Auto aggregation activity and antibiotic susceptibility as a potential probiotic properties of lactic acid bacteria (LAB) isolated from Malaysian fermented foods.

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Malaysia has a wide variety of fermented foods that beneficial for health. The most commonly studied beneficial microbes of fermented foods are Lactic acid bacteria (LAB). Other than developing of desired flavor and odor of those fermented food, Lactic acid bacteria also has high probiotic potential. However, the probiotic properties of lactic acid bacteria naturally occurring in Malaysian fermented foods still remains unexplored. Therefore, present study aimed on isolation of lactic acid bacteria from Malaysian fermented foods and examination of various phenotypic identification and study characteristics related to the probiotic potential of these microorganisms. Pekasam (fermented fish), jeruk maman (fermented vegetable), tapai (fermented glutinuous rice) and tempoyak (fermented durian) were used as a source of Lactic acid bacteria. Isolated strains were identified their colonies morphologies, physiological and biochemical test to confirm their genus. All strains were matched with characteristics of LAB by showing a negative catalase test, gram positive and rod shaped bacteria. They were mesophilic bacteria and survived at 1.5%, 2.5%, 5%, 7.5% and 10% of NaCl concentration. Further twenty selected strains were determined antibiotic susceptibility to selected eight antibiotics and auto aggregation activity. All the selected strains showed various susceptibilities toward antibiotics. All selected strains showed high auto aggregation percentage after being incubated at room temperature for 24 h. Among the samples used, lactic acid bacteria isolated from pekasam (fermented fish) showed the best probiotic potential with high auto aggregation percentage and resistance to all antibiotics. The availability of lactic acid bacteria isolated from pekasam (fermented fish) had potential application in functional foods and health-associated products. This fermented foods are commercially available and information obtained from this study could contribute to the potential use of these LAB isolates as a probiotic in the food and pharmaceutical industries.

Keywords: Fermented foods; Lactic Acid Bacteria; auto-aggregation activity; antibiotic susceptibility; probiotic properties

1. Introduction

Fermentation is the oldest method of food preservation that has been used worldwide. This method of preservation produced a variety of fermented foods and beverages with desired and edible microbes which are beneficial for health. Lactic acid bacteria originate from raw material itself, or apparatus used or environment is the responsible microbes that initiate the fermentation process (Weerkamp et al., 1996). Moreover, the growth of lactic acid bacteria could be favor by addition of salt in fermentation process (Swain et al., 2014).

Although historically the fermented products associated with beneficial Lactic Acid Bacteria were milk-based, recent research has shown promising probiotic activity of LAB isolated from fermented food (Rhee et al., 2011). They are recognized as "generally regarded as safe" and desirable micro flora in gastrointestinal tract (Tannock, 1997). Hence, they are most commonly studied probiotic for the past few decades. Probiotics are viable microorganisms, when ingested alive in a sufficient amount, have a positive effect on the health if they are able to resist in gastric juice and grow in the presence of bile (Nualkaekul et al., 2012). Therefore, it is important to determine their ability to adhere with intestines epithelial cells for successful colonization. In addition, the presence of antibiotic resistance determinants in their genome must be considered as an important parameter for the selection of the probiotic strains (Aliabadi & Chakoosari, 2013) in overcome antibiotic associated diarrhea (Angmo et al., 2016).

Research in the field of exploring a probiotic potential strain from Malaysian fermented foods are blooming. A few studies of lactic acid bacteria isolated from Malaysian fermented foods have been investigated. (Thanh et al., 2010) studied the inhibitory activity of metabolites produced by strains of *Lactobacillus plantarum*, meanwhile (Liasi et al., 2009) reported about antibiotic sensitivity from three isolates isolated from fermented fish product known as "budu". Besides that, previous work on tempoyak (fermented durian) and tapai (fermented tapioca) as sources of lactic acid bacteria have been done by (Adnan & Tan, 2007) and (Leisner et al., 2001).

