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SCREENING OF POTENTIAL PROBIOTIC CHARACTERISTICS OF LACTIC ACID BACTERIA ISOLATED FROM MALAYSIAN FERMENTED PEKASAM

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Abstract

Lactic acid bacteria (LAB) from fermented foods are proven to be able to hinder the growth and activities of some foodborne pathogens. Antagonistic effects and sensitivity to antibiotics are important factors that need to be considered during the screening of potential probiotic strains. This study aims to evaluate the *in vitro* antagonistic activities with hemolytic activity and antibiotic susceptibility of LAB isolated from Malaysian fermented food, Pekasam Senek. Twenty LAB isolates were assessed for their antagonistic activities against *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae,* and *Shigella sonnei* via the spot overlay method. LAB isolates with positive results in antagonistic proceeded with subsequent assay, hemolytic assay, and antibiotic susceptibility test. Bacterial cultures were streaked on a fresh blood agar plate to examine the signs of β-hemolysis, α-hemolysis, and γ-hemolysis. The antibiotic susceptibility patterns of the strains to six types of antibiotics were assessed through the disc diffusion method. Antagonistic evaluation tests showed that all LAB isolates were able to inhibit the growth of pathogenic bacteria with the highest inhibition zone (31 mm) produced by PS26 against *E. coli*. Twelve (12) isolates showed negative hemolytic activity which indicates that they are safe and screened out for their antibiotic susceptibility testing. Furthermore, all twelve isolates were susceptible to ampicillin, bacitracin, chloramphenicol, and erythromycin while resistant to streptomycin. This study indicates that LAB isolated from Pekasam Senek had significant antagonism ability against the tested pathogens with negative hemolysis. Meanwhile, the resistance patterns of the isolates varied depending on the different types of antibiotics.

INTRODUCTION

Lactic acid bacteria are non-motile, Gram-positive, and non-sporulating bacteria in the human gastrointestinal tract [1]. They are non-pathogenic bacteria and live naturally in gastrointestinal tracts that confer health benefits, making them a viable alternative to antibiotics or conventional therapies for intestinal diseases [2]. Intestinal lactic acid bacteria (LAB) were first introduced as probiotics at the turn of the 20th century and have been widely used as a natural alternative to antibiotic supplementation due to their health-promoting properties such as the ability to produce antimicrobial substances that inhibit the activity of pathogenic bacteria [3]. There has been a spike in interest in probiotics over the last decade, and there is now growing evidence recognizing the value of probiotic products as promising beneficial foods for gut well-being, disease prevention, and therapy [4]. The discovery of new probiotic strains from functional food such as fermented foods has lately gotten a lot of attention for their health benefits [5]. The need for non-dairy probiotics to combat infections in the individual's gut is also driving research on novel probiotic strains [5]. Lactic acid bacteria are majorly investigated probiotic strains and they are the most significant bacterial probiotic used in functional food [6]. The ability of LAB in stimulating gastrointestinal (GI) tract development, and

digestive function, improve the immune response. Lower cholesterol content also has made the microorganism become a potential probiotic [1]. Lactobacilli, *Bifidobacteria,* and *Streptococcus* are examples of probiotic bacteria that have been tested for the prevention or treatment of different diseases and determined to be safe [6; 7]. According to the World Health Organization, probiotics are "live microorganisms which when administered in adequate amounts, grant health benefit to the host" [8]. Many potential probiotic strains are found in fermented foods and proven to be eligible as probiotics and portray great food bio-preservative characteristics [9]. Probiotics and their association with human health have been an area of interest in this respect for years [8]. Research has shown that microorganisms from fermented foods are able to penetrate the gastrointestinal tract, however, this is likely to vary by product, and their presence in the gut appears to be impermanent [10]. However, these microorganisms might still be able to provide a physiological advantage in the gut, via competition with pathogenic bacteria and the production of immune-regulatory and neurogenic fermentation by-products [11]. Secondly, metabolites created from fermentation may have health advantages [12]. Lactic acid bacteria, for example, produce bioactive peptides and polyamines with potential impacts on cardiovascular, immunological, and metabolic health (applicable to both dairy and non-dairy fermented foods) [13]. Since there are many Lactobacillus strains, each animal's ideal probiotic strain should be isolated and characterized from the homologous host. Probiotic bacteria isolated from fermented foods were able to colonize the human gastrointestinal tract and showed the best growth-stimulating performance in the host [14]. Screens of probiotic bacteria usually focus on commercially fermented shrimp food. Despite the widespread usage of Pekasam Senek as a traditional side dish in Malaysian cuisine, limited research has been conducted to evaluate the probiotic potentials of its bacterial isolates. To date, few attempts have reported the characterization of bacteria isolated from fermented food; Sari *et al*. (2012) reported the physiological characteristics, acid and bile tolerance, and antimicrobial activity of LAB strains isolated from *pekasam ale-ale* [15]; Amalia et al. (2018) focused only on physiological and biochemical characteristics of LAB isolated from *terasi,* but lack of other probiotic features [16]. For Pekasam Senek, there has been no report on the probiotic potential of its bacterial isolates. Therefore, the present study was conducted to identify the probiotic characteristics of LAB isolated from Pekasam Senek.

