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The Potency of Soil *Actinomycetes* Extracts as Antibiofilm Agents against *Enterococcus faecalis*

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ABSTRACT

Enterococcus faecalis is a bacterium commonly associated with root canal infections. Its virulence and biofilm formation capacity contributes to the persistence of apical periodontitis. *Actinomycetes*, a group of Gram-positive bacteria, are well-known to produce a wide range of bioactive compounds, including antibacterial, antitumor, immunosuppressive agents, and enzyme inhibitors. This study aimed to evaluate the antibiofilm potential of *Actinomycetes* extracts isolated from soil samples collected in Gunungkidul and Pontianak, Indonesia against *E. faecalis* and to identify the primary bioactive compounds present in the active extracts. Extracts from two *Actinomycetes* strains, *Streptomyces* sp. GMR22 (from Gunungkidul) and *Streptomyces* sp. ACT214 (from Pontianak) were tested for its antibacterial and antibiofilm activity against *E. faecalis* through microbroth dilution assay and scanning electron microscopy (SEM) imaging. The primary compounds in the active extracts were identified through untargeted liquid chromatography-tandem high-resolution mass spectrometry (LC-HRMS). *Streptomyces* sp. ACT214 exhibited strong antibacterial activity with a minimum inhibitory concentration (MIC) of 1,250 µg/mL and a minimum bactericidal concentration (MBC) of 5,000 µg/mL. In contrast, GMR22 did not reach MIC or MBC at the maximum concentration tested (5,000 µg/mL). *Streptomyces* sp. ACT214 extract also demonstrated dose-dependent antibiofilm activity, completely inhibiting *E. faecalis* biofilm formation at 582 µg/mL (MBIC90) and significantly disrupting existing biofilm as observed under SEM. Conversely, GMR22 exhibited markedly lower antibiofilm activity. LC-HRMS analysis identified several major compounds in the ACT214 extract, including bis(2-ethylhexyl) phthalate, ethyl myristate, pentadecanoic acid, oleic acid, and palmitoleic acid. These findings indicate that the *Actinomycetes* extract derived from Pontianak soil possesses promising antibiofilm activity

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against *E. faecalis.* As a potential source of novel antibiotic agents, soil-derived *Actinomycetes* merit further exploration as alternative therapeutic strategies for managing *E. faecalis* biofilm in recalcitrant apical periodontitis cases.
Keywords: *Enterococcus faecalis*, biofilm, antibacterial, antibiofilm, *Actinomycetes*

INTRODUCTION

Apical periodontitis is an inflammatory condition affecting the periapical tissues of a tooth, typically resulting from root canal infections where residual microorganisms persist following suboptimal endodontic treatment, or due to reinfection of the treated site (Dioguardi et al., 2019; Ye et al., 2023). *Enterococcus faecalis*, a Gram-positive facultative anaerobic bacterium, is frequently isolated in such cases of apical periodontitis, with a high prevalence of 24-77% found in endodontic re-treatments (Stuart et al., 2006; Wang et al., 2012; Zoletti et al., 2011). The failure to effectively eradicate this pathogen can lead to sustained inflammation and a delayed healing process (Alghamdi & Shakir, 2020).

Enterococcus faecalis exhibits a range of virulence factors that contribute to its persistence and pathogenicity within root canals. These include its ability to invade dentinal tubules, outcompete with other microbial species, survive in nutrient-limited environments, and form robust biofilms (Hussein et al., 2020; Khalifa et al., 2016; Wong et al., 2021). Biofilm formation significantly enhances bacterial resistance to antimicrobial agents and environmental stress, allowing *E. faecalis* biofilms to withstand elevated concentrations of intracanal medicaments such as calcium hydroxide. This combination of virulence and resistance renders *E. faecalis* a principal cause of persistent apical periodontitis in cases where root canal disinfection is inadequate (Alghamdi & Shakir, 2020; Seguel et al., 2020). Therefore, the search for novel antibiofilm agents is critical to improving outcomes in endodontic therapy.

Actinomycetes, a group of Gram-positive filamentous soil have emerged as a prolific source of structurally diverse bioactive secondary metabolites, including antibiotics, antitumor agents, immunosuppressants, and enzyme inhibitors (Barka et al., 2016; Waturangi et al., 2016). *Actinomycetes* isolated from diverse ecological niches have demonstrated the ability to produce a wide range of antibiofilm compounds spanning multiple chemical classes such as alkaloids, peptides, polyketides, and terpenoids (Miller et al., 2022; Oja et al., 2015; Setiawati & Yusan, 2022). Terrestrial *Actinomycetes* from specific microhabitats—such as unique soil environments—remain an underexplored reservoir for discovering new bioactive agents (Jakubiec-Krzesniak et al., 2018).

The *Actinomycetes* strain used in this study was isolated from the rhizospere soil of Cajuput plant in the Wanagama Forest, located in Gunungkidul region, Yogyakarta, Indonesia. Genomic analysis of this strain revealed a relatively large genome size (11.4 Mbp) and the presence of numerous biosynthetic gene clusters (BGCs) associated with the synthesis of diverse secondary metabolites. Further genetic characterization indicated the presence of multiple polyketide synthase (PKS) genes, which are known to play key roles in the production of secondary metabolites (Melinda et al., 2021; Nirwati et al., 2022). In addition to the Gunungkidul isolate, this study also employed an *Actinomycetes* strain isolated from soil collected in an oil palm plantation area in Pontianak, West Kalimantan, Indonesia. These two *Actinomycetes* strains originated from ecologically distinct tropical soil environments. The primary objective of this study was to evaluate the antibiofilm activity of *Actinomycete* extracts derived from these two soil sources against *E. faecalis*, with the aim of identifying potential alternative anti-infective agents to improve the management of apical periodontitis. Furthermore, the study sought to identify the major bioactive compounds responsible for the observed antibiofilm effects in the active extracts.

