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Antibacterial and Antibiofilm Activities of Crude Extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 against Foodborne Bacterium, *Yersinia enterocolitica*

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Authors' contributions

This work was done in collaboration between both authors. Author MMJT designed the study, performed the analysis and wrote the first draft of the manuscript. Author ID supervised the study and analyzed the data. Both authors managed the literature search writing of the final manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

The antibacterial and antibiofilm activities of crude extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 was studied and tested against a foodborne pathogenic bacterium, *Yersinia enterocolitica*. The ethyl acetate extract exhibited favorable antibacterial activity with the zone of inhibition was 20.3±0.6 mm compared to dichloromethane (15.0±0.3 mm) and butanol (9.0±0.3 mm) extracts. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of the extract were 125 and 250 µg/mL, respectively. Structural degeneration studies through scanning electron microscopy (SEM) and transmission electron microscope (TEM) micrographs exhibited major abnormalities that occurred on the bacterial cells after exposure to the extract were complete alterations in their morphology and collapsed of the cells beyond repair. The findings showed that the extract possesses antibiofilm activity against the initial and preformed biofilm of *Y. enterocolitica* with the highest inhibition value of 69.12% and 58.70%, respectively.

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The results also revealed the initial biofilm was more susceptible to the extract as compared to pre-formed biofilm. The light microscopy (LM) and SEM photomicrographs proved that the fungal extract significantly eliminates extracellular polysaccharide (EPS) matrices and hinder the attachment of the bacterial cells for biofilm formation. Therefore, the current study suggested the ethyl acetate crude extract from an endophytic fungus, *L. pseudotheobroma*e IBRL OS-64 may be an effective antibacterial and anti-biofilm agent to treat foodborne pathogens.

Keywords: Antibacterial and antibiofilm activities; Lasiodiplodia pseudotheobromae; Yersinia enterocolitica; Minimum Inhibition Concentration (MIC); Minimum Bactericidal Concentration (MBC).

1. INTRODUCTION

Foodborne diseases are widespread and attracting public health concerns worldwide including developed and developing countries. *Yersinia enterocolitica* is a Gram-negative non-spore-forming rod bacteria belongs to the genus *Yersinia*, family Enterobacteriaceae that is widely distributed throughout the environment and has been isolated from soil, seafood, humans, raw milk, poultry, pigs and raw milk [1]. It is a zoonotic bacterial species that causes food-transmitted infections and the most common clinical manifestation of this bacterial infection is gastroenteritis, but extraintestinal manifestation and postinfectious sequelae such as reactive arthritis were also reported [2]. The prevalence of gastrointestinal illnesses such as yersiniosis is reported in developing countries including Iran, Iraq and Nigeria highlights the major underlying food safety problems in low- and middle-income countries [3]. Yersiniosis is a foodborne illness that has become more prevalent in recent years due to human transmission via the fecal-oral route and prevalence in farm animals and primarily caused by *Y. enterocolitica* [3]. Yersinia infections in humans may cause several diseases symptoms such as diarrhea, vomiting, fever, abdominal pain and some pathologies such as septicemia, acute mesenteric lymphadenitis and self-limiting enteritis [4].

Biofilms are food safety concerns as they could increase the resistance of several bacteria towards many chemical and physical factors used in controlling hygiene in the food industry [5]. According to Bridier [6], the role of biofilms as a source of foodborne human pathogens is well established. Biofilms are communities of microorganisms that attach to a surface and play a significant role in the persistence of bacterial infections [7]. They are supported by the structure of extracellular polymeric substances (EPS) matrix that comprises one or more of extracellular polysaccharides, protein and DNA [8]. Besides that, the channels within biofilm allow air, water and nutrients to go through to all parts of the structure [9]. Although biofilms play an important positive role in a variety of ecosystems, they also have many negative effects including biofilm-related infections in medical settings. The ability of pathogenic bacteria to form biofilms and survive in the presence of a high concentration of antibiotics is known as recalcitrant and this property leads to treatment failure and infection recurrence [10]. Many previous studies reported the ability of *Y. enterocolitica* to form biofilm, adhesion and its prevalence. For instance, Lenchenko [11] reported that *Y. enterocolitica* strains forming S-shaped colonies are more capable of forming biofilms than R-forms and the adhesion as well as biofilm formation increase the pathogenicity of the strains and could cause persistent infections.

Due to the emergence of the prevalence of bacterial strains towards antibiotic and biofilm formation, the search for a new natural antibacterial and antibiofilm agent is a need. According to Levy [12], Endophytes are a new finding for potentially beneficial pharmaceutical substances to overcome the antibiotic resistance phenomenon. Endophytes exist widely inside the healthy tissues of living plants and are an important component of plant micro-ecosystems [13]. An endophytic fungus, *Lasiodiplodia pseudotheobromae* is one of the endophytes that have the capability to produce antimicrobial activity towards pathogenic bacteria. This endophyte has been reported to produce some pharmacological substances and showing anticancer and antimicrobial activities [14,15]. Hence, this study was carried out to evaluate the endophytic fungus, *Lasiodiplodia pseudotheobromae* IBRL OS-64 that previously isolated from a medicinal plant, *Ocimum sanctum* leaves, that possessed bioactive compounds could be a new antibiofilm agent.

2. MATERIALS AND METHODS

2.1 Maintenance of Endophytic Fungus

The endophytic fungus, *Lasiodiplodia pseudotheobromae* IBRL OS-64 which was previously isolated from the leaf of medicinal plant, *Ocimum sanctum* L. was provided by the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The fungal isolate was cultured on potato dextrose agar (PDA) supplemented with host plant (2 g/L), incubated at 30°C for 7 days and stored at 4°C prior to use. The culture was subcultured regularly on sterile fresh media to ensure its purity and survivability.

