

Antimicrobial compounds from *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* inhibited growth of *Ralstonia syzygii* subsp. *celebesensis* causal pathogen of banana blood disease

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Abstract: Banana blood disease is a banana wilt disease caused by *Ralstonia syzygii* subsp. *celebesensis* (RSC) poses a serious threat to banana plantations, particularly in Indonesia and Malaysia. Yet, the current control methods are inadequate to mitigate the disease. Biological control agents (BCAs) offer a promising solution by using beneficial microorganisms to control pathogens. Among BCAs, lactic acid bacteria (LAB) have gained increasing attention in agriculture due to their ability to produce a variety of antimicrobial compounds. This study investigates the antimicrobial substances produced by *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* against RSC using cell-free supernatants (CFS) and liquid-liquid extraction (LLE). The inhibitory effect of the LAB extracts against RSC was evaluated using agar well diffusion method. Liquid chromatography-mass spectrometry analysis confirmed the bioactive metabolites with potential antibacterial activity against RSC are monoglucosyl-enterobactin, (6Z,9Z,12Z)-octadecatrienoic acid, and 2-(hydroxymethyl)-3-(acetamidomethylene)succinate from CFS-*L. plantarum*; canavanine, norspermidine and 4-guanidinobutanamide in CFS-*L. paracasei*; sinapic acid, lecanoric acid and D-glucono-1,5-lactone in LLE-*L. plantarum* and lithocholic acid, orotic acid and sterculic acid in LLE-*L. paracasei*. A scanning electron microscope has shown disruption and damage to the RSC cells during LAB treatment. These findings highlight the potential of *L. plantarum* and *L. paracasei* metabolites for biocontrol applications.

Keywords: biological control; lactic acid bacteria (LAB); banana wilt pathogen; bioactive metabolites

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Banana blood disease (BBD) caused by *Ralstonia syzygii* subsp. *celebesensis* (RSC) poses a major threat to banana cultivation in Southeast Asia, particularly in Indonesia, Malaysia, and the Philippines, resulting in significant economic losses (Blomme et al. 2017; Ray et al. 2021). Characterised by wilting, leaf necrosis, and vascular discoloration, the disease has spread from Sulawesi, Indonesia, via infected planting materials, tools, and possibly insect vectors. Its impact is especially severe in Malaysia and the Philippines, where banana farming is vital to both local livelihoods and export markets, disproportionately affecting smallholder farmers (Ray et al. 2022).

Biological control agents (BCAs) play a vital role in managing banana diseases, such as Fusarium wilt and anthracnose, by inhibiting pathogens and enhancing plant defences (Bubici et al. 2019). *Trichoderma* and *Bacillus* species, which act as BCAs, have shown a 74% reduction in *Fusarium* wilt incidence, largely due to their ability to induce systemic resistance by upregulating defence genes and degrading pathogen cell walls (Da Costa et al. 2021; Becker et al. 2023; Izquierdo-García et al. 2024). However, studies on the efficacy of BCA against RSC, the causative agent of BBD, remain scarce.

Lactic acid bacteria (LAB) are emerging as promising BCAs due to their ability to produce a broad spectrum of antimicrobial compounds that inhibit the growth of plant pathogens (Ibrahim et al. 2021). These Gram-positive, non-spore-forming microbes, widely known for their roles in fermentation and probiotics, are generally regarded as safe (Zheng et al. 2020). Beyond their metabolic functions, LAB produce secondary metabolites with antimicrobial, antioxidant, and immunomodulatory properties, which are essential for survival in competitive environments (Garcia-Gonzalez et al. 2021; Leroy & De Vuyst 2022). Jaffar et al. (2023a) demonstrated the inhibitory activity of LAB species, including *L. plantarum*, *Leuconostoc holzapfelii*, *Weissella cibaria*, and *W. paramesenteroides*, against *Pantoea stewartii*. In addition, LAB effectively colonise plant-associated habitats, including the phyllosphere, endosphere, and rhizosphere, and exhibit adaptive traits that enhance plant growth and suppress phytopathogens (Jaffar et al. 2023b).

LABs synthesise diverse antimicrobials, including bacteriocins (e.g., nisin and class II variants), organic acids, exopolysaccharides, and bioactive peptides, with applications in food preservation,

biomedicine and agriculture (Perez-Ramos et al. 2023). Nisin from *Lactococcus* and *Streptococcus* is a well-established food-grade preservative (Ahansaz et al. 2023), while *Lactobacillus* and *Leuconostoc* produce class II bacteriocins (Yi et al. 2022). LAB metabolites, such as organic acids, hydrogen peroxide, and diacetyl, inhibit pathogens by reducing pH and inducing oxidative stress (Zapašnik et al. 2022). Bioactive peptides (e.g., lactoferricin) and short-chain fatty acids additionally modulate immunity and gut microbiota (Garcia-Gonzalez et al. 2021; Leroy & De Vuyst 2022). Reuterin, a broad-spectrum antimicrobial, further expands LAB's biocontrol potential (Sun et al. 2022). Due to their multifunctionality, LAB-derived compounds are promising alternatives to antibiotics against resistant pathogens (Perez-Ramos et al. 2023). This study evaluates LAB antimicrobial compounds for biocontrol of RSC, aiming to develop sustainable crop protection strategies.

