

Unlocking the Probiotic with Antioxidant-Rich Potential from Wine Coffee: *In vitro* Screening and Characterization

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ABSTRACT

Food companies are becoming interested in lactic acid bacteria (LAB) due to their promising health benefits. LAB can be isolated from several bioresources; nothing is known about the probiotic potential of fermented coffee products like wine coffee. The study aimed to determine the probiotic potential of LAB isolated from wine coffee with antioxidant properties. Characterization of probiotics included antioxidant activity, acidity resistance, bile salt tolerance, antibacterial activity, antibiotic sensitivity, and haemolytic activity. The promising probiotics were identified using 16S rDNA sequences. The current results demonstrated that twenty-five isolates were found in wine coffee, and all showed antioxidant activities. Isolate E1 has the highest antioxidant activity (64.31%) among others. Isolates D4 and E2 were proposed as potential probiotics due to survival at low pH (pH 2) and bile salt (2%), inhibit *Bacillus cereus* ATCC 1178, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Salmonella* Typhimurium TRD1, and do not display haemolytic activity. Isolates D4 and E2 demonstrated resistant to Kanamycin and Aztreonam. Based on molecular identification, isolates E2 and D4 were identified as *Lactiplantibacillus plantarum*, E1 was identified as *Lactiacaseibacillus paracasei*. These LAB isolates are promising probiotic candidates and require further validation of their strain-specific potential.

Key words: Fermentation; functional food; *Lactiacaseibacillus paracasei*; *Lactiplantibacillus plantarum*; probiotic potential.

1 INTRODUCTION

Coffee (*Coffea* sp.) is a global plantation product due to high societal demand. The global production of coffee in 2022/23 increased by 396.0 million tons compared with previous years and became the second-most traded commodity after oil traded in the world (Silva et al., 2024). The variations of coffee that are quite well-known include Arabica (*Coffea arabica* L.), Robusta (*Coffea canephora*), Excelsa (*Coffea excelsa*), and Liberica (*Coffea liberica*). However, Arabica species reached 70%, and Robusta species reached 26% of the global coffee consumption (Gaspar; Ramos, 2016). Furthermore, food and beverage innovations that use coffee as the main ingredient continue to be developed, including wine coffee. Wine or fermented coffee is a type of coffee processing that has a very long post-harvest process until it can produce a unique taste and aroma. The fermentation process can also reduce the harmful effects of ochratoxin, a fungal toxin produced by *Aspergillus niger* (Lee et al., 2023).

Wine coffee originated from the highlands of Aceh and North Sumatra, Indonesia and is processed by local farmers through the fermentation process of coffee beans. The high altitude is reported to influence the taste of wine coffee (Chairuddin et al., 2023). The final coffee is processed using one of three techniques immediately after harvest to allow spontaneous fermentation (Elhalis; Cox; Zhao, 2023). The three unique wine-coffee processing techniques are dry,

wet, and semi-dry. The final processing of wine coffee is to achieve a 13% moisturization, producing a unique sensory characteristic (Muzaifa; Abubakar; Nilda, 2024; Sulaiman; Irfan; Syahputra, 2023). The spontaneous fermentation of wine coffee involved collaboration from various microorganisms, such as bacteria, yeast, and filamentous fungi, resulting in bean mucilage degradation and changes in the sensory quality of coffee beans and beverages (Elhalis; Cox; Zhao, 2023). Bacteria isolated from coffee grounds fermentation can increase chlorogenic acid, 4,5-dicaffeoylquinic acid, caffeine, and glycerophosphocholine, which in turn increase the antioxidant activity of fermented coffee grounds than unfermented coffee grounds (Therdatha et al., 2023). The LAB converted glucose and fructose into lactic acid during the fermentation process. Several LABs are categorized as probiotics (Sadiq, 2022).

Considering the LAB in several natural resources, several previous studies reported a diversity of LAB in fermented coffee. *Enterococcus mundtii* was successfully isolated from the fermented mixture of green coffee beans and coffee pulp (Zhao et al., 2023). Another study reported that LAB from laboratory-scale coffee fermentation was successfully identified as *Levilactobacillus brevis* and *Pediococcus pentosaceus* (Junqueira et al., 2022). Based on that, discovering new bacteria with probiotic properties is still interesting because they are strain-specific and might vary from region to region (Amaral et al., 2017). As mentioned before, wine coffee underwent spontaneous fermentation, increasing the likelihood

of discovering novel lactic acid bacteria with probiotic characteristics that might vary and region-specific (Leong et al., 2014; Silva et al., 2000; Vilela et al., 2010). Identifying LAB in wine coffee beans, which have the potential as probiotic candidates and antioxidant activity, is essential so that further development can be carried out regarding the promising application of probiotic LAB in the future.

2 MATERIAL AND METHODS

2.1 Collection of coffee beans and fermentation process

Robusta coffee samples were obtained from coffee farmers in Sukapura Village, Sukapura District, Probolinggo Regency, East Java Province, Indonesia. Coffee cherries were first sorted by soaking in water for 30 minutes, and the floating cherries were discarded. A total of 250 g of cherries was placed into a glass jar and sealed tightly. Fermentation was carried out at 25–30 °C under dark, anaerobic conditions (Kwak; Jeong; Kim, 2018; Wafaretta et al., 2024). After fermentation, the cherries were sun-dried for 7 hours. Sampling was performed at five points: before fermentation (day 0) and during fermentation at days 21, 28, 46, and 49.

2.2 Isolation of lactic acid bacteria

The isolation of LAB was carried out using the serial dilution method for coffee fermentation samples. A 25 g coffee beans were dissolved in 225 mL of sterile 0.85% NaCl solution and then diluted into serial dilution from 10^{-1} until 10^{-6} . A 1 mL of each dilution series was inoculated in de Man Rogosa Sharpe (MRS) agar medium (cat. no. 1.10660.0500, Merck KGaA, Darmstadt, Germany) added with 1% calcium carbonate (CaCO_3) (cat. no. 471-34-1, Merck KGaA, Darmstadt, Germany) using the spread plate method, then incubated at 37 °C for 48 hours (Zhao et al., 2023). Bacterial colonies with a clear zone were calculated. A single LAB colony was purified using the streak plate and dilution methods until pure isolate was obtained. Subsequently, Gram staining and catalase tests were performed on purified isolates to confirm their identity as LAB, characterized by Gram-positive and catalase-negative reactions.