2.2 Culture Medium

The culture medium was prepared according to Jalil [16]. Yeast extract sucrose (YES) broth containing 40 g/L sucrose, 20 g/L yeast extract (Merck, Germany), 0.5 g/L magnesium sulfate, and aqueous extract of *O. sanctum* leaf was used as a growth medium to cultivate *L. pseudotheobromae* IBRL OS-64 in a shake flask fermentation system.

2.3 Cultivation and Extraction

Cultivation and extraction of the fungal secondary metabolite were performed according to Taufiq and Darah [17]. Two mycelial agar plugs were then inoculated into 250 mL Erlenmeyer flasks containing 100 mL of YES (yeast extract sucrose) broth and were cultivated in dark-static conditions at 30°C for 16 days. The fermentative broth and fungal biomass were separated using Muslin paper and Whatman No.1 filter paper after the cultivation period. The fermentative broth was extracted thrice with equal amounts of hexane, dichloromethane, ethyl acetate, and butanol (1:1; v/v), accordingly. The extracts were concentrated under reduced pressure to dryness by using a rotary evaporator and subsequently dried in a fume hood until dried crude paste obtained.

2.4 Test Microorganism and Inoculum Preparation

The food-borne bacterium, *Y. enterocolitica* was obtained from the culture collection of IBRL, School of Biological Sciences, Universiti Sains Malaysia, Penang. The test bacterium was grown on nutrient agar (NA) and incubated at 37°C for 24 hours. The culture was subcultured monthly on freshly prepared NA to ensure its viability. The bacterial suspension of *Y. enterocolitica* was prepared by inoculating aseptically two or three single colonies from 24 –h-old cultures into 5 ml of 0.85% sterile physiological saline (w/v). The inoculum size was standardized by matching its turbidity to the 0.5 McFarland standards which is equivalent to 1×10^8 CFU/mL.

2.5 Disc Diffusion Assay

Disc diffusion assay was performed to screen the antibacterial activity of the extract towards test bacteria according to the method described by NCLLS [18]. The bacterial suspension (previously explained) was swabbed on the surface of the Muller Hinton agar (MHA) using sterile cotton swabs. Whatman antibiotic disc that previously impregnated with 20 µL of the fungal extract was placed on the seeded agar plate and the plate was then inversely incubated at 37^{o}C for 24 hours. Twenty percent of ethyl acetate and chloramphenicol (30 µg/mL) were used as negative and positive controls, respectively. The diameter of the inhibition zone was measured and recorded after the incubation period. The experiments were carried out in triplicate on different occasions.

2.6 Determination of MIC and MBC Values

The minimum inhibition concentration (MIC) of the extract was determined in the broth microdilution assay as described by NCLLS [18]. The stock extract was prepared by a two-fold dilution of 3.90 – 2000.00 µg/mL. The microtiter wells were loaded with 5.0 µL of bacterial inoculum, 95.0 µL of nutrient broth (NB, Merck, Germany) and 100 µL of extract. The final volume in each well was 200 µl and the final concentration of bacteria in each well was 5×10^5 CFU/ml. Meanwhile, the final concentrations of the extract were in the range of 1.95 – 1000.00 µg/mL. The microtiter plate was mixed thoroughly and then incubated for 24 h at 37°C. After the incubation period, 40 µl of 0.6 mg/ml colourless p-iodonitrotetrazolium violet (INT) salt (Sigma, Germany) was loaded into each well as growth indicator and the plate was re-incubated for 30 minutes at 37°C. Any changes (from colorless to purple) within the wells indicated the presence of living bacteria. The lowest dilution (concentration) of the crude extract showing no

color change was recorded as the MIC value of the extract. The streak plate method was performed in order to determine the minimum bactericidal concentration (MBC) value of the extract. A loopful of inoculum from each of the colorless wells were streaked onto fresh NA and then incubated for 24 h at 37°C. The lowest concentration of the extract resulted in a reduction of 99.9% bacterial growth (no growth observed on NA) relative to the growth control was recognized as the MBC value of the extract. The experiment was performed in triplicate.

2.7 Structural Degeneration of Bacterial Cells

The effect of the extract on *Y. enterocolitica* was studied under scanning electron microscope (SEM) and transmission electron microscope (TEM) observations. The bacterial suspension was prepared as previously described. Two flasks were prepared for control and 24 h time bacterial cells exposed to the extract. A volume of 0.1 mL of bacterial cells was inoculated into each 50 mL Erlenmeyer flasks containing 18.9 mL MHB. Then, 1.0 mL of the extract was added into the flasks to give a final volume of 20 mL with the final concentration in the flask was at the MIC value. The treated cultures were then incubated at 37°C for the required incubation time (0 and 24 h). The sample preparation for SEM and TEM analysis were done following the method described by Ibrahim [19].

2.8 Inoculums Preparation for Anti-biofilm Assay

The inoculum of *Y. enterocolitica* was prepared according to Burnolle [20]. Five colonies of 24-h-old bacteria were inoculated into the 250 mL Erlenmeyer flask containing 50 mL Mueller Hinton broth (MHB) and incubated at 37°C for 24 hours with an agitation speed of 150 rpm. The bacterial broth culture was then centrifuged (ALC Centrifugette 4206) at 4000 rpm, 4°C for 30 min to separate the supernatant and the pellet. The cell' pellet was then collected and re-suspended with 10 mL (MHB) and the supernatant was discarded. The cell densities of the suspensions were adjusted to an OD of 0.15 at 600 nm by dilution with fresh MHB.

