Journal of
Food Chemistry & Nanotechnology

https://doi.org/10.17756/jfcn.2023-s1-079

Research Article

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Isolation and Antimicrobial Activity of Plant-associated Lactic Acid Bacteria against *Pantoea stewartii* Subsp. stewartii of Jackfruit Bronzing Disease

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Received: October 11, 2023
Accepted: November 10, 2023
Published: November 15, 2023

Citation: Jaffar NS, Jawan R, Chong KP. 2023. Isolation and Antimicrobial Activity of Plant-associated Lactic Acid Bacteria against *Pantoea stewartii* Subsp. stewartii of Jackfruit Bronzing Disease. *J Food Chem Nanotechnol* 9(S1): S627-S634.

Published by United Scientific Group

Abstract

Pantoea stewartii subsp. stewartia (Pss) is the causative agent of "Jackfuit Bronzing," an emerging disease in jackfruit crops (*Artocarpus heterophyllus* L.). The disease was first discovered in Malaysia in 2017, affecting mostly the J33 clone, tekam yellow. This disease degrades the quality of fresh jackfruit, leading to economic losses, and has remained a serious problem for the Malaysian jackfruit trade. In this study, lactic acid bacteria (LAB) isolated from fruits and vegetables were screened and characterized for their antimicrobial potential against Pss. The LAB isolation process was carried out using the standard method, and the isolated colonies were initially distinguished by their morphological features on de Man, Rogosa and Sharpe (MRS) agar, Gram-positive staining characteristics, and catalase-negative responses. These colonies were then selected for identification through polymerase chain reaction (PCR) amplification of the 16S rDNA gene and sequencing. Fourteen cell-free supernatants (CFS) of the LAB isolates were found to significantly inhibit the growth of Pss *in vitro* ($p < 0.05$). LAB isolates showing promising potential as biocontrol agents were identified as *Lactiplantibacillus plantarum*, *Leuconostoc holzapfelii*, *Weissella cibaria,* and *Weisella paramesenteroides*. The findings of this study suggested that the isolated LAB may have antibacterial characteristics that could be investigated further for the treatment of jackfruit-bronzing disease.

Keywords

Jackfruit, Lactic acid bacteria, Bronzing, Bacterial disease, *Pantoea stewartii*

Introduction

Jackfruit, scientifically known as *A. heterophyllus* Lam., is a non-seasonal tropical fruit from the Moraceae family. Jackfruit is believed to have originated from the rain forests of the Western Ghats in Southwestern India [1]. Its cultivation has since spread to other parts of India and other regions around the world throughout the South and Southeast Asian region, the Caribbean, Latin America, and parts of Africa [2]. Jackfruit is closely related to several other species, including the cempedak (*Artocarpus integer*), tarap (*Artocarpus odoratissimus*), breadfruit (*Artocarpus a*ltilis), tempunai (*Artocarpus rigidis*), and tarap bulu (*Artocarpus sericarpus*) [3]. Approximately 12.2 million metric tons of jackfruit were produced worldwide in 2021, according to the Food and Agriculture Organization of the United Nations [4]. India is the leading producer of jackfruit, accounting for more than 50% of global output. Other significant producers include Bangladesh and Indonesia, which account for approximately 10% and 8% of global production, respectively [5].

In Malaysia, jackfruit cultivation has been commercialized on a large scale, making it among the top ten economically important fruit crops [6]. Jackfruit

production has grown from 29,578 mt in 2015 to 41,047 mt in 2021 [7], however, its output saw a fluctuation between 2016 and 2018 due to a new disease outbreak known as jackfruit-bronzing, caused by phytopathogenic bacteria, Pss. The jackfruit-bronzing disease was first discovered in the Malaysian state of Pahang in 2017 [8], impacting nearly 10,000 hectares (ha) of jackfruit plantations with a prevalence of 50 to 80% in most infected areas [5, 9]. Similar incidents have previously been reported in the Philippines [10] and have since spread to Mexico [11] and, most recently, China [12]. Indicated by yellow to red discoloration on affected fruit pulp and rugs, the disease poses an immediate threat to jackfruit industries. It has diminished the quality of raw jackfruit and has a negative economic impact on the marketing of jackfruit.

