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Isolation and characterization of lactic acid bacteria from sugarcane waste

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ABSTRACT

Aims: Researchers are focusing more on the isolation of new probiotic bacteria to increase varieties for the growing market demand. This study aimed to isolate lactic acid bacteria (LAB) strains from sugarcane waste materials and evaluate its characteristic.
Methodology and results: In the present study, two strains of LAB (Isolates A and B) were isolated from sugarcane waste and investigated *in vitro* for their characteristics as potential probiotics. These isolates were evaluated on their characteristics based on four biochemical tests (acid tolerance, bile tolerance, microbial adhesion, and phenol resistance), with the commercial strain *Lactobacillus* isolated from Yakult® served as a positive control. Both isolated strains (>8 $\log_{10}$ CFU/mL) displayed higher survivability than control (>6 $\log_{10}$ CFU/mL) in simulated gastrointestinal conditions at pH 2.0 and pH 6.9 after 24 h. Furthermore, both isolated LABs were resistant to inhibitory substances which are 0.05-0.3% bile and 0.4% phenol. For bile tolerance, isolate A (OD 6.83) had a higher absorbance at 0.3% bile concentration as compared to isolate B (OD 2.20). However, isolate B (7.49 $\log_{10}$ CFU/mL) showed higher resistance towards 0.4% phenol than isolate A (7.11 $\log_{10}$ CFU/mL) after 24 h. Both isolate A and isolate B displayed low cell surface hydrophobicity, strong electron donor, and basic characteristic.
Conclusion, significance and impact of study: Both isolates were able to survive under gastrointestinal stress conditions, implying their potential as probiotics. This study demonstrated that valuable products such as probiotic strain could be isolated from sugarcane wastes to use in food production or medical treatment.

Keywords: Food waste, gastrointestinal tolerance, lactic acid bacteria, microbial adhesion, phenol resistance

INTRODUCTION

There has been increasing attention to the health benefits of consuming probiotics such as lactic acid-producing bacteria worldwide. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill *et al.*, 2014). Probiotics are often linked with gut health where they aid in maintaining health and the balance of intestinal microbiota (Pundir *et al.*, 2013). Furthermore, probiotics also play an important role in various medical conditions, such as diarrhea, hypercholesterolemia, type-2 diabetes, inflammatory bowel disease, lactose intolerance, poor immune function, irritable bowel syndrome, cancer, and oral health (Yoon *et al.*, 2014; Li *et al.*, 2016; Sharif *et al.*, 2017; Gayathri and Vasadha, 2018; Jia *et al.*, 2018; Abdo *et al.*, 2019; Asan-Ozusaglam and Gunyakti, 2019; Kaklamanos *et al.*, 2019; Mostafa *et al.*, 2020).

However, several criteria need to be met by lactic acid bacteria prior to be declared as a probiotic. Probiotics should be non-toxic and generally recognized as safe (GRAS) (Lin *et al.*, 2006). Besides that, the ability to adhere to the intestinal epithelium of the host is also an important characteristic of probiotics as they are required to survive and colonize the gastrointestinal tract (Kesen and Aiyegoro, 2018). Furthermore, probiotics should be able to exert an antagonistic effect against pathogens (Halder *et al.*, 2017). Most importantly, at least 10^6-10^7 CFU/mL probiotics should be consumed in order to confer a good health benefit to the host (Jayamanne and Adams, 2006).

The discovery of new and functional ingredients such as probiotic strains could improve human diet variation (Khedid *et al.*, 2009). In the recent years, probiotic strains were widely researched on its symbiotic with prebiotic or incorporated into food or beverages as a value-added ingredient (Yee *et al.*, 2019; Lai *et al.*, 2020a; Lai *et al.*, 2020b; Lai *et al.*, 2020c; Chean *et al.*, 2021; How *et al.*, 2021; Lai *et al.*, 2021). Furthermore, researchers are often searching for new isolated probiotics strains that

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could exert better characteristics such as higher survivability under acidic or gastrointestinal conditions than available probiotics. This is to ensure a high number of viable probiotics survive through the gastrointestinal tract to confer health properties towards the host (Alkalbani *et al.*, 2019).

Sugarcane is considered one of the main crops in the world with 1.8 to 2.0 billion tonnes of production annually (Figueroa-Rodríguez *et al.*, 2019; Pipitpukdee *et al.*, 2020). The high production of sugarcane eventually increased the production of waste. Hence, beneficial components such as sugars in sugarcane waste could be utilized as a carbohydrate or energy source for the growth of lactic acid bacteria (Apás *et al.*, 2008; Younis *et al.*, 2010). Sugarcane waste had been utilized as animal feed and nutrient source for the cultivation of microorganisms (Apás *et al.*, 2008). However, valuable products such as potential probiotic strain could also be isolated from these readily available wastes and evaluated for probiotic characteristics. These potential isolated strains can be used in food production or medical treatment as an alternative source of probiotics to satisfy its growing demand in the market (Sarao and Arora, 2017).

To date, limited studies are isolating and characterizing lactic acid bacteria (LAB) from sugarcane waste. Therefore, this study was conducted to isolate and evaluate the lactic acid bacteria from sugarcane waste. The lactic acid bacteria strain was tested on its tolerance to gastric juice and bile, adherence to solvent, and its resistance to phenol.

