PROBIOTIC LACTIC ACID BACTERIA FROM PAPAYA, BANANA FRUITS AND FERMENTED RED RAW RICE BATTER: ISOLATION, SCREENING AND ANTIMICROBIAL PROPERTY

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ABSTRACT: *Probiotic lactic acid bacteria are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Therefore, the purpose of the study was to isolate the lactic acid bacteria from papaya fruit, banana fruits, fermented papaya fruit, fermented banana fruit and fermented red raw rice batter to screen the probiotic potential of isolated strains. The gram staining and catalase tests were performed to identify the presumptive lactic acid bacteria. Further, the isolated strains were subjected to diverse biochemical and physiological tests, such as different carbohydrate fermentation test, gas production from fructose and glucose and ability to survive under different temperature, pH concentration, bile salt concentration and sodium chloride concentration. The antimicrobial test was done for all selected isolated bacterial strains against E. coli, Pseudomonas aeruginosa and Staphylococcus aureus. Eight bacterial strains were isolated, and all isolated bacteria strains were gram positive, catalase negative, non-spore forming and non-motility strains. Based on the antimicrobial activity, the R2 isolated from fermented red raw rice batter and FB1 and FB2 isolated from fermented banana fruit bacterial strains have ability to control E. coli, Pseudomonas aeruginosa and Staphylococcus aureus than other bacterial strains. Therefore, the R2, FB1 and FB2 bacterial strains identified as probiotic lactic bacterial strains due to antimicrobial activity. Based on the biochemical and physiological tests indicated that R2 can survive at low pH (1.5 to 4), high temperature (47^{0}C), different bile salt concentrations (0.3%, 0.8% and 1.0%) and different sodium chloride concentrations (2% ,6% and 10%). Therefore, this study concluded that R2 isolated from fermented red raw rice batter considered as potential probiotic lactic acid bacteria. Further study could be performed to confirm its probiotic potential and identify strain based on molecular techniques.*

Keywords: Antimicrobial activity, Characterization, Lactic acid bacteria, Probiotic

1. INTRODUCTION

The lactic acid bacteria are widespread diverse group of microorganisms can be found in any environment, such as plant (fruits and vegetables), fermented foods, insects, animals and mucosal surface of humans. Moreover nowadays, lactic acid bacteria play vital role in food industry for the production of fermented food products (Florou-paneri et al., 2013). Lactic acid bacteria are Gram positive; catalase negative, non-spore former, usually non-motile rods or coccobacilli and the microorganisms can ferment carbohydrates to produce lactic acid as main fermented products (Khalid, 2011).

Probiotic microorganisms are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Lactic acid bacteria are considered as commercial probiotics due to their health promoting effects on humans. Major health benefits of probiotics are exerting antimicrobial effect on enteric pathogens, lowering serum cholesterol, scavenging free radicals, enhancing immune system and exhibiting antimutagenic

activities (Ljungh & Wadstrom, 2006). The probiotics in food able to transit through stressful gastrointestinal environment and exert beneficial effects in host. But successful delivery of these bacteria in to the human intestine through food matrix is challenging due to stresses encountered during processing, storage and gastric transition cause loss of viability (Nag, 2011).

Lactic acid bacteria can be isolated from natural ecological niches. Already published studies revealed that, the heterogeneous lactic acid bacteria are associated with fruits, fermented plant beverages, pickles and traditional fermented food products (Abubakr & Al-Adiwish, 2017 & Nuraida, 2015). The use of lactic acid bacteria and their metabolic products are generally considered as safe (Wu et al., 2017). Presently very fewer probiotic genera such as *L*actobacillus, *Bifidobacterium Lactiplantibacillus and Saccharomyces* are widely utilized (Tarapatzi et al., 2022). Therefore, there is a great need for new lactic acid bacteria strains that exhibit probiotic potential and that have a beneficial impact on human. Therefore, this study was aimed to isolate lactic acid bacteria from banana fruits, papaya fruits, fermented papaya fruits, fermented banana fruits and fermented red raw rice batter to screen the probiotic potential.

2. METHODOLOGY

2.1. Collection of Samples

Ripen red lady papaya fruit (*Carica papaya*) and kadali banana fruit (*Musa paradisiaca*) were randomly collected from Jaffna market, Sri Lanka aseptically. The fruits were packed into the sterile bag and transported to the laboratory aseptically.