MATERIALS AND METHODS

Biological material

Streptomyces sp. GMR22 was isolated from the rhizosphere *soil* of the Cajuput plant (*Melaleuca leucodendron L.*) in the Wanagama I Forest, Gunungkidul, Yogyakarta, Indonesia. The isolate has been deposited in the Indonesian Culture Collection (InaCC A148), National Research and Innovation Agency (Nirwati et al., 2022; Tambunan

et al., 2020). *Streptomyces* sp. ACT214 was isolated from soil collected in a palm oil plantation area in Pontianak, West Kalimantan. Both *Streptomyces* strains were maintained in International Streptomyces Project-2 (ISP2) medium (Nirwati et al., 2022). *Enterococcus faecalis* ATCC 29212 was maintained in Brain Heart Infusion (BHI) agar (Kim et al., 2020; Luz et al., 2019) at the Microbiology Laboratory, Dental Learning Center, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Fermentation and extraction

The fermentation process for secondary metabolite production was adapted from the method described by Alimuddin et al. (2011), with slight modifications. Isolates of *Streptomyces sp*. GMR22 and *Streptomyces* sp. ACT214 were first cultured on ISP2 agar for 7 days at 28 °C. The agar was cut into pieces (2 × 2 cm). Agar blocks (2 × 2 cm) were then transferred to 100 mL Erlenmeyer flask containing 25 mL Tryptic Soy Broth (TSB) and incubated for 3 days at 28 °C on a rotary shaker (150 rpm) to prepare the seed culture. Subsequently, the seed cultures were inoculated into 1,000 mL Erlenmeyer flasks containing 475 mL starch nitrate broth (SNB), composed of 20 g soluble starch, 0.5 g NaCl, 1 g KNO3, 0.5 g $MgSO_4.7H_2O$, 0.5 g $K_2HPO_4.3H_2O$, and 0.01 g $FeSO_4.7H_2O$ per liter of distilled water (pH 7.4). Cultures were incubated for 11 days at 28°C on a rotary shaker (150 rpm). After incubation, cells and supernatants were separated by centrifugation at 5.000 rpm for 10 minutes and then filtered by Whatman No.1 filter paper. The supernatant of *Streptomyces* sp. ACT214 was extracted twice with an equal volume of chloroform, based on prior extraction optimization studies. In contrast, the supernatant of *Streptomyces* sp. GMR22 was extracted twice with an equal volume of ethyl acetate to optimize the recovery of bioactive compounds, considering the metabolic differences between the strains. The solvent phase was concentrated by evaporation to obtain the crude extract (Melinda et al., 2021; Nirwati et al., 2022; Tambunan et al., 2020). The resulting concentrated extract was stored in a tightly sealed vials for subsequent testing.

Preparation of extract and bacterial species

Crude extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare aqueous solution at room temperature and sterilized using a 0.22 µm syringe filter (Nirwati et al., 2022). DMSO was used at a final concentration of 5% in each well, a level previously shown to be effective in solubilizing the extracts without exerting significant antibacterial activity against *E. faecalis*.

A single colony of *E. faecalis* ATCC 29212 was inoculated into 5 mL of BHI broth and incubated anaerobically at 37 °C for 24 hours. Overnight cultures were harvested by centrifugation at 3,000 rpm for 15 min, and the supernatant was discarded. The bacterial pellet was resuspended in sterile saline, and the suspension was adjusted to match the turbidity of 0.5 McFarland standard (approximately $1.5x10^9$ CFU/mL) (Ali et al., 2021; Luz et al., 2019)

Antibacterial assay

The antibacterial activity against *E. faecalis* was initially assessed using the agar disk diffusion method, with 100 µg extract loaded per disk. Following preliminary screening, minimum inhibitory concentrations (MICs) were determined using a broth microdilution method based on the Clinical and Laboratory Standards Institute (CLSI) 2015 (CLSI, 2015). Briefly, serial two-fold dilutions of the extracts were prepared in a 96-well microtiter plate. Each well contained 100 µL of Brain Heart Infusion (BHI) broth with varying concentrations of the extracts, ranging from 5,000 to 39.0625 µg/mL. A 10 µL aliquot of *E. faecalis* inoculum (adjusted to 0.5 McFarland standard) was added to each well. As controls, wells containing 5% dimethyl sulfoxide (DMSO) without extract served as the negative control, and 3% sodium hypochlorite (NaOCl) was used as the positive control. The microplates were incubated at 37°C for 24 hours. MIC values were determined visually as the lowest concentration of the extract that prevented visible bacterial growth compared to the control wells. In addition, absorbance was measured at 620 nm using a microplate reader to quantify bacterial inhibition (Al-Madi et al., 2019; Windaryanti et al., 2022). MIC is the lowest concentration at which bacterial cells are not visually observed but is determined based on the value of optical density (Zeng et al., 2021). The lowest concentration of each experimental solution inhibiting 90% of *E. faecalis* growth compared with the negative control group was defined as the MIC value. Each experiment was performed in triplicate (Al-Madi et al., 2019; Windaryanti et al., 2022).

The minimum bactericidal concentration (MBC) was determined by subculturing samples from MIC wells with no visible growth onto BHI

agar plates. After 24 hours of incubation at 37°C, the plates were examined for colony formation. The MBC was defined as the lowest concentration of the extract that resulted in a ≥99.9% reduction in viable bacterial cells (Al-Madi et al., 2019; She et al., 2021; Windaryanti et al., 2022).