MATERIAL AND METHODS

Chemicals. De Man, Rogosa, and Sharpe (Hi-Media, India) agar and broth were used to culture LAB, while Kelman Tetrazolium Chloride (TZC) agar and broth were used for RSC. TZC medium was prepared using 10 g peptone (Gibco Bacto™, Thermo Fisher Scientific, US), 1 g of casamino acids (BD Biosciences, US), 5 g of glucose (Sigma, US) and 16 g of agar powder (HmbG® Chemicals, Germany) for TZC agar in 1 000 mL of distilled water, with addition of 5 mL of 1% 2,3,5-triphenyl tetrazolium chloride (Sigma, US). Solvents, including hexane (Fisher Scientific, US), toluene (Merck, Germany), dichloromethane (Merck, US), ethyl acetate (System, Malaysia), and dimethyl sulfoxide (DMSO) (Fisher Scientific, US), were used for liquid-liquid extraction of antimicrobial compounds. HPLC-grade acetonitrile and water (Sigma-Aldrich, Germany) were used as mobile phase in the LC-MS analysis.

Microorganisms. *Lactiplantibacillus plantarum* and *L. paracasei* were isolated from fermented cabbage and fermented milk, respectively and used as BCAs (Sam et al. 2024). The bacterial pathogen RSC was obtained from the Plant Pathology Laboratory at the Horticulture Research Centre, MAR-DI, Serdang, Selangor, Malaysia.

***In vitro* bioassay of cell-free supernatant of lactic acid bacteria against *Rastonia syzygii* subsp. *celebesensis*.** The potential antagonism of *L. plantarum* and *L. paracasei* isolates against RSC was evaluated using the agar well diffusion assay. RSC and LAB were inoculated in TZC broth and MRS broth, respectively, for 48 h at 28 °C. The cell-free supernatants (CFS) of LABs were obtained by removing the cells following centrifugation at $2\,737 \times g$ for 15 min at 4 °C, and then filtering through a 0.22 µm hydrophilic PTFE syringe filter. Sterile 1 mL pipette tips were used to cut a 7 mm-diameter well in an agar plate, and 80 µL of each CFS-LAB was loaded into individual wells. Sterile MRS broth served as the negative control, and streptomycin (20 mg/mL) as the positive control. All treatments were performed in triplicate. Plates were incubated at 28 °C for 72 h. Following incubation, the diameters of the inhibition zones around each well were measured.

Determination of minimum inhibitory concentration (MIC). The MIC of the CFS was determined by the microdilution method (EUCAST 2003). Stock solutions of the extracts were made by dissolving them in 1 mL of 3% DMSO. Following aseptic procedures in 96-well microtitre plates, 100 µL of CPG was added to each well, followed by 100 µL of 50 mg/mL CFS of LABs. Following a series of two-fold dilutions, the initial concentration of the solution in the first well was reduced to a range of 0.39–25 mg/mL. A volume of 50 µL of bacterial suspension containing the target strain was added to each well, resulting in a final concentration of 5×10^5 CFU/mL. Streptomycin, 3% DMSO, and sterile MRS broth served as positive and negative controls. The bacterial growth was monitored at 590 nm after 24 h of incubation at 28 °C. The MIC, defined as the lowest concentration of each extract that showed no observable growth, was determined visually and measured at 590 nm using a Multiskan Go Microplate Spectrophotometer (Thermo Scientific, USA).

Extraction of bioactive antimicrobial compounds from lactic acid bacteria. The liquid–liquid extraction (LLE) methodology, adapted from Abdel-Nasser et al. (2023), was performed with minor modifications. *Lactobacillus plantarum* and *L. paracasei* were cultured on MRS agar at 28 °C for 24–36 h, and a single colony of both strains were inoculated into MRS broth and incubated at 28 °C in an orbital shaker at 120 rpm for 3 days.

Cultures were centrifuged at $8\,867 \times g$ for 20 min at 4 °C to obtain the CFS. Sequential LLE was conducted using solvents of increasing polarity, hexane, toluene, dichloromethane and ethyl acetate added to the CFS at a 1 : 1 (v/v) ratio. Each mixture was vigorously shaken for 30 min at 150 rpm, transferred into separating funnels, and allowed to stand for 30–60 min for phase separation. The organic phase was collected and evaporated to dryness using a rotary evaporator. The same CFS was subsequently extracted with each solvent in sequence.

***In vitro* bioassay of bioactive antimicrobial compounds of lactic acid bacteria against *Rastonia syzygii* subsp. *celebesensis*.** The *in vitro* disc diffusion bioassay of the antimicrobial substances against RSC was conducted as described by Bukhari et al. (2020) with slight modifications. RSC was cultured in fresh TZC broth for 48 h to reach the exponential phase, then adjusted to approximately 1.0×10^8 CFU/mL. The diluted RSC were then spread onto TZC agar plates using sterile cotton buds. LLE-LAB extracts were diluted to 20 mg/mL in 3 % sterile DMSO and filtered through a 0.22 µm hydrophilic PTFE syringe filter. Filter paper discs were cut to 7 mm in diameter, and each disc was loaded with 80 µL of LLE-LAB on an empty Petri dish. The paper discs were allowed to air-dry before being placed on the TZC agar. It was incubated at 28 °C for 3 days. Sterile streptomycin (20 mg/mL) and 3% DMSO were used as positive and negative controls, respectively. Each treatment was performed in triplicate, and the inhibition zone diameters were measured.

