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Isolation and characterisation of probiotic lactic acid bacteria from Malaysian fermented shrimp product *pekasam senek*

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Abstract

Lactic acid bacteria (LAB) from fermented foods have been proven as able to hinder the growth and activities of some foodborne pathogens. The present work thus aimed to isolate and characterise the potential LAB isolated from Malaysian fermented shrimp, *pekasam senek* (PS). Twelve isolates were obtained and molecularly identified *via* 16S rRNA gene sequencing, followed by acid and bile salt tolerance assessment, and cholesterol-lowering activity evaluation. The isolates showed satisfactory tolerance in a harsh acidic condition of pH 3.0; ten isolates (PS13, PS14, PS16, PS17, PS20, PS24, PS26, PS27, PS31, and PS33) exhibited over 90% of acid tolerance, while isolate PS22 exhibited 85.37% of acid tolerance, and isolate PS15 exhibited 75.73% of acid tolerance. For bile salts tolerance, nine isolates (PS13, PS14, PS15, PS16, PS17, PS24, PS27, PS31, and PS33) demonstrated survival rates ranging from 50 to 95% after 6 h of exposure to 0.3% bile salts. Despite showing a low tolerance to bile salt in comparison to the control, isolates PS20, PS22, and PS27 were still growing. The strains were identified as *Lactiplantibacillus plantarum* (PS13, PS15, PS17, PS20, PS24, PS31, and PS33), *Bacillus cereus* (PS14, and PS16), and *Bacillus* sp. (PS22, PS26, and PS27), using 16S rRNA gene sequencing. Lastly, PS24 and PS31 showed the highest cholesterol-lowering activity, with reductions of 80.50 and 80.19%, respectively. Meanwhile, PS13, PS14, PS16, PS17, PS20, PS26, PS27, and PS33 showed cholesterol-lowering activity of above 70%, and PS22 showed only 50.55% reduction. Each strain showed characteristics that influenced its ability to survive in acidic conditions, bile salts, and reduce cholesterol levels. These results proved that the isolates could have good properties as probiotics with positive cholesterol-lowering activity.

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Introduction

The use of probiotics in modern medicine has become increasingly important due to their positive effects on health and increased antimicrobial resistance (Huang *et al.*, 2020). As a natural alternative to antibiotic supplementation, probiotics have gained popularity in recent years. As a result of their ability to strengthen the barrier function of the gut, probiotics are becoming increasingly popular in food products, feeds, and supplements (Byakika *et al.*, 2020). The World Health Organization defines probiotics as "live microorganisms that, when administered in adequate amounts, grant a health benefit to the host". Many Asians and Europeans consume probiotics to achieve better growth performance (Zielińska and Kołożyn-Krajewska, 2018). Probiotics have various functional properties such as reducing cholesterol content. Therefore, probiotics could reduce the risk of hypercholesterolemia in consumers, and promote healthier food intake.

Pekasam senek is a fermented shrimp food product originating from Sarawak, Malaysia (Nillian *et al.*, 2021). It consists of shrimp, cooked rice, and raw rice that is ground and toasted, and is usually consumed as a side dish by the locals. Increasing public awareness of gastrointestinal disorders has fostered a growing demand for functional fermented foods that offer a variety of health benefits. Indirectly, novel LAB strains with health-promoting properties have been discovered in fermented foods (Jawan *et al.*, 2019). Probiotics are often associated with good bio-preservative characteristics in foods, such as the production of lactic acid, bacteriocins of hydrogen peroxide, and short-chain fatty acids. In addition,

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probiotics are beneficial because they can compete for adhesion to intestinal epithelial cells, improve the host's immune system, and eliminate harmful components, such as bacteriocins (Ida Muryany *et al.*, 2017; Huang *et al.*, 2020). The present work was thus focussed on the isolation and characterisation of potential probiotics from fermented Malaysian *pekasam senek*. This included probiotic potential testing such as acid tolerance and bile salt tolerance, bile salt hydrolase (BSH), and cholesterol-lowering activity of the isolated LAB.

Materials and methods

Sample collection

Pekasam senek samples were obtained from Saratok, Sarawak. They were collected aseptically in sterilised containers, and transferred to the microbiology laboratory at Universiti Teknologi MARA Cawangan Negeri Sembilan, Kampus Kuala Pilah, Malaysia. They were then stored in a refrigerator (4°C) until further analyses.

Bacterial isolation and purification from fermented pekasam senek

Bacteria from fermented *pekasam senek* were isolated using MRS agar (de Man, Rogosa and Sharpe; Oxoid, UK). Samples were subjected to bacterial isolation and serial dilution (10-fold) in sterile MRS broth (Mohamad *et al.*, 2020). A loopful of homogenised enriched culture was spread aseptically onto MRS agar containing 0.8% calcium carbonate ($CaCO_3$), and the plates were incubated at 37°C for 48 h (Afrin *et al.*, 2021). Selected isolates were preserved at -80°C in MRS broth medium containing (20%, v/v) glycerol stocks (Difco BD, USA) (Ida Muryany *et al.*, 2017; AlKalbani *et al.*, 2019).