2.3 Antioxidant activity test

One loop for each isolate was inoculated into 10 mL of MRS broth (cat. no. 1.10661.0500, Merck KGaA, Darmstadt, Germany) and incubated at 37°C for 24 hours. Then, 0.5 mL of LAB suspension was transferred to 4.5 mL MRS broth and incubated at 37°C for 16 hours (Kaga et al., 2021). The LAB cultures were centrifuged at 8,000 rpm, 4°C for 10 minutes. The pellet obtained was then washed twice using phosphate

buffer saline (PBS) and adjusted at 10^8 CFU/mL (Son et al., 2018). Each isolate was centrifuged at 8,000 rpm g at 4°C for 10 minutes, and the supernatant was filtered using a filter with a pore size of 0.45 µm. A 50 µL filtrate was diluted with 50 µL of 100% methanol. Furthermore, 700 µL of 50 µM DPPH solution (cat. no. D9132-5G, Merck KGaA, Darmstadt, Germany) was added and homogenized using a vortex mixer. The homogenate was incubated at room temperature for 30 minutes in the dark. The absorbance was measured at $\lambda = 517$ nm using a UV-VIS spectrophotometer. The control was the DPPH solution, while the blank was methanol (Bustos et al., 2017). Free radical scavenging activity was determined by Equation 1.

$$\text{Scavenging activity(\%)} = \left(1 - \frac{(A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \right) \times 100 \quad (1)$$

2.4 Acidity test and bile salt tolerance test

The acidity test was conducted to determine the level of LAB tolerance to acidic conditions. LAB culture (10 mL) with 10^7 cells/mL density at logarithmic growth phase were inoculated into MRS broth media at pH 2.0, 3.0, and 4.0 and incubated at 37°C for 3 hours (Alang et al., 2019, 2020). The cell density was calculated using a haemocytometer. The bile salt tolerance was performed by incubating the LAB culture in MRS broth media containing 1%, 1.5%, and 2% bile salt ox gall (cat. No. 70168, Merck KGaA, Darmstadt, Germany). Determination of cell density was carried out after incubation at 37°C for 4 hours. The survival rate of the isolate was calculated using Equation 2 (Akinyemi et al., 2024).

$$\text{Survival Rate(\%)} = \frac{\log \text{CFU Nt}}{\log \text{CFU N}_0} \times 100\% \quad (2)$$

Nt is the total viable count of LAB after treatment, and N_0 is the total viable count of LAB before treatment.

2.5 Antibacterial assay

Antibacterial activity was assayed using four pathogenic bacteria: *Bacillus cereus* ATCC 1178, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Salmonella* Typhimurium TRD1. *S. Typhimurium* was an isolated collection from the previous study (Gaffar; Jatmiko; Prihanto, 2022). One loop of selected LAB isolates was inoculated into 10 ml MRS broth and incubated at 37 °C for 24 hours. The LAB culture with a density of 10^7 CFU/mL was centrifuged at 10,000 rpm for 15 minutes at 4°C and neutralized at pH 7 to obtain neutralized cell-free supernatant (CFS), then it was filtered with a Millipore with a pore diameter of 0.22 µm. One loop for each of the four species of pathogenic bacteria was cultured in 5 mL of nutrient broth (NB) media (cat. no. 1.05443.0500, Merck KGaA, Darmstadt, Germany) and then incubated at 37 °C for 24 hours. Briefly, 0.1 mL of pathogenic

bacteria at 10^7 CFU/mL was inoculated by a spread plate on nutrient agar (NA) media (cat. no. 1.05450.0500, Merck KGaA, Darmstadt, Germany) and incubated at 4°C for 1 hour. Furthermore, the paper disk was dripped with 60 μL of each CFS and then incubated for 15 minutes. The paper disk containing CFS was placed on the surface of the NA media of pathogenic bacteria, and incubated at 37°C for 24 hours. The diameter of the inhibition zone was measured. The antibacterial activity (AU/mL) was determined by calculating the difference between the inhibition zone area and the paper disk area (both expressed in mm^2), which was then divided by the volume of the cell-free supernatant (Shehata et al., 2016).

2.6 Antibiotic susceptibility test

An antibiotic sensitivity test of LAB was conducted to determine the level of sensitivity of selected LAB isolates to several antibiotics. This test used four types of antibiotic disks, including 30 μg Kanamycin (cat. No. CT0026B, Oxoid), 15 μg Erythromycin (cat. no. CT0020B, Oxoid), 30 μg cefazolin (cat. no. CT0011B, Oxoid), and 30 μg Aztreonam (cat. no. CT0264B, Oxoid). One loop of selected LAB isolates was inoculated into 1 mL (10%) MRS broth and incubated at 37°C for 24 hours. The 0.1 mL of LAB fresh culture with a 10^7 cells/mL density was spread on MRSA in a petri dish using a glass rod spreader. Each petri dish was divided into four quadrants. Each type of antibiotic disk was placed on the surface of each quadrant and incubated at 37°C for 24 hours. The diameter of the inhibition zone was measured at the 24th hour of incubation. Based on inhibition zone diameter, the LAB isolates were categorized as sensitive, S (≥ 20 mm); intermediate, I (15-19 mm); and resistant, R (≤ 14 mm) (Sengun et al., 2024).

2.7 Haemolytic activity test

The bacterial isolates were tested for pathogenicity on blood agar media. The one loop of selected bacteria was streaked on blood agar plate (BAP) media (cat. no. 1.10886.0500, Merck KGaA, Darmstadt, Germany) containing 5% sheep blood and incubated at 37°C for 24 hours. Colonies that show a clear zone (β -haemolysis) indicated positive haemolysin, while colonies that form a slightly dark zone indicate (α -haemolysin), and while no clear zone, it indicated that the isolate could not lyse red blood cells (Alang et al., 2019).