Antibiofilm assay

The antibiofilm activity of the *Streptomyces* extracts was evaluated using a microtiter plate assay. Extracts were serially diluted two-fold in BHI broth containing 5% DMSO and dispensed into 96-well plates. A standardized bacterial suspension of *E. faecalis* was added to each well, and the plates were incubated at 37°C for 24 hours under static conditions to allow biofilm formation. Following incubation, non-adherent cells were removed by gently washing the wells with phosphate-buffered saline (PBS). The remaining adherent biofilms were stained with 1% crystal violet for 15 minutes. Excess stain was rinsed off with PBS, and the stained biofilms were fixed with absolute ethanol. Subsequently, 100 µL of acetic acid was added to each well to solubilize the stain, and absorbance was measured at 540 nm using a microplate reader (Thermo Scientific Multiskan Sky). Blank controls were included to correct for background absorbance and potential color interference from the extracts. The minimum biofilm inhibitory concentration ($MBIC_{90}$) was defined as the lowest extract concentration that inhibited ≥90% of biofilm formation compared to the vehicle (DMSO) control. All experiments were conducted in triplicate. (Christensen et al., 1985; Peeters et al., 2008; She et al., 2021).

Scanning electron microscopy (SEM)

The morphological effects of *Streptomyces sp*. ACT214 extract on *E. faecalis* biofilm were examined using scanning electron microscopy (SEM), following the method described by Nirwati et al. (2022) with modifications. *E. faecalis biofilms* were cultivated at 37 °C for 24 hours on sterile acrylic discs (12 mm in diameter) placed in 24-well microplates (NEST Scientific), in the presence of an inhibitory concentration of *Streptomyces sp*. ACT214 extract. Biofilms without extract exposure served as negative controls, while those treated with 3% sodium hypochlorite (NaOCl) served as positive controls. After incubation, the acrylic discs were gently washed twice with sterile phosphate-buffered saline (PBS) and dehydrated through a graded ethanol series (70% for 10 minutes, 95% for 10 minutes, and 100% for 20 minutes), followed by overnight air-drying. The samples were then gold-coated (Au) and examined using a JEOL JSM IT-200 SEM operated in high vacuum mode with an accelerating voltage of 5 kV, spot intensity of 30%, and at a magnification of 5,000× (Liu et al., 2017; Nirwati et al., 2022; Purbowati et al., 2023).

Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis

Untargeted metabolomic profiling of the extracts was performed using an ultra-high-performance liquid chromatography (UHPLC) system coupled with high-resolution mass spectrometry (HRMS). Chromatographic separation was carried out on a Hypersil Gold aQ analytical column at a flow rate of 40 µL/min, with an injection volume of 5 µL and a total run time of 30 minutes. The mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The gradient elution profile was programmed as follows: 5% B for 2 min; ramped to 60% B at 15 min; to 95% B at 22 min; held at 95% B until 25 min; reduced to 5% B at 25.1 min; and equilibrated at 5% B until 30 min. UHPLC was performed using the Thermo Scientific Dionex Ultimate 3000 RSLC Nano UHPLC system, and mass spectrometry was conducted using a Thermo Scientific Q Exactive instrument with a resolution of 35,000 full width at half maximum (FWHM). Data acquisition and analysis were carried out using Thermo Scientific XCalibur software (Melinda et al., 2021; Nirwati et al., 2022).

Statistical analysis

Data from the antibacterial and quantitative biofilm assays were expressed as mean ± standard deviation (SD). Statistical significance of differences between extract types and concentrations was assessed using two-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) post hoc test at a 95% confidence level ($p < 0.05$) (Almeida et al., 2018; Ferreira et al., 2021). All statistical analyses were conducted using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Antibacterial activity

The antibacterial activity of *Actinomycete* extracts, assessed using the broth microdilution method (Figure 1). The data indicate that the extract from *Streptomyces sp*. ACT214 exhibited significantly greater antibacterial activity against *E. faecalis* compared to the extract from *Streptomyces sp*. GMR22. Statistical analysis using two-way

ANOVA revealed a highly significant difference in the percentage of *E. faecalis* growth inhibition between the two extracts ($p < 0.001$). These findings are consistent with preliminary results obtained from the Kirby–Bauer agar disk diffusion method (unpublished data), which demonstrated a strong inhibition zone for *Streptomyces sp*. ACT214 (11.2–12.8 mm), while *Streptomyces sp*. GMR22 exhibited only a moderate inhibition zone (7.3–7.9 mm). These results suggest that *Streptomyces sp*. ACT214 has greater potential as an antibacterial agent against *E. faecalis* than *Streptomyces sp*. GMR22.

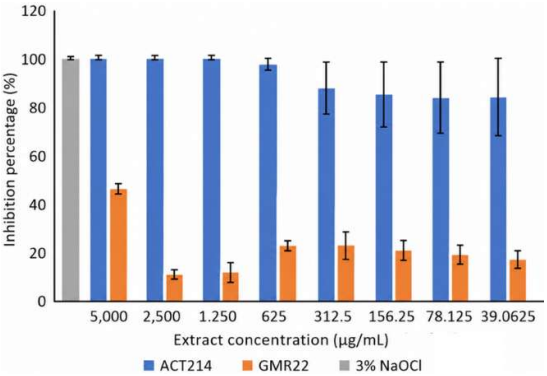


Figure 1. Antibacterial activity of *Streptomyces* sp. ACT214 and *Streptomyces* sp. GMR22 extract at various concentrations (5,000–39.06 µg/mL) on *E. faecalis*. Positive control (3% NaOCl) showed 100% inhibition. Inhibition percentage of *E. faecalis* bacterial growth from *Streptomyces* sp. ACT214 is higher than *Streptomyces* sp. GMR22 extract.

The statistical analysis with two-way ANOVA showed a significant interaction effect between bacterial strain and extract concentration on growth inhibition ($p = 0.002$), indicating that the antibacterial efficacy of the extract is dependent on both the strain and its concentration. In addition, extract concentration had a significant main effect on bacterial inhibition ($p < 0.001$). Post-hoc Tukey's HSD tests confirmed that the 5,000 µg/mL concentration resulted in the highest inhibition rate, with statistically significant differences observed between this and all lower concentrations. Based on the broth microdilution assay, *Streptomyces sp*. ACT214 exhibited a minimum inhibitory concentration (MIC) of 1,250 µg/mL, while *Streptomyces sp*. GMR22 did not reach its MIC even at the highest concentration tested (5,000 µg/mL). Furthermore, *Streptomyces sp*. ACT214 demonstrated a minimum bactericidal concentration (MBC) of 5,000 µg/mL, whereas *Streptomyces* sp. GMR22 failed to achieve MBC within the tested concentration range.

