Pulau Indah 2



Prang



Serdang



Serkat



A2: Inhibition zones of kaolinite (Sigma-Aldrich, Seelze, Germany) leachates prepared with P3 methods with varying mineral concentrations against *S. Typhimurium* and *S. aureus*

Concentration (mg/mL)	Inhibition zone (mm)	
	<i>S. Typhimurium</i>	<i>S. aureus</i>
25	0.00 ± 0.00	0.00 ± 0.00
125	0.00 ± 0.00	0.00 ± 0.00
250	0.00 ± 0.00	0.00 ± 0.00
500	0.00 ± 0.00	0.00 ± 0.00

Positive controls with ampicillin (10 µg) against *S. Typhimurium* and with ciprofloxacin (5 µg) against *S. aureus* produced zones of growth inhibition of 30.69 ± 0.96 and 26.35 ± 0.67 mm, respectively. Results were expressed as means ± standard deviation (SD).

B. Figures

B1. Soil series information from Paramanathan (2000)

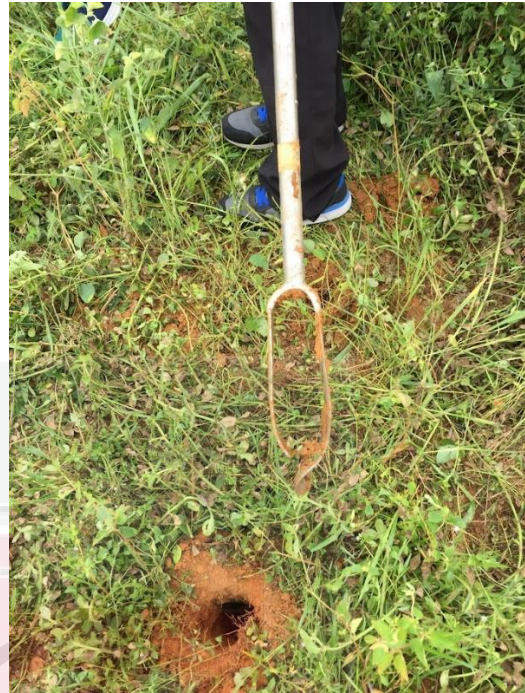
Munchong Series: Analytical Data

Horizon	Ap	Bo ₁	Bo ₂
Depth in cm	0–20	20–81	81–152
Clay%	72	74	75
Silt%	7	7	6
Fine sand%	21	19	19
Coarse sand%	—	—	—
Loss on ignition%	nd	nd	nd
pH–H ₂ O	4.36	4.96	4.57
pH–KCl	nd	nd	nd
Organic carbon%	2.46	0.65	0.75
Nitrogen%	nd	nd	nd
Free iron%	0.01	0.05	0.12
Exchangeable aluminum–1N-KCl cmol(+)kg ⁻¹ soil	1.90	0.13	0.20
Exchangeable cations–1N-NH ₄ OAc–pH-7 cmol(+)kg ⁻¹ soil			
calcium	0.07	0.07	0.05
magnesium	0.30	0.27	0.02
sodium	0.66	0.69	0.17
total bases	1.12	1.11	0.26
Cation exchange capacity–1N-NH ₄ OAc–pH-7 cmol(+)kg ⁻¹ soil	8.5	5.2	4.6
cmol(+)kg ⁻¹ clay	11.8	7.0	6.1
Base saturation%	13	21	6

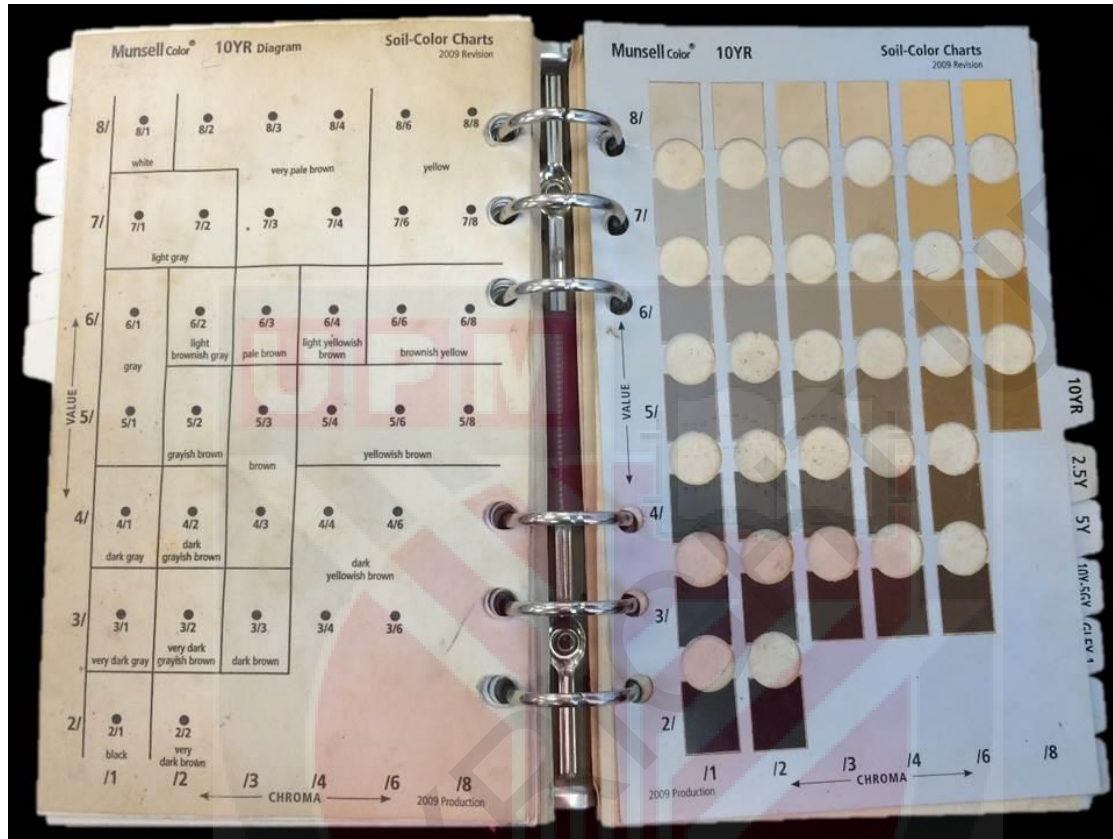
nd=not determined

Horizon	Depth in cm	Description
Ap	0–20	Brownish yellowish (10YR6/6); clay; strong fine and medium subangular blocky and crumb structures; friable; abundant pores; abundant roots; diffuse smooth boundary.
Bo ₁	20–81	Strong brown (7.5YR5/8); clay; moderate coarse subangular blocky parting into medium and fine subangular blocky structures; friable; abundant pores; many roots; diffuse smooth boundary.
Bo ₂	81–152	Reddish yellow (7.5YR6/6); clay; moderate coarse subangular blocky parting into medium and fine subangular blocky structures; friable; many pores; few roots.