Determination of antimicrobial compounds in lactic acid bacteria solvent extracts. Antimicrobial compounds extracted from CFS-LAB and LLE samples were putatively identified using liquid chromatography-mass spectrometry (LC-MS) following the method of Veettil and Chitra (2022) with modifications. Samples were diluted to 1 000 ppm using methanol, filtered through a 0.22 µm hydrophilic PTFE syringe filter and transferred into HPLC vials. LC-MS analysis was performed using a single quadrupole mass spectrometer [LC-MS 2050 (Shimadzu, Japan)] equipped with a Shim-pack Velox C18 (Shimadzu, Japan) column (1.8 µm, 2.1×150 mm). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), applied isocratically at a 40 : 60 (A : B) ratio for 15 min. The injection volume was 10 µL, the flow rate was 0.3 mL/min, and the column

temperature was maintained at 40 °C. MS analysis was conducted in both positive and negative ionisation modes using DUIS, with the following parameters: nebuliser gas flow, 2.0 L/min; drying gas flow, 5.0 L/min; heat gas flow, 7.0 L/min; desolvation line temperature, 250 °C; and desolvation temperature, 400 °C. Data were processed and analysed using MZmine 2.53, with online database searches via the PubChem library.

Mode of action of lactic acid bacteria in *Rastonia syzygii* subsp. *celebesensis*. Morphological changes of RSC treated with LAB were examined following the method of Zhang et al. (2017) with minor modifications. Freshly cultured RSC suspensions were adjusted to 1×10^8 CFU/mL and centrifuged for 5 min at $5\,000 \times g$ to obtain the cell pellet. The cell pellet was further treated with the CFS of *L. plantarum* and *L. paracasei*. Meanwhile, water-treated RSC served as a negative control. Samples were incubated at 28 °C for 24 h, washed twice with 0.1 M Phosphate-Buffered Saline (PBS) (pH 7.4) by centrifugation at $5\,000 \times g$ for 10 min, and fixed in 2.5 % (v/v) glutaraldehyde at 4 °C for 2 h. Fixed cells were dehydrated through a graded ethanol series (30%, 50%, 80%, 90%, 100%), each step followed by centrifugation at $5\,000 \times g$ for 10 min, and subsequently suspended in isopropyl alcohol. Specimens were dried using liquid nitrogen, sputter-coated, and observed by scanning electron microscopy (SEM).

RESULTS

Both CFS of *L. plantarum* and *L. paracasei* showed clear inhibition zones on the agar plate (Figure 1). The mean inhibition zones measured 35.33 ± 3.21 mm and 33.67 ± 1.53 mm for *L. plantarum* and *L. paracasei*, respectively (Table 1). Statistical analysis via the Kruskal-Wallis test revealed no significant difference in inhibitory efficacy between the strains ($P = 0.082$). These results suggest that both strains exhibit similar antimicrobial effects under the tested conditions.

Figures 2 and 3 reveal the antimicrobial activity of different solvent extracts of both *L. plantarum* and *L. paracasei*. Both toluene extracts produced clear inhibition zones of 33.67 ± 1.35 mm and 31.33 ± 2.89 mm, respectively (Table 1). In contrast, no activity was observed for hexane, dichloromethane, ethyl acetate extracts, or re-

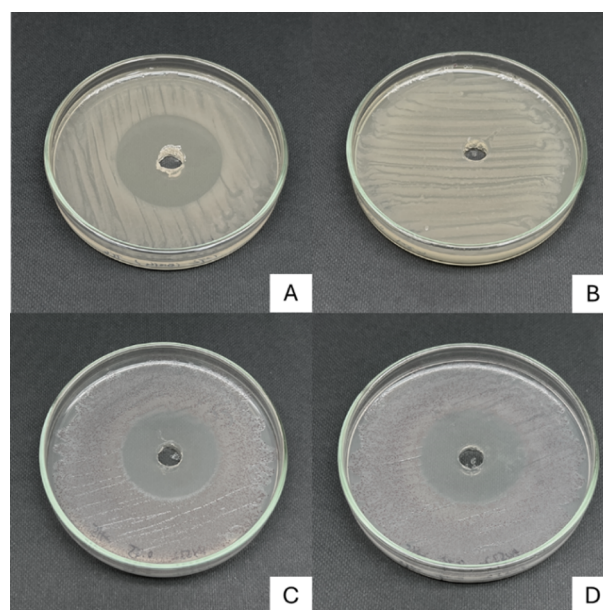


Figure 1. Antagonistic assay between RSC and CFS-LAB using agar well diffusion method

(A) positive control (Streptomycin); (B) negative control; (C) *L. plantarum*-CFS; (D) *L. paracasei*-CFS

sidual supernatant. The toluene extract's efficacy was comparable to the positive control. Kruskal-Wallis analysis revealed no significant difference between strains ($P = 0.059$), indicating similar antimicrobial potency. The positive con-

Table 1. *In vitro* antagonistic assay of CFS-LAB against RSC using the agar well diffusion method

Sample	Diameter of inhibition zone (mm)
<i>Lactiplantibacillus plantarum</i>	35.33 ± 3.21
<i>L. paracasei</i>	33.67 ± 31.53
Positive control	38.00 ± 37.55
Negative control	0.00 ± 30.00

The diameter of the inhibition zone (mm) was measured twice from the middle point of the wells to the inhibition zone. The values are the means of three replicates. The data presented in the table indicate the mean $\pm$ standard deviation. The antimicrobial activity of the CFS was further evaluated by determining the MIC against RSC. The MICs of CFS from *L. plantarum* and *L. paracasei* were 3.125 mg/mL and 6.25 mg/mL, respectively. These values demonstrate that *L. plantarum* exhibited stronger inhibitory activity than *L. paracasei*. The MIC findings provide quantitative confirmation of the antimicrobial potential of LAB-derived metabolites, thereby strengthening the preliminary evidence obtained from inhibition zone assays