Antibiofilm activity

The antibiofilm activity of the *Streptomyces sp*. ACT214 extract against *E. faecalis* was found to be concentration-dependent, with higher extract concentrations yielding greater inhibition (Figure 2). Quantitatively, the ACT214 extract inhibited *E. faecalis* biofilm formation by 79.70% to 100% across concentrations ranging from 156.25 to 5,000 µg/mL. In contrast, the extract from *Streptomyces* sp. GMR22 exhibited substantially lower antibiofilm activity, with inhibition ranging from 0% to 20.04% within the same concentration range. Importantly, the ACT214 extract achieved complete inhibition of biofilm formation at a concentration as low as 582 µg/mL, indicating a minimum biofilm inhibitory concentration ($MBIC_{90}$) at this level. In comparison, the GMR22 extract failed to reach the 90% inhibition threshold at any concentration tested.

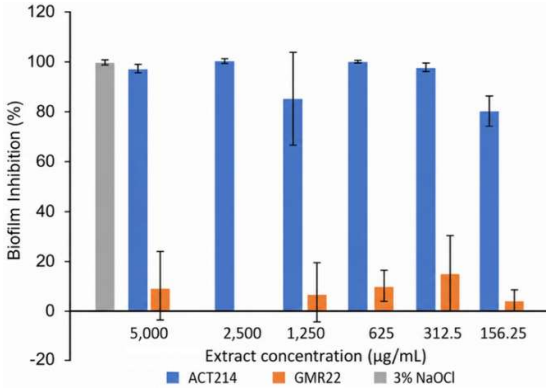


Figure 2. Antibiofilm activity of *Streptomyces sp*. ACT214 and Streptomyces sp. GMR22 extract at various concentrations (5,000–156.25 µg/mL) on *E. faecalis*. Positive control (3% NaOCl) showed 100% inhibition. Biofilm inhibition percentage of E. faecalis from *Streptomyces sp*. ACT214 is higher than *Streptomyces sp*. GMR22 extract.

The two-way ANOVA revealed a statistically significant main effect of both the *Actinomycetes* strain ($p < 0.05$) and extract concentration ($p < 0.05$) on the inhibition of *E. faecalis* biofilm formation. In addition, a significant interaction effect was observed between the strain type and extract concentration ($p < 0.05$).

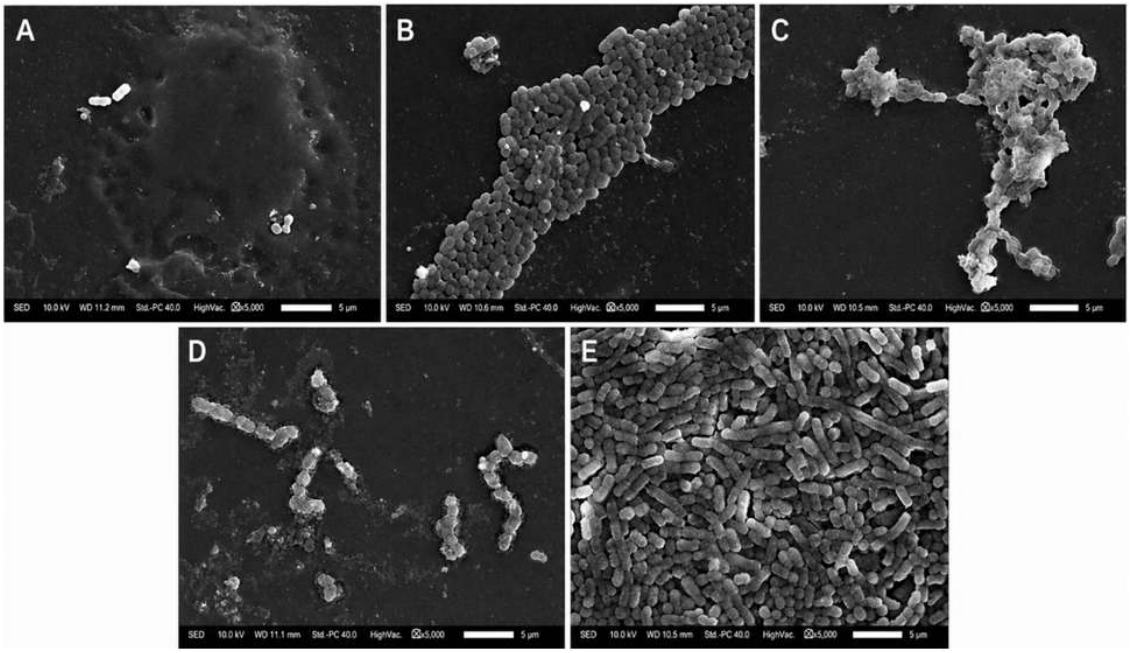


Figure 3. Scanning electron microscope of *E. faecalis* biofilm formation treated by NaOCl 3% (A), 625 µg/mL extract of *Streptomyces* sp. ACT214 (B), 1,250 µg/mL (C), 2,500 µg/mL (D), and *E. faecalis* biofilm formation in negative control (BHIB) (E). *Streptomyces* sp. ACT214 showed potential inhibition of *E. faecalis* biofilm formation.

Overall, the extract derived from *Streptomyces sp*. ACT214 demonstrated significantly greater antibiofilm activity compared to that from *Streptomyces sp.* GMR22 across all tested concentrations. Moreover, the degree of biofilm inhibition increased significantly with higher extract concentrations. Results from the post-hoc Tukey HSD test indicated that extract concentrations ranging from 156.25 to 5,000 ppm produced significantly greater biofilm inhibition than concentrations between 39.0625 and 78.125 ppm.