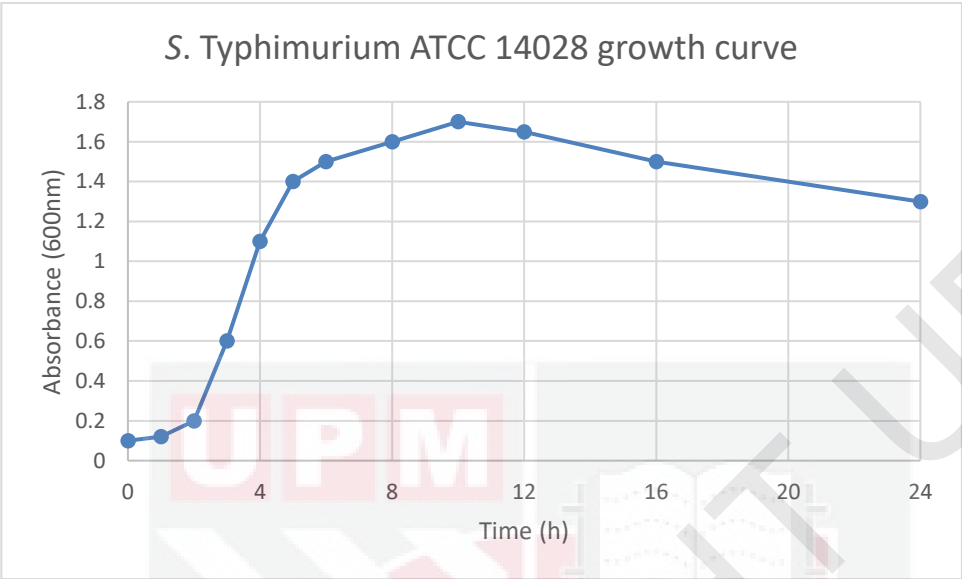
B2. Sampling procedure using hand auger



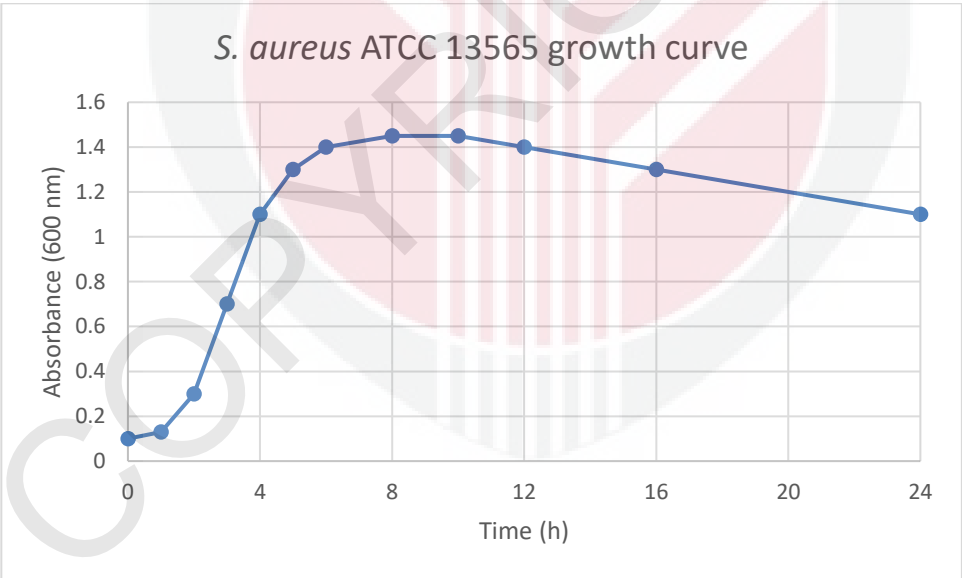
B3. Munsell colour chart



B4. Bacterial growth curve

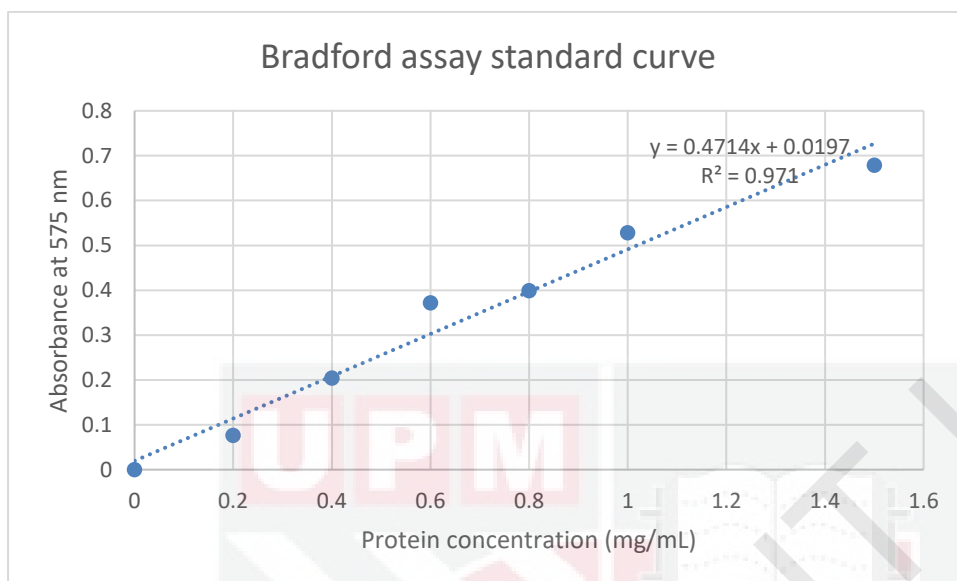


Growth curve of *S. Typhimurium* for determination of log phase



Growth curve of *S. aureus* for determination of log phase

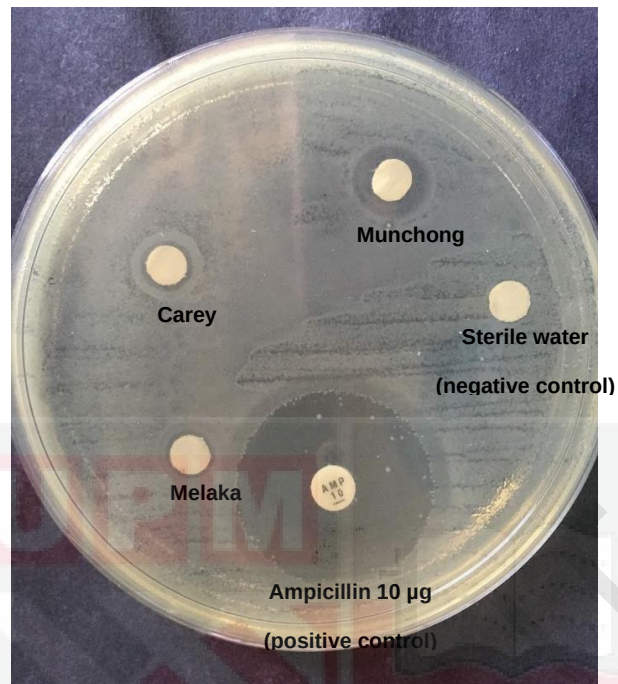
B5. Standard curve for Bradford assay



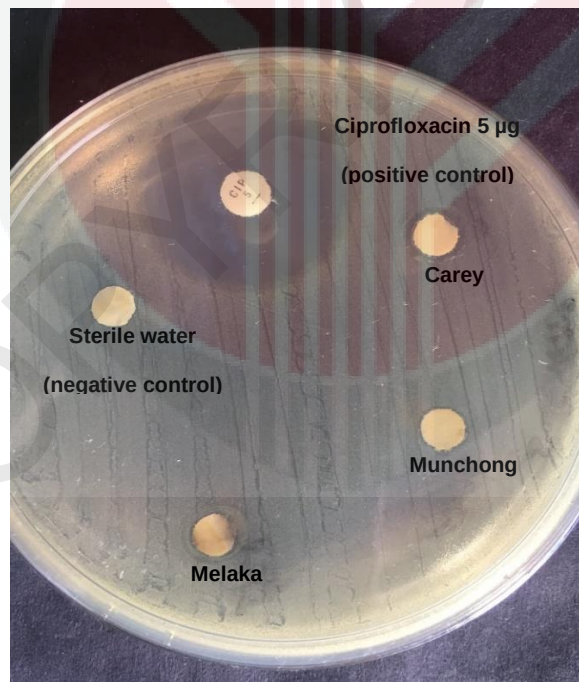
B6. Preparation for treated clay



B7. Inhibiton zone by P3 clay leachates



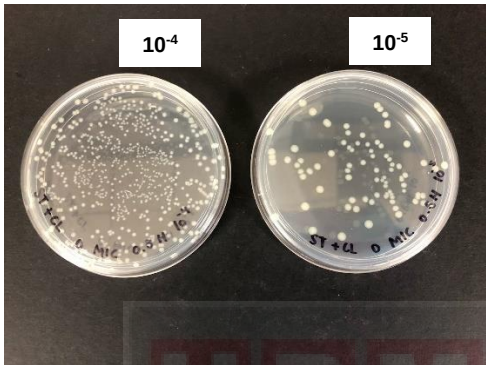
Salmonella Typhimurium ATCC 14028



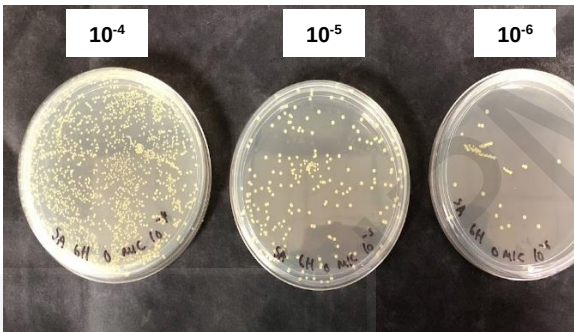
Staphylococcus aureus ATCC 13565