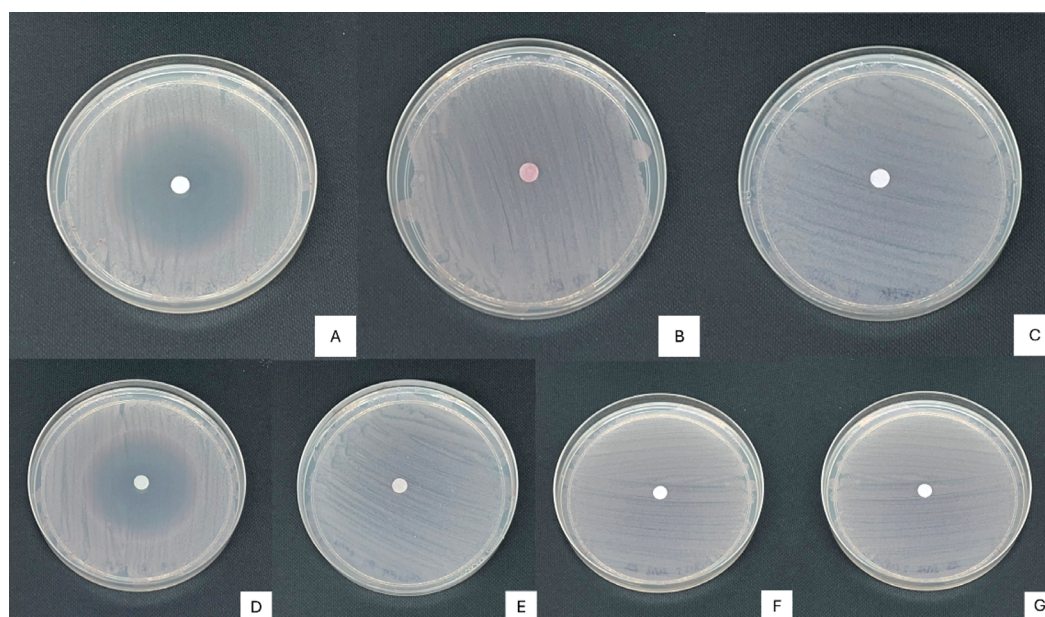


Figure 2. *In vitro* bioassay of *Lactiplantibacillus plantarum* extracts against RSC

(A) streptomycin (positive control); (B) 3% of DMSO (negative control); (C) hexane extraction of *L. plantarum*; (D) toluene extraction of *L. plantarum*; (E) dichloromethane extraction of *L. plantarum*; (F) ethyl acetate extraction of *L. plantarum*; (G) residual extraction of *L. plantarum*

trol in both experiments (Figures 1A, 2A and 3A) exhibited the largest inhibition zones, confirming their strong antimicrobial effect against RSC. In contrast, the negative control (Blank MRS broth in Figure 1B and 3 % DMSO in Figures 2B and 3B)

displayed no inhibition, indicating that the solvent at this concentration does not contribute to antimicrobial activity.

LC-MS analysis putatively identified distinct antimicrobial profiles in the CFS and toluene extracts

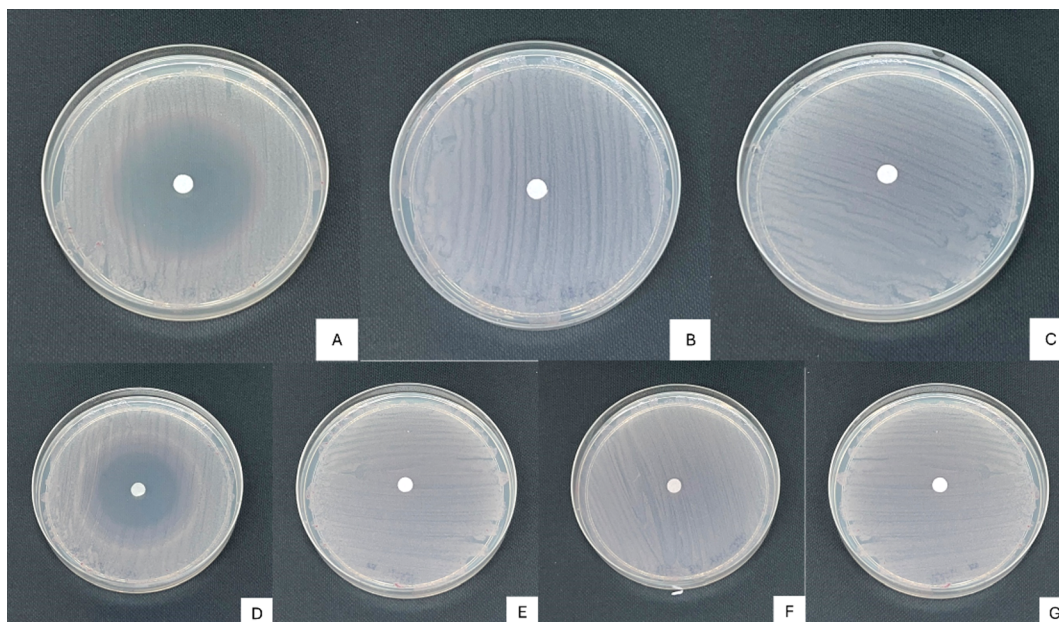


Figure 3. *In vitro* bioassay of *Lactiplantibacillus paracasei* extracts against RSC

(A) streptomycin (positive control); (B) 3% of DMSO (negative control); (C) hexane extraction of *L. paracasei*; (D) toluene extraction of *L. paracasei*; (E) dichloromethane extraction of *L. paracasei*; (F) ethyl acetate extraction of *L. paracasei*; (G) residual extraction of *L. paracasei*