Determination of acid tolerance

Assessment of acid resistance of bacterial isolates was performed using the *in vitro* method described by Ahmad *et al.* (2018), with a slight modification in pH. The MRS broth was initially inoculated with 100 µL of 10^8 CFU/mL of overnight-grown cultures, and for activity comparison of the isolates, *Lactobacillus casei* strain Shirota from Yakult (Tokyo, Japan) was selected as the reference strain (Haitham *et al*., 2017; Ilyanie *et al.*, 2022). To determine the tolerance rate of isolates in an acidic environment, 100 µL of each culture was transferred to 900 µL of MRS broth adjusted to pH 3 with 1 M hydrochloric acid (HCl), and then incubated at 37°C (Li *et al.*, 2020). The numbers of viable bacteria were determined at 0 and 3 h of incubation. The experiment was carried out in triplicate. Results were expressed as the mean ± SD of viable isolate in plates counted at 3 h compared to the control plates counted at 0 h (Haitham *et al*., 2017; Ahmad *et al.*, 2018).

Determination of bile salt tolerance

Bile salt tolerance test of isolated LAB was performed following the method described by Haitham *et al*. (2017) with slight modifications. Bile salt tolerance assay was conducted using a bile salt concentration of 0.3% (w/v) for 4 h (Bassyouni *et al*., 2012). Overnight cultures of the chosen LAB cultured in MRS broth were incubated at 37°C for 24 h. The optical density (OD) of the cultures was measured at 600 nm after overnight incubation at 37°C using a UV-Vis Spectrophotometer (PG Instruments Ltd), and was compared to a control cultures (without bile salt). The findings were computed using Eq. 1, and presented as a percentage of tolerance to bile. The experiment was repeated three times for each isolate, and the bile tolerance of commercial probiotic *Lactobacillus casei* strain Shirota from Yakult (Japan) was examined as well to compare activity with LAB isolates.

$$\text{Percentage (\%) tolerance of bile} = [(A_1/A_0] \times 100 \quad \text{(Eq. 1)}$$

where, A_1: absorbance reading at OD_{600} of isolate in MRS broth with 0.3% bile at 6 or 24 h; and A_0: absorbance reading at OD_{600} of isolate in MRS broth without 0.3% bile after 24 h.

Genotypic identification of LAB

All isolates were molecularly identified using 16S rRNA gene sequence analysis. The genomic DNA of each potential isolate was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia) following the manufacturer's procedure with minor modifications. Amplification of the 16S rRNA gene was performed using universal prokaryotic primers 127F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' ACG GCT ACC TTG TTA CGA CTT 3') (Hajar and Hamid, 2013). For 16S rRNA gene PCR amplification, the cycle was performed as follows: a cycle of initial denaturation at 95°C (1 min), followed by 35 cycles of

denaturation at 95°C (15 s), annealing at 50°C (15 s), and extension at 72°C (10s). The final extension was set at 72°C for 5 min before the reaction ended, and was held at 12°C. The PCR product size was targeted to be around 1,500 bp, and it was sequenced by Apical Scientific Laboratory (Selangor, Malaysia). Using the nucleotide BLAST program, all sequences were further examined and compared with other 16S rRNA genes stored in the GenBank database accessible online.

Bile salt hydrolase (BSH) assay

Twelve isolates isolated from *pekasam senek* were tested for BSH production using a qualitative assay (direct plate assay) (Alash and Lafy, 2018). All Isolates were cultured in MRS broth, and incubated at 37°C for 24 h. Following that, 5 µL overnight culture of isolates and the reference strain, *Lactobacillus casei* strain Shirota (10^8 CFU/mL), was spotted on MRS agar plate supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA) and 0.37 g/L of calcium chloride ($CaCl_2$). The plates were incubated anaerobically at 37°C for 72 h. The presence of precipitated bile acid (cholic acid) around colonies (opaque halos) was observed in each culture site. The precipitation zone around colonies indicated bacterial bile salt hydrolase activity (Shehata *et al*., 2016). Inoculated isolates on MRS agar plate without bile salt supplementation served as a negative control.

Cholesterol-lowering assay

The cholesterol-lowering ability of the growing potential probiotic LAB was analysed according to Tomaro-Duchesneau *et al.* (2014) with minor modifications. MRS broth was incubated for 24 h at 37°C with 100 µg/mL water-soluble cholesterol (Cholesterol-PEG 600; Sigma, US) and 1% of overnight isolates (10^8 CFU/mL). Uninoculated modified MRS broth was prepared under the same conditions, and served as a control. Following incubation period, bacterial cells were removed by centrifugation at 10,000 rpm for 15 min, and the remaining cholesterol in the cell-free supernatant was determined calorimetrically using the cholesterol quantitation kit (MAK043; Sigma, US). In a 96-well plate, cholesterol standard solutions at concentrations of 0 (blank), 1, 2, 3, 4, and 5 µg/well were added. The 30 µL uninoculated modified MRS broth and the filtered cell-free supernatant were also additionally filled into the well plate. The final volume of each well was brought to a final volume of 50 µL with cholesterol assay buffer. Each well was added with 50 µL of reaction mix. The amount of cholesterol present in the samples were calculated using Eq. 2:

$$\text{Cholesterol lowering activity, \%} = \frac{\text{Cholesterol added} - \text{Cholesterol recovered}}{\text{Cholesterol added}} \times 100 \quad \text{(Eq. 2)}$$

Statistical analysis

All data were expressed as mean ± SD. For statistical analysis, IBM SPSS (Statistical Package for the Social Sciences) statistics version 27.0 was used. One-way ANOVA was used to analyse the data (normally distributed and equally variance), followed by Tukey's test for *post hoc* comparisons. Data with probability value of less than 0.005 ($p < 0.05$) were considered statistically significant.