B8. Microbial counts

Control (no treatment)

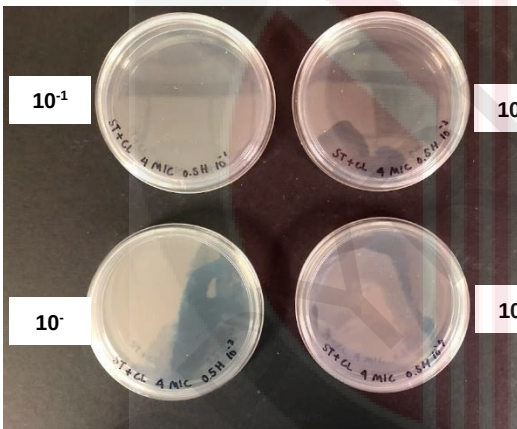


S. Typhimurium on nutrient agar after 30 minutes incubation with sterile deionised water (negative control)

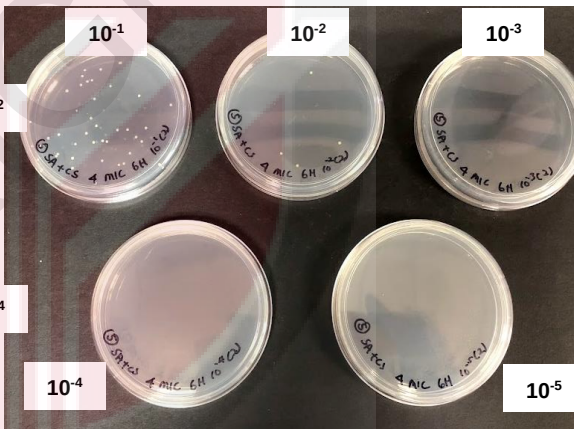


S. aureus on nutrient agar after 6 hours incubation with sterile deionised water (negative control)

Treated



S. Typhimurium on nutrient agar after 30 minutes incubation with Carey leachate at 50% concentration (4 MIC)



S. aureus on nutrient agar after 6 hours incubation with Carey suspension at 50% concentration (4 MIC)

C. Statistical Analysis

C1. Disc diffusion test for P1, P2 and P3 soil leachates against *S. Typhimurium* (Section 4.2.2)