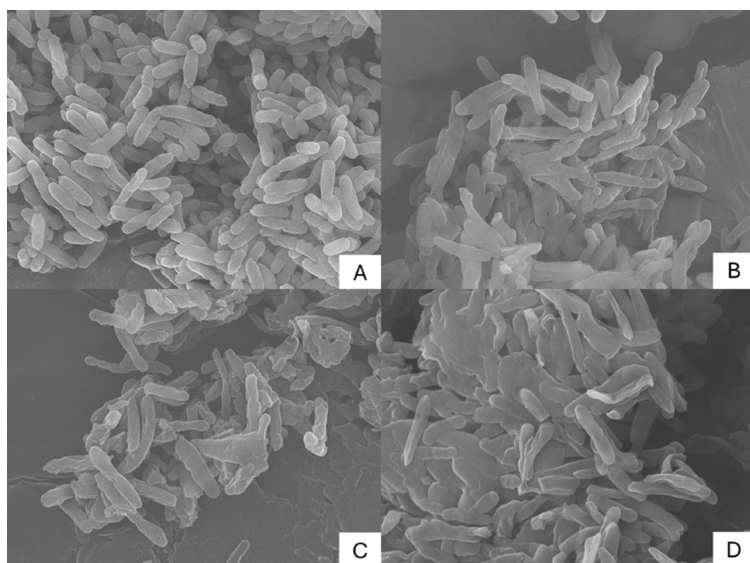


Figure 4. Scanning electron microscope (SEM) images of *Ralstonia syzygii subsp. celebesensis* (RSC) in different treatments with magnification of 10 000× (A) untreated cells; (B) cells treated with streptomycin; (C) cells treated with CFS-*Lactiplantibacillus plantarum*; (D) cells treated with CFS-*L. paracasei*

of *L. plantarum* and *L. paracasei*. The CFS-*L. plantarum* contained notable organic acids and derivatives, including (6Z,9Z,12Z)-octadecatrienoic acid, 2-(hydroxymethyl)-3-(acetamidomethylene) succinate, and the bacteriocin monoglucosyl-enterobactin as shown in Table 3. In contrast, CFS-*L. paracasei* was characterised by nitrogenous compounds such as canavanine, norspermidine, and 4-guanidinobutanamide. While for the toluene extracts, *L. plantarum* exhibited a prominence of organic acids, including lecanoric acid, sinapic acid, and D-glucono-1,5-lactone. Meanwhile, *L. paracasei* produced lithocholic acid, orotic acid and sterculic acid. These species-specific metabolic differences suggest potential variations in antimicrobial mechanisms between the two LAB strains.

Table 2. *In vitro* antagonistic assay of LLE-extract LAB against RSC via the disc diffusion method

Sample	Diameter of the inhibition zone (mm)
<i>Lactiplantibacillus plantarum</i> (Toluene)	33.67 ± 31.53
<i>L. paracasei</i> (Toluene)	31.33 ± 32.89
Positive control (Streptomycin)	38.00 ± 37.55
Negative control (3% DMSO)	0.00 ± 30.00

The diameter of the inhibition zone (mm) was measured twice from the middle point of the wells to the inhibition zone. The values are the means of three replicates. The data presented in the table indicate the mean ± standard deviation

Figure 4 shows the SEM images of the RSC after being treated with CFS-LAB. In untreated RSC, cells are rod-like, tiny, and have intact membrane structure (Figure 4A). Meanwhile, RSC cells treated with streptomycin and LABs were damaged and irregular (Figure 4B–D), possibly due to cell content leakage. Figures 4C and 4D showed clear separation of two rod-shaped RSC cells, with some not properly segregated due to cell wall damage, leading to membrane breakdown and cell wall degradation. Figure 4D showed that some cells had begun to shrink due to RSC degradation, leading to membrane breakdown and, eventually, cell content leakage.

DISCUSSION

Both CFS-*L. plantarum* and CFS-*L. paracasei* show significant inhibition zones against RSC as shown in Figures 1C and 1D. Among the solvent extracts (Figures 2C–F and 3C–F), only toluene extracts exhibited a large inhibition zone, indicating that the compounds in these extracts may be potent enough against RSC. Whereas other solvents, including hexane, dichloromethane, ethyl acetate, and residual extracts, showed minimal or no inhibition, suggesting that those fractions may not significantly contribute to antimicrobial activity. These results are consistent with a report by Padalia and Chanda (2015), which demonstrated that the toluene extract of *Tagetes erecta* flower, which consists of alkaloids, flavonoids, tannins, triterpenes, and cardiac glycosides, has successfully inhibited 43%

Table 3. Potential compounds exhibited antimicrobial activity in CFS and toluene extract of *L. plantarum* and *L. paracasei* detected using LC-MS

Extract Sample	Metabolite	Observed m/z	Retention time (min)	Charge	Database	Chromatograms (see full size in the Electronic Supplementary Material – ESM)	Spectrum (see the full size in the Electronic Supplementary Material – ESM)
CFS <i>L. plantarum</i>	(6Z,9Z,12Z)-Octadecatrienoic acid	279.21	11.15	[M+H] ⁺	PubChem CID 5280933		
	2-(Hydroxymethyl)-3-(acetamido)-domethylsuccinate	218.06	1.18	[M+H] ⁺	PubChem CID 9548691		
	Monoglucosyl-enterobactin	832.19	4.37	[M+H] ⁺	PubChem CID 101763505		

*All compounds were analysed using MzMine 2.52 and tentatively identified using the PubChem library

Table 3. To be continued...