Current study on *jeruk maman* (fermented vegetable) and *pekasam* (fermented fish) as a source of potential probiotic still remains unexplored. Moreover, the potential properties of LAB isolated from Malaysian fermented foods need to be expanded since each species of LAB has unique characteristics and thus can be used for different purposes. The availability of LAB strains isolated from this product could develop starter cultures and able to enhance the health aspect of local fermented products. Therefore, the present study is to determine the auto aggregation activity and antibiotic susceptibility of twenty isolates of LAB from various Malaysian fermented foods, which were *Tapai*, *Tempoyak*, *Pekasam* and *Jeruk Maman*. These fermented foods are commercially available and information obtained from this study could contribute to the potential use of LAB as a probiotic in the food and pharmaceutical industries.

2. Material and methods

2.1 Source of sample

Four different fermented foods were purchased from a local night market in Kuantan, Malaysia. *Tapai* is a fermented glutinous rice mixed with yeast and salt, meanwhile *Tempoyak* is fermented durian flesh mixed with salt, *Jeruk Maman* or its scientific name *Cleome gynandra* is a fermented vegetables

mixed with rice and salt and lastly, fermented fish covered with roasted rice with salt known as *pekasam.* All samples were kept refrigerated, so that microbial load does not change during storage, and processed between 12 to 16 h after the collection.

2.2 Enumeration and Isolation of lactic acid bacteria

At first, 25 g of sample was mixed with 225 mL of peptone water to obtain 1:10 dilution. Serial dilutions of the samples were prepared in peptone water. The diluted sample was spread on MRS (de Mann Rogosa Sharpe) agar, and the plates were anaerobically incubated at 37°C for 24 h. Colonies with different morphologies on the MRS agar plate were selected and further sub cultured by streaking on MRS agar in order to obtain a pure colony. The stock cultures of LAB were maintained in MRS broth supplemented with 25% sterile glycerol and stored at −80 °C. Working cultures were prepared on slants MRS agar and stored at 4°. Prior to its use during experiments, the LAB cultures were transferred twice into the appropriate medium.

2.3 Morphological, physiological and biochemical of LAB

All isolated strains were confirmed their genus by morphological, physiological and biochemical examination. Standard procedure of gram staining, catalase test, gas production test and NaCl tolerance test were performed.

2.4 Autoaggregation activity

Auto-aggregation assay were performed according to method described by (Del Re et al., 2000) and with some modification as refer to (Kumar et al., 2012) and (Collado, Meriluoto, et al., 2008). Nine milliliters of an overnight culture were centrifuged at 8000 g for 10 min. Pellet was re-suspended with phosphate buffered saline (PBS) and adjusted to OD_{600} = 0.25 ± 0.005. Four milliliters of suspension was mixed by vigorous vortexing for 10 s and then incubated at room temperature for 24 hour. At 1 h intervals, 0.1 ml of upper cell suspension was mixed with 3.9 ml of PBS and then the absorbance at 600 nm (A_{600}) was measured. The auto-aggregation percentage was calculated using the formula $(A_0\text{-}A_t)/ A_0$ x 100, where A_t represents the absorbance at any time (1, 2, 3 and 24 h), and A_0 the absorbance at time t = 0 h. In the co-aggregation test bacterial suspensions were prepared as described in the autoaggregation analysis above. 2 ml cell suspension was mixed with same volume of other cell suspension by vortexing for 20 s. Absorbance was monitored for the mixture and for the bacterial cell suspension at 600 nm was measured after mixing and after 5 h incubation at room temperature. Coaggregation was calculated as: $[(A_{pat} + A_{probio})/(A_{mix})]/(A_{pat} + A_{probio})/*100\backslash$, where A_{pat} and A_{probio} represent $A_{600\ nm}$ of the separate bacterial suspensions in control tubes, A_{mix} represents the absorbance of the mixed bacterial suspension at different times tested (Collado, Isolauri, et al., 2008).

2.5 Antibiotic assay

Antibiotic susceptibility of the selected LAB strains was determined according to method described by (Bauer et al., 1966). This method was used to determine antibiotic susceptibility against clinically important antibiotics, such as Chloramphenicol, Vancomycin, Tetracycline, Streptomycin, Ampicillin, Kanamycin, Rifampicin, and Penicillin. After complete incubation of the strains on the Mueller-Hinton agar plate, the antibiotic discs were manually place on the plates by using sterile forceps. After 24h incubation at 37°C, the clear zones were measured in accordance with the guidelines provided by the disk manufacturer.