2.2. Fermented Rice Batter Preparation

The approximately 100 g of red raw rice (Addakari variety) was washed with the water and 250 ml of water was added in to rice. After that it was cooked at 100^{O}C for 10 min and allowed to cool at room temperature (30±2^{O}C). After cooling, 100 ml of sterile water was added to cooked rice and allowed to ferment in the earthen pot for 24 hours at room temperature (30± 2^{0}C).

2.3. Isolation of Lactic Acid Bacteria Strains

2.3.1. Isolation of strains from banana and papaya fruits

The ripen banana fruits were cut in to pieces with peel and it was mashed by using motor and pestle. Approximately 10 g of mashed banana fruit sample was mixed with 90 ml of peptone water (0.1% w/v) and diluted serially. The dilutions of mixed solution with 10 to 1000-fold were used to isolate the strains. The same procedure was repeated to isolate the strains from ripen papaya fruits.

2.3.2. Isolation of strains from fermented banana and papaya fruits

The ripen banana was cut in to the small pieces and it was mashed by using mortar and pestle. Then the mashed banana fruit was put in to sterilized beaker and the beaker top part was covered with aluminum foil. It was kept for 24 hours for the fermentation under room temperature (30 ± 2 C). The approximately 10 g of fermented banana fruit was mixed with 90 ml of peptone water (0.1% w/v) and serially diluted to 10 to 1000 fold to isolate strains . The same procedure was repeated to prepare fermented papaya sample. The serial dilution procedure was repeated to isolate strains from fermented papaya fruit sample and fermented red raw rice batter.

The method described by Abubakr & Al-Adiwish, 2017 was used to isolate lactic acid bacteria from diluted sample. The 0.1ml of each diluted sample was spread on sterilized MRS (De man, Rogosa and Sharpe) agar medium. The inoculated media were incubated in an anaerobic jar at 37^0C for 24 hours.

2.4. Characterization of Isolated Lactic Acid Bacteria Strains

2.4.1. Morphological and biochemical characterization of isolated lactic acid bacteria strains

Gram staining and catalase test were performed as presumptive lactic acid bacteria test. The overnight culture of each of the isolated strain was Gram stained and observed under the microscope. The catalase test was performed by using 3% H_2O_2. The endospore staining and hanging drop method of motility test were performed to isolated lactic acid bacteria strains.

The abilities of isolated strains to utilize different carbohydrates such as glucose, lactose, galactose, sucrose, fructose, mannitol, starch, arabinose, xylose and sorbitol were determined by adding specific sugar to the basal medium composed of peptone, sodium chloride and phenol red and incubating isolated strains separately at 37^0C for 72 hours. The colour change from red to yellow reflecting the test as positive.

Citrate utilization test, urease test, Voges-Proskauer test and gas production from glucose and fructose were determined by the methods described by Thakur et al., 2017.

2.4.2. Physiological characterization of isolated lactic acid bacteria strains

Growth of isolates was determined in nutrient broth at 37^0C and 47^0C for 72 hours and at pH 1.5, 2, 4, 6, 8, 10 and 11 by incubating culture at 37^0C for 48 hours. The bile salt tolerance was assessed by incorporating bile salt at 0.3%, 0.8% and 1% in nutrient broth and incubating overnight culture at 37^0C for 48 hours. The salt (sodium chloride) tolerance was assessed by incorporating salt at 2%, 6% and 10% in nutrient broth and incubating overnight culture at 37^0C for 48 hours. The microbial growth was determined by measuring the absorbance at 600 nm.

2.5. Antimicrobial Activity of Isolated Strains

The antimicrobial activities of isolated lactic acid bacteria strains against indicator microorganisms of *E. coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* strains were tested by using the agar well diffusion assay. The single colony of each lactic acid bacteria strain was cultured in 20 ml of nutrient broth medium and incubated for 24 hours at 37°C. The fermented nutrient broth tubes centrifuged (13 000 rpm, 15 min) and filtered using 0.2 µm filter to prepare cell free supernatant. A suspension of 10^6 CFU /ml of *indicator microorganism* was spread onto the nutrient agar, into which 10 mm wells had been dug. About 100 µl of supernatant from each of lactic acid bacteria isolate was poured into each well, and plates were incubated for 24 h at 37°C. *Un-inoculated* nutrient broth was used as control. The diameter of the clear zone was measured after 24 hours of incubation.