Due to the scarcity of chemical treatments and the lack of resistant papaya varieties, there is an increasing interest in biological control agents (BCAs) for disease management. Biological control is a viable strategy for delivering long-term results that contribute to sustainable agriculture. Recently, there has been a surge of interest in the study of LAB as a potential new class of BCA against plant diseases [13]. Because of their broad application in the food industry, knowledge of their physiology and bioactive substances has been greatly explored [14]. This led to the designation of LAB as generally regarded as safe with few exclusions by the food and drug administration, implying that its usage in food crop cultivation poses no health risks to humans and animals [15]. Significant studies have shown that LAB has great potential and can be a key part of sustainable agriculture to regulate pests and diseases, improve soil quality, and mediate plant growth [16-18]. Thus, this study was carried out to isolate and identify LAB from plant-associated sources and to evaluate their antibacterial activity against Pss of the jackfruit bronzing disease.

Materials and Methods

Sample collection

Samples of fruits and vegetables were collected randomly from various supermarkets and wet markets around Kota Kinabalu and Kundasang, Sabah. The samples were placed in sterile bags, transported to the laboratory, and processed immediately to prevent deterioration. Fruit samples consisted of banana, papaya, strawberry, and tarap, while vegetable samples consisted of mustard green, red leaf lettuce, cherry tomato, and Japanese cucumber.

Isolation of LAB from fruits and vegetables

The samples were washed thoroughly with running water, surface sterilized with 10% (v/v) sodium hypochlorite solution (NaOCl) and washed 3 times with sterile distilled water. Ten grams of each fruit and vegetables samples were cut aseptically into small pieces and transferred into 100 ml of sterile de MRS broth (Oxoid, UK), in a sterile conical flask and mixed well. The samples were incubated at 37 °C for 72 h in aerobic conditions. Tenfold serial dilution was performed using 1 ml of the prepared sample suspensions into 9 ml sterile MRS broth and 100 µL was then spread plated on the MRS agar. Plates were incubated aerobically at 37 °C for 48 h. The number of dilutions prepared depended on the microbial density of each sample, but at least 3 consecutive dilutions that could provide single colony growth were chosen. Following the incubation period, each plate was examined, and those with single, white, or cream round colonies were chosen for sub-culturing onto fresh MRS agar. The sub-culturing procedure was repeated three times, and the isolates that retained characteristics after each sub-culture were cultured in MRS broth and supplemented with 20% glycerol (Sigma, USA) before being stored at -80 °C until further analysis.

Physiological, biochemical, and molecular identification of LAB isolates

The LAB isolates were identified based on their morphological and microscopic characteristics. Biochemical and physiological testing that includes Gram staining, catalase reaction test, growth at different pH (3.0, 4.0, 6.0, 7.0, and 8.5) Sodium chloride (NaCl) tolerance test (2%, 4%, and 8%, w/v), and growth test at varying temperatures (15 °C, 25 °C, 37 °C, 40 °C and 45 °C) were performed to further characterize the isolates as outlined by Hammes and Hertel [19]. The bacterial growth was measured by the absorbance at 600 nm using a spectrophotometer. Carbohydrate fermentation profiles were determined using an API 50 CHL kit (Biomérieux, France) in accordance with the manufacturer's instructions.

Molecular identification of LAB isolates by 16S rRNA sequencing

The genomic DNA of the isolates was extracted using the DNEasy Blood and Tissue Kit (Qiagen, USA) and amplified by PCR using 16S universal primers, 27F: 5´-AGT TTG ATC CTG GCT CAG-3´ and 1492R: 5´-GTT TAC CTT GTT ACG ACT T-3´ [20].

The PCR reaction mixture contained 25 µL DreamTaq Green PCR Master Mix (Thermo scientific), 3 µL of each primer, 3 µL extracted DNA, and PCR grade H_2O to reach a final volume of 50 µL. The PCR conditions were set as follows: 1 cycle of 95 °C for 15 s; 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s; and 1 cycle of 72 °C for 10 min. The 1.5 kb amplified PCR products were purified and sequenced by First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia). The nucleotide sequences obtained were edited by BioEdit software and analyzed using the basic local alignment search tool (BLAST) of the GenBank (NCBI) to check the identity of the sequences present in the database.

Preparation of LAB isolates and pathogenic strain for in vitro screening

The preparation of LAB culture was done by sub-cultured on MRS agar or MRS broth at 37 °C for 48 h. Suspension of LAB was incubated aerobically with shaking (110 rpm), 37 °C for 48 h. Pss strain was obtained from the stock culture of Horticulture Research Centre, Malaysian Agricultural Research and Development Institute. The pathogenic bacteria were revived from glycerol stock by sub-cultured on nutrient agar and incubated overnight at 37 °C. For bacterial suspension, the bacterial pathogen was cultured in nutrient broth at 37 °C overnight with shaking (110 rpm) REF. Both bacterial suspensions of LAB and Pss were standardized by adjusting

the turbidity equivalent to 0.5 McFarland standard (Biome Rieux, France) or approximately 1 - 2 × 108 CFU/ml.