Results and discussion

Acid tolerance of LAB

Potential probiotics were evaluated in stimulated gastric juice at pH 3.0 to determine their ability to survive in the harmful low pH concentrations of the stomach (Meena *et al.*, 2022). Ingested food helps improve the survival rate of LAB in the stomach during the gastric transit by raising the pH of the stomach, and making it more tolerable for the LAB (Boricha *et al.*, 2019). Apart from withstanding gastric stress, tolerance to the low pH of the gastric juice in the stomach is important for LAB survival.

All 12 isolates were incubated for 3 h in MRS broth adjusted to pH 3.0, followed by resistance assay. This corresponded to the time spent in the stomach. Selected isolates were tested for viable counts at pH 3.0, at 0 and 3 h (Meena *et al.*, 2022). A general comparison of all isolates was examined for significant differences in cell viability, and plate counts at 0 and 3 h of treatment showed similar pH tolerance.

The acid resistance results of isolates are shown in Figure 1. The findings indicated that isolates were able to grow and survive under acidic conditions at pH 3 initially; however, the growth slowed down after 3 h. All isolates showed different resistance rate patterns when exposed to pH 3, and the survival rate of the isolates was compared with *L. casei* Shirota

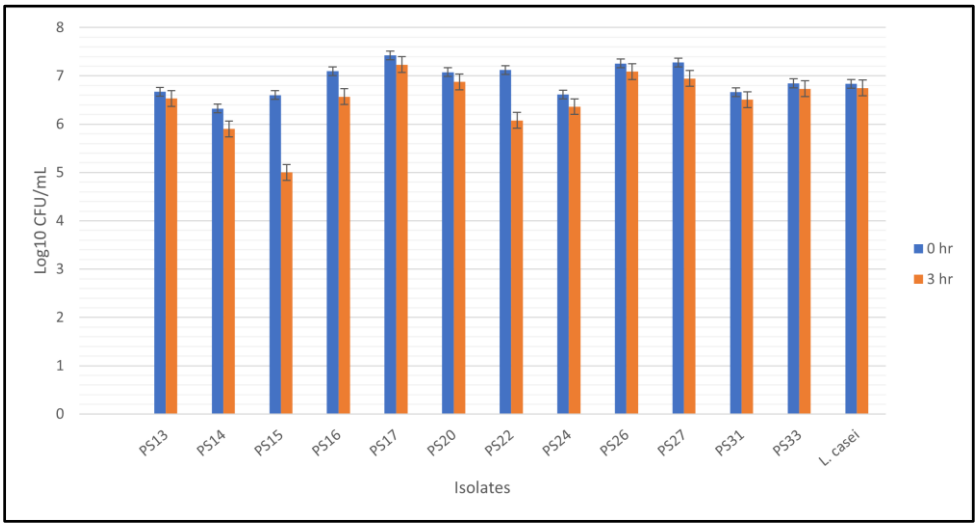

Figure 1. Effect of pH 3.0 on survival of 12 isolates and *L. casei* strain Shirota following 3 h incubation at 37°C.

strain under acidic conditions. Therefore, the results showed that all isolates were resistant to an acidic environment. Similarly, Ahmad *et al.* (2018) reported that *L. plantarum* isolated from *tempoyak* (a traditional fermented durian in Malaysia) at pH 3.0 showed a high survival rate after 3 h of exposure. Lee *et al.* (2016) successfully isolated *L. plantarum* from the famous Korean traditional fermented cabbage, *kimchi*, and reported that the strains showed a moderate survival rate even after being exposed to pH 3 for 3 h.

The highest survival rate of a potential isolate after 3 h of exposure to low pH 3.0 was shown by isolate PS33 (6.73 $\log_{10}$ CFU/mL), and the lowest by isolate PS15 (5.00 $\log_{10}$ CFU/mL). All selected isolates were able to survive in an acidic environment at pH 3, and maintain varying levels of viability after 3 h of incubation. The viable counts of all isolates after 3 h of exposure at pH 3.0 were approximately 5.00 to 7.23 $\log_{10}$ CFU/mL, indicating high acid tolerance. The final count ranges of all isolates tested after 3 h of exposure to pH 3.0 are shown in Figure 1. In total, at pH 3.0, 11 isolates (excluding isolate PS15) showed sufficient tolerance to low pH, and survived after 3 h of incubation, with the final population exceeding 5.90 $\log_{10}$ CFU/mL. Although isolate PS15 remained viable after being incubated for 3 h with live cells, the value reduced from 6.60 ± 1.4 (0 h) to 5.00 ± 0.7 $\log_{10}$ CFU/mL (3 h). This suggested that the isolate PS15 was probably killed by the harsh acidic conditions (Sahadeva *et al.*, 2011).

The survival rate of *L.* casei under acidic conditions was observed to be significantly the highest ($p < 0.05$) at pH 3.0. In general, the survival rates of all isolates and *L. casei* were observed to decrease with an increase in the incubation period. The results obtained showed that 12 isolates were resistant to pH 3.0. The results in Figure 1 show that the number of viable bacteria is strongly suppressed at pH 3.0. The greater reduction in bacterial numbers observed for isolates with longer incubation times may be due to their adaptability to acid during the presence of the strains in MRS broth. The excellent acid resistance properties exhibited by isolates complemented their strain specifications, as LAB is classified as strain-dependent (Sahadeva *et al.*, 2011). Contrarily, a study reported by Shehata *et al.* (2016) demonstrated that all LAB isolates were able to survive in simulated gastric juice at pH 2.0 after 3 h of incubation. These strains maintained different levels of viability (68 - 88.3%), with the highest survival rate observed for BO35 isolate, and the lowest survival rate for the RM28 isolate (Shehata *et al.*, 2016).