2.6. Statistical Analysis

All the experiments were performed in triplicate. Data were statistically analysed with Minitab 19 statistical software. One-way analysis of variance (ANOVA) was used to analysis the significant difference between means (significance level at $p = 0.05$).

3. DISCUSSION AND RESULTS

3.1. Isolation of Lactic Acid Bacteria Strains

The eight bacterial strains were isolated from banana fruits, papaya fruits and fermented banana fruits, fermented papaya fruits and fermented red raw rice batter. Among eight strains, two bacterial strains (B1 and B2) were isolated from banana fruits, two bacterial strains (FB1 and FB2) were isolated from fermented banana fruits, one bacterial strain (PO) was isolated from papaya fruits, one bacterial strain (FP2) was isolated from fermented papaya fruits and two bacterial strains (R1 and R2) were isolated from fermented red rice batter.

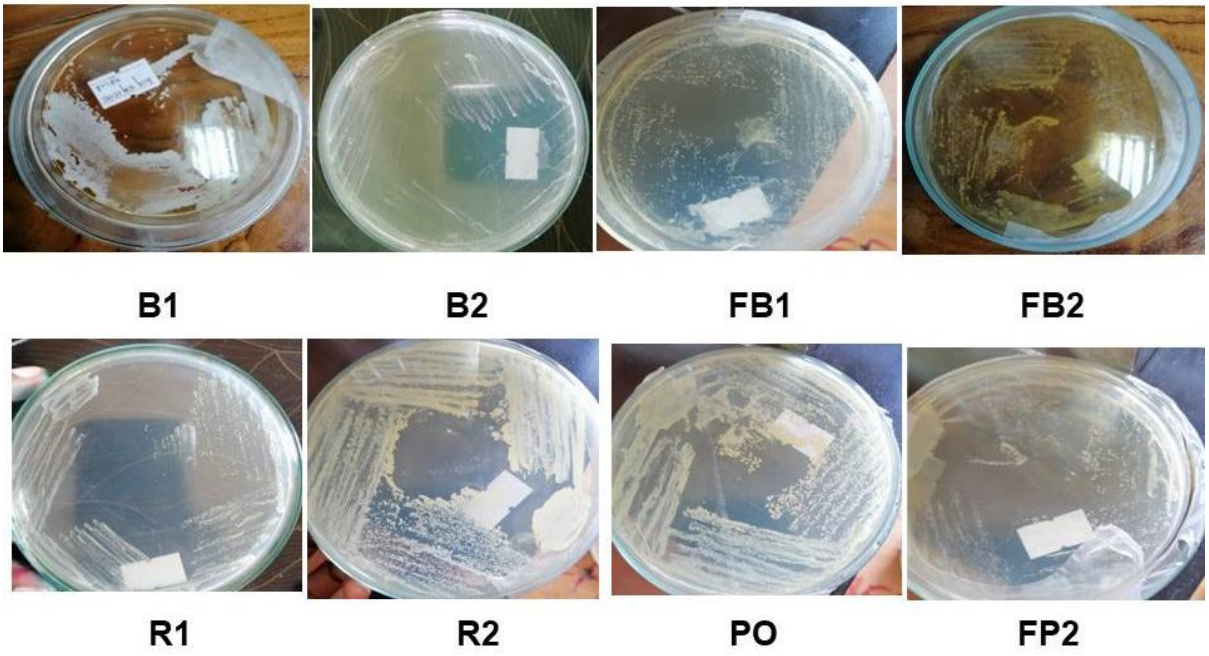

Figure1. The Isolated Lactic Acid Bacteria Strains

3.2. Characterization of Isolated Lactic Acid Bacteria Strains

The isolated bacterial strains were Gram positive and catalase negative. Therefore, the all isolated strains were considered as presumptive lactic acid bacteria. Further, all isolated strains were non-motile and non-spore formers with shape of cocci. The lactic acid bacteria are Gram positive, catalase negative, non-motile and non-spore formers with shape of cocci or rod (Khalid, 2011, Abubakr & Al-Adiwish, 2017 & Thakur et al., 2017).

