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Anti-biofilm mechanism of Malaysian natural clay against food-borne *Staphylococcus aureus* and *Salmonella Typhimurium*

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ABSTRACT

Natural clay is useful for cleaning the environment due to its antibacterial properties. Malaysian natural clay of Kuala Gula was investigated for its anti-biofilm activity against single biofilm formation of *Staphylococcus aureus* and *Salmonella* Typhimurium. Clay suspension (50.0 %) reduced *S. aureus* biofilms by 4.4 log at 24 h, respectively, while it reduced (25.0 and 12.5 %) by a 4.2 and 3.91 log reduction, respectively, at 6 h. Despite being less effective, clay leachate (50.0 %) was effective in removing *Salmonella Typhimurium* biofilm with a decrease of $>$ 3 log in 24 h. An anti-biofilm mechanism was conducted by imaging microbial morphology using SEM, which revealed abnormalities, including ruptured cell walls, after exposure to Kuala Gula clay and supported by CLSM images. Additionally, the anti-biofilm activity was associated with Reactive Oxygen Species (ROS) production. The present study reveals that Malaysian clay has potential as a natural anti-biofilm agent in the food industry.

Introduction

Clay is easily accessible as it is a constituent of the soil, which is abundant in our environment. Prior studies have investigated the bactericidal efficacy of clay. The antibacterial efficacy can be attributed to metal ions, which can inhibit bacterial growth and viability (Azmi et al., 2021; Godoy-Gallardo et al., 2021). According to Morrison et al. (2016), clay releases metal ions of Iron (Fe) and Aluminum (Al). Once released, they stick together and damage the bacteria's outer layer, a protective coating of fats and proteins that causes the proteins to misfold into improper shapes. As a result, the cell responds to the damage, breaks down those misfolded proteins, and removes them from the cell wall so the cell can function properly. Recent research continues to highlight the potential of clay-based materials in antimicrobial applications. Singh et al. (2022) showed that metal ion-exchanged bentonite clay exhibited strong antibacterial effects against *E. coli* and *S. aureus*, while Ibrahim et al. (2023) demonstrated that silver-loaded kaolinite clay effectively inhibited biofilm formation and bacterial growth, suggesting its promise in food safety and medical fields. These findings support the ongoing interest in clay as a sustainable antimicrobial agent.

Food-borne pathogens are a significant hazard to public health because they may survive in various conditions and resist standard cleaning procedures. *Staphylococcus aureus* is a Gram-positive (Bonny et al., 2020), while *Salmonella Typhimurium* is a Gram-negative bacterium linked to serious food-borne illness (Heredia and García, 2018). The capability of both pathogens to form biofilms. Biofilms are structured communities of microbial cells that adhere to surfaces and are embedded within a self-produced extracellular polymeric matrix (Alam et al., 2020; Wei et al., 2019). They enable bacteria to survive harsh conditions by protecting against antimicrobials, desiccation, and host immune responses (Bhasme et al., 2020). Foodborne pathogens such as *Staphylococcus aureus* and *Salmonella Typhimurium* form persistent biofilms on surfaces like stainless steel, commonly used in food processing environments, making them highly resistant to conventional sanitization methods (Zhang et al., 2023; Crull et al., 2011). These biofilms, consisting of aggregated microcolonies, facilitate substrate exchange, diffusion of metabolic products, and removal of toxic byproducts supporting bacterial survival and community development. Their resistance

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to cleaning and sanitizing procedures poses a serious public health concern by enabling the propagation of resistance genes and contamination of food surfaces (Liu et al., 2023; Abebe, 2020).

This study aimed to identify natural clay in Malaysia and subsequently explore the anti-biofilm efficacy of clay leachate and suspension. It explored its anti-biofilm mechanism of action in terms of morphological changes and Reactive Oxygen Species (ROS) production. Hence, identifying new inhibitory agents has the potential to produce an inexpensive alternative for the utilization of soil in Malaysia. This could have implications for developing new sanitizer-tolerant biofilm agents and their potential use in the food industry. The findings from this study can be used to guide future research focused on assessing their efficacy as cleaning and sanitizing agents. It also provides a theoretical framework for developing anti-biofilm products from natural clays.