2.8 Molecular identification of isolates

The selected LAB isolates were molecularly identified based on 16S rDNA sequences. The chromosomal DNA of LAB isolates were extracted using the Zymo Research ZR fungal/bacterial Miniprep Kit (Zymo Research, USA). The 16S rDNA region was amplified using primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-CTACGGCTACCTTGTACGA-3'). The PCR mix and reaction program can be seen in Tables 1 and 2 (Garner et al.,

2010). The amplicon was electrophoresis on 1.5% agarose gel, and the DNA bands were observed using a UV transilluminator. The 16S rDNA amplicon was purified and sequenced at 1st BASE, Malaysia. Each 16S rDNA sequence was aligned with the reference 16S rDNA sequence from GenBank of the National Centre for Biotechnology Information (NCBI). The phylogenetic tree was constructed using the Neighbour-Joining algorithm with 1000 bootstrap replicates using MEGA 7.0 software (Alang et al., 2019).

Table 1: Master mix composition.

Reagent	Volume (μL)	Final concentration
MyTaq® Master Mix, 2x	25	-
Forward primer	2	10 pmol
Reverse primer	2	10 pmol
DNA template	2	12.06 ng/ μL
ddH ₂ O	19	-
Total	50	

Table 2: PCR Program for 16S rDNA sequence.

No.	Reaction	Temperature ($^\circ\text{C}$)	Time	Cycle
1	Pre-denaturation	94	5 min	1
2	Denaturation	94	1 min	35
3	Annealing	54	30 sec	35
4	Extension	72	1 min	35
5	Final Extension	72	7 min	1

2.9 Data analysis

The experiments were performed in triplicate. The antibiotic sensitivity data was analyzed descriptively. The normality and homogeneity tests were performed using Shapiro-Wilk and Levene, respectively ($p > 0.05$). Data obtained, such as antioxidant activity, acid and bile salt tolerance (survival rate), and antibacterial activity, were analyzed using One-Way Analysis of Variance (ANOVA) with a $p < 0.05$ followed by Tukey Honest Significant Difference (HSD) as a post hoc test. Data were presented as mean $\pm$ standard deviation.

3 RESULTS

3.1 Characteristics of LAB Isolates from Fermented Coffee (Wine Coffee)

The result demonstrated that 25 bacteria isolated from wine coffee had similar colony characteristics. Most of the isolates had a colony morphology comprised of circular, cream-colored, contoured texture and overall edges, convex elevations, smooth-butter consistency, and opalescent optical. Those isolates

had a LAB characteristic due to forming a clear zone in MRSA added with CaCO_3 (Figure 1A). All isolates were Gram-positive, rod-shaped, and catalase-negative (Figure 1B-C).

3.2 Antioxidant Activity of LAB Isolates

The result demonstrated that all isolated LAB have antioxidant activity ranging from 36.32 to 64.31% (Figure 2). Color alterations indicated DPPH scavenging activity reactions. The highest antioxidant activity from the sample could shift the color of DPPH solutions from purple to yellow or clear appearances. Based on the results, the isolate E1 has the highest antioxidant activity, while the isolate C2 has the lowest antioxidant activity.

3.3 LAB Resistance to Low Acidity

The study showed that only 14 of 25 isolates could grow and resist pH 2, with survival rates greater than or equal to 100% (Figure 3). Among the tested isolates, A3 (115.04%), E1 (107.16%), and E2 (113.75%) demonstrated the highest survival rates, while isolate A4 exhibited the lowest survival rate (77.91%). Interestingly, 17 isolates exhibited survival rates greater than or equal to 100% at pH 3 or 4. However, 11 isolates (A2, A3, A5, C2, C3, D1, D3, D4, D5, E1, and E2) survived after 3 hours of incubation at pH 2-4, with survival rates varying from 100.39 to 129.06%. These isolates were subsequently selected for further analysis.

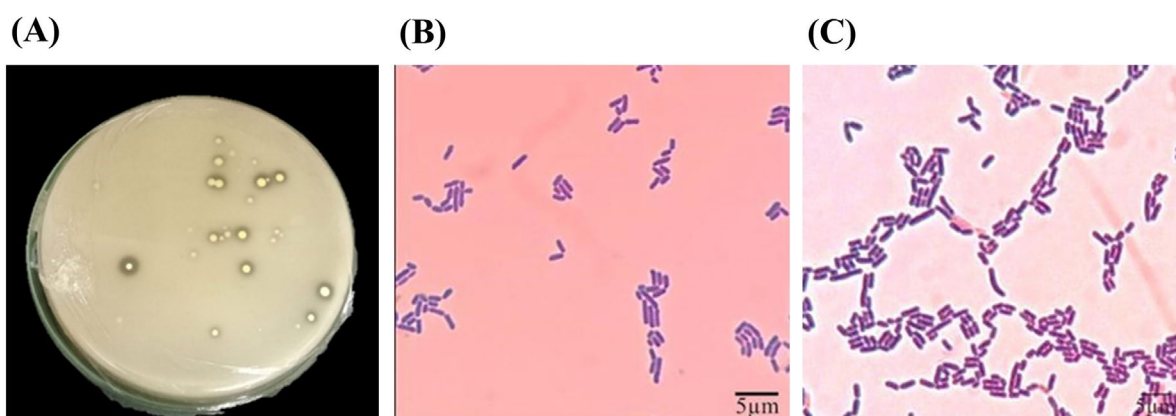


Figure 1: LAB morphology, which was isolated from wine coffee. **A)** A representative colony from sample D in MRSA containing CaCO_3 . The representative cell morphology of LAB isolates observed under a light microscope at 1000× magnification **B)** Isolate D4, **C)** Isolate E2.