The effects of *Streptomyces sp*. ACT214 extracts on *E. faecalis* biofilm formation were evaluated using scanning electron microscopy (SEM) in comparison with a negative control (no extract treatment) and a positive control (3% sodium hypochlorite, NaOCl). SEM analysis demonstrated distinct morphological differences in *E. faecalis* biofilm structure following treatment with 3% NaOCl and Streptomyces sp. ACT214 extracts, relative to the untreated negative control (Figure 3). The SEM imaging revealed that *Streptomyces sp*. ACT214 extracts reduced *E. faecalis* cell density and microcolony formation in a concentration-dependent manner. Treatment with extract concentrations of 1,250 µg/mL (Figure 3C) and 2,500 µg/mL (Figure 3D) resulted in near-complete inhibition of bacterial adhesion and aggregation. At 625 µg/mL (Figure 3B), small and discrete microcolonies remained visible, although with markedly reduced cell numbers compared to the dense, multilayered biofilms observed in the negative control (Figure 3E). The positive control treatment with 3% sodium hypochlorite (Figure 3A) also decreased cell density and microcolony formation, although the effect was less pronounced than at higher extract concentrations. Biofilms appeared more dispersed and exhibited lower structural density as the extract concentration increased. Overall, the SEM data visually support the conclusion that *Streptomyces sp.* ACT214 extracts inhibit *E. faecalis* biofilm formation by reducing cell viability, adhesion, aggregation, and the production of extracellular polymeric substances. These observations indicate that biofilm development was effectively suppressed under extract treatment.

Table I. The primary compounds detected from the chloroform extract of *Streptomyces* sp. ACT214 using untargeted liquid chromatography-tandem high-resolution mass spectrometry (LC-HRMS).

No.	Annotated Compounds	Formula	Class	RT (min)	Calculated Mass	Area (Max)	mzCloud Best Match
1	1,2-Cyclohexanediol	C6 H12 O2	Organooxygen compounds	14.852	116.0837	16,008,697	99.9
2	(2S)-2-Amino-8-oxodecanoic acid	C10 H19 N O3	Aliphatic Amines-acid	8.022	201.13629	15,976,870	99.9
3	Alanyl-N-(6-amino-2-pyridinyl)-alpha-glutamine	C13 H19 N5 O4	Aliphatic Amines-acid	8.635	309.1433	15,868,583	99.9
4	5-Nonyl-2-oxotetrahydro-3-furancarboxylic acid	C14 H24 O4	Lactones	17.4	256.16719	15,800,957	99.8
5	2,2,4-Trimethyl-1,3-pentadienol diisobutyrate	C16 H30 O4	Carboxylic acids and derivatives	17.19	286.21395	15,759,927	99.8
6	6-hydroxypseudooxynicotine	C10 H14 N2 O2	Organooxygen compounds	8.285	194.10513	15,660,409	99.8
7	Sorbic acid	C6 H8 O2	Fatty Acyls	15.432	112.0525	15,578,864	99.7
8	(Z,S)-pyrethrolone	C11 H14 O2	Vinyl/Allyl Ketones	16.568	178.09852	15,483,530	99.7
9	8-AzaG	C4 H4 N6 O	Triazolopyrimidines	4.862	152.04486	15,317,126	99.7
10	HARMOL	C12 H10 N2 O	Harmala alkaloids	11.742	198.07988	15,284,546	99.6
11	(2E)-decenoic acid	C10 H18 O2	Fatty Acyls	18.528	170.13062	15,231,367	99.6
12	Methohexital	C14 H18 N2 O3	Diazines	8.879	262.13164	15,181,959	99.6
13	N-Undecanoylglycine	C13 H25 N O3	Carboxylic acids and derivatives	15.831	243.18337	15,127,967	99.5
14	(2E)-2-Pentenoic acid	C5 H8 O2	Fatty Acyls	12.949	100.05251	15,020,218	99.5
15	1-(3-Furyl)-1,4-pentanediol	C9 H14 O3	Heteroaromatic compounds	7.114	170.09429	14,838,735	99.4
16	01700000/Ethyl levulinate	C7 H12 O3	Keto acids and derivatives	9.507	144.07851	14,679,617	99.4
17	Furfuranol	C5 H6 O2	Heteroaromatic compounds	10.785	98.037	14,551,815	99.4
18	3-Hydroxysuberic acid	C8 H14 O5	Hydroxy acids and derivatives	10.01	190.08323	14,120,663	99.4
19	UQ1000000/ triacetate lactone	C6 H6 O3	Pyrans	6.506	126.0316	13,899,569	99.3
20	Quinazoline-2,4-diol	C8 H6 N2 O2	Diazanaphthalenes	8.026	162.04288	13,819,007	99.3
21	5-(3,4-Dihydroxybenzyl)dihydro-2(3H)-furanone	C11 H12 O4	Phenols	11.719	208.07283	13,601,409	99.3
22	LQ1825000/N-cyclohexylformamide	C7 H13 N O	Carboxylic acids and derivatives	9.296	127.0997	13,596,388	99.2
23	2-Amino-6-hydroxyaminopurine	C5 H6 N6 O	Aromatic Amines Imidazoles	7.445	166.06046	13,564,247	99.2
24	4-(5,6-Dihydroxy-6-methyl-1-hepten-2-yl)-1-methyl-1,2-cyclohexanediol	C15 H28 O4	Prenol lipids	18.927	272.19872	13,355,923	99.2
25	MFCD00051313	C12 H18 O	phenols	21.964	178.13568	13,166,654	99.2
26	1-Thio-beta-D-glucopyranose	C6 H12 O5 S	Thiols(mercaptans)	5.105	196.03975	13,129,701	99.2
27	MP5900000	C6 H10 O	Organooxygen compounds	21.185	98.07324	12,978,406	99
28	Pivagabine/4-[(1-hydroxy-2,2-dimethylpropylidene)amino]butanoic acid	C9 H17 N O3	Carboxylic acids and derivatives	9.868	187.11998	12,969,942	99
29	medorinone	C9 H8 N2 O	Acrylamides	8.168	160.06374	12,895,750	99
30	Temozolomide	C6 H6 N6 O2	Imidazotetrazines	9.241	194.05564	12,855,900	98.9

Table II. The primary compounds detected from ethyl acetate fractions of Streptomyces sp. GMR22 using untargeted liquid chromatography tandem high resolution mass spectrometry (LC-HRMS).