MATERIALS AND METHODS

Materials

Two batches of sugarcane waste samples were collected from a sugarcane drink stall in the night market at Cheras, Kuala Lumpur, Malaysia. The waste samples were stored in a zip-lock bag at 4 °C prior to strain isolation within a week. The collected sugarcane waste sample was shown in Figure 1. The control Yakult® drink was purchased from a local supermarket.

Figure 1: Sugarcane waste samples obtained from sugarcane drink stall in Malaysia.

Strain isolation

The strain isolation procedures were conducted based on Ng *et al.* (2015) with modification. Approximately 2.5 g of sugarcane waste sample from each batch was mixed with 10 mL of peptone water (pH 7.0) (Merck, Germany). The sample was then centrifuged at 7500× *g* for 20 min. Then, the strain was isolated by ethanol treatment where the waste suspension was diluted with ethanol (Merck, Germany) at 1:1 (v/v) and incubated at room temperature (23-24 °C) for 30 min. Next, the suspension for sugarcane waste and control Yakult® drink was serially diluted with peptone water and plated on de Man, Rogosa, Sharpe (MRS) agar (Friedemann Schmidt, Australia) using the spread plate method. The aliquots (0.1 mL) were pipetted on the MRS agar and spread well using the L-shaped hockey stick. These plates were incubated at 37 °C for 48 h. The colony with different morphology from the sugarcane waste and control were streaked on MRS agar plates and incubated for 48 h at 37 °C. Isolate A was obtained from batch 1 of sugarcane waste, while isolate B was collected from batch 2 of sugarcane waste. Furthermore, the *Lactobacillus* strain was isolated from Yakult® and serves as the control. A colony (isolate A, isolate B, and *Lactobacillus* control strain) was randomly selected from the streak plates and added into 9.5 mL of MRS broth (Friedemann Schmidt, Australia). The bacterial suspension was activated by incubating at 37 °C for 24 h prior to subsequent analysis.

Tolerance to artificial gastric juice

The ability of isolated strains to survive in gastric conditions was observed according to Zhang *et al.* (2016) with modifications. The activated bacterial cultures (isolate A, isolate B, and *Lactobacillus* control strain) were centrifuged twice with phosphate-buffered saline (PBS, pH 7.4) (R&M Chemicals, UK) at 3900× *g* for 18 min, respectively. The cells were re-suspended into 9.8 mL of PBS. Gastric juice was prepared by dissolving sodium chloride (R&M Chemicals, UK) (2 g) and pepsin (Sigma Aldrich, Germany) (3.2 g) in 1 L of sterile distilled water. The pH 2.0 and pH 6.9 of the gastric juice were then adjusted using hydrochloric acid and sodium hydroxide solution, respectively. Furthermore, the bacterial suspension was inoculated with 0.2 mL of gastric juice at different pH (pH 2.0 and pH 6.9) respectively and mixed using a vortex mixer (VTX-3000L, LMS, Japan). The mixture was taken at 0, 1, 2, 3, 4 and 24 h of incubation and serially diluted with peptone water at 10% (v/v). The aliquots (0.1 mL) were plated on MRS agar using the spread plate method. These plates were then incubated for 48 h at 37 °C. The viable cell count (CFU/ mL) of the isolated strains and control sample were expressed as $\log_{10}$ CFU/mL.

Resistance to bile

The bile tolerance test was also carried out according to Honey Chandran and Keerthi (2018) with modification.

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Similar to the gastric tolerance test, the activated bacterial cultures (isolate A, isolate B, and *Lactobacillus* control strain) were centrifuged twice with PBS (pH 7.4) at 3900× *g* for 18 min, respectively. The cells were re-suspended into 9.8 mL of PBS. MRS with different bile concentrations (0.00, 0.05, 0.10, 0.15 and 0.30% w/v) was prepared by dissolving 13.5 g of MRS powder with 0.016, 0.031, 0.047 and 0.094 g of bile (Sigma Aldrich, Germany), respectively into 250 mL sterile distilled water. MRS without bile (0.00%) was referred to as MRSO. The bacterial suspension (0.1 mL) was added into respective 19.9 mL of MRS with bile concentration and mixed using a vortex mixer. The optical density for each mixture was tested at 37 °C during 0, 1, 2, 3, 4, 5, 6, 7 and 24 h of incubation at 560 nm using a spectrophotometer (UviLine 9400, Secoman, France).

Microbial adherence to solvents (MATS)

The microbial adhesion to solvents (MATS) test was conducted according to Bhanwar *et al.* (2014) with modifications. Similarly, the activated cultures (isolate A, isolate B, and *Lactobacillus* control strain) were centrifuged twice with PBS (pH 7.4) at 3900× *g* for 18 min, respectively. The cells were re-suspended into 5 mL of PBS. The optical density of the bacterial suspension was standardized at an absorbance of 0.4 at 600 nm (A_0). The standardized bacteria culture (1.2 mL) was treated with three solvents (0.2 mL) in microcentrifuge tubes, which are chloroform (Fisher Scientific, UK) (monopolar and acidic solvent), ethyl acetate (Friedemann Schmidt, Australia) (monopolar and basic solvent), and N-hexadecane (Sigma Aldrich, Germany) (polar solvent). The mixture was left to stand for 10 min at room temperature. The suspension with a two-phase system was then mixed using a vortex for 2 min and let to stand at room temperature for another 15 min. Subsequently, the aqueous phase of suspension was removed and the optical density at 600 nm (A_1) was measured. The percentage of MATS was then calculated using Equation 1.

$$\text{Percentage of MATS (\%)} = 1 - A_1/A_0 \times 100\% \quad (1)$$

where A_1 refers to the final absorbance of the bacterial suspension and A_0 refers to the initial absorbance of the bacterial suspension.