Materials and Methods

Soil sampling and processing

Soil samples were collected according to the potential sites identified for a clay soil sampling series in the vicinity of Perak, Malaysia (Paramananthan, 2000). Soil samples were taken from a depth of 25–50 cm after removal of 3 cm of the soil surface and then sieved to remove woody debris and plant material. Note that bulk soil samples were collected using a hand auger and stored in polythene bags. Fresh soil samples were air-dried (30–35℃) for 1–4 weeks, according to the moisture content present in the soil samples. The resulting dried soil sample was ground using a mortar and pestle before passing through a 250 μm mesh sieve.

Sieved soil samples were processed by sedimentation (clay extraction) using the pipette method (Gee and Baulder, 1986). For removal of organic matter, 20 g of soil was treated overnight with 30 % hydrogen peroxide (H_2O_2). Excess liquid was siphoned off, and wet sieving (53 μm mesh screen) was employed to remove the coarser fraction. Correspondingly, Calgon solution or sodium hexametaphosphate (SHMP) (1 % w/v sodium hexametaphosphate (SHMP), pH 8.3) was added as the soil dispersing agent, and the solution was mixed using a metal plunger. The purified clay fraction was collected from the upper layer of sediment and oven-dried for a week at 105°C.

Clay leachate and clay suspension preparation

Borquaye et al. (2016) established a method for preparing clay leachates with slight modifications. The clay samples (0.5 g/mL) were prepared by mixing 1 g of clay sample with deionized water. The mixtures were sonicated in a water bath sonicator for 1 min, followed by shaking using an orbital shaker at 180 rpm for 6 h to promote the release of metal ions. The resulting suspensions were centrifuged (4000 rpm, 30 min, 4°C) in an Eppendorf 5810 R using a fixed-angle rotor to separate insoluble and soluble fractions. The aqueous supernatant (leachate) was then filtered through a 0.45 μm cellulose membrane filter and sterilized in an autoclave (20 min, 121°C). Clay suspension (0.5 g/mL) was prepared by mixing 1 g of clay samples with deionized water. The mixtures were sonicated in a water bath sonicator for 1 min, followed by shaking using an orbital shaker at 180 rpm for 6 h. Consequently, the hydrated suspensions were sterilized in an autoclave (20 min, 121°C). The suspension was used in further investigations.

Anti-biofilm activity of clay leachate and suspension on stainless-steel surfaces

Bacterial strains and growth conditions

Staphylococcus aureus (ATCC 13565) and *Salmonella Typhimurium* (ATCC 14028) strains obtained from the American Type Culture Collection (ATCC) were used as test organisms. Bacteria were cultivated in Tryptic Soy Broth (TSB) or Nutrient Agar (NA) (Oxoid LTD, Basingstoke, Hampshire, England) at 37°C for 24 h.

Preparation of stainless-steel surface

A stainless-steel coupon (10 ×10 mm) was used for biofilm adherence. After soaking in acetone for 3 h to remove post-production debris and grease, coupons were rinsed thoroughly with deionized water, followed by soaking in 70 % ethanol for 10 min. The discs were rinsed thoroughly with deionized water and air-dried in a laminar biosafety hood for 1 h before sterilization at 121°C for 15 min (De Oliveira et al., 2010).

Anti-biofilm activity of clay leachate and suspension

Each coupon was introduced individually into a petri dish with 100 μL samples of clay leachate, or suspension. Three different concentration ranges were tested (12.5 %, 25 %, and 50 %) with exposure times of 0.5, 2, 4, 6 and 24 h. Lower concentrations were not tested if the higher concentration did not achieve more than a 3-log reduction. Consequently, if the higher exposure time of different treatments did not achieve a 3-log reduction, no further time points were monitored. After incubation at 37°C for the specified period, coupons were removed and immersed in sterile PBS for 10 sec to release unadhered cells. The cells adhered to the coupons and were collected by thoroughly rubbing their surfaces with two moistened swabs. The cells were resuspended in Sterile Peptone Water (SPW) with vigorous vertexing for 30 sec. A 10-fold serial dilution of the mixture in SPW (10^{-1}-10^{-7}) was performed, and aliquots of 100 μL spread were plated onto sterile NA plates. Plates were incubated for 24 h at 37°C (Herrera et al., 2007), the number of colonies counted, and the results expressed as Log CFU/mL.