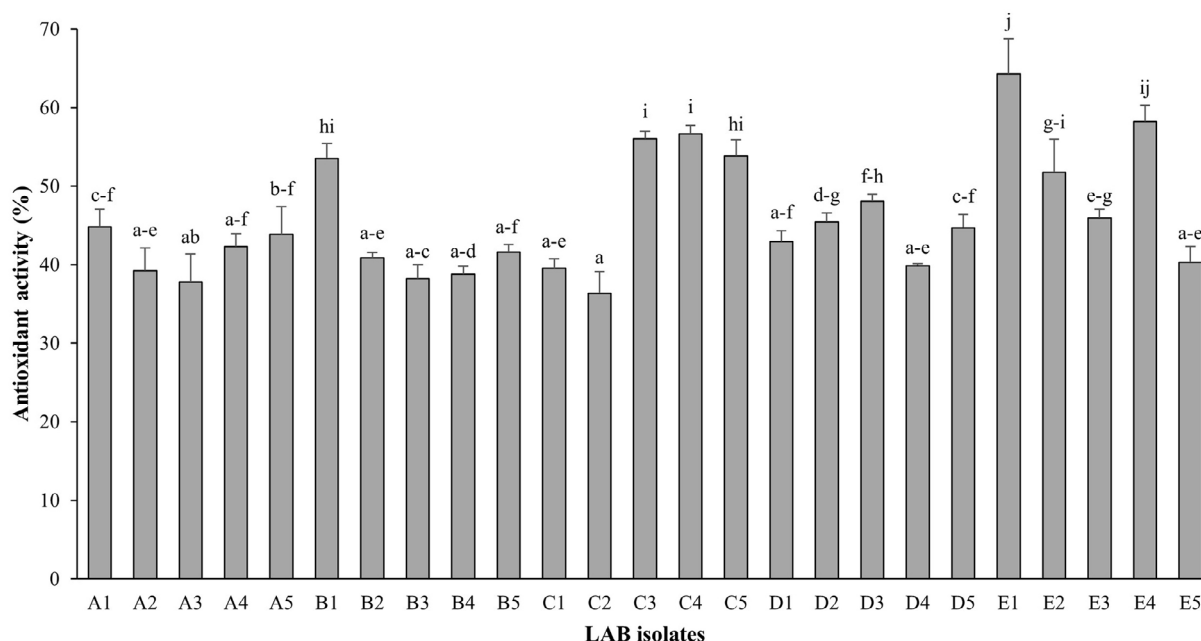


Figure 2: The antioxidant activity of LAB isolated from wine coffee. The different letters indicated significantly different antioxidant activity among isolates ($p < 0.05$) based on the Tukey HSD test ($n=3$).

3.4 Bile salt tolerance of LAB isolates

The eleven isolates selected from the low acidity test displayed a high tolerance to various bile salt concentrations. The survival rates from various bile salt concentrations (1, 1.5, and 2%) after 4 hours incubation ranged from 60 to 149% (Figure 4). Nine isolates (A3, A5, C2, C3, D3, D4, D5, E1, and E2) demonstrated the ability to survive in 2% bile salt, with survival rates greater than or equal to 100%.

The highest survival rates at 1% bile salts was exhibited by isolate E2 (119.03%), while the lowest survival rate at 1% bile salts was isolate D1 (67.86%). At 1.5% bile salts, the isolate D4 has the highest survival rate (126.24%), while the isolate D1 has the lowest survival rate (64.62%). Also, at 2% bile salt, the isolate E2 has the highest survival rate (148.03%), while the isolate D1 has the lowest survival rate (59.64%).

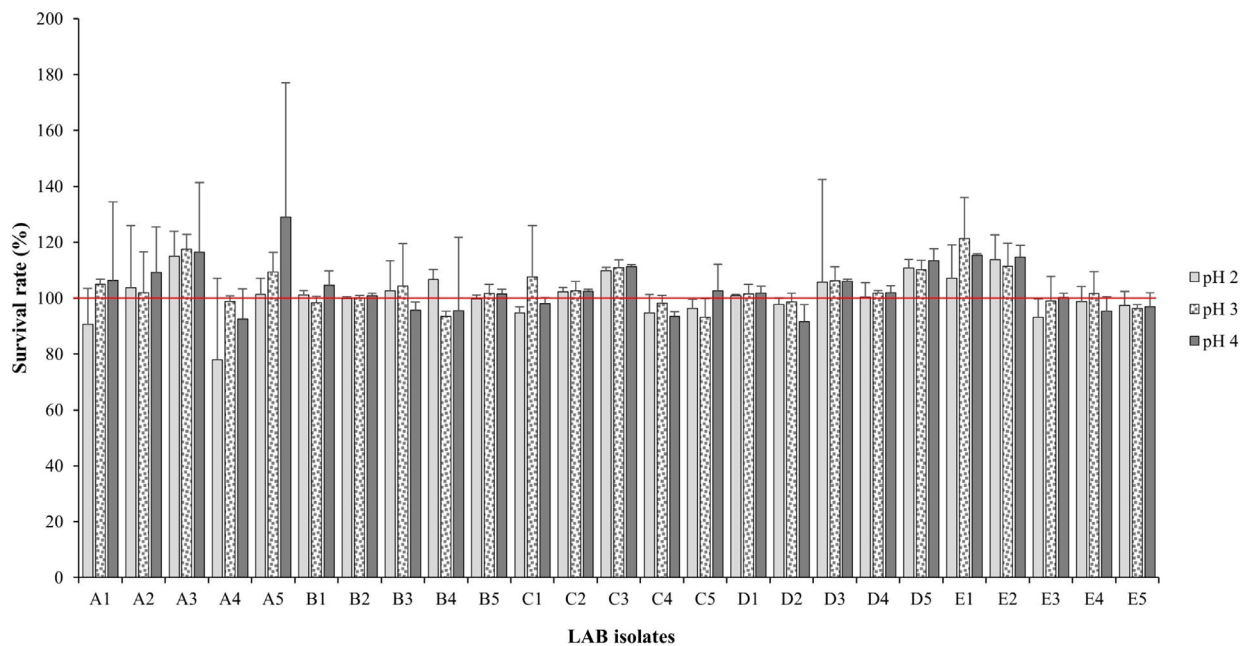


Figure 3: Survival rates of LAB after challenging under acid conditions. The survival rates did not significantly different among 25 isolates ($p>0.05$) ($n=3$).