Resistance to 0.4% (v/v) phenol

The resistance to 0.4% (v/v) phenol test was carried out based on Bhanwar *et al.* (2014). MRS broth (10 mL) with phenol (0.04 mL) and without phenol was prepared accordingly. The activated cultures (isolate A, isolate B, and *Lactobacillus* control strain as control) (0.1 mL) were added into the MRS with and without phenol, respectively. The mixture was then vortexed to mix well. The mixture sample was taken at 0 and 24 h of incubation and serially diluted with peptone water at 10% (v/v). The aliquots (0.1 mL) were plated on MRS agar using the spread plate method. The plates were then incubated at 37 °C for 48 h. The viable cell count (CFU/ mL) of the isolated and control strains were calculated using Equation 1 and expressed as $\log_{10}$ CFU/mL.

Statistical analysis

The data were statistically analyzed using Statistical Package for the Social Sciences (Version 22, Chicago, Illinois, USA). The results were presented as the mean ± standard deviation. Independent t-test, pair t-test, analysis of Variance (ANOVA), and Tukey's test as post hoc test were used to determine the significant difference between means, where $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria (LAB)

The morphology of colonies on de Man, Rogosa, Sharpe (MRS) agar from sugarcane waste was shown in Figure 2a and Figure 2b. Isolate A agar plates had a lower bacterium count as compared to isolate B plates. Furthermore, most colonies in isolate A plates appeared to be smaller than isolate B, where isolate A colonies had a diameter of approximately 1 mm while isolate B colonies had a diameter of 2 to 3 mm. In terms of colony morphology, both isolates were circular with a smooth surface and beige in color. In addition, the probiotic *Lactobacillus* strain from Yakult® (Figure 2c) was chosen as the control strain (Lin *et al.*, 2017). Similarly, the colonies from the Yakult® were also opaque, circular-shaped, and smooth in surface.

Tolerance of LAB isolates towards different pH of gastric juice

Acid tolerance test with different level of pH conditions at different incubation time is crucial to simulate the physiological aspects of the human digestive system (Sahadeva *et al.*, 2011; Hassanzadazar *et al.*, 2012). Probiotic must pass through the highly acidic stomach before it reaches the intestine and creates the proper condition for residence (Yadav *et al.*, 2013). Hence, it is crucial to evaluate the tolerance of probiotic isolates towards simulated gastric juice. Table 1 displayed the viability of LAB isolates incubated in pH 2.0 and pH 6.9 gastric juice at different hours.

From Table 1, it was observed that the control had a higher number of viable cells during the initial incubation but decreased significantly ($p<0.05$) by 32.4% and 34.3% after 24 h of incubation in pH 2 and pH 6.9, respectively. On the other hand, the reduction in viable cell count after gastric incubation for isolate A was similar to isolate B. The viable cell counts for isolate B decreased significantly ($p<0.05$) by 4.9% and 4.7% after incubated in pH 2.0 and pH 6.9 gastric juice for 24 h, respectively. Similarly, the viable cell count of isolate A reduced by 4.6% and 1.5% after incubated in pH 2.0 and pH 6.9 gastric juice for 24 h, respectively.

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Table 1: Acid tolerance of isolated strains at pH 2.0 and pH 6.9 after 24 h of incubation.

Bacterial strains	pH values	Total plate count (log_{10} CFU/mL)					
		0 h	1 h	2 h	3 h	4 h	24 h
Isolate A	2.0	9.09 ± 0.07^{bA}	9.08 ± 0.11^{bA}	9.26 ± 0.05^{aA}	9.25 ± 0.02^{aA}	9.21 ± 0.05^{abA}	8.67 ± 0.07^{cB}
	6.9	9.09 ± 0.02^{aA}	9.02 ± 0.07^{abA}	9.04 ± 0.07^{abB}	9.02 ± 0.05^{abB}	9.00 ± 0.02^{abB}	8.95 ± 0.04^{bA}
Isolate B	2.0	9.16 ± 0.01^{aA}	8.96 ± 0.04^{bB}	9.06 ± 0.03^{aB}	9.12 ± 0.03^{aA}	9.08 ± 0.05^{aA}	8.71 ± 0.05^{cA}
	6.9	9.16 ± 0.02^{bA}	9.15 ± 0.03^{bA}	9.32 ± 0.02^{aA}	9.19 ± 0.02^{abA}	8.94 ± 0.04^{cA}	8.73 ± 0.05^{dA}
Control	2.0	9.45 ± 0.05^{aA}	9.07 ± 0.09^{bB}	9.04 ± 0.02^{bA}	9.14 ± 0.04^{bA}	9.06 ± 0.06^{bA}	6.39 ± 0.02^{cA}
	6.9	9.33 ± 0.05^{aB}	9.30 ± 0.01^{aA}	8.48 ± 0.03^{cB}	8.78 ± 0.03^{bB}	8.14 ± 0.03^{dB}	6.13 ± 0.02^{eB}

Data were expressed as mean ± standard deviation (n=3).
$^{a-e}$ mean values with different superscript in the same row differs significantly (p<0.05) via one-way ANOVA and Tukey's test.
^{AB} mean values with different superscript in the same column within the same bacterial strain differs significantly (p<0.05) via one-way ANOVA and Tukey's test. *Lactobacillus* probiotic strain isolated from Yakult® serves as positive control.