Anti-biofilm mechanism of action

Morphological observations by scanning electron microscopy (SEM)

Samples were removed from the dishes at 24, 48, and 72 h, washed three times in PBS and fixed in 3 % Millonig phosphatebuffered glutaraldehyde three times for 10 min and postfixed in 2 % Millonig osmium tetroxide-buffered solution for 1 h (Serva, Germany). Correspondingly, it was washed three times for 10 min in Millonig phosphate buffer. The samples were subsequently dehydrated in increasing concentrations of acetone (50, 70, 90, and 100 %), every step for 20 min, and dried in hexamethyldisilazane for 3 h in a hood at room temperature (Sigma-Aldrich, Praha, Czech Republic). Consequently, they were placed on the carbon tabs attached to the aluminum holder and coated with platinum/palladium (Cressington sputter coater 208 HR, UK). It was observed under a Hitachi SU 8010 scanning electron microscope (Hitachi High Technologies, Tokyo, Japan) at a magnification of 15009 (at 17 kV, SE+BSE detector, working distance 8.4 mm); 60009 (at 15 kV, wd 10.9 mm); 13 0009 (at 17 kV, wd 8.4 mm); 30 0009 (at 17 kV, wd 13.6) (Makovcova et al., 2017).

Morphological observations by Confocal Laser Scanning Microscopy (CLSM)

In brief, three important solutions were prepared, which were SYTO 9 dye, 3.34 mM (Component A), propodium iodide, 20 mM (Component B), and BacLight mounting oil (Component C). Subsequently, the staining of bacteria in suspension was conducted. The equal volumes of components A and B were combined in a microfuge tube before being mixed thoroughly. Note that 3 L of the dye mixture for each mL of control and treated stainless-steel coupon were pipetted onto the stainless-steel surface. All samples were intubated at room temperature in the dark for 15 min. The live and dead cell membranes of biofilms were observed under CLSM (Nikon-$A1_R$) (microLAMDA SDN. BHD.) with PF 20x Z: 3098.850 μm (Iniguez-Moreno et al., 2018).