2.9 Inhibition Assay of the Initial and Pre-formed Biofilm

The inhibition assay of the initial/pre-formed biofilm and quantification of bacterial biofilm were performed according to the method described by Taufiq and Darah [21]. All experiments were carried out in triplicate. The inhibition activity was expressed in terms of the percentage of biofilm inhibited in comparison to the untreated control biofilms, and was calculated according to the following equation;

$$\%\ \text{biofilm inhibition} = \frac{\text{OD growth control} - \text{OD sample}}{\text{OD growth control}} \times 100$$

2.10 Light Microscopy (LM) and Scanning Electron Microscopy (SEM) of Biofilm Formation

The light microscopic and scanning electron microscopic techniques were performed according to Taufiq and Darah [21]. The biofilm formation was examined under the light microscope attached with a digital camera (Olympus U-CMAD3) and Scanning Electron Microscope (Leica Cambridge, S-360, UK).

2.11 Statistical Analysis

All the experiments were performed in triplicate (n = 3) and the experimental data were expressed as mean ± standard deviation (SD). The results obtained were analyzed by the Student t-test from comparing the effect of different extracts on *Y. enterocolitica* against control. Antibiofilm activity of initial and preformed biofilm were analyzed employing the One Way ANOVA using SPSS Version 18.0 and Duncan test was used to assess the differences between means. The results were considered statistically significant if $p < 0.05$.

3. RESULTS

3.1 Antibacterial Activity

Disc diffusion assay was performed to screen the antibacterial activity of the extracts towards test bacteria. Table 1 shows the antibacterial activity of different extracts of *Lasiodiplodia pseudotheobromae* IBRL OS-64 against foodborne bacterium, *Yersinia enterocolitica*. The results demonstrate ethyl acetate extract possesses the highest antibacterial activity against test bacterium with an inhibition zone of 20.3±0.6 mm, followed by dichloromethane extract with a value of 15.0±0.3 mm. In contrast, the butanol extract showed the lowest antibacterial activity with the inhibition zone of

9.0±0.3 mm whereas that hexane extract possessed no antibacterial activity towards test bacteria. The findings indicate that the mid-polar solvent is able to extract more bioactive compounds with antibacterial activity compared to polar and non-polar solvents. Due to its prominent activity against *Y. enterocolitica*, ethyl acetate extract was selected for further study. Table 2 shows the MIC and MBC determination of ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 against *Y. enterocolitica* on broth microdilution assay. The results demonstrate that the MIC and MBC values of the ethyl acetate extract were 125 and 250 µg/mL, respectively. The present study revealed that the extract exhibited a bactericidal effect since the MBC value two-folds higher than MIC value.

3.2 Structural Degeneration of Bacterial Cells Exposed to the Extract

Fig. 1 shows the scanning electron microscope (SEM) micrographs of *Yersinia enterocolitica* treated with 250 µg/mL of *L. pseudotheobromae* IBRL OS-64 ethyl acetate extract at 0 hour and 24 hours of exposure time. Fig. 1a exhibits control with typical rod-shaped Gram-negative bacteria. Normal binary fission of bacterial cells were also observed. After treated to the extract for 24 hours, some alterations on the bacterial cells were observed (Fig. 1b). The bacterial cells started to show multiple defects with many of the cells exhibited crumpled or shrunken cell surfaces. Some of the cells formed cavities and cell debris was observed. The cells collapsed, crumpled and hence lost their rod-shaped as compared to the control cells. Further evidence of these changes can be clearly observed from the transmission electron microscope (TEM) micrographs which reaffirm some form of disorganization of its cytoplasm and organelles (Fig. 2). Fig. 2a shows the typical structure of *Y. enterocolitica* with rigid cell walls and organelles. The cytoplasm contains elements of the cell membrane system and is enveloped by a typical cell wall of Gram-negative bacteria. After 24 hours of exposure to the extract, cells exhibited notable alterations in the cell membrane and cell wall (Fig. 2b).

3.3 Anti-biofilm Activities of Ethyl acetate Extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 against *Yersinia enterocolitica*

Fig. 3 illustrates the anti-biofilm activity of ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 against initial and pre-formed biofilm of foodborne pathogenic bacterium, *Y. enterocolitica.* At high extract concentration, the results showed a concentration-dependent pattern in which the higher concentration of extract enhanced the higher percentage of inhibition of biofilm formation for both initial and pre-formed methods. As for the initial biofilm study, low extract concentration (0.03 to 0.5 mg/mL) could trigger the formation of bacterial biofilm whereby the negative values obtained. The same occurrence also can be observed for pre-formed biofilm in which the negative values of inhibition percentage ranging from -33.55% and -10.46% obtained and this may be happened because of the bacterial adaptation towards the antibiotic treatments. At a concentration of 1.0 mg/mL, the extract started to inhibit bacterial cell formation with poor inhibitory activity for both initial and pre-formed with the inhibition values of 14.94 and 7.37 %, respectively. The concentration of 2.0 mg/mL was observed to be a turning point for a high percentage of biofilm inhibition whereby almost triple times (55.0%) of the bacterial cell was destroyed as compared to a concentration of 1.0 mg/mL. The highest percentage of inhibition for initial biofilm formation was observed when the bacterial culture was exposed to extract concentration of 8 mg/mL with an inhibition value of 69.12%. Besides that, as for pre-formed biofilm, the highest reduction of biofilm formation was observed at the extract concentration of 4 and 8 mg/mL with the inhibition values of 57.33

Table 1. Antibacterial activity of crude extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 against *Yersinia enterocolitica* on disc diffusion assay

Microorganism	Diameter of inhibition zone (mm)				
	HEX[1]	DCM[2]	EA[3]	BUT[4]	Control*
Yersinia enterocolitica	-	15.0±0.3[a]	20.3±0.6[b]	9.0±0.3[c]	29.8±0.3[d]