3. Results and discussion

3.1 Enumeration and Isolation of lactic acid bacteria

Similar counts were observed for all four samples (Table 1). *Pekasam* contains the highest level of lactic acid bacteria when determined on MRS Agar. It has been reported that Plasom (Thailand fermented fish) which has the same fermentation process also showed high number of LAB isolated (Hwanhlem et al., 2011). High number of LAB count in *Tempoyak* showed a similar result with study done by (Leisner et al., 2001) that lactic acid bacteria was dominant micro flora in *tempoyak*. Meanwhile, the population of LAB count in *jeruk maman* was at the level 10^7 cfu g^{-1}. There was no reports found on the isolation and identification of LAB from *jeruk maman*. A similar findings on fermented leafy vegetables showed LAB count at the same level as *jeruk maman* (Tamang et al., 2005). Reports on isolation and identification of LAB isolated from *tapai* are very limited in literature. However, *tapai* could be a potential source of LAB.

Table 1

Total colony count on MRS Agar

Sample	Colony Count (cfu/g)
Tapai	5.6×10^5
Tempoyak	5.3×10^7
Pekasam	3.2×10^8
Jeruk Maman	4.4×10^7

3.2 Morphological, physiological and biochemical of LAB

Out of these isolates grown on MRS agar, twenty strains were then picked based on colony morphology of lactic acid bacteria on MRS agar. Most colonies on MRS agar plates were small, circle, grey or white. All the selected strains were Gram positive, catalase negative and rod shaped bacteria. They were mesophilic and survived at range from 1.5% until 7.5% of NaCl concentration. Six strains produced CO_2 from glucose (Table 2). (Nuraida, 2015) reviewed that LAB in Asian fermented foods include *Lactobacillus sp, Leuconostoc sp* and *Weisella sp* were gram positive, non-spore forming, coccus or rod shaped bacteria. They ferment carbohydrates to lactic acid (homofermentation) or mixture of lactic acid, carbon dioxide and acetic acid or/and ethanol.

Table 2

Phenotypic Identification of Isolates

Isolates Code	Shape	Catalase	NaCl tolerance					Sugar Fermentation		
			1.5%	2.5%	5%	7.5%	10.0%	0.5%	1.0%	gas
M3Bi	rod	-	+	+	+	+	-	+	+	-
M5Aii	rod	-	+	+	+	+	-	+	+	-
M4Ai	rod	-	+	+	+	+	+	+	+	-
M5Bi	rod	-	+	+	+	+	+	+	+	-
P4Bii	rod	-	+	+	+	+	+	+	+	+
P5Bii	rod	-	+	+	+	+	+	+	+	-
P4Bi	rod	-	+	+	+	+	+	+	+	-
P4Biii	rod	-	+	+	+	+	+	+	+	-
P5Aii	rod	-	+	+	+	+	+	+	+	+
P5Bi	rod	-	+	+	+	+	+	+	+	-
P4Ai	rod	-	+	+	+	+	+	+	+	-
P5Ai	rod	-	+	+	+	+	+	+	+	+
P5Aiii	rod	-	+	+	+	+	+	+	+	+
T5Ai	rod	-	+	+	+	+	+	+	+	-
T6Aiii	rod	-	+	+	+	+	+	+	+	-
T6Aii	rod	-	+	+	+	+	-	+	+	-
T6Ai	rod	-	+	+	+	-	-	+	+	-
Ta2Ai	rod	-	+	+	+	+	+	+	+	-
Ta3Ai	rod	-	+	+	+	+	+	+	+	+
Ta3Aii	rod	-	+	+	+	+	+	+	+	-
Ta5Ai	rod	-	+	+	+	+	+	+	+	+
Ta2Aii	rod	-	+	+	+	+	+	+	+	-

(+)= growth; (-) = no growth

3.3 Auto aggregation activity

Auto aggregation means the clumping of bacterial cells from the same strains. Auto aggregation has been correlated with the adhesion capacity of the strain to the intestinal epithelial cells. One of the important property for probiotics is the ability to adhere to epithelial cells and mucosal surfaces. With this regard, LAB isolates were examined for auto aggregation activity.