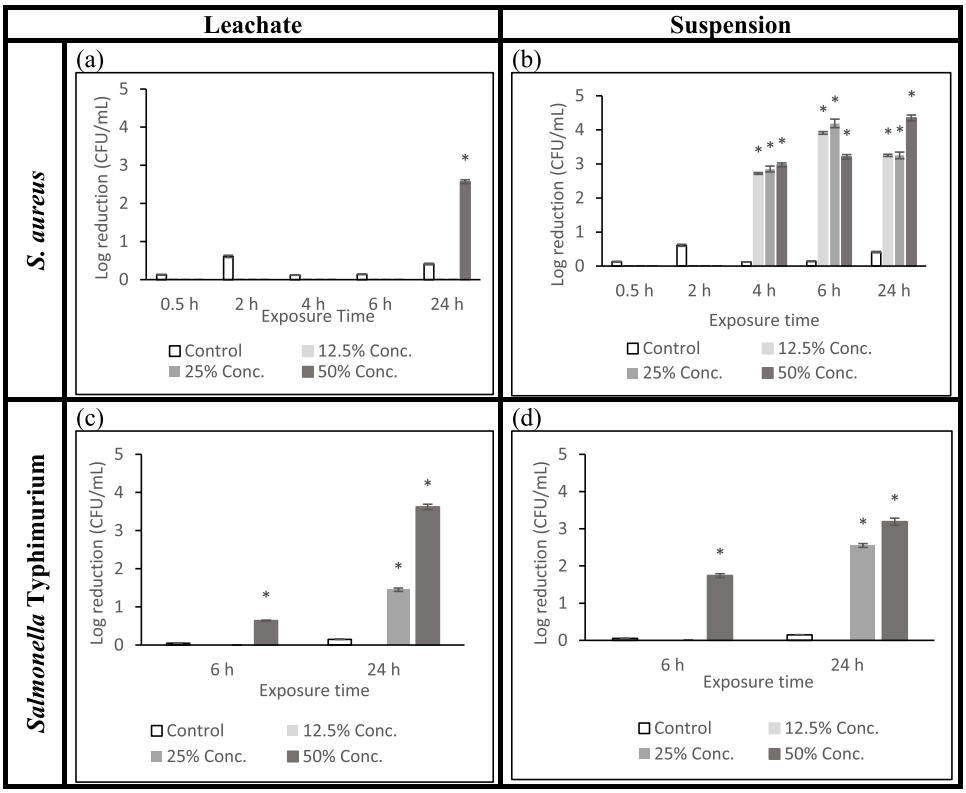

Fig. 1. Anti-biofilm effectiveness of Malaysian natural clay (Kuala Gula sample) against *S. aureus* and *Salmonella Typhimurium* single biofilm formation on stainless-steel coupons. Samples were tested at 50 %, 25 %, and 12.5 % concentration, and the log reduction in viability was measured for the clay samples (leachate and suspension). The log reduction was determined by subtracting the final Log CFU/mL with biofilm on Day 4; the maximum number of viable cell adhesions was 7.31 CFU/mL (10^7 for *S. aureus)* and 6.96 CFU/mL (10^6 for *Salmonella Typhimurium*) on Day 4 of incubation (Wan Omar et al., 2023). Error bars represent the mean and standard error of at least three biological replicates. Error bars represent the mean and standard error of at least three biological replicates. Treatments marked with an asterisk (*) indicate statistically significant differences ($p < 0.05$) compared to respective controls.

Reactive oxygen species (ROS) production by Nitroblue tetrazolium (NBT)

Superoxide anion radicals were measured using an NBT assay (Jung et al., 2016). Samples were added to bacterial cells in the exponential growth phase and incubated for 15 min only. Prolonged incubation after adding samples could allow the cells to express oxidative stress defenses and clay-induced oxidative stress. Cells were harvested by centrifugation, and the cell pellet was dissolved again in 0.5 mL of water of 1 mg/mL NBT for 30 min. Consequently, 0.1 mL of 0.1 M HCl was added, and the cells were centrifuged at 15,000 ×g for 1 min. Dimethyl sulfoxide (0.4 mL) was added to the cell pellet to solubilize the reduced NBT. The absorbance was measured at 575 nm and normalized by the protein concentration from the same number of cells in the same sample.

Statistical analysis

Statistical evaluation of the data was performed using Minitab Software (Minitab 17.0 for Windows, Minitab, USA). A one-way Analysis of Variance (ANOVA) was used for the analytical variation. Meanwhile, the Tukeys' test was utilized for comparison of means with a level of significance of 0.05 ($p < 0.05$). Data was presented as a mean ± standard deviation.

Results and discussion

Anti-biofilm effectiveness of clay samples against single biofilm formation

Leachate and suspension of Kuala Gula clay were prepared, and its anti-biofilm properties were investigated against Gram-positive (*S. aureus*) and Gram-negative *(Salmonella Typhimurium*) at 50 %, 25 % and 12.5 % concentrations. Suspension-preparation: three concentrations achieved more than 3 logs reduction ($p \geq 0.05$). For 6- and 24-hour exposure times, the suspensions successfully killed 99.9 % of the biofilm population on the stainless-steel surfaces ($p \leq 0.05$), in which 12.5 % and 25 % drastically reduced *S. aureus* biofilm to 4.16 and 4.19 $\log_{10}$ reductions at and 6 h, and 4.35 $\log_{10}$ reductions at 24 h (Fig. 1). The clay leachate effectively killed 99.9 % of the biofilm population on the contact surface at the highest concentration (50 %) for 24 h of *Salmonella Typhimurium* biofilm viability ($p \geq 0.05$). However, the 12.5 and 25 % concentrations were generally much less effective in single biofilm eradication with *Salmonella Typhimurium* (Fig. 1). It verified that the *S. aureus* biofilm is much more susceptible than the *Salmonella Typhimurium* . Consequently, the differing composition and morphology of the cell wall in the Gram-negative species is likely responsible for improved tolerance, probably due to the relatively impermeable outer membrane that is absent in Gram-positive (Breijyeh et al., 2020). Biofilms are structured communities of microbial cells that adhere to surfaces and are embedded within a self-produced extracellular polymeric matrix. Suspensions had superior efficacy compared to leachates in eliminating bacteria from the biofilms. Clay mineralogy is known to enhance antibacterial efficacy over leachates containing metals in solution alone (Caflisch et al., 2018; Morrison et al., 2016), consistent with the findings reported here with bacteria in a biofilm.