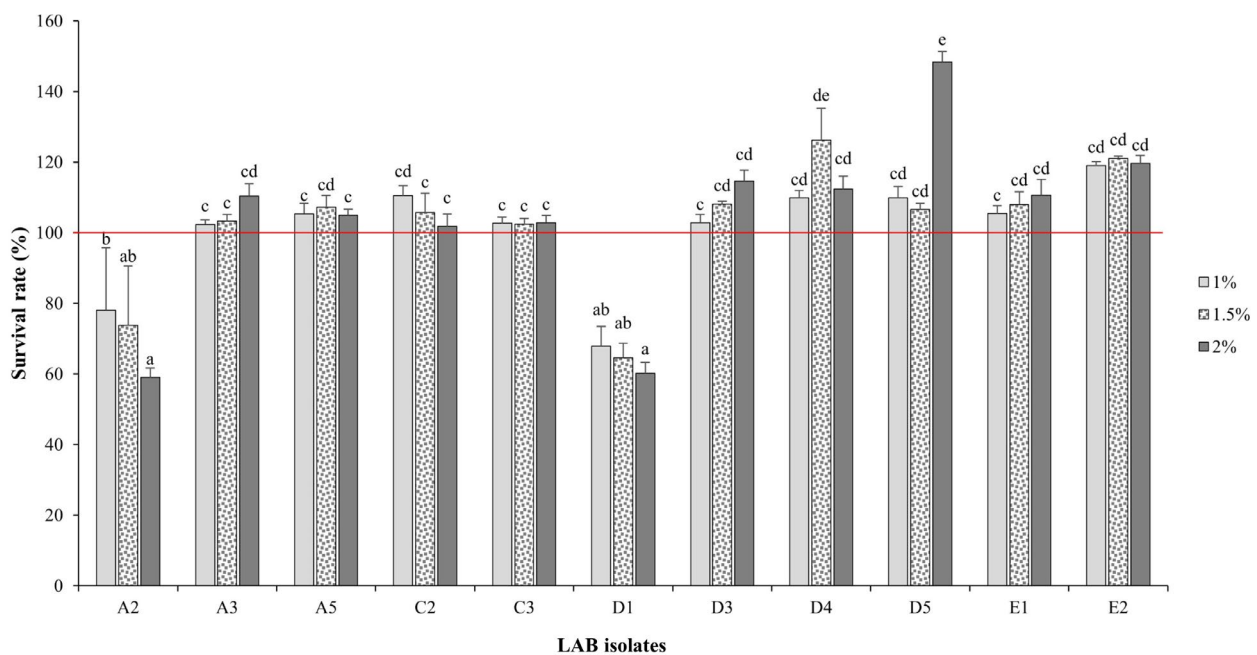


Figure 4: Survival rates of LAB after challenges under various bile salt concentrations. The different letters indicated significant differences in survival rate among isolates ($p<0.05$) based on the Tukey HSD test ($n=3$).

3.5 Antibacterial Activities of LAB Isolates

The antibacterial activity of selected LAB from wine coffee against different pathogens was evaluated and displayed in Figure 5. Nine isolates exhibit inhibitory activity against *Bacillus cereus* ATCC 1178, ranging from 50 to 95.5 AU/mL. Five isolates demonstrated inhibitory activity against *S. aureus* ATCC 29213, ranging from 0-106.2 AU/mL. Eight isolates demonstrated inhibitory activity against *E. coli* ATCC 25922, ranging from 0–122.2 AU/mL. For *S. Typhimurium* TRD1, only six isolates exhibited inhibitory activity ranging from 0-120.72 AU/mL. Interestingly, only two LAB isolates, D4 and E2, could inhibit all pathogens (Figure 5).

3.6 Antibiotic Sensitivity of LAB Isolates

The antibiotic sensitivity test measured the multidrug resistance of LAB isolated from wine coffee. The representative of nine isolates (A3, A5, C2, C3, D3, D4, D5, E1, and E2) was challenged with kanamycin 30 µg, erythromycin 15 µg, cefazolin 3 µg, and aztreonam 30 µg. The current result showed that each isolate has a different antibiotic sensitivity (Table 3). All isolates were resistant to the antibiotic's kanamycin and aztreonam. For erythromycin, isolates were classified into sensitive and intermediate categories, but all were sensitive to the antibiotic cefazolin. However, based on the sensitivity test results, no isolates could be classified as multidrug-resistant (MDR) since no isolates could resist more than three different classes of antibiotics.

3.7 Haemolytic activity of LAB isolates

Haemolytic activity was performed to determine whether the isolate had pathogenic potential. The nine isolates

(AE, A5, C2, C3, D3, D4, D5, E1, and E2) resulted in a negative result on the BAP medium (Figure 6).

3.8 The species of Selected LAB Isolates

Based on the probiotic characterization results, three potential isolates, namely E1, E2, and D4, were selected for species identification. Isolates D4 and E2 have fulfilled the requirement as probiotics. Meanwhile, isolate E1 has the highest antioxidant activity (Figure 2). The PCR results displayed that the amplicon of 16S rDNA was around 1500 bp. Based on 16S rDNA sequence similarity, D4 and E2 were identified as *Lactiplantibacillus plantarum* (Figure 7A), while E1 isolate was identified as *Lactocaseibacillus paracasei* (Figure 7B).

4 DISCUSSION

The present study aims to identify the probiotic properties of LAB isolated from wine coffee. The probiotic properties were investigated regarding their characteristics, bile salt tolerance, antibiotic sensitivity, antioxidant, antibacterial, and haemolysis activity. Probiotics must possess the capability to withstand the acidic environment in the stomach and the presence of bile salt in the small intestine, as these factors play a crucial role in their selection (Cong et al., 2024). The discovery of new bacteria with probiotic properties is still interesting because they are strain-specific and might vary from region to region (Amaral et al., 2017). In this context, the functional properties of LAB strains were evaluated and selected from wine coffee. LAB produces organic acid that influences the coffee liquor quality and the desired/undesirable sensory taste of the final product (Elhalis; Cox; Zhao, 2023).