In vitro screening for detection of antimicrobial activity

Preliminary screening for antimicrobial activity was conducted by agar overlay and agar well diffusion assays with modification [21]. For agar overlay assay, the LAB isolates of 48 h were streaked on MRS agar. Subsequently, 1 ml of overnight suspension of Pss strain (~108 CFU/ml) was inoculated to a soft potato dextrose agar (PDA) (Difco) and placed onto the MRS agar. The plate was incubated under aerobic conditions at 37 ˚C for 48 h. Isolates with inhibition zones were randomly selected. Three replicates per each isolate were tested. In the agar diffusion assay, LAB cell-free supernatant (LAB-CFS) was used to screen against the target pathogen Pss according to Lin et al. [22] with slight modifications. Previously selected LAB isolates were incubated with shaking (110 rpm) in MRS broth at 37 ˚C for 48 h. A 15 ml of overnight culture of each LAB was centrifuged at 7,500 rpm for 20 min at 4 ˚C. The pellet was discarded and the CFS was filter-sterilized through a sterile 0.22 µm pore size filter (Sartorius Stedim, France). Prior to the screening, 1 ml of overnight suspension of Pss strain (~108 CFU/ml) was inoculated to 15 ml soft PDA agar (Difco) and wells of 7 mm in diameter were prepared after the agar solidified. 100 µL of the LAB-CFS were pipetted into the wells and allowed to diffuse into the agar for 2 h at 4 ˚C ± 2 ˚C; with sterile MRS broth and kanamycin (0.25 mg/ml) as the negative and positive control. The Petri dishes were then incubated overnight at 37 ˚C and the diameter of inhibition zones was recorded. Three replicates per each isolate were tested.

Determination of the LAB antimicrobial substances

The LAB-CFS was utilized to determine the production of antimicrobial substances via the agar-well diffusion assay. Following the preparation as in 2.5, the LAB-CFS was then divided into four portions prior to screening evaluation as follows: (i) untreated CFS (pH 3.75 to pH 4.15); (ii) pH neutralized CFS (adjusted pH 6.50 to 7.0 using sterilized 1 N NaOH); (iii) CFS treated with 1 mg/ml of catalase (Sigma-Aldrich, USA) at 25 ˚C ± 2 ˚C for 1 h and; (iv) CFS treated with 1 mg/ml of proteinase K (Sigma-Aldrich, USA) at 37 ˚C for 2 h. Prior to the screening, 1 ml of overnight suspension of Pss strain (~108 CFU/ml) was inoculated to 15 ml soft PDA agar (Difco) and wells of 7 mm in diameter were prepared after the agar solidified. 100 µL of CFS (i), (ii), (iii), and (iv) were pipetted into the wells and allowed to diffuse into the agar for 2 h at 4 ˚C ± 2 ˚C, with sterile MRS broth as control. The Petri dishes were then incubated overnight at 37 ˚C and the inhibition zones were recorded. Three replicates per each isolate were tested.

Statistical analysis

The data were analyzed using XLSTAT 2019 statistical software (Boston, United States). A one-way analysis of variance (ANOVA) by Tukey's pairwise multiple comparison test was performed to evaluate the significance of sample mean differences. The significance level was set at $p < 0.05$. Unless otherwise specified, all experimental results were expressed as the mean value of three replicates (n = 3).

Results and Discussion

Isolation, morphological and biochemical characterization LAB

LAB has been commonly isolated from fruits and vegetables, as evidenced by various research [23-25]. In this study, a total of 112 bacterial isolates were obtained from 9 samples of fruit and vegetables. After Gram staining and screening to eliminate catalase-positive organisms, 54 presumptive LAB isolates remained. The biochemical reactions of Gram staining and catalase reaction are frequently employed as initial means of identifying LAB. The Gram staining technique is utilized for the purpose of bacterial identification, relying on the distinctive features of their cell walls. LAB is a group of Gram-positive bacteria, characterized by the presence of a cell wall that is rich in peptidoglycan and is commonly recognized as catalase-negative microorganisms due to their limited production of catalase enzymes. As a result, they are unable to effectively catalyze the breakdown of hydrogen peroxide into water and oxygen [26].