**Drug control, Chloramphenicol, HEX[1] = Hexane extract, DCM[2] = Dichloromethane extract, EA[3] = Ethyl acetate extract, BUT[4] = Butanol extract, Alphabet shows the significant difference between the extracts*

Table 2. Minimum inhibition concentration and minimum bactericidal concentration determination of ethyl acetate extract against *Yersinia enterocolitica*

Microorganism	MIC and MBC (µg/mL)			
	MIC	MBC	Ratio	Conclusion
Yersinia enterocolitica	125	250	2	Bactericidal

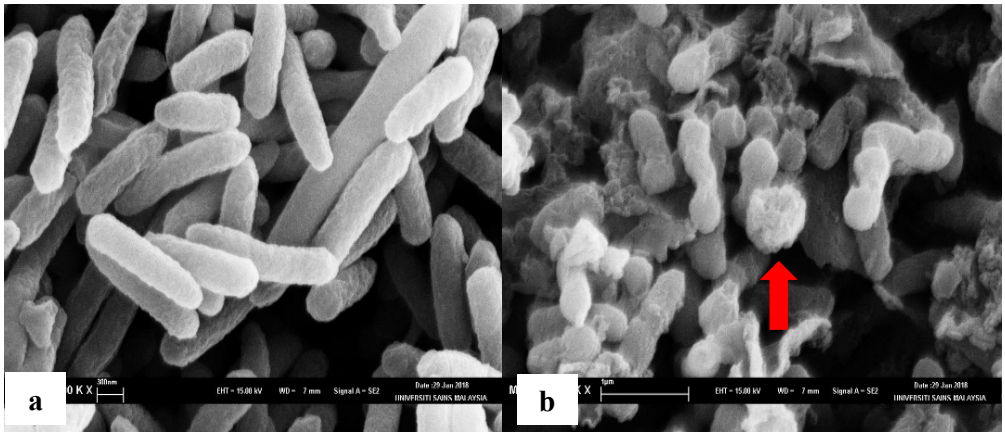

Fig. 1. SEM micrographs of *Yersinia enterocolitica* treated with 250 µg/mL of *Lasiodiplodia pseudotheobromae* IBRL OS-64 ethyl acetate extract at different exposure time. (a) 0 hour [control] (b) 24 hours. Scale bars: 200nm

and 58.70%, respectively, since it was not significantly different (Duncan, $p < 0.05$). As shown in Fig. 3, the percentage of inhibition slightly decreased at the highest extract concentration which was 16 mg/mL.

3.4 Inhibition of *Yersinia enterocolitica* Biofilm by Ethyl acetate Extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 on Initial and Preformed Biofilm Formation Observed under Light Microscope (LM) and Scanning Electron Microscope (SEM)

Fig. 4 shows a light microscopy view of the biofilm formation of *Y. enterocolitica* and effect of ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 towards biofilm formation. Figs. 4(a) and 4(c) show the control sets for both initial biofilm and pre-formed. The Figures indicate the biofilm formation by *Y. enterocolitica* with an abundance of microcolonies forming microbial matrices. Fig. 4(b) illustrates the initial biofilm formation treated with the fungal extract. The result revealed that even though the extract is able to reduce and attenuate the biofilm formation, some of the cells are still able to adhere and attach to the glass coverslip. The result also in agreement with the anti-biofilm activity in microtiter plate assay that showed less than 70% biofilm of *Y. enterocolitica* was successfully inhibited by the fungal extract. As for preformed biofilm, the bacterial cell exposed with the fungal extract was observed to be able to form a biofilm (Fig. 4d). However, the formation of microbial matrices is slightly less dense as compared to control (Fig. 4c). This indicates that the fungal extract is still able to reduce biofilm formation by inhibiting new bacterial growth. The biofilm cycles of certain strains might play a significant role in biofilm formation and thus affect the antibiofilm activity of the extract.

The SEM micrographs show apparent destruction of bacterial biofilm after being exposed to the fungal extract at a concentration of 16.0 mg/mL for initial and preformed biofilm treatments (Fig. 5). Fig. 5a shows a very high density of microcolonies tied together by extracellular polymeric substances (EPS) matrices at a low magnification view. A clearer view is observed in Fig. 5b, where at higher magnification the bacterial cells were seen to be glued together in the EPS complex. Fig. 5c and 5d show the initial biofilm treatment of bacterial cells with the fungal extract. The Figures reveal the decrement of the EPS matrix, however a small portion of it (red arrow) still can be observed (Fig. 5c). The EPS layers that

entrapped the microcolonies vanished resulting in unprotected bacterial cells. Furthermore, at a higher magnification view (Fig. 5d), it is clearly seen that the disruption of the bacterial cell occurred by the formation of hole and cavity (red arrow) that leads to cell death. Fig. 5e and 5f show the biofilm formation after exposure to the fungal extract for pre-formed biofilm treatment. Fig. 5e reveals the biofilm formation and bacterial cells were significantly decreased as compared to the control and slightly greater as compared to initial biofilm. Meanwhile, Fig. 5f exhibits biofilm formation and bacterial cells at a higher magnification view. Observations revealed that the extract degraded the EPS matrix formation (red arrow) by disturbing the layer integration and also destroyed bacterial cells into small particles (black arrow).