MATERIALS AND METHODS

Materials

Pekasam Senek was collected from Saratok Wet Market, Saratok, Sarawak. de Man, Ragosa and Sharpe (MRS) agar, MRS broth, and Mueller Hinton agar were purchased from Nextgene, Malaysia. Bacterial test strain *Bacillus cereus (B. cereus,* ATCC 1178), *Escherichia coli (E. coli,* ATCC 25922), *Klebsiella pneumoniae (K. pneumoniae,* ATCC 700603), and *Shigella sonnei (S. sonnei*, ATCC 25931) were obtained from the Culture Bank Unit, School of Biology, Faculty of Applied Sciences Kuala Pilah Campus, UiTM Cawangan Negeri Sembilan. All reagents and solvents used in this study were of the analytical grade.

Isolation of Lactic Acid Bacteria (LAB)

In this current study, for the isolation of lactic acid bacteria, the spread plate method was used. Briefly, samples of Pekasam Senek were weighed 10 g, this amount was then crushed together with 250 mL of MRS broth. This was followed by the serial dilution of the mixture up to a 10^{-5} dilution factor [17]. From each dilution, 100 µL was taken out by a micropipette and spread onto MRS agar media which has been prepared in a petri dish by pour plate method. Furthermore, the culture was incubated for 24-48 hours at 37°C aerobically. The purification of lactic acid bacteria isolates was performed through the quadrant method until pure isolates were obtained. The potential probiotic isolates were stored in MRS broth containing 20% glycerol at -20°C for long preservation and further analysis [7].

Morphological Observation of Lactic Acid Bacteria Cells (Gram Staining)

The potential probiotic LAB were first biochemically analyzed based on their morphology. The isolates will be recognized by utilizing Gram-staining according to the standard procedure [18]. The glass slide was blot dried and observed under a light microscope to identify the Gram-positive strain, confirming the isolate as lactic acid bacteria.

Biochemical Tests of Lactic Acid Bacteria (Catalase Test)

The catalase test was performed to initially identify the lactic acid bacteria strain. A single colony was streaked on a glass slide and a drop of 3% hydrogen peroxide was added to the colony [19]. Negative catalase isolates were selected for subsequent assay to determine the potential probiotic characteristics of the isolates [20].

Antagonistic Activity of Lactic Acid Bacteria (LAB) via Spot Overlay Method

The antagonistic activity of presumptive strains of LAB isolates against pathogenic bacteria (*Bacillus cereus, Escherichia coli, Klebsiella pneumoniae,* and *Shigella sonnei*) was screened via the spot overlay method described by Jomehzadeh et al. (2020) with a slight modification [2]. Then, five µL of LAB (10^9 CFU/mL) were spotted onto previously prepared MRS agar and incubated afterward at 37°C for 24 hours. After the overnight incubation, 10 mL of

molten Mueller Hinton Agar (MHA) seeded with pathogenic bacteria (10^7 CFU/mL) were poured onto the agar plate and let solidify. After the incubation at 37°C for 24 hours, the antagonistic activity of the LAB strains was observed by measuring the diameter of the inhibition zone (mm) around the colony. 10 µg of streptomycin (10 µg) was used as positive control while commercial strains of *Lactobacillus casei* strain Shirota (Yakult, Japan) were used as the reference strain [9]. The inhibition zones were measured in diameter (mm).