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Munchong 1	3	7.387	A
Serkat 1	3	7.000	A B
Carey 1	3	6.667	B C
Bernam 1	3	6.300	C
Batu Anam 1	3	6.1333	C
Melaka 1	3	6.0933	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_1	N	Mean	Grouping
Serkat 2	3	7.333	A
Munchong 2	3	7.000	A
Melaka 2	3	7.000	A
Carey 2	3	6.833	A
Bernam 2	3	6.833	A
Batu Anam 2	3	6.400	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_2	N	Mean	Grouping
Munchong 3	3	11.167	A
Batu Anam 3	3	8.8333	B
Serkat 3	3	8.783	B
Bernam 3	3	8.500	B
Carey 3	3	8.0833	B
Melaka 3	3	7.833	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Batu Anam 3	3	8.8333	A
Batu Anam 2	3	6.400	B
Batu Anam 1	3	6.1333	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_1	N	Mean	Grouping
Bernam 3	3	8.500	A
Bernam 2	3	6.833	B
Bernam 1	3	6.300	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_2	N	Mean	Grouping
Melaka 3	3	7.833	A
Melaka 2	3	7.000	A
Melaka 1	3	6.0933	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_3	N	Mean	Grouping
Munchong 3	3	11.167	A
Munchong 1	3	7.387	B
Munchong 2	3	7.000	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_4	N	Mean	Grouping
Carey 3	3	8.0833	A
Carey 2	3	6.833	B
Carey 1	3	6.667	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_5	N	Mean	Grouping
Serkat 3	3	8.783	A
Serkat 2	3	7.333	B
Serkat 1	3	7.000	B

Means that do not share a letter are significantly different.

- C2. Disc diffusion test for P1, P2 and P3 soil leachates against *S. aureus* (Section 4.2.2)

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Melaka 1	3	6.500	A
Serkat 1	3	6.400	A
Bernam 1	3	6.400	A
Batu Anam 1	3	6.400	A
Munchong 1	3	6.3333	A
Carey 1	3	6.300	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_1	N	Mean	Grouping
Carey 2	3	7.167	A
Serkat 2	3	6.700	A B
Melaka 2	3	6.400	B
Batu Anam 2	3	6.400	B
Munchong 2	3	6.3333	B
Bernam 2	3	6.300	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_2	N	Mean	Grouping
Carey 3	3	7.900	A
Batu Anam 3	3	6.400	B
Munchong 3	3	6.3333	B
Serkat 3	3	6.300	B
Melaka 3	3	6.300	B
Bernam 3	3	6.300	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Batu Anam 3	3	6.400	A
Batu Anam 2	3	6.400	A
Batu Anam 1	3	6.400	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_1	N	Mean	Grouping
Bernam 1	3	6.400	A
Bernam 3	3	6.300	A
Bernam 2	3	6.300	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_2	N	Mean	Grouping
Melaka 1	3	6.500	A
Melaka 2	3	6.400	A
Melaka 3	3	6.300	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_3	N	Mean	Grouping
Munchong 3	3	6.3333	A
Munchong 2	3	6.3333	A
Munchong 1	3	6.3333	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_4	N	Mean	Grouping
Carey 3	3	7.900	A
Carey 2	3	7.167	B
Carey 1	3	6.300	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Sample_5	N	Mean	Grouping
Serkat 2	3	6.700	A
Serkat 1	3	6.400	A
Serkat 3	3	6.300	A

Means that do not share a letter are significantly different.

C3. Effect of untreated and treated on leachates pH (Section 4.3.1.1)

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Untreated Leachate	3	2.7967	A
Carey Treated Leachate	3	2.5133	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Untreated Suspension	3	2.84333	A
Carey Treated Suspension	3	2.7500	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Treated Leachate	3	6.19333	A
Munchong Untreated Leachate	3	3.6633	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

C4	N	Mean	Grouping
Munchong Treated Suspension	3	6.1000	A
Munchong Untreated Suspension	3	3.4833	B

Means that do not share a letter are significantly different.

C4. Effect of untreated and treated on sample CEC (Section 4.3.1.2)

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C14	N	Mean	Grouping
Carey Treated CEC	3	15.000	A
Carey Untreated CEC	3	9.6667	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Treated CEC	3	21.328	A
Munchong Untreated CEC	3	7.1111	B

Means that do not share a letter are significantly different.

C5. Effect of untreated and treated on soluble metal content in leachates (Section 4.3.1.3)

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated Ag	3	0.004500	A
Carey Untreated Ag	3	0.001500	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated Al	3	92.63	A
Carey Untreated Al	3	9.92	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated As	3	0.06467	A
Carey Untreated As	3	0.02100	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated Cu	3	0.015500	A
Carey Untreated Cu	3	0.001500	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated Fe	3	67.457	A
Carey Untreated Fe	3	43.45	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated Mg	3	88.38	A
Carey Untreated Mg	3	56.88	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated Mn	3	0.2060	A
Carey Untreated Mn	3	0.11900	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated Ni	3	0.06350	A
Carey Untreated Ni	3	0.010000	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated Pb	3	0.004000	A
Carey Untreated Pb	3	0.002000	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Carey Treated Zn	3	0.1425	A
Carey Untreated Zn	3	0.05050	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Untreated Ag	3	0.002333	A
Munchong Treated Ag	3	0.000500	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Treated Al	3	36.45	A
Munchong Untreated Al	3	0.1305	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Treated As	3	0.4010	A
Munchong Untreated As	3	0.00500	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Treated Cu	3	0.009000	A
Munchong Untreated Cu	3	0.002000	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Treated Fe	3	6.38	A
Munchong Untreated Fe	3	1.795	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Untreated Mg	3	0.27200	A
Munchong Treated Mg	3	0.1093	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Untreated Mn	3	0.01400	A
Munchong Treated Mn	3	0.001000	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Treated Ni	3	0.001000	A
Munchong Untreated Ni	3	0.000500	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Treated Pb	3	0.00550	A
Munchong Untreated Pb	3	0.00400	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Munchong Untreated Zn	3	0.004000	A
Munchong Treated Zn	3	0.002500	B

Means that do not share a letter are significantly different.