LAB isolates with high acid tolerance were selected because they meet the criteria of withstanding the initial barrier of a harsh acidic environment without loss of viability. It is important for LAB to tolerate an acidic environment to reach and colonise the intestinal tract, and subsequently exert their benefits. LAB trigger acid tolerance responses under acid stress, and trigger repair mechanisms that contribute to pH homeostasis and the development of low pH tolerance (Ilyanie *et al.*, 2022).

Bile salt tolerance of LAB

Resistance to bile salts is one of the criteria studied *in vitro* to evaluate the probiotic properties of potential LAB isolates that allow them to proliferate and survive within the gastrointestinal tract (GIT) (Huang *et al.*, 2020). Twelve isolates with high tolerance to acidic pH were further tested for their resistance at 0.3% bile salt concentration for 6 h, reflecting the duration in the intestines, and at 24 h to allow bacterial growth. After 6 h of incubation in MRS broth containing 0.3% bile salts, nine out of 12 isolates survived at rates between 51 to 95%. The other three isolates showed very low bile salt resistance of less than 50%.

As shown in Figure 2, exposure to 0.3% bile salts for a long incubation period of 24 h significantly ($p < 0.05$) reduced the viability of six isolates (PS13, PS14, PS24, PS26, PS31, and PS33). However, PS15, PS16, PS17, PS20, PS22, and PS27 showed an increase after 24 h of exposure to 0.3% bile salts. PS20, PS22, and PS27 isolates also grew, as evidenced by an increase in absorbance compared to 6 h, although 50% survival was not achieved compared to the control. The resistance patterns of PS15 (62.6% at 6 h; 70.2% at 24 h), PS16 (50.7% at 6 h; 78.9% at 24 h), and PS17 (62.4% at 6 h; 63.8% at 24 h) appeared contradictory with significant ($p < 0.05$) increments at 24 h. The commercially available probiotic *L. casei* strain Shirota survived almost completely in a 0.3% bile salt environment, exhibiting resistance values of 86% after 6 h and 78% after 24 h.

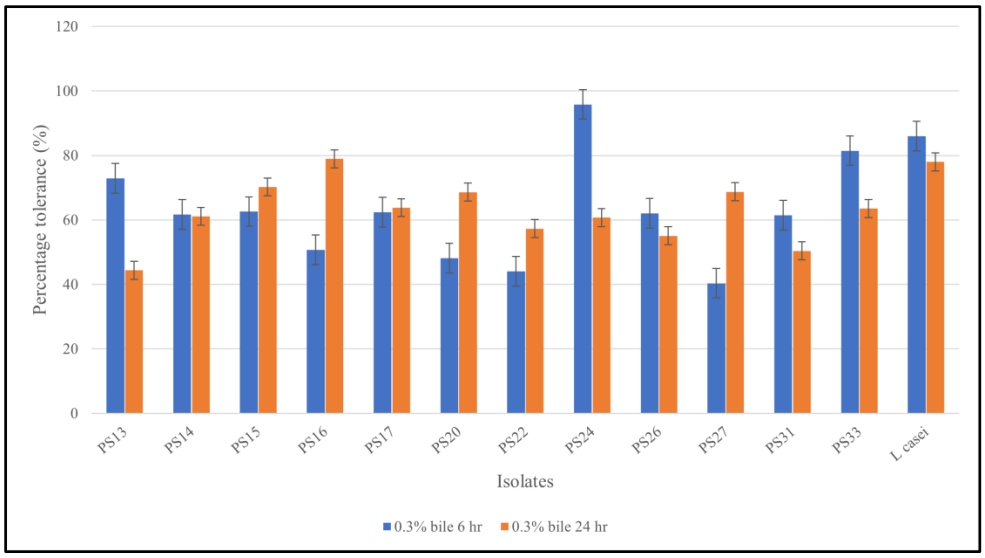

Figure 2. Effect of 0.3% bile salt on survival of 12 isolates, and *L. casei* strain Shirota following 6 h incubation for 24 h at 37°C.

Liver is an organ known to naturally produce bile salts, which are toxic substances. Bile salts are harmful to living cells because they disrupt the structure of cell membranes. However, bile salts are essential in supporting the digestion of lipids in the gastrointestinal tract. Since bacterial membranes are made of lipids, bacterial resistance to bile salts is important for their gastrointestinal colonisation (Sakoui *et al.*, 2022). The resistance rate determines the ability of bacteria to survive until viable cells reach the small and large intestines. This ultimately improves the balance of the gut microbiota (Shehata *et al.*, 2016). *Lactobacillus* has been reported to be a strain that can survive the gastrointestinal passage because it can grow in the bile environment. Bile salt hydrolase activity clearly influences bacterial resistance to bile salts. Huang *et al.* (2019) also reported that the feeding in the gastrointestinal tract could protect the LAB strain, and increase its tolerance to bile salts.

In the present work, six of the isolates (PS13, PS14, PS24, PS26, PS31, and PS33) achieved higher survival rates at 24 h as compared to 6 h, and another six isolates showed improved survival in bile salts. The low rate suggested that the bile resistance characteristics might be strain-specific. The higher resistance of these isolates to longer incubation periods might have been due to their ability to cope with stress conditions over longer incubation periods compared to other isolates tested (Sahadeva *et al.*, 2011). LAB with protective mechanism against bile may be due to the production of bile salt hydrolase

(BSH), which can hydrolyse the bile, and reduce its lethal effects. Additionally, isolates may have the ability to alter cell membrane composition, and cause active bile efflux to counteract the lethal effects of bile (Ilyanie *et al.*, 2022).

