Antibiotic susceptibility of Lactic Acid Bacteria (LAB) Isolated from Malaysian Fermented Foods

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ABSTRACT

One of important probiotic properties of Lactic Acid Bacteria (LAB) is the ability to resist towards antibiotics. However, the antibiotic susceptibility of LAB associated in Malaysian fermented foods is less explored. Therefore, this study aims to investigate the antibiotic susceptibility of isolated LAB from Pekasam (fermented fish), jeruk maman (fermented vegetable), tapai (fermented glutinuous rice) and tempoyak (fermented durian). Twenty selected strains growth on MRS agar were confirmed LAB by showing negative result in catalase test, gram positive and rod shaped bacteria in gram staining test. All isolated LAB were determined their antibiotic susceptibility towards Penicillin, Ampicillin, Kanamycin, Vancomycin, Streptomycin, Tetracylin, Chloramphenicol and Rifampicin. All strains showed different degree of resistance towards all eight antibiotic used. Three LAB strains which were P-8, P-1 isolated from pekasam and M-3 isolated from jeruk maman showed ability to resist towards all antibiotics used. It can be concluded that the results obtained from this study could contribute to the potential use of isolated LAB as a probiotic in the food and pharmaceutical industries.

Keywords: Aggregation activity, Lactic Acid Bacteria, Probiotic, Fermented food

1. Introduction

Probiotics are live microorganisms, when ingested in an adequate amount will contribute to a positive effect on the health of the consumer (Hotel et al., 2001). They are recognized as “generally regarded as safe” and desirable micro flora in the gastrointestinal tract (Tannock, 1997). The common used probiotics are Lactic Acid Bacteria from genera *Lactobacillus* and *Bifidobacterium* (Saez-Lara et al., 2015). However, not all *Lactobacillus* and *Bifidobacterium* are probiotics since the probiotic properties are for each strains not genera or species. There are numerous criteria to be met before claiming any strain as a probiotics. One of the important property for probiotics is the ability to resist against clinically important antibiotics.

Antibiotics are normally used as drugs to treat bacterial infection prescribed by a medical officer or added as food additives to control microbial spoilage in food product. However, one of the most adverse effect associated with antibiotics is diarrhea (Neut et al., 2017). According to McFarland (2008), this occurs due to the destruction of all beneficial micro biota by a usage of broad spectrum of antibiotics in treating bacterial infection. Modi et al. (2014) reported that inappropriate or overuse antibiotics has led to the emergence of resistance bacteria. The antibiotic-associated diarrhea allows pathogens to colonize the gut when all protective host gut micro biota were destroyed. Hence probiotics are one approach to prevent colonization and reduce the antibiotic-associated

diarrhea. Therefore, it is important for any LAB strains to undergo antibiotic resistance assay before it can be consumed as probiotic.

Recent research has shown a promising probiotic activity of LAB isolated from fermented food (Rhee et al., 2011). However, research in exploring a probiotic potential strain from Malaysian fermented food is less explored. Most previous studies were conducted on isolation of LAB without further testing on their probiotic potential and it need to be expanded since each LAB strains has unique characteristics and thus can be used for different purposes. Therefore, the present study aims to investigate the antibiotic susceptibility of LAB isolated from *Tapai*, *Tempoyak*, *Pekasam* and *Jeruk Maman* against various antibiotics. Hence, LAB isolated from Malaysian fermented food has potential as a probiotic particularly in food and pharmaceutical industries.

2. Materials and methods

2.1 Source of sample

Tapai, tempoyak, pekasam and *jeruk maman* were purchased from a local night market in Kuantan, Malaysia. *Tapai* is a fermented glutinous rice mixed with yeast and salt, meanwhile, *Tempoyak* is a fermented durian flesh mixed with salt, *Jeruk Maman* is a fermented *Cleome gynandra* mixed with rice and salt and lastly, fermented fish covered with roasted rice and salt known as *pekasam*. All samples were kept aseptically refrigerated at 4°C to protect from contamination

and deterioration, so that microbial load does not change during storage, and processed between 12 to 16 h after the collection (Edalatian et al., 2012).

2.2 Enumeration and Isolation of lactic acid bacteria

About 25 g of fermented food 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 single 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 of MRS agar and stored at 4°C. Prior to its use during experiments, the LAB cultures were transferred twice into the appropriate medium.

2.3 Morphological, physiological and biochemical testing of LAB

The morphological, physiological and biochemical examination of the isolates were determined by the standard procedure of gram staining, catalase test and gas production test. Circle, small, white or creamy white colonies were selected and re-streaked onto MRS agar plates to obtain pure cultures. Each pure culture was tested for cell morphology, gram and catalase reaction. All isolates were selected for other biochemical tests such as sugar fermentation and salt tolerance assay.