C6. Factor loadings for biplot analysis between available metals and pH on the antibacterial activity against *S. Typhimurium* and *S. aureus* (Section 4.3.3)

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
Log Reduction (ST)	0.9676	0.1891	0.0042	-0.1183	-0.1165	0.0064	-0.0150	-0.0078	-0.0114	0.0010	-0.0015
Log Reduction (SA)	0.9219	0.3540	-0.0285	-0.1177	-0.0998	-0.0103	-0.0103	0.0045	0.0031	-0.0014	0.0016
pH	-0.6616	0.7300	-0.1451	-0.0089	0.0874	0.0120	-0.0178	-0.0118	0.0010	0.0030	0.0010
Ag	0.8325	-0.0765	0.4687	-0.2281	0.1706	0.0024	-0.0134	0.0133	-0.0025	0.0002	0.0000
Al	0.8576	0.4944	-0.1169	-0.0469	-0.0612	-0.0094	-0.0127	0.0083	0.0099	0.0007	-0.0005
As	-0.3403	0.8786	-0.2723	0.1710	0.0912	-0.0213	-0.0013	0.0092	-0.0059	-0.0018	-0.0009
Cu	0.7367	0.6460	-0.1135	-0.1563	0.0202	0.0391	0.0269	0.0007	0.0005	-0.0010	0.0000
Fe	0.9270	-0.1748	-0.1388	0.3007	-0.0075	0.0057	0.0102	0.0173	-0.0016	0.0032	0.0001
Mg	0.9250	-0.2411	-0.1421	0.2555	0.0191	0.0133	-0.0144	-0.0034	-0.0056	-0.0018	0.0016
Mn	0.9537	-0.2208	-0.0681	0.1763	0.0748	0.0101	-0.0106	-0.0100	0.0085	-0.0007	-0.0015
Ni	0.9894	0.1052	0.0137	-0.0879	0.0270	-0.0307	0.0190	-0.0094	-0.0014	0.0014	0.0006
Pb	-0.0591	0.6245	0.7139	0.3056	-0.0585	0.0019	0.0045	-0.0044	0.0009	-0.0002	0.0000
Zn	0.9950	-0.0352	-0.0191	0.0426	0.0773	-0.0155	0.0096	-0.0143	0.0000	-0.0002	0.0000

C7. Available metals in treated Carey clay leachates in different concentrations (Section 4.4.4.1)

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Ag 4 MIC	3	0.004500	A
Ag 2 MIC	3	0.002000	B
Ag 1 MIC	3	0.001000	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C4	N	Mean	Grouping
As 1 MIC	3	0.06450	A
As 2 MIC	3	0.01800	B
As 4 MIC	3	0.00800	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C7	N	Mean	Grouping
Al 4 MIC	3	92.63	A
Al 2 MIC	3	53.22	B
Al 1 MIC	3	30.53	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C10	N	Mean	Grouping
Cu 4 MIC	3	0.015500	A
Cu 2 MIC	3	0.006500	B
Cu 1 MIC	3	0.003000	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C13	N	Mean	Grouping
Fe 4 MIC	3	67.457	A
Fe 2 MIC	3	44.035	B
Fe 1 MIC	3	23.965	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C16	N	Mean	Grouping
Mg 4 MIC	3	88.38	A
Mg 2 MIC	3	79.980	B
Mg 1 MIC	3	51.48	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C19	N	Mean	Grouping
Mn 4 MIC	3	0.2060	A
Mn 2 MIC	3	0.1290	A B
Mn 1 MIC	3	0.0745	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C22	N	Mean	Grouping
Ni 4 MIC	3	0.06350	A
Ni 2 MIC	3	0.03600	A B
Ni 1 MIC	3	0.02250	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C25	N	Mean	Grouping
Pb 1 MIC	3	0.009500	A
Pb 2 MIC	3	0.008500	A
Pb 4 MIC	3	0.004000	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C28	N	Mean	Grouping
Zn 4 MIC	3	0.1425	A
Zn 2 MIC	3	0.0745	B
Zn 1 MIC	3	0.0440	B

Means that do not share a letter are significantly different.

- C8. Available metals in treated Carey clay suspensions in different concentrations (Section 4.4.4.2)

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Ag 4 MIC	3	0.282500	A
Ag 1 MIC	3	0.28100	A
Ag 2 MIC	3	0.27850	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C4	N	Mean	Grouping
Al 4 MIC	3	57.81	A
Al 2 MIC	3	29.430	B
Al 1 MIC	3	18.05	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C7	N	Mean	Grouping
Cu 4 MIC	3	0.010000	A
Cu 1 MIC	3	0.01000	A
Cu 2 MIC	3	0.00633	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C10	N	Mean	Grouping
Fe 4 MIC	3	25.64	A
Fe 2 MIC	3	18.405	B
Fe 1 MIC	3	9.533	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C13	N	Mean	Grouping
Mg 4 MIC	3	105.01	A
Mg 2 MIC	3	57.525	B
Mg 1 MIC	3	33.965	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C16	N	Mean	Grouping
Mn 4 MIC	3	0.2495	A
Mn 2 MIC	3	0.13850	B
Mn 1 MIC	3	0.074000	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C19	N	Mean	Grouping
Ni 4 MIC	3	0.05350	A
Ni 2 MIC	3	0.030500	B
Ni 1 MIC	3	0.017000	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C22	N	Mean	Grouping
Pb 1 MIC	3	0.004000	A
Pb 4 MIC	3	0.003500	A
Pb 2 MIC	3	0.00200	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C25	N	Mean	Grouping
Zn 4 MIC	3	0.1310	A
Zn 2 MIC	3	0.08100	B
Zn 1 MIC	3	0.04600	C

Means that do not share a letter are significantly different.

C9. Leakage of cellular DNA for *S. Typhimurium* (Section 4.5.2.1)

1. Control

One-way ANOVA: C2 versus C1

Method

Null hypothesis All means are equal
Alternative hypothesis Not all means are equal
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C1	5	D0, D0.5, D1.0, D1.5, D2.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C1	4	0.000284	0.000071	1.22	0.364
Error	10	0.000585	0.000058		
Total	14	0.000869			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0076461	32.71%	5.79%	0.00%

Means

C1	N	Mean	StDev	95% CI
D0	3	0.04025	0.00182	(0.03041, 0.05009)
D0.5	3	0.03845	0.00904	(0.02861, 0.04829)
D1.0	3	0.04358	0.00624	(0.03375, 0.05342)
D1.5	3	0.03465	0.00435	(0.02481, 0.04449)
D2.0	3	0.03108	0.01222	(0.02125, 0.04092)

Pooled StDev = 0.00764613

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
D1.0	3	0.04358	A
D0	3	0.04025	A
D0.5	3	0.03845	A
D1.5	3	0.03465	A
D2.0	3	0.03108	A

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C2 vs C1

2. Leachate

One-way ANOVA: C5 versus C4

Method

Null hypothesis All means are equal
Alternative hypothesis Not all means are equal
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C4	5	L0, L0.5, L1.0, L1.5, L2.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C4	4	0.026766	0.006692	57.11	0.000
Error	10	0.001172	0.000117		
Total	14	0.027938			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0108241	95.81%	94.13%	90.56%