(a) **(b)** **(c)**

Figure 2: Bacterial colonies from (a) batch 1 sugarcane waste, (b) batch 2 sugarcane waste, (c) positive control (*Lactobacillus* probiotic strain isolated from Yakult®).

Nevertheless, all 3 strains decreased significantly (p<0.05) in viable cell count after 24 h of incubation in pH 2.0 gastric juice which was in agreement with numerous studies. The viable cell count of *Lactobacillus* isolates exposed to acid tolerance test was reported to decrease after 24 h of incubation (Sahadeva *et al.*, 2011; Bhanwar *et al.*, 2014). Besides that, various studies also showed that exposing bacteria to gastric juice with pH less than pH 2.0 had a reduction in viable cells as the incubation period increases (Lin *et al.*, 2006; Ashraf *et al.*, 2009; Sadrani *et al.*, 2014; Jose *et al.*, 2015).

Based on the result, both isolate A and B are found to be sensitive to an acidic environment as the overall plate count in pH 2.0 was lesser than those of pH 6.9 (Table 1). It was reported that the viable LAB counts were affected by the acidity (Shori, 2017). In comparison to this, the viable cells of the control at pH 6.9 were lower than at pH 2.0. Generally, the bacteria cells were supposed to be more tolerable under the neutral condition as compared to acidic conditions. A possible cause for the control strain exerting high tolerance in acidic conditions could be due to the acid tolerance mechanism developed by the *Lactobacillus* control strain after subjected to acidic conditions for 24 h (Guan and Liu, 2020). Moreover, the reduction of the control under pH 6.9 could be due to the lack of available nutrients after 24 h (Karimi *et al.*, 2011).

In addition, a huge reduction in the final plate count of control was presented in Table 1. After 24 h of acid exposure (pH 2.0), the viable bacteria of both isolate A and B were more than 8 log_{10} CFU/mL, while the tolerance of control has decreased by 32.4% to approximately 6 log_{10} CFU/mL. This result is supported by other studies that revealed that the survival rate of *L. casei* Shirota decreased by approximately 40% after 4 h of pH 2.5 exposure (Guo *et al.*, 2009; Wills, 2012), which was comparable to the findings of this study. Moreover, the bacterial counts of *Lactobacillus* strains at pH 2.0 were reported to be in the range of 1-6 log_{10} CFU/mL

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(Puniya *et al.*, 2012; Chang *et al.*, 2013; Vijayakumar *et al.*, 2015). Although the acid tolerance for control was poor, it demonstrated the ability to survive and remain viable in pH 2.0 gastric juice.

The isolates from the sugarcane waste in this study demonstrated a lower reduction (< 0.45 $\log_{10}$ CFU/mL) after 24 h of incubation under pH 2.0 as compared to other studies. Ng *et al.* (2015) reported that various *Lactobacillus* strains exposed to pH 2.0 conditions for 24 h reduced more than 7 $\log_{10}$ CFU/mL. The reduction of LAB in the result of the acidic condition was also found to be in the range of approximately 3 to 9 $\log_{10}$ CFU/mL at pH 2.0 (Millette *et al.*, 2008; Boke *et al.*, 2010; Puniya *et al.*, 2012; Chang *et al.*, 2013). Despite the higher reduction reported by these studies, Mirlohi *et al.* (2009) presented similar results for the acid tolerance of *Lactobacillus* strains whereby the reduction of bacterial count after 24 h of incubation under acidic conditions was approximately 1 $\log_{10}$ CFU/mL.

As a result, isolate A was shown to be the most resistant to acid stress among the three strains. Nonetheless, since both isolate A and isolate B was able to retain their viability above 8 $\log_{10}$ CFU/ mL at pH 2.0 and pH 6.9 of simulated gastric juice, these strains were considered to be acid tolerant and able to overcome the harsh condition of gastric juice.

Tolerance of LAB isolates towards different bile concentration

Besides surviving in the gastric environment, probiotics should also survive in the intestinal bile salts in order to exert beneficial activity (Yang *et al.*, 2020). As the bile salts and pancreatin could contribute to the adverse condition in the small intestine, hence probiotic strain should be able to adapt to high concentrations of bile salt (Mulaw *et al.*, 2019). Figure 3 illustrated the capability of isolated strains from sugarcane waste and *Lactobacillus* control strain from Yakult® in surviving different bile concentrations at intervals of 0, 1, 2, 3, 4, 5, 6, 7 and 24 h.

According to Figure 3, the absorbance of three strains in MRS broth without bile (MRSO) increased significantly (p<0.05) over time at a different rate. All isolates were observed to have a similar growth rate during the first 3 h. However, from the 4th h to the 7th h, control (Figure 3a) had grown at a higher rate (538.9%) as compared to isolate A (Figure 3b) and isolate B (Figure 3c) (209.1% and 212.7%, respectively). Besides that, a drastic acceleration in the growth of control and isolate A was detected across the 7th h to 24th h of incubation by 569.6% and 1785.3%, respectively. In contrast, there was only an increment of 28.5% in isolate B from the 7th to 24th h in MRSO. The final growth rate of the control strain was the highest, followed by isolate A and isolate B in MRSO (Supplementary Table S1).