From the results of the resistance of isolates to 0.3% bile salts shown in Figure 2, isolates were found to have a higher survival rate after 6 h than after 24 h. Bile is a toxic digestive fluid that can inhibit bacteria by emulsifying lipids. In addition, when ingested in large quantities, it becomes extremely hydrophobic, which can change the structure of the cell membranes, and cause tissue disorganisation. Therefore, the ability of a strain to survive in the intestine is more important than its tolerance to acidic environments, as a strain can only be considered a probiotic if it can grow and survive in harsh intestinal conditions. LAB can withstand severe small intestinal condition because its cell membrane is made of lipid, and can change the composition of its cell membrane. This enhances the ability of LAB to colonise the GIT, and remain active in the small intestine. Once the LAB can withstand normal physical bile concentrations, it can withstand stressful conditions, and reach the small and large intestines. An aggressive bile flush is then performed to neutralise the deadly effects of bile, and ultimately improve the balance of the intestinal flora.

Molecular identification of potential probiotic isolates though 16S rRNA

The PCR of the LAB isolates using the 16S rRNA gene with forward and reverse primers revealed a band size of approximately 1,500 bp for all amplicons, which was the expected size. Table 1 shows the result of 12 isolates identified using 16S rRNA sequences, including their corresponding accession numbers. The BLAST results of 16S rRNA gene sequences showed that the isolates were molecularly identified as *Lactiplantibacillus plantarum, Bacillus cereus*, and *Bacillus* sp. It was identified with 98 to 100% similarity to the GenBank nucleotide sequences database. A phylogenetic tree of 16S rRNA sequences of the 12 isolates and seven bacterial strains obtained from the GenBank was constructed using MEGA X. This is shown in Figure 3. *Escherichia coli* (NR_024570.1) was used as an outgroup. Identification of isolates using the 16S rRNA gene identified all isolates as *L. plantarum* and *Bacillus* species with 98 - 100% similarity to the GenBank nucleotide sequence database. However, molecular identification of *Bacillus* isolates did not yield satisfactory results.

16S rRNA gene sequence identified the Bacillus strain as Bacillus sp.

Lactobacillus is one of the established Gram-positive bacteria that is facultative anaerobic or microaerophilic. It is a rod-shaped, non-spore-forming bacterium, acid-tolerant, and catalase-negative (Huang *et al.*, 2019). *Lactobacillus* such as *L. casei* and *L. acidophilus* are commonly used as probiotics, and are available in probiotic drinks such as Yakult and Vitagen. It is not surprising that the LAB was isolated from the lactic acid bacteria group from fermented seafoods. Previously, Khalil *et al.* (2018) successfully isolated *L. fermentum*, an exopolysaccharide-producing LAB strain, from *budu*. Haitham *et al*. (2017) were able to isolate 13 probiotic LAB from *belacan*. These were identified as

Table 1. Identified LAB isolates by 16S rRNA gene sequencing with their GenBank accession number.

Isolate	Name	GenBank accession number (NCBI)
PS13	*Lactiplantibacillus plantarum*	OQ326933.1
PS14	*Bacillus cereus*	MK578217.1
PS15	*Lactiplantibacillus plantarum*	OQ346373.1
PS16	*Bacillus cereus*	LC189362.1
PS17	*Lactiplantibacillus plantarum*	OQ301750.1
PS20	*Lactiplantibacillus plantarum*	OQ002418.1
PS22	*Bacillus* sp.	MK600540.1
PS24	*Lactiplantibacillus plantarum*	OQ301751.1
PS26	*Bacillus* sp.	MT611870.1
PS27	*Bacillus* sp.	MT538528.1
PS31	*Lactiplantibacillus plantarum*	OQ002415.1
PS33	*Lactiplantibacillus plantarum*	OQ927570.1