Hemolytic Activity of Lactic Acid Bacteria (LAB)

Isolates were screened on blood agar plates containing 5% sheep blood and incubated at 37°C for 48 hours. Fresh overnight bacterial cultures were streaked on 5% sheep blood agar and incubated at 37°C for 24-48 hours [21]. Hemolytic activities of the bacterial culture were examined for signs of β-hemolysis (clear zones around colonies), α-hemolysis (green zones around colonies), or γ-hemolysis (no clear zones around colonies) on human blood agar plates. Hemolytic activity was detected as the presence of a clear zone around bacterial colonies [22].

Antibiotic Susceptibility Assay of Lactic Acid Bacteria (LAB) using Disc Diffusion Method

Antibiotic susceptibility assay was performed by the disc diffusion method using commercially available antibiotic discs (Oxoid™, UK) including ampicillin (10 µg/disc), bacitracin (10 µg/disc), chloramphenicol (30 µg/disc), erythromycin (15 µg/disc), streptomycin (10 µg/disc) and vancomycin (30 µg/disc) [23]. Lactic acid bacteria strains were inoculated in MRS broth and grow overnight. When the broth cultures reached the turbidity equal to 0.5 McFarland standards at 37°C, the suspensions were streaked onto previously prepared MRS agar plates with sterile cotton swabs, and then antibiotic discs were seeded onto the agar [24]. After 48 hours of incubation at 37°C, the inhibition zone diameters were measured, and results were expressed in terms of resistance (≤15 mm), moderate susceptibility (16-20 mm), or sensitive (≥21 mm) [2].

RESULTS AND DISCUSSION

All twenty (20) potential probiotic bacteria were identified as LAB based on their morphological, biochemical, and physiological characteristics. The observation showed that all LAB strains were Gram-positive bacteria. Through Gram-staining and microscopy, the isolates were differentiated into Gram-positive bacillus, coccus, or coccobacillus. After morphological studies, a catalase test was performed with isolated bacteria to identify isolates on the basis of their biochemical characteristics. All 19 tested presumptive isolates were catalase negative. Nineteen isolates were Gram-positive and catalase-negative properties as shown in Table 1. All the Gram-positive bacteria studied did not pose any catalase activity, indicating that the strains fit with the important prerequisite for the probiotics [25]. All isolates were further analyzed to study their potential probiotic properties.

In the present study, all of the LAB isolates exhibited a varying degree of antagonism against *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae,* and *Shigella sonnei*. Table 2 shows the inhibition zone of twenty isolates against four pathogenic bacteria via the spot-overlay method [1;28]. The spot overlay method was performed to determine the antibacterial activities of the strains against *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae,* and *Shigella sonnei*. All the isolates exhibited the inhibition of the growth of four indicator pathogens, which had a broad spectrum of bacteriosis. According to Jomehzadeh et al. (2020), isolates having clearance zones less than 11 mm and more than 23 mm diameter against the tested pathogens stipulated poor and strong antagonistic activity, respectively. Among them, two strains (PS15 and PS26) had the strongest antibacterial effect on *E. coli* with 31mm of inhibition zone in diameter [2]. Accordingly, all the selected potential probiotic LAB strains currently studied exhibited strong antagonistic activity against the food-borne pathogens, where PS26 displayed the highest antagonistic activity against *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae,* and *Shigella sonnei* with the inhibition zone ranged from 28 to 31 mm in diameters. In accordance with the current study, Hajar and Hamid (2013) have demonstrated that 5 out of 7 *Lactobacillus* isolates obtained from Malaysian traditional fermented shrimp 'Cincaluk' have a strong antagonistic activity against *E. coli* except for isolates 4 and 5 which did not show any inhibition zone against the pathogenic bacteria [27]. PS16 also displayed the strongest bacteriostatic effect on the growth of *K. pneumoniae.* A recent review has a similar result on the antagonistic activity exhibited by *Lactobacillus* spp. against the pathogenic food-borne bacteria, *Klebsiella pneumoniae* [12]. *Enterococcus, Pediococcus, Leuconostoc*, and *Weissella* strains from the study portrayed great inhibitory effects against *Klebsiella pneumoniae*. However, PS31 showed the weakest antibacterial effect on *B. cereus,* and the values are given in Table 2. LAB isolates from the current study successfully exhibited antagonistic activity against food-borne pathogens, *Bacillus cereus*, unlike LAB isolated from Koozeh cheese which has no ability and active effect to inhibit the growth of *E. coli* and *B. cereus* [28]. The isolates exhibited excellent antibacterial activities on the four selected indicator pathogens and were selected for the subsequent analysis of the hemolytic activity. The different degrees of antagonistic activity of LAB towards food-borne bacteria might be due to several factors such as the production of organic acid or from the bacteriocins.