In our study, LAB isolates showed wide differences in their auto aggregation activity (Table 3). The highest auto aggregation was observed in P5Ai with aggregation percentage reached to 98.8% after incubated at room temperature for 24 hour which was followed by T5Ai by 91.1% of auto aggregation activity. Whilst, auto aggregation ability of all isolates was increased with time and was higher at 24 h of incubation than 3 h. (Goh & Klaenhammer, 2010) explained that aggregation promoting factors increase self-aggregation with incubation. Similar findings was also reported by (Dias et al., 2013), that the auto aggregation of *L. plantarum* strains improved with the increase in time of incubation. This result indicates that the P5Ai strain possess high potential ability to adhere to epithelial cells and mucosal surfaces.

Table 3

Auto-aggregation ability of LAB strains

Strains	Auto-aggregation (%)	
	3 h	24 h
Ta2Ai	4.8 ± 0.0075	44.3 ± 0.0010
Ta3Aii	12.8 ± 0.0062	53.8 ± 0.0021
Ta5Ai	8.8 ± 0.0023	90.2 ± 0.0003
Ta3Ai	13.1 ± 0.0121	57.6 ± 0.0035
P5Ai	18.2 ± 0.0092	98.8 ± 0.0006
P4Ai	12.4 ± 0.0036	57.3 ± 0.0044
P5Bi	0.75 ± 0.0034	66.9 ± 0.0040
P4Bi	11.9 ± 0.0076	37.4 ± 0.0121
P4Biii	25.8 ± 0.0059	49.6 ± 0.0015
P5Bii	4.4 ± 0.0046	85.9 ± 0.0015
P4Bii	11.4 ± 0.0012	65.4 ± 0.0040
P5Aiii	10.4 ± 0.0050	78.2 ± 0.0082
T5Ai	7.5 ± 0.0016	91.1 ± 0.0012
T6Ai	3.8 ± 0.0010	61.1 ± 0.0025
T6Aii	12.5 ± 0.0020	52 ± 0.0015
M3Bi	7.7 ± 0.0020	67.8 ± 0.0017
M5Bi	11.2 ± 0.0016	69.6 ± 0.0020
M4Ai	6.5 ± 0.0017	67.7 ± 0.0031
M5Aii	5.5 ± 0.0032	57.1 ± 0.0027

3.4 Antibiotic Assay

The ability of microorganisms to withstands the bacteriostatic and bactericidal effects of antibiotics is known as antibiotic resistance (Katzung et al., 2004). Transfer of resistance to antimicrobial agents such as antibiotic is an essential mechanism if the LAB are to adapt and survive in specific environment (Herreros et al., 2005). Hence in view of the above, before a LAB strain can be consumed as a probiotic it must undergo antibiotic resistance screening to ensure its safe application. In this study, all twenty isolates have been tested their antibiotic resistance towards Chloramphenicol, Vancomycin, Tetracycline, Ampicillin, Kanamycin, Rifampicin and Penicillin. Different degree of susceptibility towards various antibiotics are shown in Table 4. All strains were resistance to Vancomycin, common antibiotic used for the treatments of bacterial infection. (Gotcheva et al., 2002) reported that vancomycin resistance is an intrinsic property of many LAB. Many Lactobacillus species show a high level of resistance to Vancomycin (Aliabadi & Chakoosari, 2013).

Findings by (Angmo et al., 2016) also showed that most of LAB isolated were susceptible to all antibiotics such as penicillin G, erythromycin and ampicillin except vancomycin. Three potential LAB strains are resistance to all antibiotics which are P5Ai, P4Bii and MBi. Some potential LAB strains are resistance against other antibiotics, especially for penicilin, ampicillin, chloramphenicol and tetracyclin,

are variable depending upon specific LAB strains. For example LAB isolated from tapai (Ta3Aii) showed sensitivity to all antibiotics except Vancomycin.