The growth trend of the isolates A, B, and control in MRS broth with 0.05% bile, 0.10% bile, and 0.15% were found to be similar to the growth pattern in MRSO (Figure 3). The absorbance of isolate B was found to be lower than isolate A and control strain after 24 h of incubation in both MRS with and without bile at approximately OD 2. This indicates that isolate B displayed slower growth in MRS as compared to isolate A and control. Similar results were reported by Mirlohi *et al.* (2009) and Sekse *et al.* (2012) where different strains from the same species (*L. rhamnosus* and *Escherichia coli*) displayed different growth in their same respective cultivation media (MRS, Syncase, Luria-Bertani broth). Besides that, Sekse *et al.* (2012) also reported that the growth rate of *E. coli* in Syncase and Luria-Bertani broths varies with strain types due to different available nutrients. This showed that nutrients in MRS broth may not be favorable for the growth of isolate B in this study.

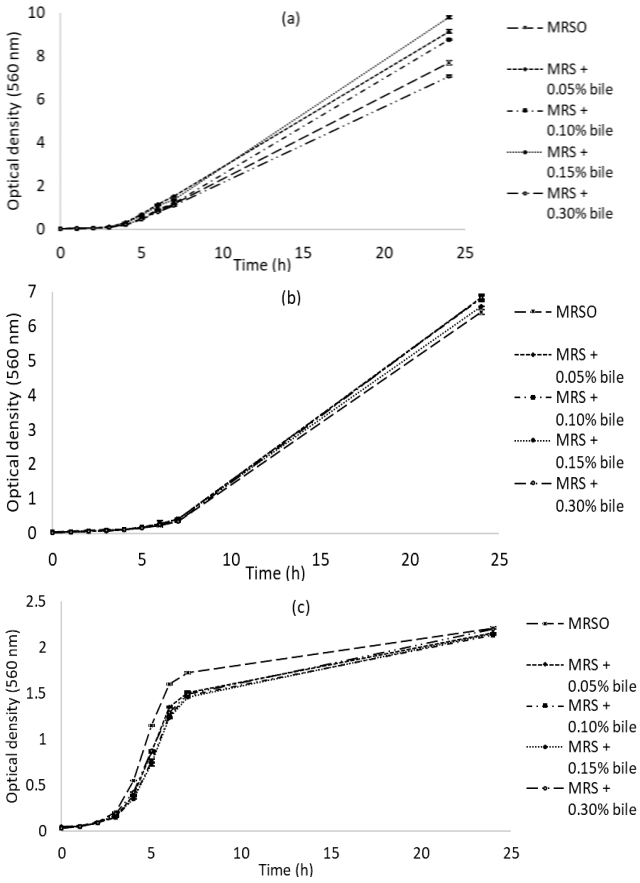

Figure 3: Optical density (OD 560 nm) of (a) control, (b) isolate A, and (c) isolate B in different bile concentration over a period of 24 h incubation. Error bars indicate the standard deviation of triplicate experiments (n=3). *Lactobacillus* probiotic strain isolated from Yakult® serves as positive control.

In addition, the bacterial suspension of control in MRS broth containing 0.15% bile has the highest absorbance as compared to other bile concentrations and MRSO. Similar to control, the turbidity of isolate A in MRS broth with bile was higher than MRSO, with the highest absorbance found in MRS broth with 0.30% bile. Greater

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emphasis was given to bacterial strains in MRS broth with 0.3% bile concentration as probiotic bacteria should be able to endure up to 0.3% bile (Widaningrum *et al.*, 2019).

However, isolate B had lower absorbance of 0.45–3.6% in MRS with bile than MRSO after 24 h of incubation. Similar results were reported by Boke *et al.* (2010), Sahadeva *et al.* (2011), Ren *et al.* (2014) and Ng *et al.* (2015) where LAB strain had lower viability after incubation with the presence of bile as compared to without the bile. The decline in strains viability with the presence of bile was due to the ability of bile salt in inducing complete cell permeabilization, stopping glucose uptake, and severely altering the cell surface (Taranto *et al.*, 2006). As the bacteria were exposed to bile salts, the disruption of the cellular homeostasis and membrane integrity may occur. This effect could further lead to leakage of bacterial content and followed by cell death (Hassanzadazar *et al.*, 2012).

The growth pattern was different between isolate A and B after incubating in MRS with bile, where isolate A displayed better tolerance in bile than isolate B. A number of studies indicated that bacterial strains that could tolerate bile would continue to grow under high bile conditions, thus resulting in the increase of absorbance (Hoque *et al.*, 2010; Hassanzadazar *et al.*, 2012). Besides that, the depressant effect of bile salts on bacterial growth could be influenced by the bacteria characteristics and bile salt concentration (Deshpande *et al.*, 2014).