Table 1. Biochemical characterization of isolates; Gram Staining, Catalase Test

Isolates	Gram staining	Catalase test
PS13	+	-ve
PS14	+	-ve
PS15	+	-ve
PS16	+	-ve
PS17	+	-ve
PS18	+	-ve
PS19	+	-ve
PS20	+	-ve
PS21	+	-ve
PS22	+	-ve
PS24	+	-ve
PS25	+	-ve
PS26	+	-ve
PS27	+	-ve
PS28	+	-ve
PS29	+	-ve
PS30	+	-ve
PS31	+	-ve
PS32	+	-ve
PS33	+	-ve
***Lactobacillus casei* strain Shirota**	+	-ve

Table 2. The mean ± SE of the inhibition zone (mm) of isolates (10^9 CFU/ml) against pathogenic bacteria (10^7 CFU/ml) after 24 h incubation period at 37°C

Isolates	Inhibition zone (mm)			
	B. cereus ATCC 11778	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 700603	*S. sonnei* ATCC 25931
PS13	26 ± 1.4	30 ± 0.7	27 ± 1.4	30 ± 1.4
PS14	23 ± 1.4	28 ± 0.7	20 ± 1.4	26 ± 1.4
PS15	25 ± 1.4	31 ± 0.7	24 ± 2.1	26 ± 1.4
PS16	26 ± 1.4	28 ± 0.7	31 ± 0.7	28 ± 1.4
PS17	26 ± 1.4	26 ± 1.4	22 ± 0.7	26 ± 2.8
PS18	14 ±2.1	17 ± 0.7	16 ± 2.1	18 ± 2.8
PS19	20 ± 1.4	23 ± 0.7	24 ± 2.8	25 ± 1.4
PS20	19 ± 3.5	25 ± 0.7	21 ± 2.1	21 ± 1.4
PS21	21 ± 2.1	24 ± 0.7	22 ± 0.7	23 ± 2.8
PS22	22 ± 1.4	26 ± 0.7	20 ± 0.7	22 ± 1.4
PS24	26 ± 2.8	31 ± 0.7	28 ± 0.7	29 ± 0.7
PS25	26 ± 4.2	29 ± 0.7	28 ± 0.7	29 ± 0.7
PS26	28 ± 2.1	31 ± 1.4	30 ± 1.4	28 ± 2.1
PS27	16 ± 1.4	21 ± 1.4	19 ± 3.5	18 ± 2.1
PS28	15 ± 0.7	21 ± 2.1	17 ± 1.4	18 ± 2.1
PS29	17 ± 0.7	22 ± 0.7	20 ± 1.4	13 ± 2.8
PS30	14 ± 0.7	23 ± 2.1	17 ± 0.7	16 ± 1.4
PS31	6 ± 0.7	15 ± 2.8	22 ± 2.8	19 ± 2.8
PS32	22 ± 0.7	27 ± 1.4	22 ± 1.4	21 ± 3.5
PS33	24 ± 1.4	27 ± 2.8	24 ± 2.8	23 ±3.5
Streptomycin (10µg)	9 ± 2.8	9 ± 3.5	14 ± 0.7	17 ±0.7
***Lactobacillus casei* strain Shirota**	22 ± 2.1	25 ± 1.4	25 ± 1.4	23 ± 1.4

Pathogenic bacteria: *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae* and *Shigella sonnei*

One of the important characteristics of probiotics for consumption is to be safe in the host [29]. The findings and use of non-hemolytic strains as probiotics demonstrate their non-virulent nature. Table 3 displays the results of twelve (12) out of 20 LAB strains that showed negative hemolysis test, indicating that 12 presumptive strains had no hemolysis and were safe for further use. Similar to the majority of LAB strains reported [29], the LAB strains derive from fermented Pekasam Senek in the current study are non-hemolytic. 8 LAB isolates examined for hemolytic activity were β-hemolytic and showed clear zones around the colony which indicates that they are positive hemolytic strains. Those 8 isolates were immediately screened out, as they were not considered safe according to the important characteristics of probiotics [21]. The findings were supported by a study by Reuben et al. (2019) where positive hemolytic activity was shown among the isolates [30]. In this study, 12 of the 20 LAB strains tested for hemolytic activity were found to be non-hemolytic, and this explains their safety usage as probiotics.