Means

C4	N	Mean	StDev	95% CI
L0	3	0.14355	0.00355	(0.12963, 0.15747)
L0.5	3	0.25102	0.01115	(0.23709, 0.26494)
L1.0	3	0.25992	0.00424	(0.24599, 0.27384)
L1.5	3	0.23318	0.01444	(0.21926, 0.24711)
L2.0	3	0.24322	0.01491	(0.22929, 0.25714)

Pooled StDev = 0.0108241

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C4	N	Mean	Grouping
L1.0	3	0.25992	A
L0.5	3	0.25102	A
L2.0	3	0.24322	A
L1.5	3	0.23318	A
L0	3	0.14355	B

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C5 vs C4

3. Suspension

One-way ANOVA: C8 versus C7

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C7	5	S0, S0.5, S1.0, S1.5, S2.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C7	4	0.011639	0.002910	29.96	0.000
Error	10	0.000971	0.000097		
Total	14	0.012610			

Model Summary

	S	R-sq	R-sq(adj)	R-sq(pred)
	0.0098545	92.30%	89.22%	82.67%

Means

C7	N	Mean	StDev	95% CI
S0	3	0.14118	0.00381	(0.12851, 0.15386)
S0.5	3	0.21632	0.01297	(0.20364, 0.22899)
S1.0	3	0.21245	0.00473	(0.19977, 0.22513)
S1.5	3	0.20732	0.01011	(0.19464, 0.21999)
S2.0	3	0.20398	0.01335	(0.19131, 0.21666)

Pooled StDev = 0.00985447

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C7	N	Mean	Grouping
S0.5	3	0.21632	A
S1.0	3	0.21245	A
S1.5	3	0.20732	A
S2.0	3	0.20398	A
S0	3	0.14118	B

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C8 vs C7

C10. Leakage of cellular DNA for *S. aureus* (Section 4.5.2.1)

1. Control

One-way ANOVA: C17 versus C16

Method

Null hypothesis All means are equal
Alternative hypothesis Not all means are equal
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C16	5	D0, D10, D24, D6, D8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C16	4	0.000231	0.000058	0.88	0.511
Error	10	0.000657	0.000066		
Total	14	0.000888			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0081066	25.98%	0.00%	0.00%

Means

C16	N	Mean	StDev	95% CI
D0	3	0.03523	0.00690	(0.02480, 0.04566)
D10	3	0.04250	0.00504	(0.03207, 0.05293)
D24	3	0.03887	0.00713	(0.02844, 0.04930)
D6	3	0.03703	0.00413	(0.02660, 0.04746)
D8	3	0.03067	0.01370	(0.02024, 0.04110)

Pooled StDev = 0.00810658

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C16	N	Mean	Grouping
D10	3	0.04250	A
D24	3	0.03887	A
D6	3	0.03703	A
D0	3	0.03523	A
D8	3	0.03067	A

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C17 vs C16

2. Leachate

One-way ANOVA: C11 versus C10

Method

Null hypothesis All means are equal
Alternative hypothesis Not all means are equal
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C10	5	L0, L10, L24, L6, L8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C10	4	0.045538	0.011385	84.00	0.000
Error	10	0.001355	0.000136		
Total	14	0.046893			

Model Summary

	S	R-sq	R-sq(adj)	R-sq(pred)
	0.0116414	97.11%	95.95%	93.50%

Means

C10	N	Mean	StDev	95% CI
L0	3	0.10817	0.01096	(0.09319, 0.12314)
L10	3	0.23927	0.00259	(0.22429, 0.25424)
L24	3	0.24890	0.01031	(0.23392, 0.26388)
L6	3	0.2463	0.0207	(0.2313, 0.2612)
L8	3	0.24817	0.00382	(0.23319, 0.26314)

Pooled StDev = 0.0116414

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C10	N	Mean	Grouping
L24	3	0.24890	A
L8	3	0.24817	A
L6	3	0.2463	A
L10	3	0.23927	A
L0	3	0.10817	B

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C11 vs C10

3. Suspension

One-way ANOVA: C14 versus C13

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C13	5	S0, S10, S24, S6, S8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C13	4	0.028359	0.007090	36.40	0.000
Error	10	0.001948	0.000195		
Total	14	0.030306			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0139555	93.57%	91.00%	85.54%

Means

C13	N	Mean	StDev	95% CI
S0	3	0.10470	0.01238	(0.08675, 0.12265)
S10	3	0.20570	0.01452	(0.18775, 0.22365)
S24	3	0.2169	0.0231	(0.1989, 0.2348)
S6	3	0.21257	0.00876	(0.19461, 0.23052)
S8	3	0.216600	0.000819	(0.198647, 0.234553)

Pooled StDev = 0.0139555

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C13	N	Mean	Grouping
S24	3	0.2169	A
S8	3	0.216600	A
S6	3	0.21257	A
S10	3	0.20570	A
S0	3	0.10470	B

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C14 vs C13

C11. Leakage of protein for *S. Typhimurium* (Section 4.5.2.2)

1. Control

One-way ANOVA: C2 versus C1

Method

Null hypothesis All means are equal
Alternative hypothesis Not all means are equal
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C1	5	C0, C0.5, C1, C1.5, C2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C1	4	0.001444	0.000361	1.74	0.217
Error	10	0.002070	0.000207		
Total	14	0.003514			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0143876	41.10%	17.53%	0.00%

Means

C1	N	Mean	StDev	95% CI
C0	3	0.21525	0.00324	(0.19674, 0.23375)
C0.5	3	0.23434	0.00803	(0.21583, 0.25285)
C1	3	0.2315	0.0253	(0.2130, 0.2500)
C1.5	3	0.24565	0.01530	(0.22714, 0.26416)
C2	3	0.23504	0.00925	(0.21654, 0.25355)

Pooled StDev = 0.0143876

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
C1.5	3	0.24565	A
C2	3	0.23504	A
C0.5	3	0.23434	A
C1	3	0.2315	A
C0	3	0.21525	A

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C2 vs C1

2. Leachate

One-way ANOVA: C5 versus C4

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C4	5	L0, L0.5, L1, L1.5, L2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C4	4	0.026422	0.006605	18.21	0.000
Error	10	0.003627	0.000363		
Total	14	0.030049			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0190449	87.93%	83.10%	72.84%