Different pH, temperature, and other environmental factors might cause the bacteria to be more sensitive to bile salt or enhance their survival rate (Li, 2012). As both strains were isolated from different batches of sugarcane waste, the initial conditions subjected to the isolated strain could be different. In short, isolate A displayed better tolerance than isolate B in all bile concentrations, but both isolates were able to grow under all different bile concentrations. This shows that both isolates from sugarcane waste could be potential probiotic candidates.

Microbial adhesion of LAB isolates towards different types of solvents

Figure 4 displayed the microbial adhesion of sugarcane waste isolates and control strain to solvents (MATS test). All three bacterial strains have a similar pattern in terms of the affinity towards different solvents where they exhibited high affinity to chloroform, followed by ethyl acetate, and lastly N-hexadecane. This shows that both isolates from sugarcane waste and control have electron donors and basic characteristics with poor adhesive properties.

The microbial adhesion to the non-polar solvent, N-hexadecane often showed the cell surface's hydrophobic or hydrophilic nature and its ability to adhere to host intestinal epithelium cells due to the absence of electrostatic interactions (Farniya *et al.*, 2019). Besides that, the monopolar solvents of chloroform and ethyl acetate reflected the Lewis acid-base properties at the bacteria cell surface (Sánchez-Ortiz *et al.*, 2015). Furthermore, affinity towards chloroform was considered

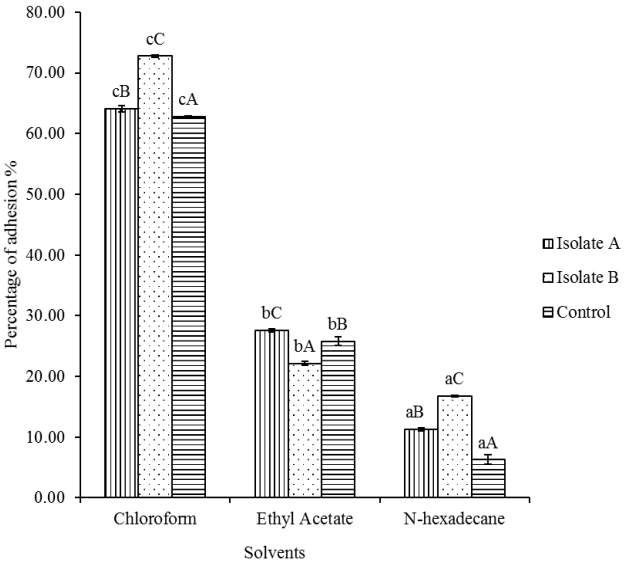

Figure 4: The percentage of adhesion (MATS %) of bacterial strains to different type of solvents. Error bars indicate the standard deviation of triplicate experiments (n=3). $^{a-c}$ mean values with different superscript differs significantly within the same bacterial strains (p<0.05) via one-way ANOVA and Tukey's test. $^{A-C}$ mean values with different superscript differs significantly within the same type of solvents (p<0.05) via one-way ANOVA and Tukey's test. *Lactobacillus* probiotic strain isolated from Yakult® serves as positive control.

to be an indication of electron donor and basic characteristics. On the other hand, affinity towards ethyl acetate showed electron acceptor and acidic characteristics (Farniya *et al.*, 2019).

High cell surface hydrophobicity suggests a high level of adhesion to epithelial cells due to van der Waals interactions (Krausova *et al.*, 2019). According to the result of N-hexadecane in Figure 4, isolate B (16.49%) has the highest hydrophobicity as compared to isolate A (11.30%) and control (6.29%). This indicated that isolate B could have a greater capability to adhere to the host's epithelial cell. With this adhesion ability, the microorganisms can prevent the entrance of pathogens through steric interaction or definite blockage on cell receptors (Monteagudo-Mera *et al.*, 2019).

Even though isolate B has the greatest affinity towards N-hexadecane, but the definition of high hydrophobicity was not clear. Ng *et al.* (2015) reported that *Lactobacillus* strains with more than 40% affinity to N-hexadecane generally are more hydrophobic. As a result, this shows that all three isolates in this study possessed the hydrophilic nature of the cell surface. This is supported by several studies where *L. lactis*, *L. paracasei*, *L. plantarum*, *L. fermentum*, *L. casei*, and *L. rhamnosus* were found to have hydrophilic surfaces (Bhanwar *et al.*, 2014; Sadrani *et al.*, 2014; Petrova *et al.*, 2019).

Similar to N-hexadecane, isolate B (72.78%) displayed higher adhesion to chloroform in comparison

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Table 2: Viable cell counts of bacterial strains towards 0.4% phenol solution.

Bacterial strains	Medium	Viable cell count (log_{10} CFU/mL)	
		0 h	24 h
Isolate A	MRS broth	7.23 ± 0.01^{aA}	9.26 ± 0.04^{bB}
	MRS broth + 0.4% Phenol	7.22 ± 0.04^{aA}	7.11 ± 0.06^{aA}
Isolate B	MRS broth	7.18 ± 0.04^{aA}	9.47 ± 0.05^{bB}
	MRS broth + 0.4% Phenol	7.18 ± 0.02^{aA}	7.49 ± 0.06^{bA}
Control	MRS broth	7.24 ± 0.01^{aA}	10.07 ± 0.03^{bB}
	MRS broth + 0.4% Phenol	7.22 ± 0.07^{aA}	8.10 ± 0.01^{bA}

Data were expressed as mean ± standard deviation (n=3).
[ab]mean values with different superscript in the same row differs significantly (p<0.05) via pair t-test.
[AB]mean values with different superscript in the same column within the same bacterial strains differs significantly (p<0.05) via independent t-test. *Lactobacillus* probiotic strain isolated from Yakult® serves as positive control.

isolate A (64.09%) and control strain (62.83%). The adhesion affinity of both isolates towards chloroform was in agreement with Bhanwar *et al.* (2014), Sadrani *et al.* (2014) and Kirillova *et al.* (2017) where *Lactobacillus* strains were described as strong electron donor and non-acidic character with 45% to 97% percentage of adhesion towards chloroform.