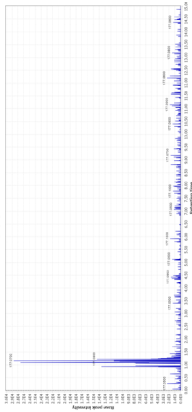
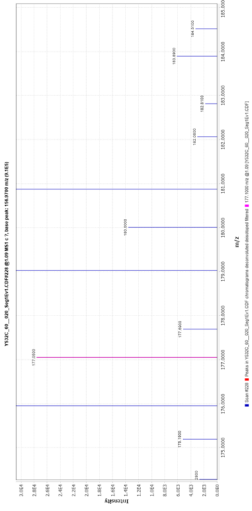
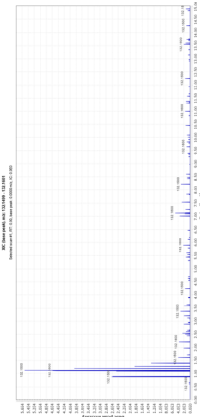
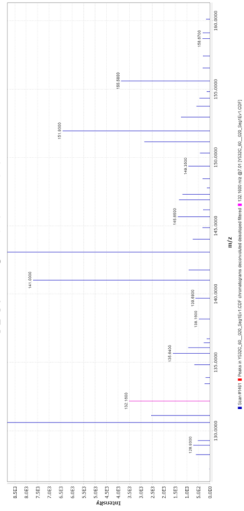
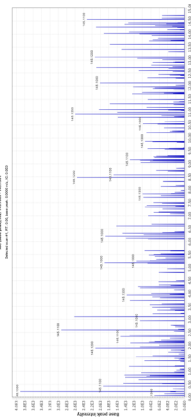
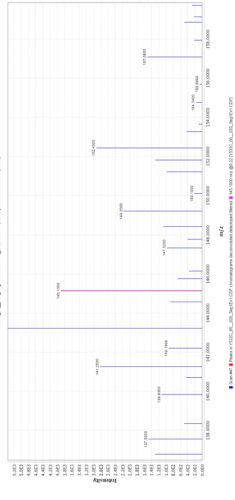
Extract	Sample	Metabolite	Observed m/z	Reten- tion time (min)	Charge	Database	Chromatograms (see full size in the Electronic Supplementary Material – ESM)	Spectrum (see the full size in the Electronic Supplementary Material – ESM)
CFS	<i>L. para- casei</i>	Canavanine	177.1	1.09	[M+H] ⁺	PubChem CID 439202		
		Norspermi- dine	132.16	7.01	[M+H] ⁺	PubChem CID 5942		
		4-Guanidin- obutanamide	145.1	0.22	[M+H] ⁺	PubChem CID 123		

Table 3. To be continued...

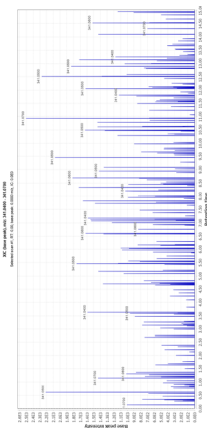
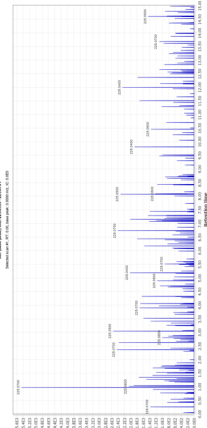
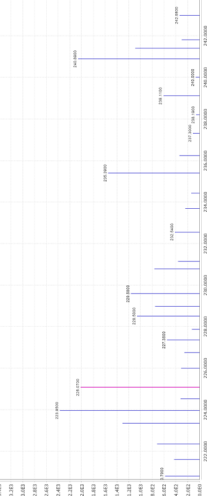
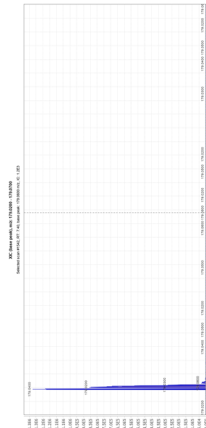
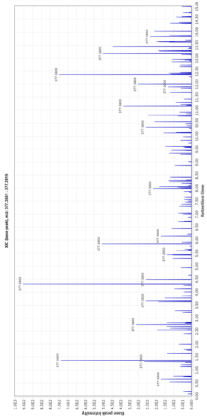
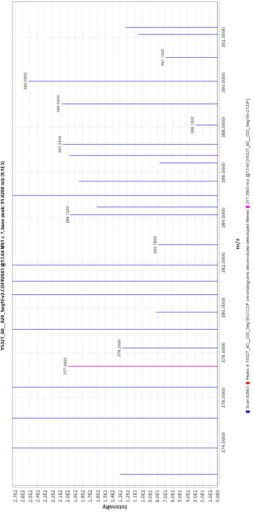
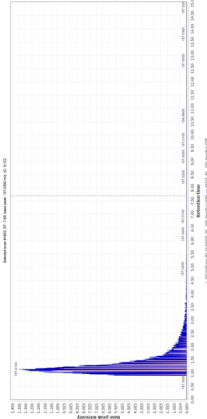
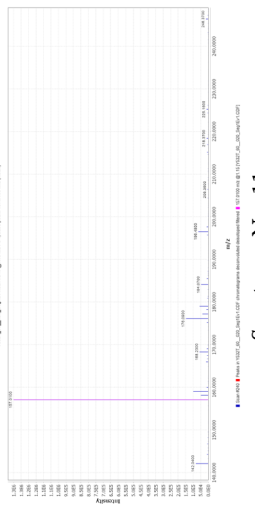
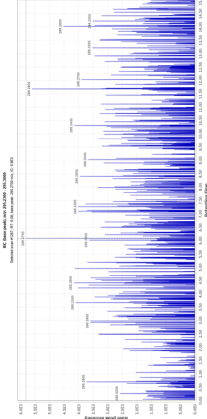
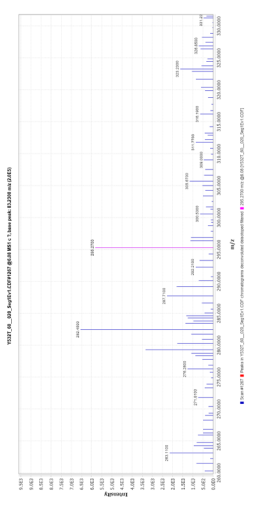
Extract	Sample	Metabolite	Observed m/z	Reten- tion time (min)	Charge	Database	Chromatograms (see full size in the Electronic Supplementary Material – ESM)	Spectrum (see the full size in the Electronic Supplementary Material – ESM)
Tolu- ene extract	<i>L. plan- tarum</i>	Lecanoric acid	341.06	7.38	[M+Na]	PubChem CID 99613		
		Sinapic acid	225.065	7.15	[M+H] ⁺	PubChem CID 10743		
		D-gluco- no-1,5-lac- tone (GDL)	179.04	0.94	[M+H] ⁺	PubChem CID 7027		