In the preliminary screening by agar overlay assay, 14 of the presumptive LAB isolates were selected based on their distinct colony morphology and inhibitory effect against Pss. Growth at different temperatures was to determine whether the isolated bacteria were psychrophilic, mesophilic, or thermophilic. Psychrophilic microorganisms thrive at 20 ˚C and below 15 ˚C. The mesophilic group grows at 15 ˚C - 45 ˚C, while the thermophilic group grows at 45 ˚C - 80 ˚C. In addition to growth at different temperatures, growth at different pH and in the presence of various NaCl concentrations was to determine whether the isolated LAB can resist high acidic and halophilic conditions respectively and to detect their optimal growth condition [27]. Turbidity was observed as an indicator of cellular growth following a 48 h incubation period on MRS media. The majority of the isolates were single, chained, or clustered rods, and cocci, able to grow between the temperature of 25 ˚C to 40 ˚C (mesophilic bacteria) except for isolates B2 - 17 and B3 - 34 which had the thermophilic character and were able to grow at 45 ˚C. Table 1 shows the characteristics of these isolates.

The pH effect revealed that most isolates could grow ideally between pH 6.0 and 7.0, however at low pH, only 7 isolates could grow at pH 4.0, and no growth was detected at pH 3.0 for all isolates (Figure 1). It was also observed that all isolates were able to grow at 2% and 4% NaCl concentrations and only 4 isolates were able to slightly grow at 8% NaCl (Figure 2). The physiological trait of LAB to thrive within specific ranges of temperature, acidity, and salinity is a crucial aspect that determines their survival within the challenging environment of plant-microbiome. For instance, Fhoula et al. [28] discovered that the physiochemical features of the isolated rhizosphere LAB, such as their ability to break down proteins, their ability to tolerate high levels of NaCl, and their ability to produce exopolysaccharide (EPS), could explain how they were able to survive in oligotrophic environments [29, 30]. EPSs are often linked to bacteria's ability to fight stress and protect themselves from conditions like high salinity, and UV radiation. However, it is also implicated in their adhesion to biological surfaces and the decrease in sodium toxicity [31].

Table 1: Phenotypic characteristics of the isolated LAB.

Isolates	Source	Cell morphology	Gram reaction	Catalase reaction	Growth temperature (°C)				
					≤ 15 °C	28 °C	35 °C	40 °C	45 °C
B1 - 5	Banana (pulp)	Small, rod, in-chain, white-creamy	+	-	-	+	+	+	-
B1 - 6	Banana (pulp)	Small, rod, in-chain, white-creamy	+	-	-	+	+	+	-
B1 - 8	Banana peel	Small, rod, creamy	+	-	-	+	+	+	-
B1 - 9	Banana (pulp)	Small, rod, white-creamy	+	-	-	+	+	+	-
B2 - 10	Banana (peel)	Medium, creamy-white, short rod	+	-	-	+	+	+	-
B2 - 14	Red leaf lettuce	Small, creamy-white, ovoid	+	-	-	+	+	+	-
B2 - 17	Red leaf lettuce	Small, creamy-white, sightly thick coccoid	+	-	-	+	+	+	+
B2 - 23	Sawi	Small, rod, creamy	+	-	-	+	+	+	-
B2 - 29	Banana (peel)	Small, white-creamy, ovoid	+	-	-	+	+	+	-
B2 - 34	Red leaf lettuce	Small, creamy-white, coccoid	+	-	-	+	+	+	+
B3 - 36	Papaya (pulp)	Small, creamy-white, coccoid	+	-	-	+	+	-	-
B3 - 39	Papaya (pulp)	Small, creamy-white, coccoid	+	-	-	+	+	+	-
B3 - 40	Papaya (pulp)	Small, creamy-white, coccoid	+	-	-	+	+	+	-
B3 - 41	Papaya (pulp)	Small, rod, in-chain, white-creamy	+	-	-	+	+	+	-

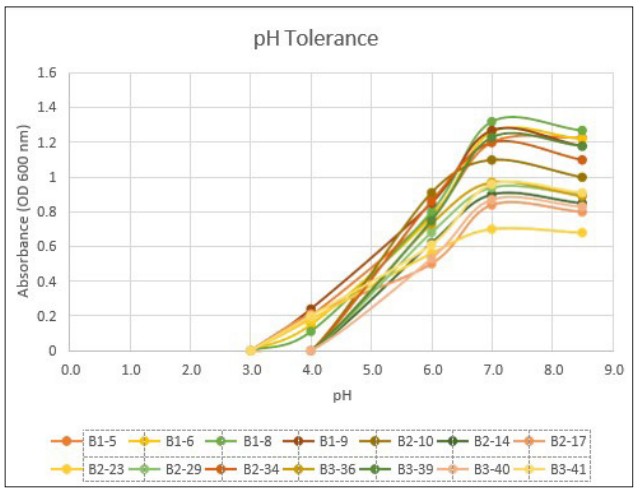

Figure 1: Effect of difference pH on the growth of LAB isolates.