Compared to other natural antibiofilm agents, Malaysian natural clay offers distinct advantages. Essential oils and plant extracts (e.g., thymol, eugenol, polyphenols) act by disrupting quorum sensing, membranes, or EPS formation (Hassan et al., 2021; dos Santos et al., 2022), but they often face issues like instability, strong odor, and limited industrial applicability. In contrast, clay suspensions achieved consistent > 4 log reductions, showing stable efficacy on food-contact

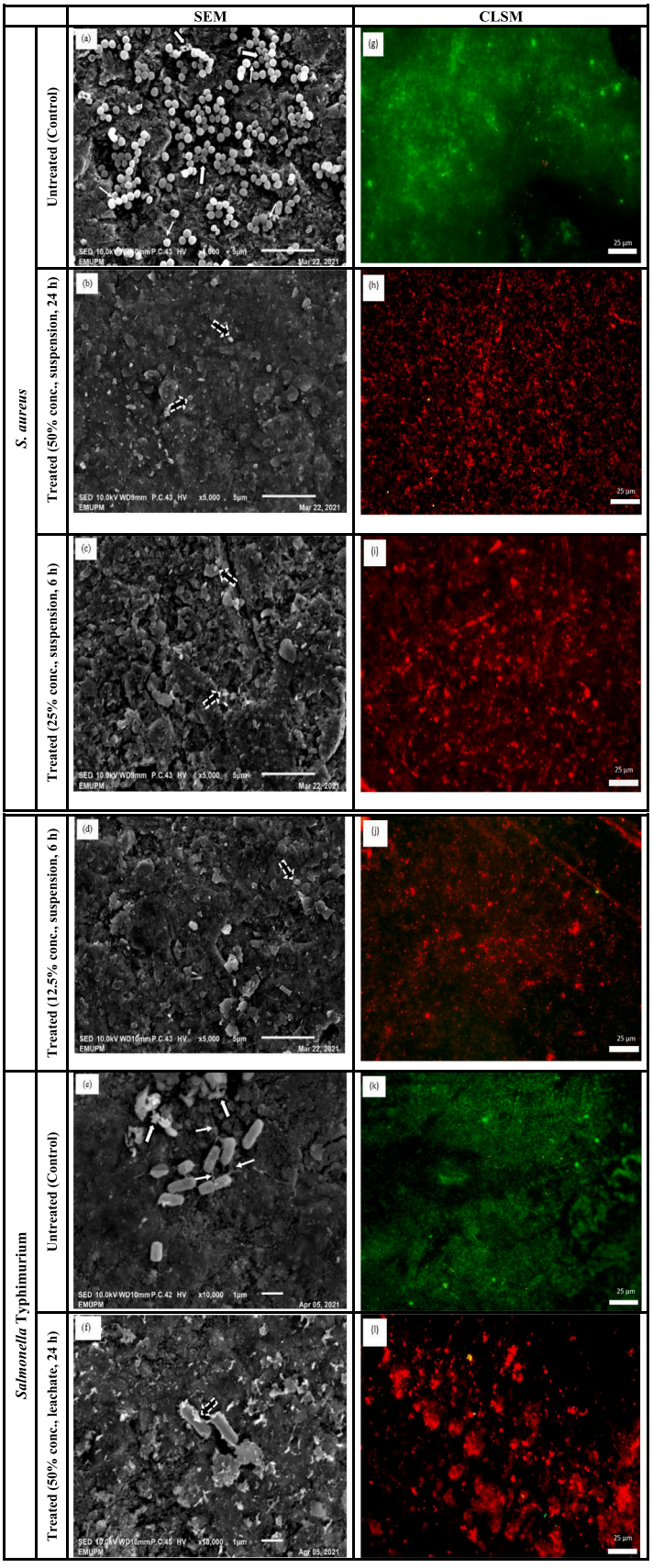

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Fig. 2. SEM images of single biofilm formation by *S. aureus* (a, b, c, d) and *Salmonella Typhimurium* (e, f) before and after treatments; (a) *S. aureus* biofilm without treatments, (b) after treated with 50 % concentration clay suspension at 24 h exposure time, (c) after treated with 25 % concentration clay suspension at 6 h exposure time, (d) after treated with 12.5 % concentration at 6 h exposure time, (e) *Salmonella Typhimurium* before treatments, (f) after treated with 50 % concentration clay leachate at 24 h exposure time. Black arrow with a white outline: amorphous extracellular matrix; white arrow: adhesive fibers; black with dash line arrow: the destruction of the cell wall structure. CLSM images (scale 25 μm) of single biofilm formation by *S. aureus* (g. h. i. j) and *Salmonella Typhimurium* (k, l) before and after treatments with Kuala Gula clay samples.

surfaces. Nonetheless, variability in mineral composition due to geographical origin may affect clay performance (Fu et al., 2021). Furthermore, scaling up for industrial use would require standardized processing and regulatory validation. Despite these challenges, natural clay remains a cost-effective and promising option for biofilm control in food and logistics applications. Future studies could include facultative and obligate anaerobes, using anaerobic workstations and modified protocols to better evaluate the clay treatment's broad-spectrum efficacy.