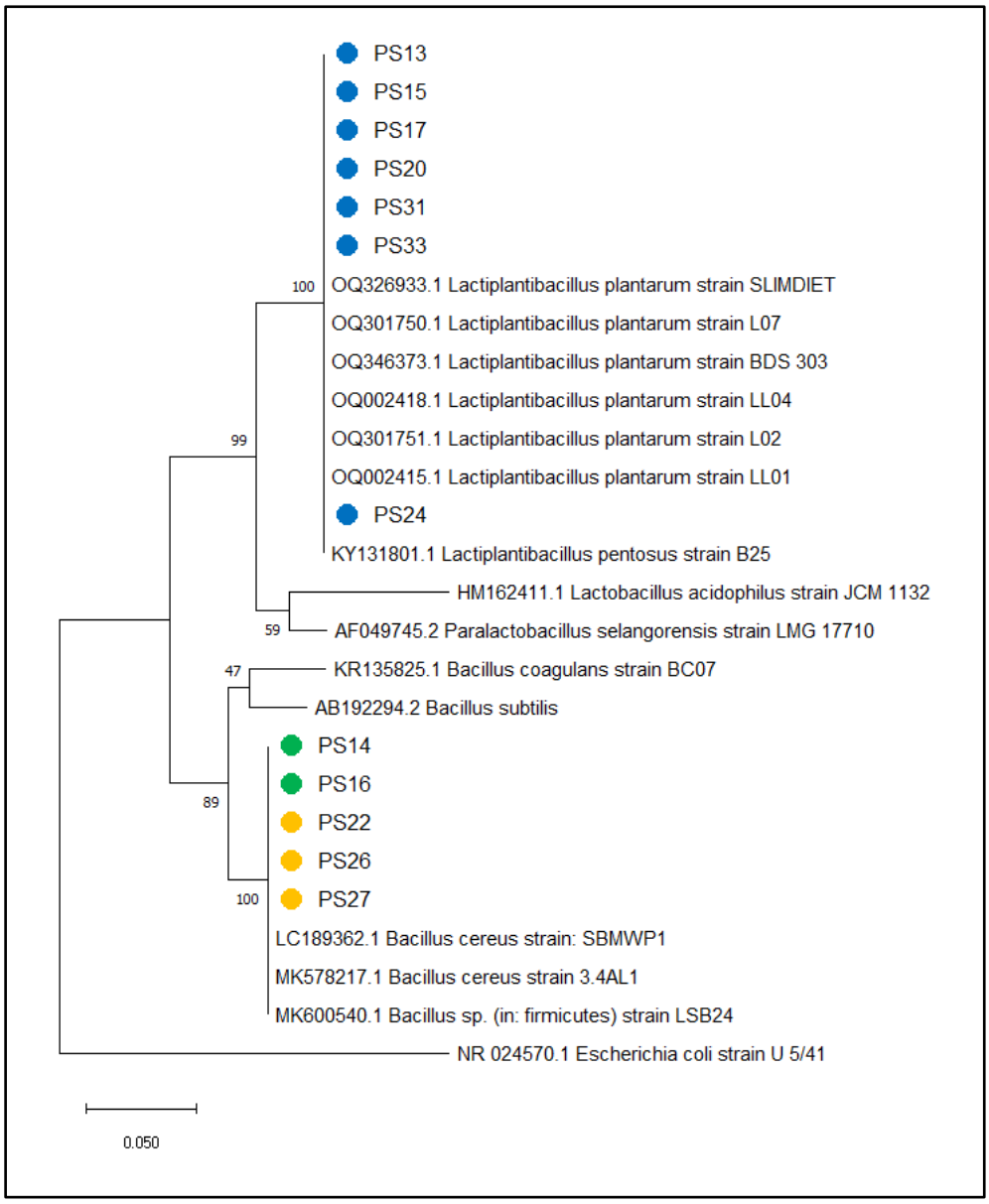

Figure 3. Phylogenetic tree of isolates and reference strains based on the 16S rRNA gene sequences (blue: *Lactiplantibacillus plantarum*; green: *Bacillus cereus*; and yellow: *Bacillus* sp.).

L. plantarum and *L. acidophilus*, among two other species from different genera named *Enterococcus faecium* and *Pediococcus acidilactici*. Similarly, in a study by Le and Yang (2018), one of the major isolates of salt-fermented shrimp was *L. plantarum*.

The present work discovered *Bacillus* spp. from fermented *pekasam senek*. Although the species is known as a cause of food poisoning, it is also recognised and approved as a probiotic in human medicine and livestock production, although the standard of safety evaluation is low (Zhu *et al.*, 2016). *Bacillus* strains are widespread in nature, typically found in fermented foods and the human intestine. Apart from accidental outbreak, no outbreaks of foodborne *B. cereus* infections caused by probiotics have been recorded. Therefore, future safety regulation of probiotics should consider the potential for antibiotic resistance gene transfer in addition to the lack of observable toxicity.

Bacillus cereus is used as a probiotic in humans, and some of the available commercial *Bacillus cereus* strain for human use are Biosubtyl^{AD} (*B. cereus*), Bactisubtil (*B. cereus* IP 5832), and Subtyl (*B. cereus* var. *vietnami*) (Lee *et al.*, 2019). *Bacillus* strains are associated with the risk of enterotoxin production, but are often inadvertently ingested through fermented foods. *Bacillus* species with antibiotic resistance properties are commonly found in vegetables, grains, and meat products. A disadvantage of using *Bacillus* strains as probiotics is their ability to introduce genes associated with antibiotic resistance. Nevertheless, sporulation of

Bacillus strains allows long-term survival under extreme environmental conditions, and prevents them from being killed, due to germination, growth, and new formation within the GIT (Jawan *et al.*, 2019).

The use of probiotic strains has resulted in consumer affinity and acceptability while maintaining probiotic properties. The findings of the present work supported previous reports regarding *Bacillus* spp. as probiotics that are safe for human use (Zhu *et al.*, 2016). In contrast to LAB, probiotic *Bacillus* strains have the advantage of stability as they can be used in spore form under storage conditions (Lee *et al.*, 2019). Furthermore, the evaluation of the gut microbiota implementing *Bacillus* probiotics is necessary to discover novel probiotics that alter the microbiota. The results also showed that MEGA's maximum likelihood method can be used to determine the evolutionary history of an organism.

Bile salt hydrolase (BSH) activity of LAB

The clinical benefit of BSH-active bacteria in decreasing hypercholesterolemia is regularly documented. Intestinal bacteria produce BSH to deconjugate glycol-conjugated and tauro-conjugated bile-acids (BA) by hydrolysing their amide bonds, and releasing free bile acids, including cholic and chenodeoxycholic acids, as well as amino acids like taurine and glycine. It has been demonstrated by Bourgin *et al.* (2021) that intestinal bacteria can deconjugate bile-acids, which reduce cholesterol absorption by absorptive cells known as enterocytes, and increase cholesterol excretion through the faeces. In humans, probiotic LAB play a significant role in the prevention of metabolic illnesses.