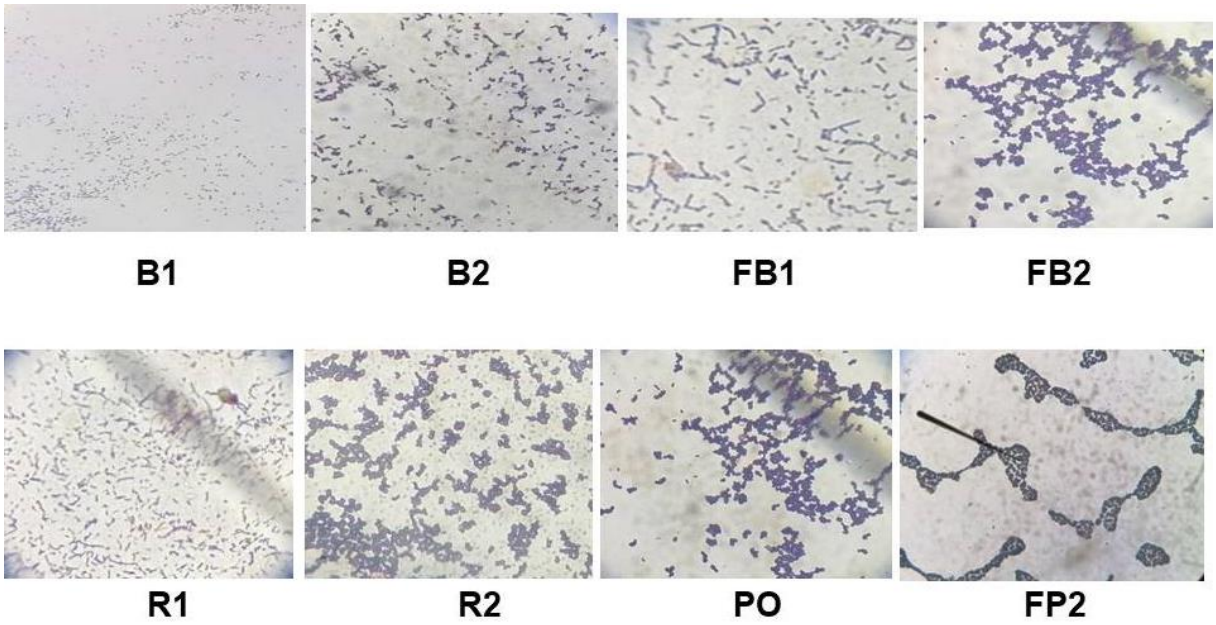

Figure 2. Microscopic Picture of Gram-Stained Isolated Lactic Acid Bacteria
Strains of B1 and B2 isolated from banana fruit, FB1 and FB2 isolated from fermented banana fruit, R1 and R2 isolated from fermented red raw rice batter, PO isolated from papaya fruit and FP2 isolated from fermented papaya fruits

The lactic acid bacteria are associated with fruits naturally. Verón et al (2017) isolated 17 strains of lactic acid bacteria from *O. ficus-indica* fruits and identified the isolated strains as *Lactobacillus plantarum* and *Fructobacillus fructosus*. The already published studies revealed that strains of *Fructobacillus fructosus, Lactobacillus kunkeei, F. Pseudoficulneus* and *F. Durionis* isolated from chinese fruits and flowers, *Lactiplantibacillus plantarum, Lacticaseibacillus casei, Weissella cibaria and Pediococcus* isolated from fruits processing residues, *Pediococcus pentosaceus* and *Lactobacillus plantarum* isolated from grape fruits (Sakandar et al., 2019, De Amorim Trindade et al, 2022 & Taroub et al., 2019). Therefore, lactic acid bacteria could be isolated from natural environment to assess the probiotic potential of lactic acid bacteria.

3.2.1. Physiological characterization of isolated lactic acid bacteria strains

pH tolerance test

The probiotic need to be survived under the stomach pH of 1.5 – 2 for 4 hours or more before transport to small intestine. Table 1 shows the abilities of isolated bacterial strains to grow at different pH conditions, Such as pH 1.5, pH 2, pH 4, pH 6, pH 8, pH 10 and pH 11. The B1, FB1, FB2, R2 and FP2 bacterial strains are able to grow in these pH ranges. Therefore these bacterial strains have the probiotic ability, and it can be survived in gastric pH of 1.5 -2. The B2 bacterial strain able to grow in low pH range, but it doesn't have the ability to tolerate the high pH conditions. The R1 and PO bacterial strains can be survived in high pH conditions, but doesn't grow in low pH ranges of 1.5 and 2.0.