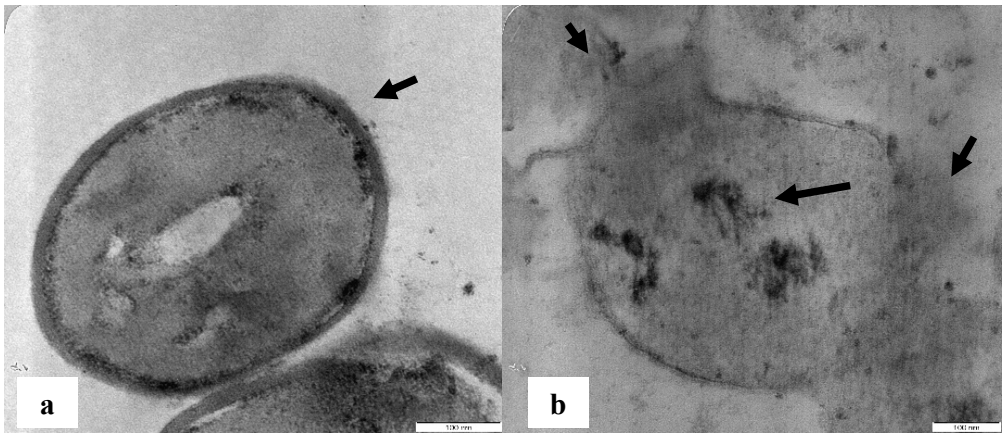

Fig. 2. TEM micrographs of *Yersinia enterocolitica* treated with 250 µg/mL of *Lasiodiplodia pseudotheobromae* IBRL OS-64 ethyl acetate extract at different exposure time. (a) 0 hour [control] (b) 24 hours. Scale bars: 200nm

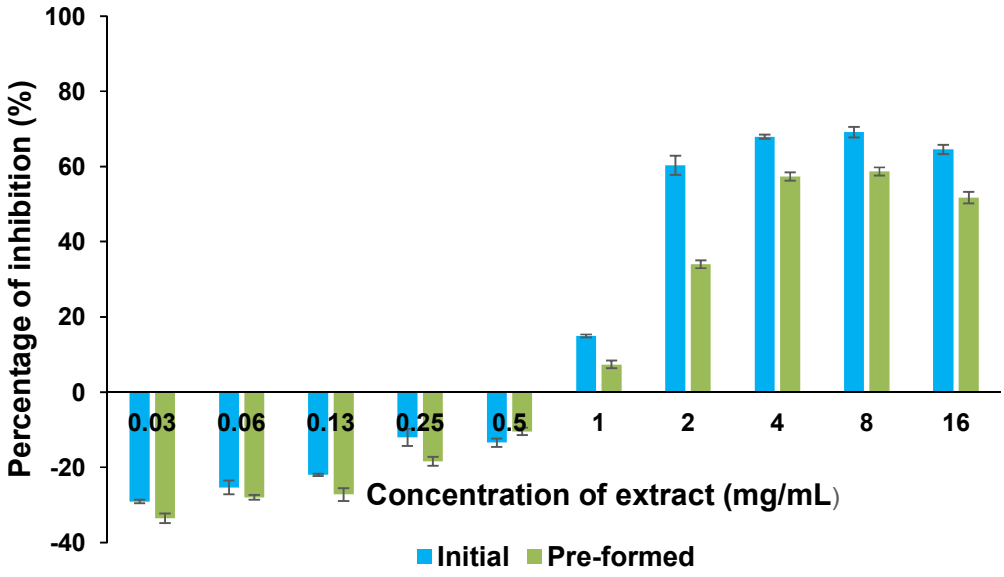

Fig. 3. Anti-biofilm activity of ethyl acetate extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 against the initial and pre-formed biofilm of foodborne pathogenic bacteria, *Yersinia enterocolitica*. The highest inhibition percentage was obtained at extract concentration of 8.00 mg/mL for both biofilms types

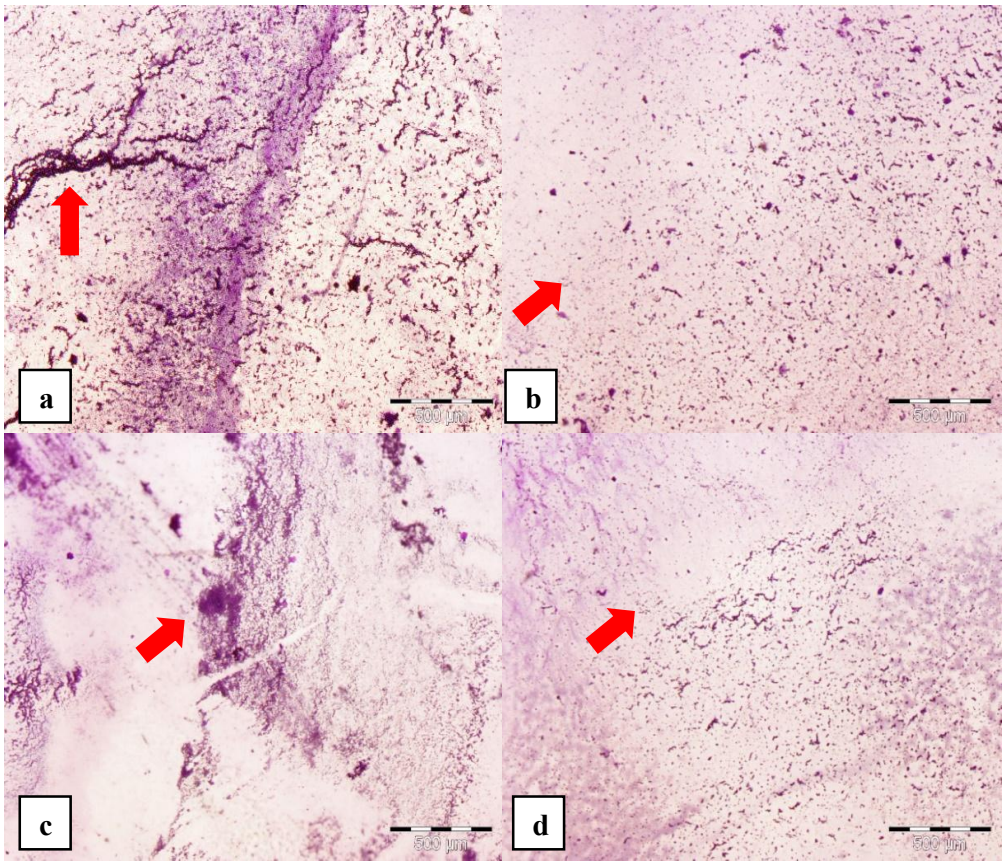

Fig. 4. Light microscopy view of *Yersinia enterocolitica* biofilm showing the effect of ethyl acetate extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64