No	Annotated Compounds	Formula	Class	RT (min)	Calculated Mass	Area (Max)	mzCloud Best Match
1	Ethyl myristate	C16 H32 O2	Fatty Acyls	20.07	256.23941	3,129,463,569	100
2	Oleic acid	C18 H34 O2	Fatty Acyls	20.832	282.25496	720,430,478	100
3	Maltol	C6 H6 O3	Pyrans	6.159	126.03151	680,172,717	100
4	3-Hydroxy-2-methylpyridine	C6 H7 N O	Pyridines	1.964	109.05277	53,822,110	100
5	Pentadecanoic acid	C15 H30 O2	Fatty Acyls	22.464	242.2236	3,087,882,201	99.9
6	Palmitoleic acid	C16 H30 O2	Fatty Acyls	22.184	254.22367	708,765,108	99.9
7	trans-10-Heptadecenoic acid	C17 H32 O2	Fatty Acyls	22.964	268.23939	261,627,602	99.9
8	Linoleic acid	C18 H32 O2	Fatty Acyls	23.011	280.2393	195,116,226	99.9
9	Norharman	C11 H8 N2	Indoles and derivatives	7.776	168.0685	139,958,205	99.9
10	8-Hydroxyquinoline	C9 H7 N O	Quinolines and derivatives	7.158	145.05245	100,790,998	99.9
11	2,3,5,6-Tetramethylpyrazine	C8 H12 N2	Diazines	9.095	136.09976	502,736,500	99.8
12	Nicotinic acid	C6 H5 N O2	Pyridines and derivatives	1.779	123.0319	141,518,414	99.8
13	Uracil	C4 H4 N2 O2	Diazines	1.397	112.02726	79,312,555	99.8
14	Hypoxanthine	C5 H4 N4 O	Imidazopyrimidines	1.37	136.0383	63,204,448	99.8
15	Stearamide	C18 H37 N O	Carboximidic acids and derivatives	20.587	283.28672	266,641,650	99.7
16	4-Methoxycinnamic acid	C10 H10 O3	Cinnamic acids and derivatives	21.004	178.06253	253,891,346	99.6
17	2-Amino-3-methoxybenzoic acid	C8 H9 N O3	Benzene and substituted derivatives	11.844	167.05797	180,259,891	99.6
18	Bis(2-ethylhexyl) phthalate	C24 H38 O4	Benzene and substituted derivatives	21.197	390.27533	5,825,648,888	99.5
19	2-(Methylthio)benzothiazole	C8 H7 N S2	Benzothiazoles	16.461	181.00171	7,950,250	99.5
20	7-hydroxy-6-methoxy-2H-chromen-2-one	C10 H8 O4	Coumarins and derivatives	10.112	192.04151	274,311,650	99.4
21	4-Indolecarbaldehyde	C9 H7 N O	Indoles and derivatives	10.742	145.05245	258,134,266	99.4
22	N-Acetyltyramine	C10 H13 N O2	tyramines (tyramines alkaloid)	7.856	179.0941	175,509,034	99.4
23	Octhilinone (OIT)	C11 H19 N O S	1,2-thiazole	17.076	213.118	45,425,417	99.4
24	Myristic acid	C14 H28 O2	Fatty Acyls	21.474	228.20781	474,405,063	99.3
25	10-Propoxydecanoic acid	C13 H26 O3	fatty acid	16.936	230.18719	194,452,041	99.3
26	[3,2-c]Pyrazole-5α-androstan-17β-ol	C20 H30 N2 O	Steroid	21.631	314.23467	28,184,469	99.3
27	Dibenzylamine	C14 H15 N	Benzene and substituted derivatives	8.792	197.12032	7,572,293	99.3
28	2-methyl-1,2-dihydrophthalazin-1-one	C9 H8 N2 O	Hydrazines	7.547	160.06322	140,429,682	99.2
29	Benzophenone	C13 H10 O	Benzene and substituted derivatives	16.244	182.0728	57,348,020	99.2
30	Phthaldialdehyde	C8 H6 O2	Benzene and substituted derivatives	9.095	134.03665	53,643,595	99.2

Primary compounds of active extract

Untargeted liquid chromatography–high-resolution mass spectrometry (LC-HRMS) analysis of the *Streptomyces sp*. ACT214 chloroform extract identified a total of 402 chemical compounds. Among these, 142 compounds achieved an mzCloud best match score exceeding 70% (Table I).

The major compounds identified, which include members of diverse chemical classes such as fatty acyls (e.g., ethyl myristate, oleic acid), saturated and unsaturated fatty acids, pyrans, pyridines, indoles, quinolines, diazines, benzothiazoles, coumarins, tyramine alkaloids, 1,2-thiazoles, steroids, hydrazines, and various substituted benzene derivatives (Table I). Additional compounds detected in notable quantities included maltol, pyridines, quinolines, pyrazines, nicotinates, uracil, hypoxanthine, cinnamaldehydes, benzothiazoles, coumarins, indoles, tyramines, and steroids. The predominant metabolites, as determined by both peak area and mzCloud match score, were bis(2-ethylhexyl) phthalate (retention time: 21.197 min), followed by ethyl myristate (20.070 min), pentadecanoic acid (22.464 min), oleic acid (20.832 min), and palmitoleic acid (22.184 min).