Table 3. To be continued...

Extract	Sample	Metabolite	Observed m/z	Reten- tion time (min)	Charge	Database	Chromatograms (see full size in the Electronic Supplementary Material – ESM)	Spectrum (see the full size in the Electronic Supplementary Material – ESM)
Tolu- ene extract	<i>L. para- casei</i>	Lithocholic acid	377.39	13.64	[M+H] ⁺	PubChem ID 9903		
		Orotic acid	157.01	1.15	[M+H] ⁺	PubChem ID 967		
		Sterculic acid	295.27	6.08		PubChem 12921		

of the bacterial strains tested and 25% of the fungal strains. This study highlighted the role of solvent polarity in extracting bioactive compounds, noting that non-polar solvents like toluene can effectively extract certain antimicrobial agents.

However, the inhibition zones varied slightly, suggesting potential differences in the composition of bioactive compounds between the two LAB strains. Yang et al. (2024) categorised metabolites from *Lactobacillus delbrueckii* and *Streptococcus thermophilus* into 20 classes, including amino acids, organic acids, flavonoids, and aldehydes. Similarly, Shokryazdan et al. (2014) reported strain-specific differences in antimicrobial activity among *Lactobacillus* strains, with *L. casei* BF1 showing strong inhibition and *L. acidophilus* HM1 being less effective. These findings suggest that antimicrobial efficacy may vary significantly among strains, possibly explaining the greater variability in *L. paracasei*'s activity compared to *L. plantarum*.

LC-MS analysis putatively identified several metabolites in CFS-*L. plantarum*, including (6Z,9Z,12Z)-octadecatrienoic acid, 2-(hydroxymethyl)-3-(acetamidomethylene) succinate, and monoglucosyl-enterobactin, which may contribute to the observed antimicrobial activity. It has been reported to disrupt bacterial membranes, increasing fluidity and permeability, and may also induce oxidative stress via lipid peroxidation and interfere with metabolic enzymes (Yoon et al. 2018; Desbois & Smith 2010). These effects could weaken RSC's membrane integrity and reduce its ability to maintain energy metabolism during host infection. Similarly, 2-(hydroxymethyl)-3-(acetamidomethylene) succinate has been suggested to disrupt the tricarboxylic acid (TCA) cycle via hydrogen bonding or metal-ion chelation, potentially impairing energy production (He & Ouyang 2016; Arnold & Finley 2022). Monoglucosyl-enterobactin, a siderophore-like bacteriocin, is thought to act through a "Trojan horse" mechanism, exploiting siderophore-specific receptors such as FepA to enter competitor bacteria and disrupt iron metabolism (Miethke & Marahiel 2007; D'Onofrio et al. 2010). This compound may also compete for available iron, inducing iron starvation in microbial communities (Sassone-Corsi & Raffatellu 2015). RSC requires iron for survival and xylem colonisation; this compound could effectively restrict its proliferation. However, further validation using MS/

MS or targeted assays will be required to confirm these mechanistic roles.

In CFS-*L. paracasei*, several metabolites with antimicrobial potential were also detected. Canavanine, an arginine analogue, disrupts protein synthesis and induces oxidative stress (Rosenthal 2001; Lee et al. 2013), which could impair RSC's protein turnover and reduce its survival under host-induced oxidative stress. While norspermidine inhibits biofilm formation and destabilises existing biofilms in Gram-negative bacteria (McGinnis et al. 2009; Sobe et al. 2017), thereby weakening RSC's capacity for stable xylem colonisation. Additionally, 4-guanidinobutanamide exhibits multi-target antimicrobial effects, including membrane destabilisation, enzyme inhibition, and potential DNA binding, thereby disrupting nitrogen metabolism and gene expression (Harris et al. 2000; Feng et al. 2013). Its guanidinium group enhances interaction with negatively charged bacterial surfaces, increasing permeability and impairing transcription and replication (Feng et al. 2013). Such combined effects could compromise RSC's membrane integrity and genetic regulation, ultimately limiting its growth and virulence. A better view is discussed in the future in the SEM analysis.