Table1. Growth of Isolated Lactic Acid Bacteria Strains at Different pH Range

Bacterial strains	PH 1.5	pH 2	pH 4	pH 6	pH 8	pH 10	pH 11
B1	+	+	+	+	+	+	+
FB1	+	+	+	+	+	+	+
B2	+	+	+	+	-	-	-
FB2	+	+	+	+	+	+	+
R1	-	-	+	+	+	+	+
R2	+	+	+	+	+	+	+
PO	-	-	+	+	+	+	+
FP2	+	+	+	+	+	+	+

Note: (+) : Positive growth, (-) : No turbidity. Strains of B1 and B2 isolated from banana fruit, FB1 and FB2 isolated from fermented banana fruit, R1 and R2 isolated from fermented red raw rice batter, PO isolated from papaya fruit and FP2 isolated from fermented papaya fruits

Mulaw et al. (2019) reported that the *Lactobacillus* spp isolated from traditional fermented food products survived at pH of 2.0. Szutowska and Gwiazdowska (2021) indicated that the *L. plantarum* strains exhibited the best growth abilities under low pH conditions. The bacterial strain of R2 was shown significantly higher growth rate in pH 1.5, 2, 4, 6, 8 and pH 10 compared with other isolated bacterial strains. Therefore, R2 isolated from fermented red raw rice batter is considered as potential bacteria to survive under gastric environment.

Temperature tolerance test

The all isolated bacterial strains were able to grown in 37^0C temperature. The isolated strains of bacterial cultures of B1, FB2 and R2 were shown significant growth rate at temperature of 47^0C. Therefore, these bacterial strains have the ability to tolerate the high temperature during the processing of food products. The R1 didn't grow at 47^0C temperature and it cannot survival in high temperature condition.

Sodium chloride tolerance test

According to Menconi et al. (2014), higher sodium chloride tolerance would be a requirement for probiotic lactic acid bacteria to be used as commercial strains. Table 2 shows the growth of isolated bacterial strains of B1, B2, FB1, FB2, R1, R2, PO and FP2 in different sodium chloride concentrations. The isolated bacterial strains of B1, B2, FP2 and R2 were grown in 2%, 6% and 10% of sodium chloride concentrations. Therefore these bacterial strains have the ability to tolerate the high sodium chloride concentrations. But isolated bacterial strains of FB1, FB2, R1 and PO weren't grown in high sodium chloride concentration.

Table 2. Growth of Isolated Lactic Acid Bacteria Strains in Different Sodium Chloride Concentration

Bacterial strains	2%	6%	10%
B1	+	+	+
FB1	+	-	-
B2	+	+	+
FB2	+	+	-
R1	+	-	-
R2	+	+	+
PO	+	-	-
FP2	+	+	+

Note : (+): Positive growth, (-) – No turbidity. Strains of B1 and B2 isolated from banana fruit, FB1 and FB2 isolated from fermented banana fruit, R1 and R2 isolated from fermented red raw rice batter, PO isolated from papaya fruit and FP2 isolated from fermented papaya fruits

Bile salt tolerance test

The host factors that may affect the survival of probiotic are high acid and bile salt concentration in gastrointestinal tract. The bile salt affects the metabolic activity and colonization of beneficial bacteria. Therefore the pH and bile salt tolerance tests were performed to select potential probiotic lactic acid bacteria. The bacterial strain of R1 wasn't grown in any of the bile salt concentrations. The bacterial strains of B1, FB1, B2, FB2, R2, PO and FP2 were showed significant growth in all three-bile salt concentrations (0.3%, 0.8% and 1%), therefore these bacterial strains have the abilities to tolerate high bile salt concentration in human gut.

The concentration of bile salts in the small intestine ranges from approximately 0.2 to 2 %(w/v) depending upon individual and amount of food ingested (Kristoffersen, 2007). Therefore bacterial strains of B1, FB1, B2, FB2, R2, PO and FP2 have the ability to act as probiotic lactic acid bacteria. Mulaw et al. (2019) reported that the *Lactobacillus* spp showed tolerance to bile salt at the concentration of 0.3%. Szutowska & Gwiazdowska (2021) indicated that the *L. plantarum* strains exhibited the best growth abilities under different bile concentration of 0.25%, 0.5% and 1.0%.