However, the results of chloroform treatment for all 3 isolates were different from ethyl acetate, where isolate B (22.17%) has lower adhesion to ethyl acetate as compared to isolate A (27.60%) and control (25.82%). This is because chloroform and ethyl acetate have the same monopolar properties but different Lewis acid-base characteristics. Thus, the implication of Lewis acid-base interaction from the bacterial strain may lead to the difference in adhesion percentage (Bhanwar *et al.*, 2014; Sadrani *et al.*, 2014). Among the three strains in this study, isolate B displayed the best electron donor with basic characteristics and the highest adhesion ability to the intestinal epithelial cell.

Resistance of LAB isolates towards 0.4% (v/v) of phenol

Phenol is a product of colonic protein degradation and metabolism that is able to exert a toxic effect. It can be found in the gastrointestinal tract where they may cause gut mucosal effects and inhibit the growth of some *Lactobacilli* strains (Fonseca *et al.*, 2021). Therefore, resistance towards phenol could further ensure the survivability of isolated LAB from sugarcane waste in the gastrointestinal tract (Padmavathi *et al.*, 2018). Table 2 displayed resistance of bacterial strain in this study towards phenol after 24 h of incubation.

Isolate A, isolate B, and control strain in this study had a lower viable cell count after 24 h of incubation with the presence of phenol as compared to the absence of phenol by 23.2%, 20.9% and 19.6%, respectively (Table 2). Fonseca *et al.* (2021) found that the bacterial resistance towards phenol was diverse, strain-dependent varies with phenol concentration. Higher phenol concentration in the environment may result in less ability for bacteria to survive in the presence of this toxic compound (Sadrani *et al.*, 2014; Fonseca *et al.*, 2021).

Table 2 shows the resistance of isolates from sugarcane waste and control towards 0.4% of phenol. The viability of isolate A had remained constant (p>0.05) after 24 h of incubation with the presence of phenol. However, isolate B and control strain increased significantly (p<0.05) in viable cell count by 4.32% and 12.19% respectively after 24 h of incubation with MRS and phenol. This shows that these 3 strains had different degrees of sensitivity towards phenol compound, where isolate B and control exhibited better resistance towards phenol after 24 h of incubation, whereas isolate A showed the least resistance to phenol.

Isolate B and control strain displayed better resistance towards phenol with increase growth (p<0.05) during 24 h of fermentation. Nevertheless, isolate A was also able to retain its viability with no decrement of cells in the presence of 0.4% phenol for 24 h. These results were in accordance with few studies that displayed the ability of LAB strain in tolerating 0.4% phenol after 24 h of incubation (Kiliç *et al.*, 2013; Bhanwar *et al.*, 2014; Sadrani *et al.*, 2014).

CONCLUSION

In conclusion, both isolate A and isolate B had higher tolerance against acidic conditions than control strain after 24 h of incubation. However, isolate A displayed better resistance towards 0.05-0.3% bile concentration than isolate B; while isolate B showed higher resistance towards 0.4% phenol than isolate A, after 24 h of incubation. Nevertheless, both isolated LABs were resistant to bile and phenol. Moreover, isolate A and isolate B were characterized as low cell surface hydrophobicity, strong electron donor, and basic characteristic. The ability to survive in simulated gastrointestinal stress conditions with better tolerance against acidic conditions than control strain demonstrated the potential of the isolated LAB from sugarcane waste as probiotics. Future studies could identify the isolates and further evaluate the antibiotic resistance, antimicrobial activity, anti-oxidative properties, and aggregation properties of the LAB isolated from sugarcane waste.

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SUPPLEMENTARY INFORMATION

Table S1: Optical density (OD 560 nm) of bacterial strains in different bile concentration over a period of 24 h incubation.