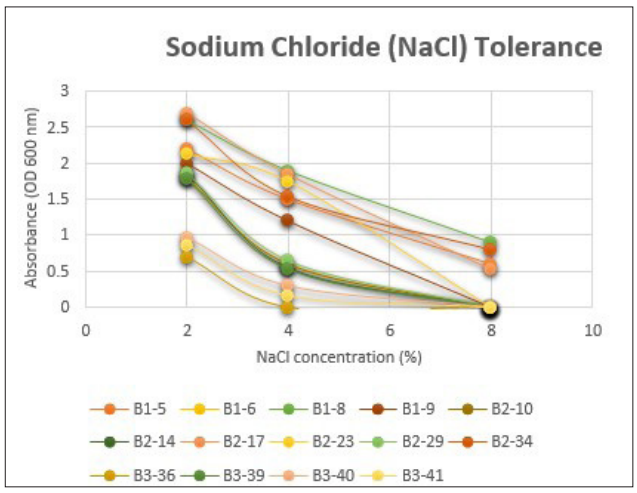

Figure 2: Effect of difference NaCl concentration on the growth of LAB isolates.

Based on the strength of the preliminary screening for antimicrobial activity, 7 isolates were selected for characterization by API 50 CHL to determine the biochemical properties of isolates in carbohydrate fermentation. The fermentative potential of the 7 isolates was assessed by subjecting them to 49 different carbohydrates present in the API 50 CHL kit. The ability of the isolates to ferment carbohydrates was demonstrated by the discoloration of the basal medium, which initially exhibited a purple hue. The color of isolates subjected to fermentation will undergo a transformation to yellow. All 7 isolates could ferment n-acetylglucosamine, esculin ferric citrate, D-maltose, D-saccharose, D-trehalose, gentiobiose, and potassium gluconate as carbon sources as presented in table 2. It was also found that not all carbohydrates could be fermented by the selected isolates of LAB. This condition was seen during discoloration, where some of the bacteria could not do the fermentation perfectly as this was possibly due to the lack of capability of enzyme produced by isolate to decompose sugar in the basal solution.

LAB with high fermentation activity will change the basal solution's color from purple to yellow, and LAB isolates without fermentation capacity would not change the color of basal solution at all. Probabilistic identification using the advanced bacterial identification software (ABIS) indicated the isolates could be classified based on morpho-biochemical characters and growth conditions of these isolates [32].

Molecular identification by 16S rRNA sequencing

The molecular identification using the universal primer 27F and 1492R showed clear bands of isolates with an approximate molecular weight 1,500 bp (Figure 3). The results of 16S rDNA sequencing identified B1 - 9, B2 - 23 and B3 - 41 isolates as *L. plantarum* based on 99% similarity to a GenBank entry NR_115605.1 and NR_104573.1. Isolate B2 - 14 was identified as *L. holzapfelii* with 99% similarity to a GenBank entry NR_042620.1, B2 - 34 has a similarity level of 99% to a GenBank entry NR_036924.1, *W. cibaria*, B3 - 36, and B3 - 40 was identified as *W. paramesenteroides* based on 99% similarity to GenBank entry, NR_104568.1 (Table 3). The identi-

Table 2: Carbohydrate fermentation profile of the LAB isolates by API 50 CHL kit. Probabilistic identification using the ABIS was used based on the morpho-biochemical characters and growth conditions for the identification of the LAB isolates.