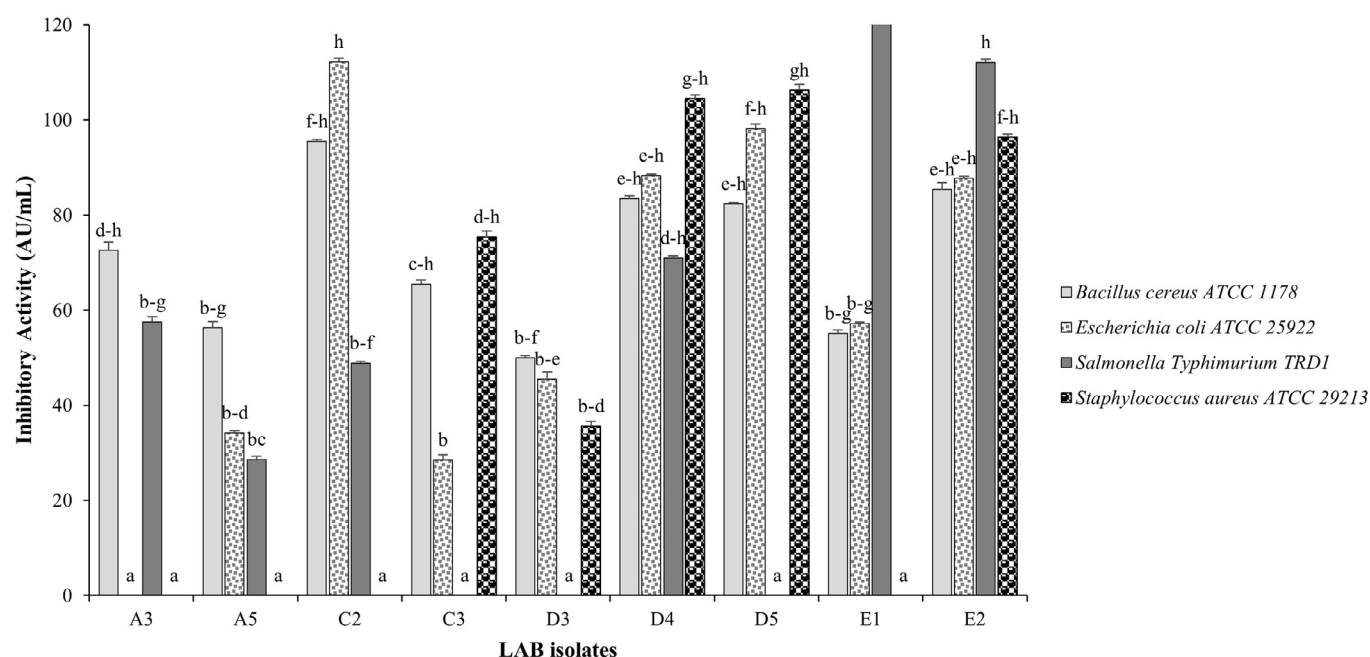


Figure 5: Antibacterial activity of selected LAB isolated from wine coffee. The different letters indicated significantly different antibacterial activity among LAB isolates and bacterial pathogens ($p < 0.05$) based on the Tukey HSD test ($n=3$).

Out of 25 LAB isolated from wine coffee, two strains with high potential as probiotics were identified as *Lactiplantibacillus plantarum* (D4 and E2), which is 100% similar to *L. plantarum* MR1 (Figure 7A). Besides, the present result found an interesting LAB with the highest antioxidant property, which was identified as *Lacticaseibacillus paracasei* (E1) with 99% similarity (Figure 7B). However, both *L. plantarum* and *L. paracasei* that were identified in the present study have the qualified presumption of safety (QPS) status and are on the Generally Recognized As Safe (GRAS) product list by the European Food Safety Authority (EFSA) and Food and Drug Administration (FDA) (EFSA et al., 2022; FDA, 2024). *L. plantarum* is a multifaceted and broadly distributed species that might be found in a variety of fruits (Trindade et al., 2022), vegetables (Bringel et al., 2005), or food (Paul et al., 2023) and is naturally resident of human mucosa (Silva-Bea et al., 2022). A previous study reported that *L. plantarum* was one of the predominant bacterial species identified during the semi-dry fermentation process of coffee (*Coffea arabica* L.) (Vilela et al., 2010). Thus, *L. plantarum* was also identified

during wet coffee fermentation (Cassimiro et al., 2023; Elhalis; Cox; Zhao, 2023). Further, *L. plantarum* was reported to attenuate gastrointestinal disturbances related to antibiotics (Lönnermark et al., 2010).

Meanwhile, *L. paracasei* inhabits diverse ecological niches, including fermented dairy products (Dehghani Champiri et al., 2023), human gastrointestinal and reproductive systems (Ljungh; Lan; Yanagisawa, 2002; Zárate; Santos; Nader-Macias, 2007), and fresh or fermented fruits (Cruz et al., 2020). However, the current study is the first report that *L. paracasei* was found during the fermentation of coffee. Interestingly, a diverse spectrum of microorganisms from wine coffee has been found in different regions, and the region-specific species have also been identified. *L. plantarum* and *Lactobacillus* sp. are major isolates detected in Brazil and India (Silva et al., 2000; Velmourougane, 2013; Vilela et al., 2010), while *Leuconostoc* species is reported in Ethiopia, Hawaii, Mexico, and Taiwan frequently (Leong et al., 2014; Schillinger et al., 2008). In West Africa, dry and wet coffee fermentation was reported to contain *Weissella*, *Leuconostoc mesenteroides*, *Lactococcus*

Table 3: The antibiotic sensitivity of LAB from wine coffee.