Means

C4	N	Mean	StDev	95% CI
L0	3	0.22797	0.00857	(0.20347, 0.25247)
L0.5	3	0.30081	0.00212	(0.27631, 0.32531)
L1	3	0.3270	0.0234	(0.3025, 0.3515)
L1.5	3	0.3291	0.0251	(0.3046, 0.3536)
L2	3	0.3475	0.0236	(0.3230, 0.3720)

Pooled StDev = 0.0190449

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C4	N	Mean	Grouping
L2	3	0.3475	A
L1.5	3	0.3291	A
L1	3	0.3270	A
L0.5	3	0.30081	A
L0	3	0.22797	B

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C5 vs C4

3. Suspension

One-way ANOVA: C8 versus C7

Method

Null hypothesis All means are equal
Alternative hypothesis Not all means are equal
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C7	5	S0, S0.5, S1, S1.5, S2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C7	4	0.022118	0.005530	9.30	0.002
Error	10	0.005946	0.000595		
Total	14	0.028064			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0243847	78.81%	70.34%	52.33%

Means

C7	N	Mean	StDev	95% CI
S0	3	0.23504	0.00765	(0.20368, 0.26641)
S0.5	3	0.3093	0.0351	(0.2779, 0.3407)
S1	3	0.31919	0.01068	(0.28782, 0.35056)
S1.5	3	0.31990	0.00561	(0.28853, 0.35127)
S2	3	0.3503	0.0392	(0.3189, 0.3817)

Pooled StDev = 0.0243847

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C7	N	Mean	Grouping
S2	3	0.3503	A
S1.5	3	0.31990	A
S1	3	0.31919	A
S0.5	3	0.3093	A
S0	3	0.23504	B

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C8 vs C7

C12. Leakage of protein for *S. aureus* (Section 4.5.2.2)

1. Control

One-way ANOVA: C2 versus C1

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C1	5	C0, C10, C24, C6, C8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C1	4	0.001720	0.000430	2.73	0.090
Error	10	0.001578	0.000158		
Total	14	0.003298			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0125620	52.16%	33.02%	0.00%

Means

C1	N	Mean	StDev	95% CI
C0	3	0.08372	0.00648	(0.06756, 0.09988)
C10	3	0.0957	0.0234	(0.0796, 0.1119)
C24	3	0.11625	0.00212	(0.10009, 0.13241)
C6	3	0.09221	0.01089	(0.07605, 0.10837)
C8	3	0.09857	0.00883	(0.08241, 0.11473)

Pooled StDev = 0.0125620

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
C24	3	0.11625	A
C8	3	0.09857	A
C10	3	0.0957	A
C6	3	0.09221	A
C0	3	0.08372	A

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C2 vs C1

2. Leachate

One-way ANOVA: C5 versus C4

Method

Null hypothesis All means are equal
Alternative hypothesis Not all means are equal
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C4	5	L0, L10, L24, L6, L8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C4	4	0.014055	0.003514	5.73	0.012
Error	10	0.006129	0.000613		
Total	14	0.020184			

Model Summary

	S	R-sq	R-sq(adj)	R-sq(pred)
	0.0247571	69.63%	57.49%	31.68%

Means

C4	N	Mean	StDev	95% CI
L0	3	0.12827	0.01089	(0.09642, 0.16012)
L10	3	0.1962	0.0208	(0.1643, 0.2280)
L24	3	0.2124	0.0369	(0.1806, 0.2443)
L6	3	0.1544	0.0306	(0.1226, 0.1863)
L8	3	0.19050	0.01470	(0.15865, 0.22234)

Pooled StDev = 0.0247571

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C4	N	Mean	Grouping
L24	3	0.2124	A
L10	3	0.1962	A
L8	3	0.19050	A B
L6	3	0.1544	A B
L0	3	0.12827	B

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C5 vs C4

3. Suspension

One-way ANOVA: C8 versus C7

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C7	5	S0, S10, S24, S6, S8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C7	4	0.028114	0.007028	11.78	0.001
Error	10	0.005967	0.000597		
Total	14	0.034081			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0244277	82.49%	75.49%	60.61%

Means

C7	N	Mean	StDev	95% CI
S0	3	0.08938	0.00442	(0.05795, 0.12080)
S10	3	0.1707	0.0217	(0.1393, 0.2021)
S24	3	0.2202	0.0449	(0.1888, 0.2516)
S6	3	0.13251	0.01380	(0.10109, 0.16394)
S8	3	0.16292	0.01697	(0.13149, 0.19434)

Pooled StDev = 0.0244277

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C7	N	Mean	Grouping
S24	3	0.2202	A
S10	3	0.1707	A B
S8	3	0.16292	A B
S6	3	0.13251	B C
S0	3	0.08938	C

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C8 vs C7

C13. Changes in membrane potential for *S. Typhimurium* (Section 4.5.3)

One-way ANOVA: C2 versus C1

Method

Null hypothesis All means are equal
Alternative hypothesis Not all means are equal
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C1	7	0 MIC, CL 1 MIC, CL 2 MIC, CL 4 MIC, CS 1 MIC, CS 2 MIC, CS 4 MIC

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C1	6	57530507	9588418	7.60	0.001
Error	14	17673919	1262423		
Total	20	75204426			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1123.58	76.50%	66.43%	47.12%

Means

C1	N	Mean	StDev	95% CI
0 MIC	3	40370	206	(38978, 41761)
CL 1 MIC	3	41842	1103	(40451, 43233)
CL 2 MIC	3	42914	966	(41523, 44306)
CL 4 MIC	3	45516	2171	(44125, 46907)
CS 1 MIC	3	43933	767	(42542, 45324)
CS 2 MIC	3	44443	310	(43052, 45834)
CS 4 MIC	3	44619	1118	(43227, 46010)

Pooled StDev = 1123.58

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
CL 4 MIC	3	45516	A
CS 4 MIC	3	44619	A B
CS 2 MIC	3	44443	A B
CS 1 MIC	3	43933	A B
CL 2 MIC	3	42914	A B C
CL 1 MIC	3	41842	B C
0 MIC	3	40370	C

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C2 vs C1

C14. Changes in membrane potential for *S. aureus* (Section 4.5.3)

One-way ANOVA: C5 versus C4

Method

Null hypothesis All means are equal
 Alternative hypothesis Not all means are equal
 Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C4	7	0 MIC, CL 1 MIC, CL 2 MIC, CL 4 MIC, CS 1 MIC, CS 2 MIC, CS 4 MIC

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C4	6	307123547	51187258	82.50	0.000
Error	14	8686150	620439		
Total	20	315809697			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
787.680	97.25%	96.07%	93.81%