Anti-biofilm mechanism of action

Morphological changes of biofilm formation after being treated with anti-biofilm clay samples

Fig. 2 illustrates the morphological changes by SEM and CLSM images of a single species of *S. aureus* and *Salmonella Typhimurium* biofilm on a stainless-steel surface after being treated with anti-biofilm clay samples. Figures S2 (a) and (e) portray the SEM image of *S. aureus* and *Salmonella Typhimurium* , which appeared as grape-like clusters and appeared as a rod-shaped cluster, respectively, with some cells embedded in an amorphous matrix. After treatments, Figures S2 (b), (c), and (d) display the deterioration of *S. aureus* biofilm by clay suspension with 50 %, 25 %, and 12.5 % concentration, respectively. There was a huge reduction and damaged or lysed cells of both pathogens in single-species of *S. aureus* biofilm, rough with individual bumps. Correspondingly, there were no EPS appeared. Besides, Fig. 2(c) and (d) exhibited ruptured biofilm cells with the appearance of a bleb-like structure. Some metal cations, such as iron, copper, and chromium, can cause DNA damage, lipid peroxidation, protein oxidation, and cell death. Furthermore, Al is believed to work by disrupting membrane-bound enzymes essential for bacterial cellular metabolism synergistically towards biofilms (Londono and Williams, 2015). Additionally, clay leachate was shown to distort *Salmonella Typhimurium* biofilm cells (Fig. 2(f)). Biofilm cells were greatly diminished and destroyed, leaving a rough surface with distinct bumps. All metals can be dangerous because, at high concentrations, they can bind irreversibly to sulfhydryl groups in proteins or enzyme metal binding sites (Balali-Mood et al., 2021).

To confirm the results of localized cell death, the effects of the clay samples against a single biofilm on a stainless-steel surface were visualized using Confocal Laser Scanning Microscopy (CLSM). Fig. 2(g) and (k) depicted biofilm spatial structure, spatial of viable bacteria, and bacteria localized cell death against single *S. aureus* and *Salmonella Typhimurium* biofilms, respectively. Both diagrams expressed thickened clusters of biofilms, possibly EPS. Meanwhile, Fig. 2(h), (i), and (j) illustrate the deterioration of biofilm by treated suspensions with 50 %, 25 %, and 12.5 % concentrations, respectively, against *S. aureus* biofilm after treatments. Fig. 2(l) expressed the degradation of *Salmonella*