Isolates	Antibiotics			
	Kanamycin (30 µg)	Erythromycin (15 µg)	Cefazolin (30 µg)	Aztreonam (30 µg)
A3	R	S	S	R
A5	R	I	S	R
C2	R	I	S	R
C3	R	S	S	R
D3	R	I	S	R
D4	R	I	S	R
D5	R	S	S	R
E1	R	S	S	R
E2	R	S	S	R

Note: R (resistance), I (intermediate), S (sensitive). The LAB isolates were categorized as sensitive, S (≥ 20 mm); intermediate, I (15-19 mm); and resistant, R (≤ 14 mm).

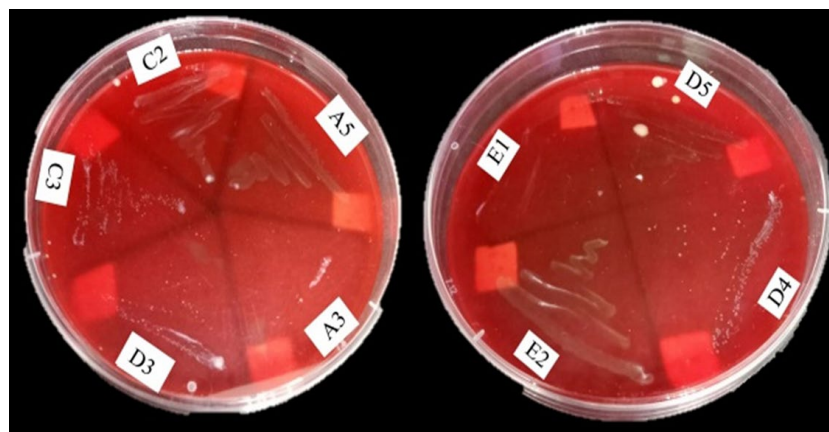
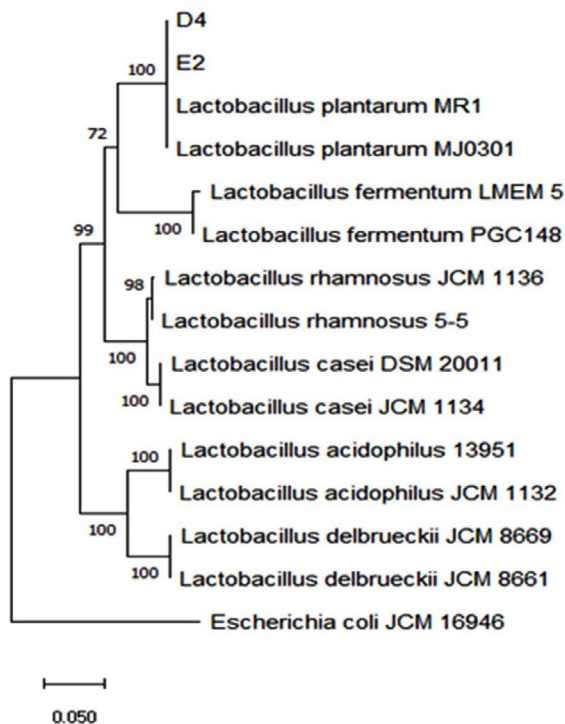


Figure 6: Haemolytic activity of selected LAB isolates from wine coffee on BAP medium.

(A)



(B)

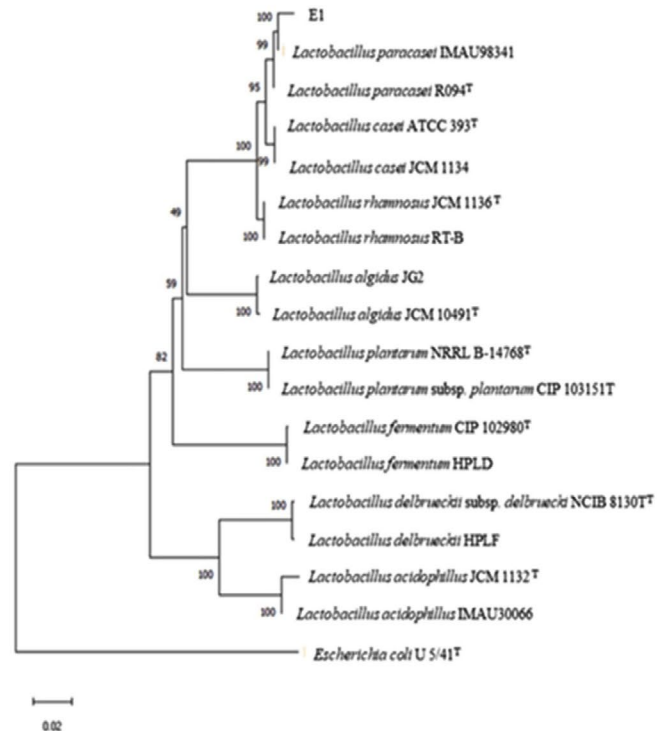


Figure 7: The phylogenetic tree of three selected isolates E1, E2, and D4, with reference strains based on 16S rDNA sequence similarity using the Neighbour-Joining algorithm and the Tamura-Nei model at 1000 bootstraps (A) D4 and E2 isolates were identified as *Lactiplantibacillus plantarum*. (B) E1 isolate identified as *Lactocaseibacillus paracasei*.

lactis subsp. *Lactis*, and *Lactobacillus fermentum* (Hamdouche et al., 2016). Civet coffee, which originated partially digested by the Indonesian palm civet, is quite popular in Indonesia, and a previous study reported that *L. paracasei* isolated from civet digestive system is the most viable bacteria that fit on coffee cherry peel flour and increase the quality of hot brewed roasted Robusta coffee (Fauzi et al., 2023). The difference bacterial found in the different regions might influence on coffee quality and characteristics.