Table 3. Growth of Isolated Lactic Acid Bacteria Strains in Different Bile Salt Concentrations

Bacterial strains	0.3%	0.8%	1%
B1	+	+	+
FB1	+	+	+
B2	+	+	+
FB2	+	+	+
R1	-	-	-
R2	+	+	+
PO	+	+	+
FP2	+	+	+

Note : (+) – Positive growth, (-) : No turbidity . Strains of B1 and B2 isolated from banana fruit, FB1 and FB2 isolated from fermented banana fruit, R1 and R2 isolated from fermented red raw rice batter, PO isolated from papaya fruit and FP2 isolated from fermented papaya fruits.

3.2.2. Biochemical characterization of isolated bacterial strains

The bacterial strain of B1 fermented the fructose, sucrose, galactose, lactose, arabinose, mannitol, starch and sorbitol, but not utilized the glucose and xylose. The bacterial strain of FB1 fermented the fructose, sucrose, galactose, lactose, arabinose and xylose, but not utilized the glucose, mannitol, starch and sorbitol. The bacterial strain of B2 fermented the fructose,

sucrose, galactose, lactose, arabinose, and mannitol, but not utilized the glucose, xylose, starch and sorbitol.

The bacterial strain of FB2 fermented the fructose, sucrose, glucose, galactose, lactose, arabinose, mannitol, starch and sorbitol, but not utilized the xylose. The strain of R1 fermented the fructose, sucrose, glucose, galactose, lactose, arabinose, starch and sorbitol, but not utilized the mannitol and xylose. The bacterial strain of R2 fermented the fructose, sucrose, glucose, galactose, lactose, arabinose and mannitol but not ferment the xylose, starch and sorbitol. The bacterial strain of PO fermented the fructose, sucrose, glucose, galactose, lactose and mannitol, but not utilized the arabinose, xylose, starch and sorbitol. The bacterial strain of FP2 fermented fructose, sucrose, galactose, lactose, arabinose, mannitol, starch and sorbitol but not utilized the glucose and xylose.

Table 4. Carbohydrate Fermentation Test of Isolated Lactic Acid Bacterial Strains

Bacterial strains	Glucose	Lactose	Galactose	Sucrose	Fructose	Mannitol	Starch	Arabinose	Xylose	Sorbitol
B1	-	+	+	+	+	+	+	+	-	+
FB1	-	+	+	+	+	-	-	+	+	-
B2	-	+	+	+	+	+	-	+	-	-
FB2	+	+	+	+	+	+	+	+	-	+
R1	+	+	+	+	+	-	+	+	-	+
R2	+	+	+	+	+	+	-	+	-	-
PO	+	+	+	+	+	+	-	-	-	-
FP2	-	+	+	+	+	+	+	+	-	+

Note : (+) : Ferment the specific carbohydrate, (-) : Not ferment the specfic carbohydrate. Strains of B1 and B2 isolated from banana fruit, FB1 and FB2 isolated from fermented banana fruit, R1 and R2 isolated from fermented raw rice batter, PO isolated from papaya fruit and FP2 isolated from fermented papaya fruits

The all isolated strains didn't utilize citrate and arginine. The isolated strains were urease test negative, Vogas-Proskauer test negative and homo-fermentative strains (didn't produce gas from fermentation of fructose and glucose).

3.3. Antimicrobial Activity of Isolated Lactic Acid Bacterial Strains

According to the table 5, B1 bacterial strain controlled the Escherichia coli but uncontrolled the Pseudomonas aeruginosa and Staphylococcus aureus. The FB1, FB2 and R2 have the ability to control the *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus.* The B2 bacterial strain controlled the *Escherichia coli* and *Pseudomonas aeruginosa* but uncontrolled the *Staphylococcus aureus*. The bacterial strain of R1 did not control the any of the tested pathogenic microorganisms. The PO and FP2 bacterial strains controlled the *Escherichia coli* and *Staphylococcus* aureus but uncontrolled the *Pseeudomonas aeruginosa.*

Based on diameter of clear zone obtained from each of the lactic acid bacteria against tested pathogens, R2 inhibited the growth of pathogens significantly higher than other isolates. Mulaw et al. (2019) reported that the *Lactobacillus* isolates obtained from Egyptian dairy product had a strong antibacterial effect against *E. coli* with zone of incubation of 20.67 mm.