All 12 isolates in the present work were grown on MRS agar plates supplemented with 0.5% taurodeoxycholic acid (TDCA) and 0.37 g/L of calcium chloride ($CaCl_2$). A control plate was made up of MRS agar plate without TDCA and $CaCl_2$. All isolates on both test plates showed normal growth, without precipitation zones, indicating a weak BSH activity. The benefits of probiotic bacteria that produce BSH are currently being debated. Although the probiotics involved in BSH production have a cholesterol-lowering effect on the host, conjugated bile salts are toxic to the host and interact with the gut microbiome, causing digestive issues. According to some researchers, the health benefits of BSH probiotics are not completely favourable due to the possibility of stimulation (Yusuf *et al.*, 2020).

All 12 isolates did not exhibit BSH activity to varying degrees. All isolates exhibited low BSH activity, as the isolates did not have a precipitation zone. BSH helps probiotics resist bile salts during gastrointestinal transit. Furthermore, Begley *et al.* (2006) also reported that BSH activity was not detected in bacteria isolated from environments where bile salts were absent.

Bile acid (BA) deconjugation by BSH enzymes is a key process in BA metabolism in the small intestine (Bourgin *et al.*, 2021). As a result, BSH regulates a variety of metabolic activities in animals, such as dietary lipid absorption, cholesterol metabolism, energy, and inflammatory homeostasis. BSH activity modulation has been reported to be a viable technique to control obesity in humans, as it can alter host lipid metabolism, energy production, and weight gain. Therefore, BSH represents a potential microbiome target in the development of novel therapeutics to control human obesity.

In accordance with Dong and Lee (2018), the six main conjugated BA in bile are the taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), glycolic acid (GCA), glycodeoxycholic acid (GDCA), and glycochenodeoxycholic acid (GCDCA). The content of conjugated BA varies across animals and humans, and the amounts in humans are as follows: 9% TCA, 9% TDCA, 8% TCDCA, 26% GCA, 22% GDCA, and 26% GCDCA, indicating the ratio of glycine to taurine; 3:1 (Dong and Lee, 2018).

On the contrary, Tarique *et al.* (2022) reported that 12 selected isolates from a traditional high-acid and low-moisture yogurt-like products (*lebaneh*) exhibited hydrolactic activity toward bile salt mixtures. The findings of the study showed that the BSH activity in LB2 and MBL5 was the highest, and that of MBL3 was the lowest. Additionally, Hernández-Gómez *et al.* (2021) reported a positive BSH activity in all probiotic strains, with the exception of *S. boulardii*, and Tsai *et al.* (2014) reported 22 isolates with precipitation zones of various sizes, ranging from 17 to 24 mm.

Deconjugation of BA by the BSH enzyme plays a significant role in human GIT by reducing potential damage to cell membranes. BSH deconjugation activity is strongly associated with *Lactobacillus* species, and is reported as strain-dependent trait. The BSH enzyme activity varies accordingly with environments, as they have wide

range of specificity toward glycol and tauro-conjugated bile acid (Bourgin *et al.*, 2021). *Lactobacillus* strains in the intestine have been reported to be able to produce both glycol and tauro-conjugated bile salts through the hydrolysis of the amide bond, and the release of free bile acids such as cholic and chenodeoxycholic acid, and the amino acids such as glycine and taurine (Xu *et al.*, 2019).

One of the screening factors for probiotics linked with hypercholesterolemia therapy is the capacity of bacteria to proliferate and hydrolyse conjugated bile salts. However, the ability to produce BSH for cholesterol reduction is not a required feature of probiotics.

Cholesterol-lowering activity of LAB

There is an increasing number of novel probiotic strains with significant cholesterol-lowering activity in humans (Choi and Chang, 2015). One of the established mechanisms for cholesterol-lowering activity in humans is the removal of intestinal cholesterol by probiotic cells. The cholesterol-lowering activity of the potential isolates was analysed and determined calorimetrically using the cholesterol quantitation kit.

Twelve bacterial strains showed an adequate reduction of cholesterol of over 50% and up to 80.50%, which surpassed the gateway value for the bacterial strain to be considered a probiotic with cholesterol-lowering properties. Considering the result obtained (Figure 4), isolate PS22 showed the least cholesterol-lowering activity with 50.55% of cholesterol removal. Meanwhile, the greatest cholesterol reduction was observed in isolate PS24 at 80.50%, followed by PS31 at 80.19%. The ability of different strains to lower cholesterol content was not significantly different ($p > 0.05$) from one another.

The result depicted in Figure 4 indicates that *Lactiplantibacillus plantarum* certainly has the potential to reduce the level of serum cholesterol in humans, and *Bacillus* spp. are also effective in lowering the cholesterol content in blood serum. Even in the absence of BSH, the isolates tested in the present work showed potential to lower the cholesterol content. The present work proposed that *Lactiplantibacillus plantarum* (PS13, PS17, PS20, PS24, and PS31) could have better ability to lower cholesterol levels as they showed a more stable reduction pattern compared to *Bacillus* spp. In terms of elucidating the cholesterol-lowering mechanism by *Bacillus* spp., the findings from the present work can only be regarded as preliminary. In comparison with the control MRS broth, all 12 isolates showed ability to lower the water-soluble cholesterol loaded into the MRS medium. This implied that the isolates hindered the cholesterol absorption by the circulatory system. The isolates with cholesterol-lowering ability might be beneficial in food preparation or pharmaceutical applications, even though the functional probiotics are strain-dependent.