Test		LAB isolate						
		B1 - 9	B3 - 41	B2 - 14	B2 - 23	B2 - 34	B2 - 34	B3 - 40
Control	C	-	-	-	-	-	-	-
Glycerol	GLY	-	-	-	-	-	-	-
Erythritol	ERY	-	-	-	-	-	-	-
D - Arabinose	DARA	-	-	-	-	-	-	-
L - Arabinose	LARA	+	+	+	+	+	+	+
D - Ribose	RIB	+	-	+	+	+	+	+
D - Xylose	DXYL	+	-	-	+	+	+	+
L - Xylose	LXYL	-	-	-	-	-	-	-
D - Adonitol	ADO	-	-	-	-	-	-	-
Methyl β-D-xylopyranoside	MDX	-	-	-	-	-	-	-
D - Galactose	GAL	+	-	+	+	+	+	+
D - Glucose	GLU	+	+	+	+	+	+	+
D - Fructose	FRU	+	+	+	+	+	+	+
D - Mannose	MNE	+	+	+	+	+	+	+
L - Sorbose	SBE	-	-	-	-	-	-	-
L - Rhamnose	RHA	-	-	+	-	-	-	-
Dulcitol	DUL	-	-	-	-	-	-	-
Inositol	INO	-	-	-	-	-	-	-
D - Mannitol	MAN	+	-	+	+	+	+	-
D - Sorbitol	SOR	+	-	+	-	-	-	-
Methyl α-D-mannopyranoside	MDM	+	-	-	-	-	-	-
Methyl α-D-glucopyranoside	MDG	-	+	+	-	-	-	+
N-Acetylglucosa mine	NAG	+	+	+	+	+	+	+
Amygdalin	AMY	+	+	+	+	+	+	-
Arbutin	ARB	+	+	+	+	+	+	-
Esculin/ferric citrate	ESC	+	+	+	+	+	+	+
Salicin	SAL	+	+	+	+	+	+	-
D - Cellobiose	CEL	+	+	+	+	+	+	-
D - Maltose	MAL	+	+	+	+	+	+	+
D - Lactose	LAC	+	-	+	+	+	+	-
D - Melibiose	MEL	+	-	+	-	-	-	+
D - Saccharose (sucrose)	SAC	+	+	+	+	+	+	+
D - Trehalose	TRE	+	+	+	+	+	+	+
Inulin	INU	-	-	-	-	-	-	-
D - Melezitose	MLZ	+	-	+	-	-	-	-
D - Raffinose	RAF	+	-	+	-	-	-	-
Starch (amidon)	AMD	-	-	-	+	+	+	-
Glycogen	GLYG	-	-	-	-	-	-	-
Xylitol	XLT	-	-	-	-	-	-	-
Gentiobiose	GEN	+	+	+	+	+	+	+
D - Turanose	TUR	+	+	+	+	+	+	-
D - Lyxose	LYX	-	-	-	-	+	-	-
D - Tagatose	TAG	-	-	-	-	-	-	-
D - Fucose	DFUC	-	-	-	-	-	-	-
L - Fucose	LFUC	-	-	-	-	-	-	-
D - Arabitol	DARL	-	-	-	-	-	-	-
L - Arabitol	LARL	-	-	-	-	-	-	-
Potassium gluconate	GNT	+	+	+	+	+	+	+
Potassium 2-ketogluconate	2KG	-	-	-	-	-	-	-
Potassium 5-ketogluconate	5KG	-	-	-	-	-	-	-
API identification by ABIS		*L. plantarum* (98.3%)	*L. plantarum* (95.2%)	*L. plantarum* (86.9%)	*L. plantarum* (92.6%)	*Weissella confusa* (84.5%)	*W. confusa* (80.5%)	*W. confusa* (83.7%)

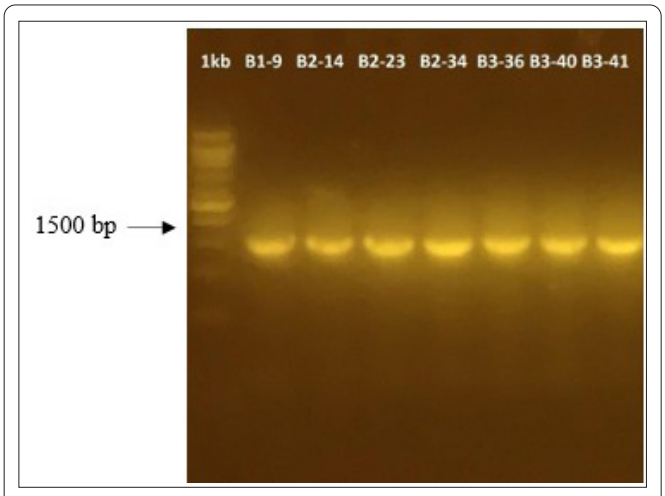

Figure 3: Gel image of the PCR product (1,500 bp) using the 16S primer, 24F and 1492R.