Table 4

Inhibition zone diameters of LAB isolated from Tapai, Tempoyak, Pekasam and Jeruk Maman on agar when antibiotic were used as indicator

Strains Code	Inhibition Zone (mm)							
	RD	K	S	AMP	C	P	Va	Te
Ta2Ai	17.5 ± 2.12	13.5 ± 0.71	13.5 ± 0.71	23.5 ± 0.71	0	0	0	0
Ta5Ai	14.5 ± 0.71	8.5 ± 2.12	12.5 ± 2.12	21 ± 1.41	19 ± 1.41	16.5 ± 0.71	0	0
Ta3Aii	17 ± 0.00	12.5 ± 2.12	17 ± 1.41	20.5 ± 3.54	22.5 ± 0.71	16.5 ± 2.12	0	11.5 ± 0.71
Ta3Ai	0	17 ± 1.41	15.5 ± 2.12	0	21 ± 0.00	0	0	20.5 ± 0.71
P5Bii	15.5 ± 2.12	11 ± 1.41	12 ± 2.83	11.5 ± 0.71	19 ± 1.41	10 ± 1.41	0	0
P4Biii	14.5 ± 0.71	10.5 ± 2.12	13 + 0.00	22 ± 2.83	15 ± 0.00	0	0	0
P4Bi	13 ± 0.00	16.5 ± 2.12	16.5 ± 2.12	19.5 ± 3.54	23 ± 2.83	16 ± 1.41	0	10.5 ± 2.12
P5Ai	0	0	0	0	0	0	0	0
P5Bi	0	16 ± 1.41	17.5 ± 0.71	14.5 ± 0.71	14.5 ± 0.71	16 ± 0.00	0	0
P4Ai	17 ±1.41	13.5 ± 0.71	13 ± 1.41	0	9 ± 1.41	0	0	0
P4Bii	0	0	0	0	0	0	0	0
P5Aiii	21 ± 1.41	21.5 ± 2.12	21 ± 1.41	14.5 ± 0.71	23.5 ± 0.71	10.5 ± 0.71	0	22 ± 0.00
T6Aii	0	0	0	10 ± 1.41	14.5 ± 0.71	0	0	0
T6Aiii	10.5 ± 0.71	13.5 ± 0.71	15 ± 0.00	18.5 ± 0.71	0	0	0	0
T5Ai	14.5 ± 0.71	0	9.5 ± 0.71	0	18 ± 2.83	12.5 ± 2.12	0	17.5 ± 0.71
T6Ai	22.5 ± 3.54	0	14.5 ± 4.95	11 ± 1.41	17 ± 1.41	0	0	0
M4Ai	0	11 ± 1.41	14 ± 0.00	18 ± 1.41	20 ± 0.00	22.5 ± 3.54	0	0
M3Bi	16.5 ± 0.71	16 ± 2.83	16.5 ± 0.71	23.5 ± 0.71	20.5 ± 0.71	20 ± 1.41	0	0
M5Bi	0	0	0	0	0	0	0	0
M5Aii	0	15 ± 1.41	0	0	13 ± 1.41	20.5 ± 0.71	0	0
A1	0	22.5 ± 0.71	16 ± 4.24	14 ± 2.83	24.5 ± 0.71	0	0	25.5 ± 0.71
A2	13.5 ± 0.71	0	0	0	26.5 ± 0.71	0	17 ± 1.41	11 ± 0.00

Values represented as mean ± SD; Control strain A1 is *E.coli*; Control strain A2 is MRSA; RD is rifampicin; K is kanamycin; S is streptomycin, AMP is ampicillin; C is chloramphenicol; P is penicillin; Va is vancomycin; Te is tetracycline.

4. Conclusion

Traditional Malaysian fermented foods are potential sources of microorganisms especially LAB. Among the samples used, LAB originating from fermented fish (pekasam) showed promosing properties of probiotic. However, the functional properties have not been fully investigated. Studies are need to be confirmed in animal and human studies. Although most Malaysian fermented foods are safe, isolating a single microorganism should also be supported with studies to confirm its safety. The potential and beneficial microorganisms for probiotic in Malaysian fermented foods needs to be more explore and extended to microorganisms other than LAB that also presents such as yeasts.

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