Table 5. Antimicrobial Activity of Isolated Lactic Acid Bacteria Strains

Bacterial strains	*Staphylococcus aureus.*	*Pseudomonas aeruginosa*	*Escherichia coli*
B1	NA	NA	17 ± 0.1^{b}

FB1	16±0.2[b]	16±0.3[b]	16±0.2[c]
B2	NA	16±0.3[b]	16±0.2[c]
FB2	16±0.1[b]	16±0.2[b]	16±0.2[c]
R1	NA	NA	NA
R2	22±0.1[a]	17±0.3[a]	23±0.3[a]
PO	16±0.3[b]	NA	16±0.2[c]
FP2	16±0.2[b]	NA	16±0.1[c]

Note : NA – No antimicrobial activity. Diameter of clear zones were measured in mm and strains of B1 and B2 isolated from banana fruit, FB1 and FB2 isolated from fermented banana fruit, R1 and R2 isolated from fermented red raw rice batter, PO isolated from papaya fruit and FP2 isolated from fermented papaya fruit. Results were represented with mean ± standard deviation in triplicate. [ab]Means within column with different superscripts are significantly different (p <0.05).

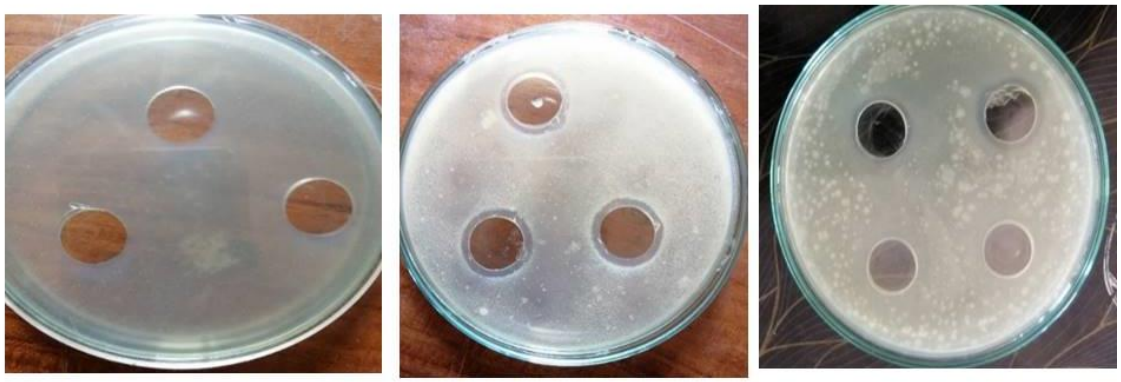

Figure 3. Antimicrobial *Activity of R2 against Pseudomonas aeruginosa, E. coli, and Staphylococcus aureus*

The isolation and identification of novel probiotic lactic acid bacteria for the production of probiotic food products to improve human health remains to be great need. Already published study indicated that the *Lactobacillus plantarum* and *Pediococcus pentosaceus* isolated from kimchi could be acted as novel probiotics due to higher degree of acid and bile salt tolerance and antimicrobial activity (Ryu and Chang, 2013).

The lactic acid bacteria isolated from traditional cheese such as *E. faecium*, *E. durans*, *E. durans* and *E. durans* have potential use as novel probiotic strains due to antibiotic resistance, bile and gastric juice tolerance, ability to survive at different pH and temperature ranges and salt concentrations (Mohammed and Çon, 2021). The probiotic lactic acid bacteria should survive under acidic environmental in the gastrointestinal tract, tolerate the bile salt concentration and inhibit the growth of pathogens (Hawaz, 2014). According to the antimicrobial activity, biochemical characterization and physiological characterization, R2 strain isolated from fermented red raw rice batter is considered as potential probiotic lactic acid bacteria.

4. CONCLUSION

The utilization of lactic acid bacteria generally considered as safe. It could be isolated from banana fruits, papaya fruits, fermented banana fruits, fermented papaya fruits and fermented red raw rice batter. Based on the biochemical and physiological characterization and wide spectrum antimicrobial activity, R2 isolated from fermented red raw rice batter was considered as potential probiotic lactic acid bacteria.

5. LIMITATIONS AND FUTURE RESEARCH VENUES

The complete probiotic potential studies and health risk studies should be done. Therefore, further studies of antibiotic resistance, undesirable biochemical characteristics, proteolytic activity and hemolytic activity and molecular characterization of R2 lactic acid bacteria isolated from fermented red raw rice batter will be conducted at Department of Biosystems Technology, Faculty of Technology, University of Jaffna.

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