fication of LAB isolates through the utilization of the API 50 CHL demonstrated a satisfactory level of concordance with the outcomes of 16S rDNA sequencing, except for B2 - 14 which differed at the genus level, and B3 - 36 which differed at the species level. It has been reported that the LAB most frequently found on plant tissues are members of the Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Streptococcus, and Weissella genera [25]. While the occurrence of LAB on plants is not commonly documented in terms of their quantities or proportions, a study by Minervini et al. [33] revealed that *L. plantarum* accounted for as much as 30% of all LAB strains that were isolated from Durum wheat. In the phyllosphere of Romaine lettuce, Leuconostoc and Lactococcus were among the six most abundant bacterial genera [34]. The successful isolation of LAB from the present study, utilizing fresh plant samples, is consistent with prior reports in the literature.

In vitro screening of LAB on Pss and characterization of the antimicrobial substances

Numerous researchers have documented the antimicrobial properties of LAB derived from plants source against various microorganisms including bacteria, yeast, and molds [35-37]. Nevertheless, their application is more common as bioprotective and bio preservative agents for food, owing to their antimicrobial properties against foodborne pathogens. This characteristic makes them viable for utilization as biocontrol agents in combatting plant diseases. Despite this, knowledge regarding the use of LAB as biocontrol agents in plant protection is scarce. LAB is known to control a few plant diseases, including bacterial soft rot, bacterial wilt, fire blight, and head blight [38-40]. However, there have been no reports on the possibility of LAB in the biological control of the jackfruit bronzing disease.

In this study, the preliminary screening by agar overlay assay showed early evidence of the antimicrobial activity of LAB isolates. It was demonstrated by their ability to inhibit bacterial pathogens and produce an inhibition or clear zone surrounding the LAB site. Out of the 54 presumptive LAB isolates tested, 13 isolates showed their ability to inhibit Pss. In the subsequent screening by agar diffusion assay using the LAB-CFS, the results were in support of the initial screening by the agar overlay assay. Table 4 summarizes the screening findings and revealed that the inhibition or clear zones' diameters varied based on the LAB isolates. The observed differences in antagonistic activity among LAB isolates are consistent with the findings of El-Mabrok et al. [41], Taha et al. [21] and Mansilla et al. [42] who described the strain-specific action of LAB against phytopathogens. Seven isolates with good inhibition based on the screening results (clear zone > 17 mm) were selected to further characterize the antimicrobial substances.

The primary mechanism by which LAB inhibits the growth of pathogens is through the production of antimicro-

Table 4: Antimicrobial activity of the isolated LAB against Pss by agar overlay and agar diffusion assay. The different in (i) reflect the degree of growth inhibition zone expressed in mm as the mean of 3 replicates as follow: (+) inhibition zone between 5 and 10 mm; (++) inhibition zone between 11 and 17 mm, and (+++) inhibition zone > 17 mm. Antimicrobial activity in (ii) are presented as mean of inhibition zone ± standard deviation from 3 replicate experiments. Superscript letters indicate significant inhibitory as determined by ANOVA ($p < 0.05$).

Isolates	Mean inhibition diameter (mm)	
	Agar overlay	Agar diffusion
B1 - 5	++	12.33 ± 2.31^{def}
B1 - 6	++	13.67 ± 1.15^{cde}
B1 - 8	+	5.00 ± 4.36^{g}
B1 - 9*	+++	18.67 ± 2.08^{ab}
B2 - 10	++	14.33 ± 2.08^{cde}
B2 - 14*	+++	17.00 ± 1.00^{bc}
B2 - 17	+	9.00 ± 0.00^{fg}
B2 - 23*	+++	17.67 ± 2.08^{bc}
B2 - 29	++	14.33 ± 2.52^{cde}
B2 - 34*	+++	18.67 ± 4.16^{ab}
B3 - 36*	+++	22.33 ± 1.15^{a}
B3 - 40	+++	15.67 ± 1.53^{bcd}
B3 - 39	++	11.00 ± 1.00^{ef}
B3 - 41*	+++	19.33 ± 4.62^{ab}
Kanamycin	-	6.67 ± 0.58^{g}

Table 3: LAB isolates identification by sequencing of 16S rDNA gene.