Probiotic microorganisms must have the ability to survive in an acidic environment in the stomach, generally ranging from 1.5 to 3.5, to prevent the entry of external bacteria (Han et al., 2017). Eleven out of 25 tested strains could survive after 3 hours of incubation at pH 2-4. Similar to the previous study, several LAB strains could tolerate the acidic pH 3 (Manovina et al., 2022). The growth of LAB was slowed down when the pH dropped below 4.5, entering the stationary phase (Liao et al., 2010). Interestingly, the previous study reported that *L. paracasei* can resist an acidic environment through neutralized acid stress (Hill et al., 2018). However, the variations of low pH resistance might be related to strain specificity and the growth phase difference.

After passing through the stomach, LAB would be exposed to bile salts in the intestine. Therefore, probiotics must

have high endurance for bile salt to survive and reach the colon (Akman et al., 2021). Additionally, a previous study reported that LAB isolated from shalgam juices, including *L. paracasei* TS14, has the highest survival rate of 0.3% bile salts, while *P. acidilactici* TS16 was resistant to 1% bile salt (Sengun et al., 2024). Also, *L. plantarum* showed a high tolerance to bile salt, up to 1.5% (Akman et al., 2021). Similarly to the present study, *L. plantarum* D4, *L. plantarum* E2, and *L. paracasei* E1 have viability above 100% in 1-2% bile salt.

Four food-borne pathogens were selected to examine the antimicrobial activity of LAB. The pathogen represents the Gram-positive and Gram-negative. The antimicrobial activity of LAB is from numerous metabolites, such as hydrogen peroxide, organic acids, diacetyl, carbon dioxide, and bacteriocin (Rajoka et al., 2017). Interestingly, all LAB isolates could inhibit *B. cereus* ATCC 1178 growth (Figure 5). *B. cereus* is a Gram-positive bacteria with a thicker cell wall but more permeable peptidoglycan, making it more sensitive to metabolite produced by LAB (Haryani et al., 2023). Interestingly, isolates D4 and E2, identified as *L. plantarum*, displayed a broad spectrum of bacterial inhibition against both types of indicator bacteria. Similar to the previous result, *L. plantarum* strain LMEMh and LMEMh1 and its CFS isolated from honey exhibited antibacterial activity against Gram-positive and Gram-negative

bacteria (Roy; Mandal, 2024). Another study also reported that *L. plantarum* BP102 and its metabolites showed antibacterial activity against *E. coli* and *B. cereus* (Suryani; Jatmiko; Mustafa, 2023). However, the result also demonstrated that some LAB isolates could not inhibit *S. aureus* ATCC 29213, *S. Typhimurium* TRD1, or *E. coli* ATCC 25922 (Figure 5). These LAB antimicrobial activity results might depend on the strain, isolation source, and target microorganism. Since the CFS was neutralized, bacteriocin inhibitory like substances are presumed to be responsible for the antimicrobial effects rather than organic acids (Obioha et al., 2021).

Safety issues are critical when considering a lactic acid bacteria as a potential probiotic before administration (Chen et al., 2020). The susceptibility to antibiotics is vital due to the opportunity to transfer antibiotic-resistant genes from probiotic candidates to food-borne pathogen bacteria (Trindade et al., 2022). The results demonstrated that all LAB isolates were resistant to kanamycin and sensitive to cefazoline. Since LAB strains are often very resistant to aminoglycosides, the fact that they are resistant to kanamycin does not restrict the probiotic potential of isolates (Zhou et al., 2012). *Lactobacillus* spp. is reported to have high sensitivity to cefazolin (Ma et al., 2020). However, the differences in susceptibility from antibiotics depended on the LAB strains (Ayivi et al., 2020). A previous study reported that *L. plantarum* derived from fermented tangerine vinegar was susceptible to cefazolin, amoxicillin, imipenem, gentamicin, azithromycin, chloramphenicol, ampicillin, and tetracyclines (Sui et al., 2021). Another study reported that *L. paracasei* TS33 was susceptible to ampicillin, chloramphenicol, erythromycin, and tetracycline (Sengun et al., 2024).

The lack of haemolytic activity suggested that LAB is promising as a probiotic candidate, which is safe for humans and gut microbiota for health benefits without side effects (Yasmin et al., 2020). *S. aureus* is well-known for its haemolytic activity, which produces enzymes that break down phospholipids and disrupt the membrane stability of erythrocytes (Lee; Kim; Kang, 2022; Zhang et al., 2016). The results demonstrated that nine isolates did not have haemolytic activity (Figure 6). In line with the previous results, *L. paracasei* and *L. plantarum* did not have haemolytic activity (Kwon et al., 2021; Martín et al., 2023). In this study, *L. plantarum* D4 and E2, and *L. paracasei* E1 derived from wine coffee demonstrated probiotic potential through antibacterial and antioxidant activity. Thus, using local products of fermented coffee, such as wine coffee, might provide a great source and functional properties for human consumption. Finally, the limitation of the present study was the probiotic potential of these strains characterized only based on *in vitro* tests.

5 CONCLUSION

The isolates D4 and E2, *Lactiplantibacillus plantarum* were regarded as promising probiotics with safety and

antimicrobial properties. The isolate E1, *Lactocaseibacillus paracasei*, also exhibited probiotic properties, albeit to a lesser extent, and showed the highest antioxidant activity among other isolates. Further research is required to validate the strain-specific probiotic potential using whole genome sequencing, and pre-or clinical trials should be conducted to verify their health benefits. These selected isolates are expected to improve the functional attributes of fermented products.

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7 AUTHORS' CONTRIBUTIONS

Conceptual Idea: Jatmiko, Y.D.; Methodology design: Jatmiko Y.D.; Suharjono, S.; Ardyati, T.; Mustafa, I.; Data collection: Mustamin, A.; Puja, L.R.; Data analysis and interpretation: Mustamin, A.; Puja, L.R.; Atho'illah, M.F.; Arifah, S.N.; and Writing and editing: Mustamin, A.; Puja, L.R.; Atho'illah, M.F.; Arifah, S.N.; Suharjono, S.; Ardyati, T.; Mustafa, I.; Jatmiko Y.D. All authors read and approved of the final manuscript.

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