Similarly, LC-HRMS profiling of the ethyl acetate extract from *Streptomyces sp*. GMR22 revealed 315 chemical entities, with 80 compounds exhibiting mzCloud best match scores above 70% (Table II). 30 compounds with the highest mzCloud best match scores detected from the ethyl acetate fraction of *Streptomyces* sp. GMR22 using untargeted LC-HRMS analysis (Table II). The extract was found to contain a broad spectrum of metabolites, including organooxygen compounds, amines, acids, lactones, fatty acyls, heteroaromatic compounds, phenols, imidazoles, prenol lipids, thiols, and heterocyclic compounds. However, unlike ACT214, the GMR22 extract demonstrated relatively uniform peak areas across the identified compounds, suggesting a lack of prominent bioactive dominance within its chemical profile.

Overall, this study highlights the significant potential of soil *Actinomycetes* extracts from distinct ecological regions of Indonesia—Gunungkidul (*Streptomyces* sp. GMR22) and Pontianak (*Streptomyces* sp. ACT214)—as sources of bioactive compounds targeting *E. faecalis*, a key pathogen in persistent apical periodontitis. Specifically, the extract of *Streptomyces* sp. ACT214 demonstrated concentration-dependent inhibition of bacterial growth, cell adhesion, aggregation, and biofilm formation. These findings corroborate previous studies that have underscored the antibiofilm capabilities of *Actinomycete*-derived metabolites (Barka et al., 2016; Raissa et al., 2020; Xie et al., 2019).

The observed variations in antibacterial and antibiofilm activities between the two *Actinomycetes* strains can be attributed to differences in their secondary metabolite profiles, as revealed by untargeted LC-HRMS analysis. The extract of *Streptomyces sp*. ACT214 exhibited a broader chemical diversity, encompassing multiple compound classes including fatty acyls, pyrans, pyridines, indoles, quinolines, diazines, benzothiazoles, coumarins, tyramine alkaloids, 1,2-thiazoles, steroids, hydrazines, and various substituted benzene derivatives. In contrast, the extract of *Streptomyces sp*. GMR22 was composed primarily of as organooxygen compounds, amines, acids, lactones, fatty acyls, heteroaromatic compounds, phenols, imidazoles, prenol lipids, thiols, and heterocyclic compounds. The distinctive metabolite profile of *Streptomyces sp*. ACT214 likely underpins its superior antibacterial and antibiofilm efficacy against *E. faecalis*. These findings are consistent with prior research by Jalaluldeen et al. (2015) and Khattab et al. (2016), which demonstrated that demonstrated differences in metabolites and compounds produced by various *Streptomyces* strains, impacting their antibacterial and antibiofilm activities (Jalaluldeen et al., 2015; Khattab et al., 2016).

The greater potency of *Streptomyces sp*. ACT214 extracts suggests the presence of bioactive secondary metabolites that are either absent or produced at lower levels in *Streptomyces sp*. GMR22. The superior antibiofilm activity exhibited by *Streptomyces sp*. ACT214 supports the notion of differential metabolite production between distinct morphotypes. The diversity of secondary metabolites—reflected in their chemical structures, molecular formulas, and molecular weights—is largely determined by the genetic composition of the species and individual strains. Previous studies have reported that even *Streptomyces* strains with identical 16S rRNA gene sequences, but isolated from geographically distinct environments, can exhibit significant differences in pigmentation and the production of accessory secondary metabolites. These findings highlight the role of strain-specific genetic factors in shaping metabolite profiles, while also acknowledging that environmental conditions can modulate the expression of genes involved in secondary metabolism. Moreover, the study has shown that a substantial proportion of

biosynthetic gene clusters (BGCs) present in *Streptomyces* genomes remain transcriptionally silent under standard laboratory conditions, indicating the complexity of secondary metabolite regulation in these bacteria (Seipke, 2015; Sottorff et al., 2019).

The major metabolites identified in the *Streptomyces sp.* ACT214 extract—namely bis(2-ethylhexyl) phthalate (DEHP), ethyl myristate, pentadecanoic acid, oleic acid, and palmitoleic acid—exhibit strong potential as antibiofilm agents. DEHP, in particular, has been reported to significantly influence bacterial biofilm development and virulence. Ghaly et al. (2023) demonstrated that DEHP interferes with bacterial intercellular communication, leading to a reduction in the production of biofilm extracellular polysaccharides, prodigiosin, haemolysin, and proteases. This disruption subsequently enhances bacterial susceptibility to conventional antibiotics (Ghaly et al., 2023). Conversely, Suhartono et al. (2023) observed that DEHP may also stimulate extracellular polysaccharide production, which plays a crucial role in maintaining biofilm structure and physicochemical properties (Suhartono et al., 2023).

In addition to DEHP, the presence of various fatty acyls (e.g., oleic acid, palmitoleic acid, linoleic acid), pyrans (e.g., maltol), and indoles (e.g., norharman) in the ACT214 extract is particularly noteworthy. These compound classes have been widely documented for their antibiofilm and anti-quorum sensing activities against diverse bacterial pathogens (Kamarudheen & Rao, 2019; Kumar et al., 2020; Lee et al., 2017; Su et al., 2020). Furthermore, the detection of coumarins and quinolines in the extract is of significant interest, as both have been associated with broad-spectrum antibacterial and antibiofilm efficacy (Abouelhassan et al., 2014; Reen et al., 2018; Senerovic et al., 2020).