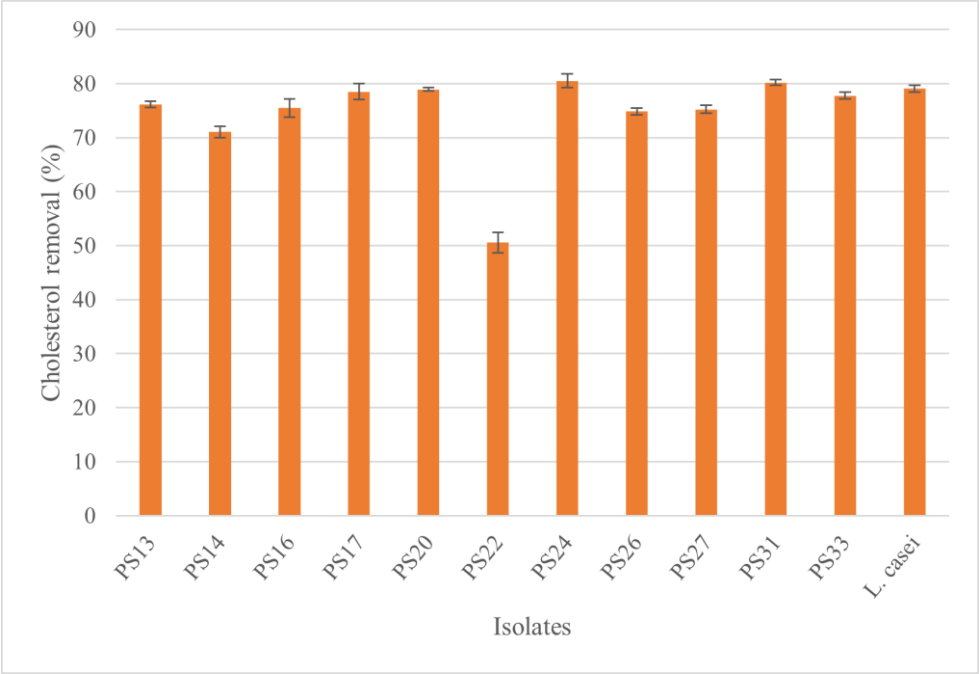

Figure 4. Cholesterol-lowering activity of 12 isolates and *L. casei* strain Shirota.

This agreed with Ding *et al.* (2017), where 44 strains of LAB displayed more than 50% of cholesterol reduction rate, and nine *L. plantarum* strains showed the highest cholesterol reduction at 75.9%. Other researchers that studied *Lactobacillus* isolated from fermented foods discovered similar outcomes: Gao and Li (2018) reported that 32 bacterial strains isolated from traditional fermented cucumber possessed cholesterol-lowering properties with a reduction rate of more than 45%; Ma *et al.* (2019) reported that eight bacterial strains showed cholesterol removal rates of more than 40%, and *L. plantarum* CAAS 18010 was recorded as LAB strain with the highest cholesterol reduction. Contradictory with a report by Shehata *et al.* (2016), where nine LAB isolates from dairy and non-dairy sources showed a small degree of cholesterol removal, ranging from 8.4 to 43.5%.

The findings were linked to the assimilation of cholesterol into cells and the adhesion of cholesterol to the cell surface (Bhat and Bajaj, 2020). The results also suggested the ability of isolates to reduce cholesterol content during the growth phase, which is influenced by the metabolic process. The growing cells might break down and take in cholesterol as a fatty acid for cell membrane formation, and the conversion of cholesterol to coprostanol (Yusuf *et al.*, 2020). Meanwhile, the differential in cholesterol reduction from the media might have been caused by cholesterol absorption by developing cells.

The findings of the present work revealed that cholesterol removal activity varied by isolates, as evidenced by varying degrees of decrease in *Lactiplantibacillus plantarum* and *Bacillus* spp.

Conclusion

Research on microorganisms with probiotic characteristics is being conducted all over the world since it has shown promising results. The need to isolate lactic acid bacterial strains with potent probiotic potential in the present work was achieved with the isolation and characterisation of 12 LAB strains from traditional Malaysian fermented food product from Sarawak, which was fermented *pekasam senek*. The characterisation and identification of LAB isolates using the 16S rRNA genes revealed that six isolates were *Lactiplantibacillus plantarum* (PS13, PS15, PS17, PS20, PS24, and PS31), two were *Bacillus cereus* (PS14 and PS16), and three were *Bacillus* sp. (PS22, PS26, and PS27). All 12 isolates demonstrated satisfactory tolerance against gastrointestinal high acidic and bile salt conditions. The viable counts of all isolates were around 5.00 to 7.23 $\log_{10}$ CFU/mL after 3 h of exposure to low pH 3.0. Meanwhile, for the bile salt assay, nine isolates survived with a 50 to 95% of tolerance rate, and three isolates (strains PS20, PS22, and PS27) survived with below 50% of tolerance rate after 6 h of exposure to 0.3% bile salt. The subsequent molecular identification using 16S rRNA genes of isolated strains showed that six bacterial strains were identified as *Lactiplantibacillus* sp. and *Bacillus* sp. with 98 to 100% of similarity in the GenBank. Furthermore, the isolated *Lactiplantibacillus* sp. displayed more stable cholesterol-lowering activity compared to *Bacillus* sp. due to their species-specific characteristic. Finally, the identified LAB have shown their potential in lowering the cholesterol content, which adds to the health benefits of probiotics from fermented food products. The results demonstrated that the identified isolates could be suitable and safe for the use in food as probiotics with health benefits for humans.

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