Table 3. Haemolytic Activity

Isolates	Haemolytic activity
PS13	γ
PS14	γ
PS15	γ
PS16	γ
PS17	γ
PS18	β
PS19	β
PS20	γ
PS21	β
PS22	γ
PS24	γ
PS25	β
PS26	γ
PS27	γ
PS28	β
PS29	β
PS30	β
PS31	α
PS32	β
PS33	α
***Lactobacillus casei* strain Shirota**	γ

The susceptibility of LAB isolates to antibiotics is an important characteristic of probiotic bacteria. In antibiotic susceptibility testing, all isolates showed different degrees of resistance and susceptibility patterns towards the selected antibiotics. The antibiotic susceptibility of all isolates was evaluated via the disc diffusion method using MRS medium and the results are shown in Table 4. All twelve (12) isolates were sensitive to ampicillin, bacitracin, chloramphenicol, and erythromycin, whilst resistant to streptomycin. Among them, four were sensitive to vancomycin while sixteen strains were resistant to it. As reported in the literature, the resistance and susceptibility of bacterial strains to several antibiotics might be desirable due to various reasons, either related to the antibiotics or bacterial strains themselves [31]. One of the important characteristics of probiotics is that they should be resistant to certain antibiotics to survive in the gastrointestinal tract. This finding is in accordance with the presence of antibiotic-resistance genes in many LABs that have been reported in *Lactobacillus* species. Various reports generally described that LAB is normally resistant to aminoglycoside (amikacin, kanamycin, streptomycin, and gentamycin) and related to their natural and intrinsic resistance [2]. Intrinsic resistance in lactic acid bacteria is not considered a risk to human health but the low transfer risk of intrinsic resistance was associated with the chromosomal mutation. Choi et al. (2018) reported that new probiotic strains; *W. cibaria* KCTC 3746 and *W. koreenis* KCCM 43060 were resistant to vancomycin. Contradict with strains *L. curvatus* KCCM 43119 and *Ln. mesenteroides* KCCM 43060 that were susceptible to vancomycin, were supposed

to be not chromosomally encoded [25]. PS20, PS22, PS31, and PS 33 strain from the current study indicates similar outcomes of the resistance towards vancomycin. In another report by A. Kumar and Kumar (2015), *L. rhamnosus* BFE 7442 was resistant against ciprofloxacin, gentamycin, and streptomycin that portrayed the probiotic strains consisted of the resistance gene but are silent [27]. *Lactobacillus* spp. are generally susceptible to chloramphenicol [25]. In accordance with this study, all isolates were susceptible to ampicillin, bacitracin, chloramphenicol, and erythromycin, whilst resistant to streptomycin. Only 4 isolates were susceptible to vancomycin.

Table 4. Susceptibility of isolates towards antibiotics using disc diffusion method after 24 h of incubation at 37°C

Antibiotic	Amp	B	C	E	S	Va
Concentration of antibiotic	**10 µg**	**10 µg**	**30 µg**	**15 µg**	**10 µg**	**30 µg**
Isolates	**Inhibition zone (mm)**					
PS13	S	S	S	S	R	R
PS14	S	S	S	S	R	R
PS15	S	S	S	S	R	R
PS16	S	S	S	S	R	R
PS17	S	S	S	S	R	R
PS20	S	S	S	S	R	S
PS22	S	S	S	S	R	S
PS24	S	S	S	S	R	R
PS26	S	S	S	S	R	R
PS27	S	S	S	S	R	R
PS31	S	S	S	S	R	S
PS33	S	S	S	S	R	S

Amp-Ampicillin, B-Bacitracin, C-Chloramphenicol, E-Erythromycin, S-Streptomycin, Va-Vancomycin
R: resistant to antibiotics, I: intermediately sensitive to antibiotics, S: sensitive to antibiotics.

In conclusion, the present study showed that fermented Pekasam Senek are sources of potential probiotics, and the isolates meet the important features to be considered as probiotics for application in food fermentation. The selected isolates can inhibit pathogenic bacteria with no hemolytic activity and are susceptible to antibiotics which are considered a great potential probiotic and safe for human use.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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