Figures (a) initial biofilm - untreated; (b) initial biofilm - treated with 8 mg/mL of extract; (c) pre-formed biofilm – untreated; (d) pre-formed biofilm – treated with 8 mg/mL of extract

4. DISCUSSION

The choice of the solvent for the extraction process depends on the intention of studying the extract and several types of organic solvents with different polarity ranges were frequently used for extraction in order to gain a diverse type of possible bioactive substances [22]. According to Synytsya [23], the successful isolation of fungal metabolites exhibiting antibacterial activity depends on extracting solvents, producing strain and the testing of microbes. In this study, it was found that the test bacterium, *Y. enterocolitica* did not respond to some organic solvents whereas it showed some activity to some other organic solvents. This phenomenon might be due to the type of organic solvent used for the extraction process affecting the presence of bioactive substances in the crude extract and thus affecting its activity [24]. Some researches concerning the effectiveness of extraction methods highlight that ethanol and methanol extraction yields higher antibacterial activity against Gram-negative and Gram-positive bacterial strains than ethyl acetate whilst others reported that ethyl acetate and ethanol are better than dichloromethane [25, 26].

The present study revealed that the test bacterium was inactive against hexane extract and this was consistent with the previous report by Tong [27]. This might be due to the low amount of non-polar compounds in the crude extract since hexane was mainly to dissolve more nonpolar compounds such as wax, lipid and fat [28]. Hexane wash also eliminated unwanted metabolites such as fatty acids that were not of interest in natural product screening [29]. Meanwhile, current findings revealed that ethyl acetate extract was the best organic solvent in extracting bioactive compounds with antibacterial activity due to higher inhibitory

activity than other solvents. A similar observation was reported by Zerroug [30] who claimed that the ethyl acetate was the best solvent used for the extraction of bioactive secondary metabolites from an endophytic fungus, *Penicillium griseofulvum* MPR1. This phenomenon might be due to its efficiency in extracting and isolating secondary metabolites of fungal origin [31]. Furthermore, this solvent has capability to extract broad range compounds such as phenolic (low molecular weight)