Fatty acids and fatty acyl derivatives such as ethyl myristate, oleic acid, pentadecanoic acid, palmitoleic acid, trans-10-heptadecenoic acid, linoleic acid, and myristic acid are known to disrupt bacterial cell membranes, causing leakage of intracellular components and subsequent cell death (Jin et al., 2021; Nicol et al., 2018). These compounds can also impair quorum-sensing mechanisms by inhibiting the synthesis or activity of autoinducer molecules (Khadke et al., 2021; Li et al., 2022), and reduce the production of extracellular polymeric substances (EPS), which are vital for the structural cohesion and resilience of bacterial biofilms (Nicol et al., 2018). The antibiofilm activity of fatty acyls is largely attributed to their ability to alter bacterial membrane composition, thereby enhancing resistance to membrane-disrupting agents. In *E. faecalis*, the incorporation of exogenous fatty acyls has been shown to confer membrane protection by modifying its phospholipid profile, which contributes to enhanced structural integrity (Saito et al., 2014). Fatty acids have also been demonstrated to inhibit biofilm formation in a range of bacterial species, including *Pseudomonas aeruginosa* (Yuyama et al., 2020). For instance, oleic acid has been reported to modulate both bacterial viability and biofilm development (Davies & Marques, 2009; Stenz et al., 2008). Several studies studies have highlighted the impact of fatty acids on quorum sensing systems, which are cell-to-cell communication mechanisms in bacteria. Fatty acids have been found to bind to quorum-sensing acyl-homoserine lactone synthases, affecting quorum-sensing communication systems (Khadke et al., 2021; Low et al., 2019). The disruption of QS signaling by fatty acids has been directly associated with impaired biofilm formation and reduced bacterial communication efficiency (Li et al., 2022; Martínez et al., 2019). Specifically, fatty acids have been found to inhibit AI-2 quorum sensing and biofilm formation in *Campylobacter jejuni* (Li et al., 2022).

Numerous studies have demonstrated that unsaturated fatty acids play a significant role in regulating biofilm formation in *Staphylococcus aureus* and other Gram-positive bacteria (Yuyama et al., 2020).These fatty acids, particularly the unsaturated types, have been shown to disrupt quorum sensing (QS) communication pathways, inhibit bacterial motility, and suppress biofilm development in various pathogenic species, including *Acinetobacter baumannii* (Nicol et al., 2018). The antibiofilm efficacy of mono-unsaturated fatty acids—such as palmitoleic and myristoleic acids—further underscores their potential to interfere with biofilm formation (Low et al., 2019; Nicol et al., 2018). In addition to their antimicrobial effects at higher concentrations, fatty acids also function as modulators of bacterial colonization and virulence at low concentrations. Both saturated and unsaturated fatty acids have been reported to hinder biofilm formation through multiple mechanisms, including altering bacterial adhesion to surfaces, modifying cell membrane fluidity, reducing the synthesis of extracellular polymeric substances (EPS), and modulating

quorum sensing systems (QSS) (Kumar et al., 2020).

The antibiofilm activity exhibited by pyrans and their derivatives is mediated through multiple mechanisms. These include the inhibition of pyrimidine biosynthesis pathways (Liu et al., 2022), disruption of bacterial cell membrane integrity, interference with the synthesis of the biofilm extracellular matrix, and modulation of bacterial virulence factors involved in biofilm formation (Abd El-Aleam et al., 2021; Su et al., 2020). Recent studies have further demonstrated that the design and synthesis of novel fused spiro pyran derivatives hold significant promise as biofilm-disrupting agents (Saigal et al., 2019). These compounds may interact with bacterial cell membranes, affecting membrane integrity and potentially interfering with the transport or assembly of biofilm matrix components. Furthermore, pyran derivatives have been proposed to suppress the biosynthesis of quorum sensing signal (QSS) molecules, effectively preventing the initiation of quorum sensing cascades that are essential for biofilm formation. Notably, this inhibitory effect is exerted primarily during the early stages of biofilm formation, with limited efficacy in eradicating preformed biofilms (Su et al., 2020).

The observed potent antibiofilm activity of the *Streptomyces sp*. ACT214 extract is likely attributable to the synergistic interplay of multiple bioactive constituents within the extract. This synergy may contribute to the inhibition of various stages of biofilm formation, including initial initial attachment, maturation, and dispersal. Such multifactorial mechanisms underscore the therapeutic potential of this extract as a novel antibiofilm agent targeting *Enterococcus faecalis*, a clinically significant pathogen implicated in persistent endodontic infections.

The findings of this study emphasize the untapped potential of terrestrial *Actinomycetes*, particularly those derived from unique environmental niches, as rich sources of novel antibacterial and antibiofilm compounds. Specifically, the *Streptomyces sp*. ACT214 isolate obtained from Pontianak soil, Indonesia, demonstrated significant inhibitory activity against *E. faecalis*, a bacterium frequently associated with refractory apical periodontitis. These results suggest the need for further research focused on the purification and structural characterization of the active constituents present in the ACT214 extract. Such compounds may serve as lead candidates for the development of novel therapeutics targeting *E. faecalis* biofilms in endodontic contexts. Moreover, future studies should explore the precise mechanisms of action and evaluate potential synergistic effects when combined with existing antimicrobial agents. Investigations into the cytotoxicity and biocompatibility of these compounds will also be essential to assess their suitability for clinical application, particularly in the context of root canal disinfection.

CONCLUSION

The extract derived from *Actinomycetes* isolated from Pontianak soil, Indonesia, demonstrated significant antibacterial and antibiofilm activities against *E. faecalis*. In particular, *Streptomyces sp.* ACT214 exhibited potent inhibition of *E. faecalis* growth, adhesion, and microcolony formation. These findings support the potential of terrestrial *Actinomycetes* as promising reservoirs of novel anti-infective agents capable of targeting key virulence factors *of E. faecalis*. The antibiofilm metabolites from *Streptomyces sp*. ACT214 may have clinical relevance as alternative or adjunctive therapeutic agents aimed at improving root canal disinfection and mitigating apical periodontitis caused by persistent *E. faecalis* biofilms. Further investigations are essential to isolate, structurally characterize, and optimize these bioactive compounds to advance their development as potential therapeutic agents in endodontic treatment strategies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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