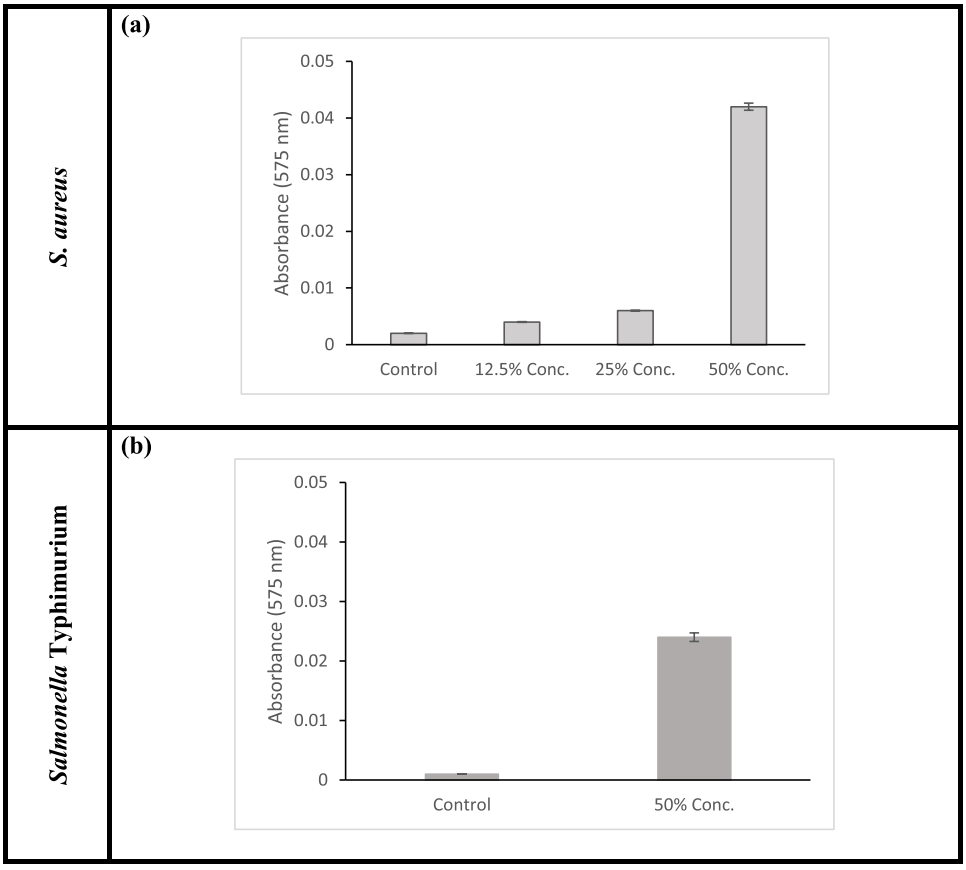

Fig. 3. Reactive Oxygen Species (ROS) production of (a) different treatments (12.5 %, 25 %, and 50 % concentration) against single-species *S. aureus* biofilm and (b) 50 % concentration of treated leachate against *Salmonella Typhimurium* biofilm.

Typhimurium biofilm by clay leachate at a 50 % concentration. Red fluorescent demonstrated the destruction and rupture of membranes in this single biofilm formation after treatments. Fig. 2(h), (i), and (j) depicted that the clusters were not as robust as compared to Fig. 2(l). This signifies that *Salmonella Typhimurium* was difficult to eradicate compared to *S. aureus* in single-species biofilm. Moreover, previous research has discovered that the ability of biofilms treated with luteolin to resist antibiotics was significantly reduced. Additionally, using CLSM demonstrated a decrease in the presence of biofilm matrix components, particularly polysaccharides and proteins (Fu et al., 2021).

Reactive oxygen species (ROS) production

The oxidative stress of single and dual-species biofilm formation on stainless-steel coupons is depicted in Fig. 3. An Nitroblue Tetrazolium (NBT) assay performed after treatments were exposed to biofilm revealed significantly increased oxidative stress. The results demonstrate that the oxidative stress of *S. aureus* biofilm (Fig. 3(a)) is the greatest in the clay suspension (50 % concentration), followed by 25 %, and 12.5 % concentration with Optical Density (OD) values of 0.042, 0.006, and 0.004, respectively. Fig. 3(b) portrays an evaluation of oxidative stress during *Salmonella Typhimurium* single biofilm formation using 50 % clay leachate, which resulted in an OD of 0.023, significantly ($P \leq 0.05$) higher than control. Consequently, this result revealed that biofilm cells were exposed to oxidative stress by the treatments. Damage to the cell surface and oxidative stress appeared to have detrimental effects on biofilm cells, although the severity of the stress may vary between species (Jiang et al., 2009; Oh et al., 2016).

Conclusion

Anti-biofilm activity against *S. aureus in vitro*, with lower but significant efficacy against single *Salmonella Typhimurium* biofilm. The clay suspension generally proved more effective at biofilm disruption and at destroying a single *S. aureus* biofilm. The mechanism of action was exemplified in SEM and CLSM images as damaged *S. aureus* and *Salmonella Typhimurium* biofilm cells were destroyed. Other than that, the stain is fluorescent red after treatment, showing dead or damaged cells. Damage to the cell surface and oxidative stress appeared to be one of the detrimental effects on biofilm cells by NBT assay. The discovery of the present study holds promise for providing a cost-effective option for soil utilization in Malaysia. Consequently, this might potentially have implications for the advancement of biofilms that are resistant to sanitizers, as well as their potential applications within the industry.

CRediT authorship contribution statement

Roslan Ismail: Writing – review & editing, Visualization. **Nor-Khaizura Mahmud Ab Rashid:** Writing – review & editing, Visualization. **Gary J. Sharples:** Writing – review & editing, Visualization, Funding acquisition. **Nur Naqiyah Azmi:** Writing – review & editing, Visualization. **Nor Ainy Mahyudin:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Wan Hasyera Wan Omar:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation.

Ethical approval and informed consent

Not applicable.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2025.100403.

Data availability

Data will be made available on request.

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