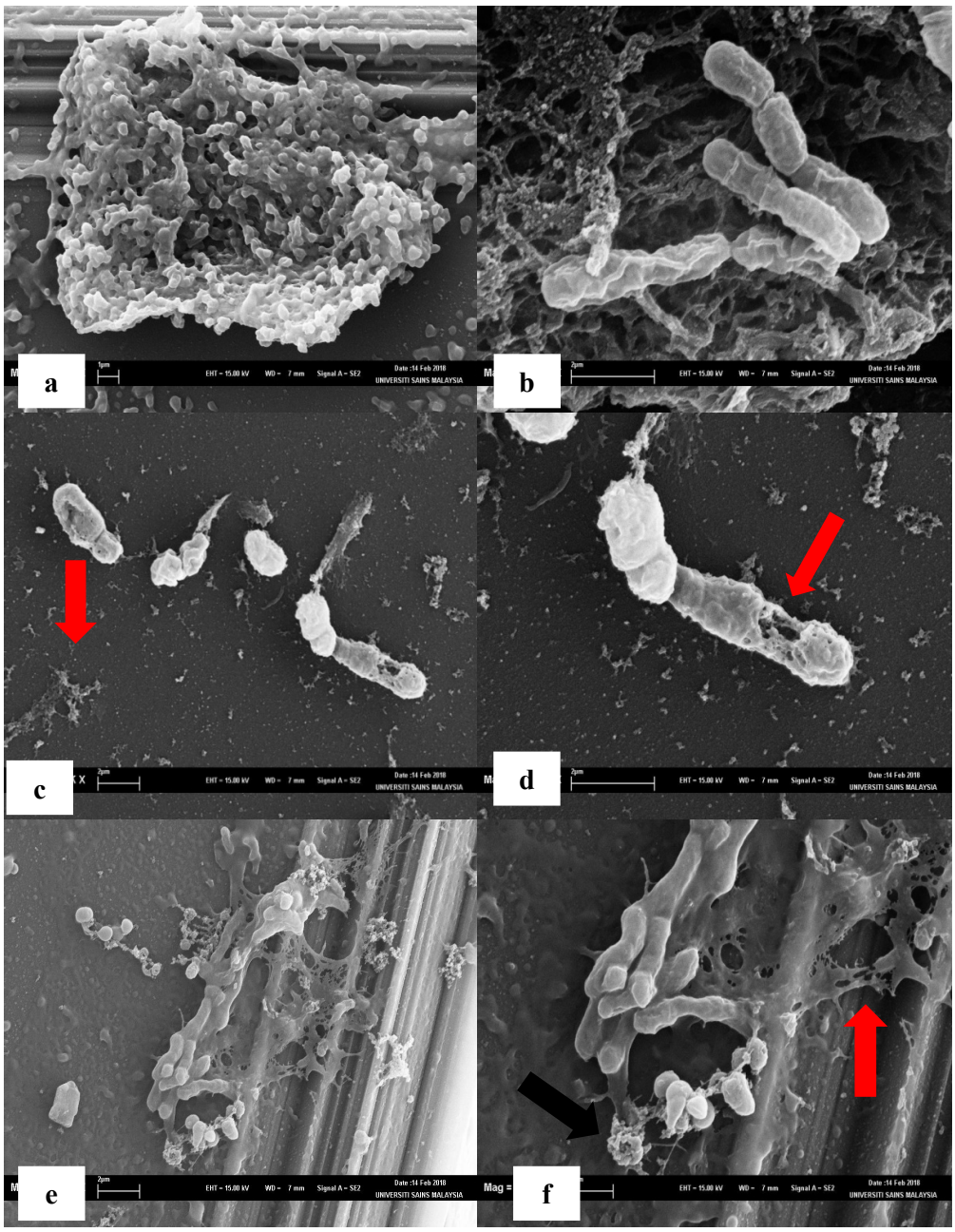

Fig. 5. SEM micrographs of *Yersinia enterocolitica* biofilm showing the effect of ethyl acetate extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64

Figures (a) control – untreated at 5000 x; (b) control – untreated at 10000 x; (c) initial biofilm – treated with 8 mg/mL of extract d at 5000 x; (d) initial biofilm - treated with 8 mg/mL of extract at 10000 x; (e) preformed biofilm treated with 8 mg/mL of extract at 5000 x; (f) treated with 8 mg/mL of extract at 10000 x.

and high molecular weight compounds, polyphenols [32]. Begtrup and Larsen [33] also suggested that mid-polar organic solvents including dichloromethane and ethyl acetate are suitable in extracting various bioactive compounds since they are easily evaporated and dried due to low boiling point.

The broth dilution assay is a common method to determine minimum inhibition concentration (MIC) values and according to Rocksin [34], this is a recommended method to test *in-vitro* susceptibility of pathogenic bacteria. This method was chosen in the present study as compared to agar dilution and broth macrodilution due to its simple and rapid procedures, low volume of extract needed and less laboratory-intensive. Besides that, this method is useful to evaluate the antimicrobial activity of new antibiotic agents and able to differentiate the effect of antibiotic agents either can kill bacterial cells (bactericidal effect) or just inhibit their growth or known as a bacteriostatic effect [35]. According to Smith-Palmer [36], determinations of MICs and MBCs value is crucial for the detailed bio-activity assessment of antimicrobial agents to remove agents that exhibit a slight inhibitory effect towards the targeted bacteria. In clinical practice, the knowledge of MICs is useful as a guide to evaluate the amount of antimicrobial drugs needed to treat a particular pathogenic infection and avoid drug resistance, indirectly [37]. The present study revealed that the MBC value of the extract towards test bacteria was two-folds higher than its MIC value and the result indicated that the extract has a tendency as a bactericidal agent. According to Krishnan [38], antimicrobial substances are considered as bactericidal agents when the MBC/MIC ratio less or equal to 4, whilst bacteriostatic agents if the MBC/MIC ratio greater than 4.

The antibacterial activity of the extract was supported by structural degeneration analysis through a scanning electron microscope (SEM) and transmission electron microscope (TEM) that revealed the abnormalities that occurred on the bacterial cells treated with the extract. The phenomenon can be summarized that cell death starts with the shrinkage of the bacterial cells and followed by the formation of dents as well as unusual cell morphogenesis. A similar observation was also reported by Nor Afifah [39] who studied the antimicrobial activity of *Halimeda discoidea* against a pathogenic bacteria, *Klebsiella pneumoniae* ATCC 13883 cells. *Yersinia enterocolitica* is a Gram-negative bacteria with a cell envelope consisting of the cytoplasmic membrane, thin peptidoglycan layer and outer cell membrane [40]. Therefore, the extract is believed to cause cleavage of the cell envelope by attacking either the cytoplasmic membrane, peptidoglycan layer or even both. The prediction was in agreement with Ibrahim [19] who revealed that the ethyl acetate extract of endophytic fungal isolate, *Nigrospora sphaerica* CL-OP 30 that exposed to bacterial cells caused the disintegration of cell wall and membrane and thus resulting in cleavage and leakage of their cytoplasmic contents.

Anti-biofilm activity of the extract was determined using broth microdilution methods and the results revealed that the anti-biofilm activity was dose-dependent with a higher dose could eliminate more bacterial growth for both initial and preformed biofilm. However, a low extract dose could enhance the negative value of antibiofilm activity. According to Nor Afifah [41], the negative value obtained for the percentage of inhibition at the low antimicrobial concentration indicated that the bacteria react to environmental changes (the exposure to antimicrobial) and try to adapt the harsh condition by producing a large amount of biofilm. The phenomenon occurred because a high concentration of extract would promote the biofilm formation and this, was in agreement with Walraven [42], who revealed that the use of high concentration of antifungal drugs might facilitate the formation of biofilm of echinocandin-resistant *Candida albicans* isolates.

Besides that, the fungal extract was observed to have significant anti-biofilm activity against *Y. enterocolitica* for both initial and pre-formed biofilm studies. This might have occurred due to the effectiveness of the fungal extract in overcoming the resistance mechanism by *Y. enterocolitica* strain such as quorum sensing (QS). According to Atkinson and Williams [43], QS is a regulatory mechanism by bacteria that is responsible for the expression of specific genes that amplify bacterial virulence by enhancing the expression of disease causing attributes including secretion of virulence factors, motility and biofilm formation. Therefore, the fungal extract might act as anti-quorum sensing that is able to eradicate biofilm formation. Truchado [44] stated that flavonoids from orange extract have the capability in inhibiting quorum sensing of *Y. enterocolitica*. They revealed that flavonoids showed a moderate anti-pathogenic activity

towards *Y. enterocolitica* by eradicating the N-acylhomoserine lactones (AHLs) production and the biofilm formation that were found to be associated with QS-regulatory genes induction such as flhDC and yenR. Furthermore, they speculated that anti-QS might be due to inhibition of AHLs synthesis at the protein level, degradation of an enzyme crucial for AHLs and inhibition of AHLs transportation and secretion to the media. In addition, the inhibitory effect of quorum sensing might be due to other regulatory mechanisms including inhibition of target AHLs-receptor binding and antagonism effect of AHLs receptors [45].