Bacterial strains	Time (h)	Optical density (560 nm)				
		MRSO	MRS + 0.05% bile	MRS + 0.10% bile	MRS + 0.15% bile	MRS + 0.30% bile
Control*	0	0.03 ± 0.00^{aA}	0.03 ± 0.00^{bA}	0.04 ± 0.00^{dA}	0.04 ± 0.00^{eA}	0.03 ± 0.00^{cA}
	1	0.04 ± 0.00^{aA}	0.05 ± 0.00^{cA}	0.04 ± 0.00^{aA}	0.05 ± 0.00^{cA}	0.05 ± 0.00^{bA}
	2	0.06 ± 0.00^{bcA}	0.06 ± 0.00^{cA}	0.05 ± 0.00^{abA}	0.06 ± 0.00^{dA}	0.05 ± 0.00^{aA}
	3	0.08 ± 0.00^{aAB}	0.10 ± 0.00^{cA}	0.09 ± 0.00^{bB}	0.13 ± 0.00^{dB}	0.11 ± 0.00^{cB}
	4	0.18 ± 0.00^{aB}	0.32 ± 0.01^{dB}	0.24 ± 0.00^{bC}	0.28 ± 0.00^{cC}	0.23 ± 0.00^{bC}
	5	0.47 ± 0.01^{abC}	0.70 ± 0.01^{dC}	0.49 ± 0.01^{bD}	0.62 ± 0.01^{cD}	0.47 ± 0.00^{aD}
	6	0.87 ± 0.00^{bD}	1.15 ± 0.02^{eD}	0.93 ± 0.02^{cE}	1.10 ± 0.02^{dE}	0.81 ± 0.02^{aE}
	7	1.15 ± 0.00^{bE}	1.52 ± 0.01^{dE}	1.18 ± 0.02^{bF}	1.36 ± 0.02^{cF}	1.09 ± 0.02^{aF}
	24	7.70 ± 0.10^{bF}	9.14 ± 0.08^{dF}	8.77 ± 0.03^{cG}	9.79 ± 0.06^{eG}	7.06 ± 0.05^{aG}
Isolate A	0	0.05 ± 0.00^{dA}	0.04 ± 0.00^{cA}	0.04 ± 0.00^{bA}	0.03 ± 0.00^{aA}	0.03 ± 0.00^{aA}
	1	0.07 ± 0.00^{dAB}	0.05 ± 0.00^{aA}	0.05 ± 0.00^{bA}	0.05 ± 0.00^{abAB}	0.05 ± 0.00^{cAB}
	2	0.08 ± 0.00^{cAB}	0.06 ± 0.00^{bA}	0.06 ± 0.00^{aA}	0.06 ± 0.00^{bB}	0.06 ± 0.00^{bAB}
	3	0.11 ± 0.00^{cB}	0.07 ± 0.00^{aA}	0.08 ± 0.00^{bA}	0.08 ± 0.00^{bC}	0.08 ± 0.00^{bBC}
	4	0.11 ± 0.00^{aB}	0.11 ± 0.00^{aAB}	0.11 ± 0.00^{aAB}	0.11 ± 0.00^{bD}	0.11 ± 0.00^{aC}
	5	0.17 ± 0.00^{bC}	0.17 ± 0.00^{bB}	0.19 ± 0.00^{dB}	0.18 ± 0.00^{cE}	0.16 ± 0.00^{aD}
	6	0.23 ± 0.00^{aC}	0.28 ± 0.01^{cdC}	0.28 ± 0.00^{dC}	0.27 ± 0.01^{bcF}	0.25 ± 0.01^{bE}
	7	0.34 ± 0.01^{aD}	0.41 ± 0.00^{bD}	0.41 ± 0.01^{bD}	0.41 ± 0.02^{bG}	0.34 ± 0.01^{aF}
	24	6.41 ± 0.06^{aE}	6.83 ± 0.10^{bE}	6.80 ± 0.09^{bE}	6.58 ± 0.00^{aH}	6.83 ± 0.04^{bG}
Isolate B	0	0.05 ± 0.00^{aA}	0.05 ± 0.00^{aA}	0.03 ± 0.00^{bA}	0.03 ± 0.00^{aA}	0.03 ± 0.00^{bA}
	1	0.06 ± 0.00^{aA}	0.05 ± 0.00^{aA}	0.05 ± 0.00^{aA}	0.05 ± 0.00^{aB}	0.05 ± 0.00^{aB}
	2	0.10 ± 0.00^{cB}	0.09 ± 0.00^{cB}	0.09 ± 0.00^{bcB}	0.09 ± 0.00^{abC}	0.09 ± 0.00^{aC}
	3	0.21 ± 0.00^{eC}	0.16 ± 0.00^{bC}	0.15 ± 0.00^{aC}	0.17 ± 0.00^{cD}	0.19 ± 0.00^{dD}
	4	0.55 ± 0.00^{dD}	0.39 ± 0.01^{bD}	0.36 ± 0.01^{aD}	0.35 ± 0.00^{aE}	0.42 ± 0.00^{cE}
	5	1.15 ± 0.01^{cE}	0.86 ± 0.01^{bE}	0.76 ± 0.02^{aE}	0.73 ± 0.02^{aF}	0.87 ± 0.01^{bF}
	6	1.60 ± 0.01^{dF}	1.35 ± 0.01^{cF}	1.26 ± 0.02^{bF}	1.23 ± 0.01^{aG}	1.29 ± 0.00^{bG}
	7	1.72 ± 0.01^{cG}	1.51 ± 0.01^{bG}	1.47 ± 0.01^{aG}	1.46 ± 0.01^{aH}	1.49 ± 0.02^{abH}
	24	2.21 ± 0.01^{dH}	2.16 ± 0.00^{bH}	2.13 ± 0.00^{aH}	2.15 ± 0.00^{bI}	2.20 ± 0.00^{cI}

Data were expressed as mean ± standard deviation (n=3).
$^{a-e}$ mean values with different superscript in the same row differs significantly (p<0.05) via one-way ANOVA and Tukey's test.
$^{A-I}$ mean values with different superscript in the same column within the same bacterial strain differs significantly (p<0.05) via one-way ANOVA and Tukey's test.
*_Lactobacillus_ probiotic strain isolated from Yakult® serves as positive control.

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