Source	Isolate	API identification (ABIS)	16S rDNA gene identification	GenBank accession no.	Identity score (% similarity)
Banana (pulp)	B1 - 9	*L. plantarum* (98.3%)	*L. plantarum*	NR_115605.1	99.93
Red leaf lettuce	B2 - 14	*L. plantarum* (86.9%)	*L. holzapfelii*	NR_042620.1	99.79
Sawi	B2 - 23	*L. plantarum* (92.6%)	*L. plantarum*	NR_104573.1	100
Red leaf lettuce	B2 - 34	*W. confusa* (84.5%)	*W. cibaria*	NR_036924.1	99.79
Papaya (pulp)	B3 - 36	*W. confusa* (86.7%)	*W. paramesenteroides*	NR_104568.1	99.79
Papaya (pulp)	B3 - 40	*W. cibaria* (84.7%)	*W. paramesenteroides*	NR_104568.1	99.79
Papaya (pulp)	B3 - 41	*L. plantarum* (95.2%)	*L. plantarum*	NR_104573.1	99.79

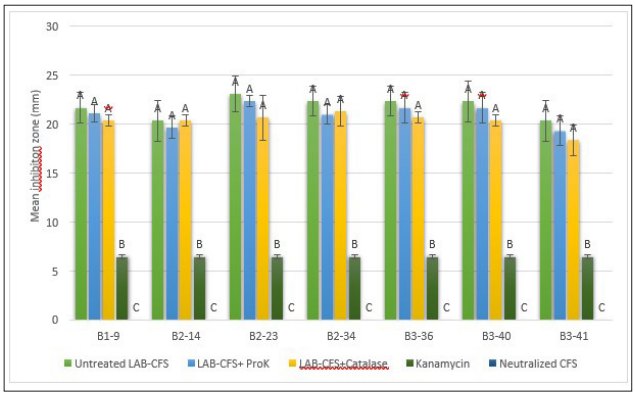

Figure 4: Antibacterial effect on untreated LAB-CFS, pH-neutralized LAB-CFS, catalase and proteinase K-treated LAB-CFS against Pss. Error bars represent the standard deviation of means (n = 3). Different uppercase letters indicate the mean value within the same LAB-CFS code are significantly different ($p < 0.05$) compared to control (Kanamycin).

bial substances, including bacteriocins, organic acids, hydrogen peroxide, and siderophores. These substances can be utilized for biocontrol purposes [43]. As shown in figure 4, all untreated CFS from 7 LAB isolates have antibacterial activity against Pss, and the diameters of the inhibition zones vary, however, the neutralized CFS of these isolates showed no inhibition against the target pathogen. This finding suggested that the acidic conditions present in the untreated CFS due to the presence of organic acid may have contributed to the antibacterial activity. A similar finding was also highlighted by Lan et al. [44], Oliveira et al. [45], Steglińska et al. [46].

It was also shown that the extracellular extract made from these isolates did not respond to the action of the enzyme's catalase and proteinase K. According to the results of the agar well diffusion assay, there was no significant reduction ($p > 0.05$) in the diameter of the inhibitory zone displayed by these enzyme-treated CFS. The catalase was incorporated to eliminate the possibility that the antibacterial efficacy of the treated CFS was attributed to the presence of hydrogen peroxide. According to Singh et al. [47], the catalase enzyme facilitates the decomposition of hydrogen peroxide into water and oxygen, thereby decreasing the concentration of hydrogen peroxide in the CFS if present. The ability of catalase-treated CFS in this study to maintain their inhibitory action against pathogens suggests that hydrogen peroxide may not contribute to the strains' antibacterial activity. In addition to organic acids and hydrogen peroxide, it is plausible that proteinaceous compounds, such as bacteriocin, may be present in the CFS as an antibacterial compound, albeit in minimal quantities. When introduced to the CFS, proteolytic enzymes such as proteinase K inactivate the proteinaceous substance [48]. When compared to untreated FS, the proteinase K-treated CFS in this study were able to sustain their inhibitory action, indicating that the proteinaceous compound may not be the source of the antimicrobial activity.

Conclusions

The LAB isolates identified from this study as *L. plantarum* (B1 - 9, B2 - 23 and B3 - 41), *L. holzapfelii* (B2 - 14), *W. cibaria* (B2 - 34) and *W. paramesenteroides* (B3 - 36) showing promising potential as biocontrol agents against Pss of the jackfruit-bronzing disease. The antibacterial activity was likely associated with the production of organic acid and further investigations are needed to establish the disease-suppressing biocontrol mechanisms.

Acknowledgements

None.

Conflict of Interest

None.

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