Overall, the SEM observations revealed that the fungal extract significantly affected the initial biofilm as compared to pre-formed biofilm. However, the reports regarding the antibiofilm activity of *L. pseudotheobromae* IBRL OS-64 towards *Y. enterocolitica* via SEM observations were very scarce. The SEM results showed the formation of EPS matrix layers called biofilm by bacterial cells in the control treatment. According to Ishida [46], the formation of biofilm provides an effective resistance mechanism of the bacterial colonies towards large molecules including antimicrobial proteins lysozyme and complement. The ability of *Y. enterocolitica* to produce a biofilm that entrapped microcolonies helps them to survive in hostile conditions within the host and thus, this factor is considered to be responsible for persistent or chronic infections [47]. Besides that, the formation of biofilm provides an attachment site for bacterial cells. Therefore, the ability of a bacterial strain to adhere to various surfaces of the host resulted in less susceptibility towards the antimicrobial drugs due to protection from the host immune system and giving rise to the chronic infection which is difficult to eradicate [48]. The difficulty in eradicating biofilm layers is one of the factors that lead to chronic infection whereby the sessile cells become more resistant to higher concentrations of antibiotics as compared to planktonic cells [49].

For initial biofilm treatment, a low density of EPS matrix layers was observed. This might be due to the high efficiency of the extract to combat young cells as compared to mature cells. Ito [50] revealed that the cells of *Escherichia coli* in the young biofilm were completely destroyed and killed by ampicillin as compared to mature biofilm, According to Nor Afifah [41], antibacterial action towards bacterial cells in initial biofilm would be the first mode of action. Besides that, the surface modification mechanism by the extract may increase the susceptibility of bacterial cells towards antimicrobial drugs. According to Rendueles [51], the extract has the capability to modify the physicochemical properties of the surfaces by altering the wettability and its surface charge which resulted in disrupting the interaction between the bacteria and surface. Generally, initial attachment is a crucial factor in biofilm formation. Donlan [52], stated that the success of bacterial attachment on the surfaces is dependent on the absorption of macromolecules. In addition, initial attachment is crucial for biofilm formation since the bacterial cells within EPS matrix layers rely on the interaction between bacterial cells and the environment's surface for their survival [53]. Therefore, the surface alteration by fungal extract might occur in the current study of initial biofilm that alters the glass coverslip surface avoiding initial attachment of bacterial cells and thus, increased their susceptibility towards the antimicrobial agents.

Anti-Quorum sensing is another strategy that can be used in order to inhibit pathogenic bacterial growth since they are capable of communicating between cells (cell to cell interaction) as a response mechanism towards antibiotics. Bacha [54] reported the potential of Ethiopian medicinal plant extracts in the interfering cell to cell communication of pathogenic bacteria and thus, inhibiting their growth. For pre-formed biofilm, according to Evans [55], a low growth rate of bacterial biofilm is one of the antibiotic resistance mechanisms found in *E. coli*. Similarly, Cerca [56] revealed that the low growth rate in sessile cells as compared to planktonic cells resulted in higher resistance towards antimicrobial drugs. They also postulated that growth rates of the cells also influenced the efficiency of antibiotic agents especially cell wall synthesis inhibitors. Furthermore, bacterial cells entrapped in the biofilm are less metabolically active than free cells (planktonic cells) and thus, they are less sensitive to the mechanism of action by many antibiotic drugs that targeting on metabolic pathways or synthesis of macromolecules including quinolones and ß-lactams [57, 58]. Other reports suggested that less susceptibility of sessile cells may be due to microenvironment created by biofilms such as low oxygen level and acidic pH that might affect the effectiveness of antibiotics [59].

The presence of persister cells also might be a great factor that increases the susceptibility of the biofilm towards antimicrobial drugs as compared to planktonic cells. According to Keren [60], the specialized persister cells such as the one that was found in Gram-negative bacteria, *Escherichia coli* are significantly responsible for the recalcitrance of biofilm infection since they neither grow nor die in the environment with the presence of antibiotic and therefore, exhibit multidrug tolerance. Besides that, Quorum sensing (QS) is another factor that might explain why pre-formed biofilms are less sensitive to antimicrobial drugs. QS is a cell to cell interaction process that regulates the behavior of most bacterial strains and it depends on extracellular signal molecules, production, detection and autoinducers [61]. Under stress conditions, bacterial cells were induced by a particular set of genes that are capable of sensing and subsequently responding by increasing cell population density [62]. As for Gram-negative bacteria, cell to cell communication involves in the production and secretion of signalling molecules known as acyl-homoserine lactones (AHLs) which is a primary signal to detect cell density [63]. This mechanism may be used by *Y. enterocolitica* in increasing their population density as a response to vulnerable conditions created by the presence of fungal extract.

5. CONCLUSION

The present study conclusively shows the antibacterial and antibiofilm potential of the ethyl acetate extract of an endophytic fungus, *L. pseudotheobromae* IBRL OS-64. Besides, it could serve as a useful source for new natural antibacterial agents. However, phytochemical studies will be necessary to isolate the active substances and evaluate the antibacterial and antibiofilm against a wide range of bacterial populations.

CONSENT

It is not applicable.

ETTHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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