Sinapic acid from *L. plantarum* toluene extract exhibits antioxidant and antimicrobial activities (Pandi & Kalappan 2021), impeding biofilm formation through multiple mechanisms that alter bacterial traits (Husain et al. 2024; Nićiforović & Abramović 2013). Biofilm is a key virulence trait of RSC for root colonisation and vascular blockage; interference with biofilm assembly could weaken its infection capability. This aligns with previous studies showing that sinapic acid interacts with microbial enzymes, hindering metabolic processes and specifically targeting Gram-negative bacteria (Hayashi et al. 2019; Giresha et al. 2022). DGL hydrolyses into gluconic acid, which acidifies the environment and chelates metal ions such as Ca^{2+} (Yim et al. 2015; Behbahani et al. 2022). Calcium ions are essential for RSC biofilm stability and motility; disruption of these ions may reduce its capacity to establish systemic infection. Chelation also destabilises the Gram-negative outer membrane, further sensitising RSC to antimicrobial attack. Lecanoric acid inhibits microbial growth by targeting essential bacterial proteins (Rajendran et al. 2020), exhibiting broad-spectrum activity against *E. coli*,

S. aureus, *Vibrio cholerae*, and *Candida albicans*, and mimicking fungicidal mechanisms effective against tea pathogens (Rajendran et al. 2023). By interfering with protein function, it may suppress metabolic enzymes critical for RSC growth and pathogenicity, potentially limiting its ability to colonise host xylem vessels.

Lithocholic acid putatively detected in *L. paracasei* compromises bacterial membranes, leading to increased permeability and cell lysis (Begley et al. 2004; González et al. 2020). Such mechanisms could directly damage RSC cell integrity and energy production, hindering survival in soil and rhizosphere niches. Similar studies proved that lithocholic acid targets pathogens such as *S. aureus*, *E. coli*, *P. aeruginosa*, *C. difficile* and *H. pylori*, while promoting oxidative stress via ROS accumulation and disrupting energy production by impairing the proton motive force and ATP synthesis (Nascimento et al. 2015; Kovács et al. 2019; Kisthardt et al. 2023; Sievers et al. 2019). Orotic acid targets pyrimidine biosynthesis by inhibiting orotate phosphoribosyltransferase and OMP decarboxylase (Santiago & West 2002; Kilstrup et al. 2005). As nucleic acid synthesis is essential for replication, such inhibition could strongly suppress RSC growth and proliferation, thereby lowering its infection potential in planta. Moreover, sterculic acid acts as a stearyl-CoA desaturase (SCD) inhibitor, preventing mono-unsaturated fatty acid biosynthesis (Peláez et al. 2020). This alters membrane fluidity and integrity, which are critical for RSC motility and colonisation. By disrupting these processes, sterculic acid could reduce bacterial spread within plant vascular tissues.

Comparison of compounds released by *L. plantarum* and *L. paracasei* reveals that those in the CFS are more potent than in the LLE extract due to the presence of monoglucosyl-enterobactin in *L. plantarum*, which exhibits a highly specific antimicrobial mechanism, with enterobactin's iron-chelating properties offering potential for novel antimicrobial strategies, particularly against Gram-negative pathogens dependent on iron acquisition. In *L. paracasei*, canavanine and norspermidine are well-documented antimicrobial compounds that inhibit bacterial growth by disrupting quorum sensing. Abouzeid et al. (2023) demonstrated that canavanine effectively suppressed *E. coli* biofilm formation, while norspermidine significantly inhibited

biofilm development in several pathogenic bacteria, including *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Cardile et al. 2016).

SEM provides detailed morphological insights into bacterial structures under different treatments with CFS-LAB, yielding more significant results in the antagonistic test and in the analysis of compounds released by CFS rather than LLE extraction. Untreated RSC cells (Figure 3A) display normal rod-shaped morphology with smooth surfaces, indicating healthy growth, while streptomycin-treated cells (Figure 3B) show elongation and surface roughness, suggesting membrane damage and intracellular leakage (Li et al. 2016; Guo et al. 2021). This aligns with previous research demonstrating that aminoglycoside antibiotics such as streptomycin disrupt bacterial protein synthesis and induce oxidative stress, leading to membrane instability (Kotra et al. 2000). Treatment with CFS-*L. plantarum* (Figure 3C) causes marked surface disruption, aggregation, and possible lysis, supporting previous findings that *L. plantarum* produces organic acids and bacteriocins that compromise membrane integrity (Sajan & Saravanan 2024).

Similarly, RSC cells were treated with CFS-*L. paracasei* (Figure 3D) exhibit irregular shapes with more pronounced membrane damage, suggesting a stronger antimicrobial effect. These findings align with prior studies indicating that probiotic-derived antimicrobial compounds effectively target Gram-negative pathogens by disrupting membranes and interfering with metabolism (Petrova et al. 2022). The results highlight the potential of LAB-derived CFS as a biocontrol agent against *Ralstonia* spp., offering an alternative or complementary approach to conventional antibiotics in agriculture and clinical applications. Further quantitative analyses, such as membrane permeability assays and viability tests, are necessary to confirm these observations. Thus, the current study suggests that antimicrobial substances produced by *L. plantarum* and *L. paracasei* effectively inhibit the *in vitro* growth of RSC, providing preliminary evidence for further evaluation. However, future work should integrate complementary approaches, such as viability and membrane permeability tests, to strengthen mechanistic insights and validate the observed structural disruptions.

CONCLUSION

This study provides preliminary evidence that metabolites produced by *L. plantarum* and *L. paracasei* exhibit antimicrobial activity against RSC under in vitro conditions. LC-MS analysis putatively identified several bioactive compounds, including fatty acids, organic acids and siderophore-like bacteriocins, that may contribute to the observed inhibitory effects. While these findings highlight the potential of lactic acid bacteria as a source of natural antagonists, their functional roles remain to be validated through targeted metabolite assays and mechanistic studies. Importantly, the current results are limited to laboratory-scale assays, and further investigation is required to assess efficacy and consistency under in planta, nursery, and field conditions. Future research should focus on confirming metabolite activity, elucidating molecular mechanisms, and evaluating practical application strategies to determine whether LAB-based formulations can be developed into sustainable biocontrol solutions for Banana Blood Disease.

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