Means

C4	N	Mean	StDev	95% CI
0 MIC	3	39838	352	(38862, 40813)
CL 1 MIC	3	43413	492	(42438, 44388)
CL 2 MIC	3	45008	590	(44033, 45983)
CL 4 MIC	3	49098	450	(48122, 50073)
CS 1 MIC	3	47829	420	(46854, 48804)
CS 2 MIC	3	48253	1296	(47278, 49228)
CS 4 MIC	3	52387	1253	(51412, 53363)

Pooled StDev = 787.680

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C4	N	Mean	Grouping
CS 4 MIC	3	52387	A
CL 4 MIC	3	49098	B
CS 2 MIC	3	48253	B
CS 1 MIC	3	47829	B
CL 2 MIC	3	45008	C
CL 1 MIC	3	43413	C
0 MIC	3	39838	D

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C5 vs C4

C15. ROS production for *S. Typhimurium* (Section 4.5.4)

One-way ANOVA: ROS ST versus Concentration

Method

Null hypothesis All means are equal
 Alternative hypothesis Not all means are equal
 Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Concentration	7	0 MIC, CL 1 MIC, CL 2 MIC, CL 4 MIC, CS 1 MIC, CS 2 MIC, CS 4 MIC

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	6	7.57922	1.26320	2422.58	0.000
Error	14	0.00730	0.00052		
Total	20	7.58652			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0228348	99.90%	99.86%	99.78%

Means

Concentration	N	Mean	StDev	95% CI
0 MIC	3	0.06733	0.00929	(0.03906, 0.09561)
CL 1 MIC	3	0.09333	0.01007	(0.06506, 0.12161)
CL 2 MIC	3	0.13800	0.01179	(0.10972, 0.16628)
CL 4 MIC	3	0.26633	0.00961	(0.23806, 0.29461)
CS 1 MIC	3	0.35633	0.01501	(0.32806, 0.38461)
CS 2 MIC	3	1.1383	0.0488	(1.1101, 1.1666)
CS 4 MIC	3	1.7547	0.0250	(1.7264, 1.7829)

Pooled StDev = 0.0228348

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
CS 4 MIC	3	1.7547	A
CS 2 MIC	3	1.1383	B
CS 1 MIC	3	0.35633	C
CL 4 MIC	3	0.26633	D
CL 2 MIC	3	0.13800	E
CL 1 MIC	3	0.09333	E F
0 MIC	3	0.06733	F

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of ROS ST vs Concentration

C16. ROS production for *S. aureus* (Section 4.5.4)

One-way ANOVA: C2 versus C1

Method

Null hypothesis All means are equal
 Alternative hypothesis Not all means are equal
 Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C1	7	0 MIC, CL 1 MIC, CL 2 MIC, CL 4 MIC, CS 1 MIC, CS 2 MIC, CS 4 MIC

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C1	6	5.4533	0.908883	120.50	0.000
Error	14	0.1056	0.007542		
Total	20	5.5589			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0868463	98.10%	97.29%	95.73%

Means

C1	N	Mean	StDev	95% CI
0 MIC	3	0.06900	0.00872	(-0.03854, 0.17654)
CL 1 MIC	3	0.107667	0.001528	(0.000125, 0.215208)
CL 2 MIC	3	0.13667	0.00929	(0.02913, 0.24421)
CL 4 MIC	3	0.3163	0.0197	(0.2088, 0.4239)
CS 1 MIC	3	0.8333	0.0371	(0.7258, 0.9409)
CS 2 MIC	3	1.1963	0.0745	(1.0888, 1.3039)
CS 4 MIC	3	1.370	0.213	(1.262, 1.477)

Pooled StDev = 0.0868463

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
CS 4 MIC	3	1.370	A
CS 2 MIC	3	1.1963	A
CS 1 MIC	3	0.8333	B
CL 4 MIC	3	0.3163	C
CL 2 MIC	3	0.13667	C D
CL 1 MIC	3	0.107667	C D
0 MIC	3	0.06900	D

Means that do not share a letter are significantly different.

Tukey Simultaneous 95% CIs

Interval Plot of C2 vs C1

BIODATA OF STUDENT

Nur Naqiyah Azmi obtained her primary education in 1998 at SK Taman Universiti in Seri Kembangan, Selangor. In 2004, she continued her secondary education at SMKA Maahad Hamidiah in Kajang, Selangor. For her pre-university studies, she attended Mara College Seremban, where she completed the International Baccalaureate Diploma Programme from 2009 to 2011. She later pursued a Bachelor's Degree in Science, majoring in Agrifood Biotechnology, at the University of Melbourne, Australia, graduating in 2014. She worked as a research assistant for Environmental Biotechnology (EB) group at Universiti Putra Malaysia from 2014-2015. In 2016, she enrolled in Universiti Putra Malaysia to pursue a Master's Degree in Food Technology (coursework mode). After graduating, she furthered her doctoral studies in Halal Products Science at Universiti Putra Malaysia in 2017.

LIST OF PUBLICATIONS

Published article:

Azmi, N. N., Mahyudin, N. A., Wan Omar, W. H., Mahmud Ab Rashid, N. K., Ishak, C. F., Abdullah, A. H., & Sharples, G. J. (2022). Antibacterial activity of clay soils against food-borne *Salmonella* Typhimurium and *Staphylococcus aureus*. *Molecules*, 27(1), 1–15.

Azmi, N.N., Mahyudin, N.A., Wan Omar, W.H., Abdullah, A.H., Ismail, R., Ishak, C.F., & Sharples, G.J. (2024). Antibacterial mechanism of Malaysian Carey clay against food-borne *Staphylococcus aureus*. *Natural Product Research*, 1-5.

Submitted for publication:

Azmi, N.N., Mahyudin, N.A., Wan Omar, W.H., Abdullah, A.H., Ismail, R., Ishak, C.F., & Sharples, G.J. Exploring hydrated clay soil treatments in inhibiting growth of *Salmonella* Typhimurium and *Staphylococcus aureus* towards effective Sertu procedure. *Food Research*. (Under review)

Conference:

Azmi, N.N., Mahyudin, N.A., Wan Omar, W.H., Abdullah, A.H., Ismail, R., Ishak, C.F., & Sharples, G.J. Exploring hydrated clay soil treatments in inhibiting growth of *Salmonella* Typhimurium and *Staphylococcus aureus* towards effective Sertu procedure. 3rd International Conference on Agriculture, Animal Sciences and Food Technology. 27 – 28th August 2023. Universiti Sultan Zainal Abidin, Terengganu, Malaysia. (Oral Presenter).

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