2.4 Antibiotic susceptibility

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 important antibiotics which were Chloramphenicol, Vancomycin, Tetracycline, Streptomycin, Ampicillin, Kanamycin, Rifampicin, and Penicillin. After complete incubation of the strains on the Mueller-Hinton agar (MHA) plate, the antibiotic discs were manually placed 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. Results were expressed as sensitive, S (≥ 21mm); intermediate, I (16-20mm) and resistant (≤ 15mm) respectively according to that described by (Vlková et al., 2006).

3. Results and discussion

3.1 Enumeration and Isolation of lactic acid bacteria

Total LAB colony count was obtained for all four fermented foods as represented in Table 1. The highest LAB count was 9.42 log CFU/g observed in *tempoyak*. It can be supported that LAB was dominant micro flora in *tempoyak* with total colony count ranging from 8.88 to 10.42 log CFU/g as reported by (L.-O. Chuah et al., 2016; Leisner et al., 2001). This is because sugar contained in durian pulp and salt added during fermentation promote the growth of LAB. Total LAB count for both *Pekasam* and *Jeruk Maman* are respectively 8.3 and 8.03 log CFU/g. It can be supported by finding from (Mahyudin et al., 2015; Ohhira et al.,

1991) reported that total LAB count obtained from *pekasam* was 6.05 to 6.20 and 8.83 log CFU/g respectively. LAB present naturally can grow and utilize the carbohydrate from the roasted rice and produce lactic and acetic acid during *pekasam* fermentation. Meanwhile, there were no report found on isolation of LAB from *jeruk maman* to support the results. However, total LAB count of *jeruk maman* obtained in this study was agreed with other report from (Tanasupawat et al., 1995) on fermented leafy vegetable that showed LAB count 7.5 to 10.72 log CFU/g. Breidt et al. (2013) proved that LAB present initially in vegetables at the lower numbers but grow rapidly with the used of salt and rice during fermentation. *Tapai* also showed a convincing number of total LAB count in this study which was 8.1 log CFU/g. This finding was supported by Tanasupawat et al. (1995) in his study on fermented glutinuous rice similar to *tapai* known as *khaomak*. He showed that *khaomak* contained total LAB count ranging from 8.1 to 8.63 log CFU/g. Even though ragi or yeast was used as a starter culture in *tapai* fermentation, LAB from the environment may enter the ferments, grow and develop the sour flavour.

3.2 Morphological, physiological and biochemical of lactic acid bacteria

A total of twenty colonies from *pekasam, jeruk maman, tapai* and *tempoyak* were selected randomly and considered as presumptive LAB based on their growth appearance on MRS agar media. All selected isolates were circular shape, creamy white or white and small size when grow on MRS agar, produced

catalase negative in catalase test and observed as gram positive and rod shaped bacteria under microscope as shown in Figure 1. This can be supported by Nuraida (2015) that LAB were gram positive, non-spore forming, coccus or rod shaped bacteria. According to Table 2, all twenty isolates were salt tolerant as they survived at 1.5 until 10 % of NaCl (w/v) concentration. LAB should tolerates to salt as it was used in fermentation process. Salt may enhance the fermentation process by promoting growth of LAB and inhibit the growth of other microorganism (Cai et al., 1997). All isolates showed positive result in sugar fermentation test. Sugar fermentation test is important in LAB identification. Sugar fermentation is detected when acid production from sugar used in growth media (Okada et al., 1983). According to Nuraida (2015), LAB ferment carbohydrates to lactic acid (homofermentative) or mixture of lactic acid, carbon dioxide, acetic acid or/and ethanol (heterofermentative). It can be concluded that, all twenty isolates were LAB.

3.3 Antibiotic susceptibility

Katzung et al. (2004) defined antibiotic resistance as the ability of microorganisms to withstand the bacteriostatic and bactericidal effects of antibiotics. Antibiotic resistance is determined by measuring the diameter of clear zone inhibition formed around antibiotic disc after incubated for 24h with blank disc as reference to clearly distinguish presence of clear zone as shown in Figure 2. All twenty LAB strains were tested their antibiotic resistance towards Chloramphenicol, Vancomycin, Tetracycline, Ampicillin, Kanamycin, Rifampicin,

Streptomycin and Penicillin. Meanwhile E. coli and MRSA were used as positive controls. As stated in Table 3, all strains were resistance to Vancomycin and showed different degree of susceptibility towards other antibiotics. 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. Gotcheva et al. (2002) reported that resistance of Vancomycin is an intrinsic property of many LAB. More than half of twenty LAB strains were resistance for each antibiotic. Antibiotic resistance are variable depending upon specific LAB strains. There were 16 strains resistant to Kanamycin, 14 strains were resisted to Streptomycin, 15 strains resistant to Ampicillin, Rifampicin, Penicillin, 18 strains were resisted to Tetracycline and 10 strains were resisted to Chloramphenicol. This can be supported by study from Pan et al. (2011) that most LAB strains were resisted towards various antibiotics. The ability to inhibit shown by these strains due to presence of inhibitory compounds produced during bacterial growth known as bacteriocin.

4. Conclusion

In this study, all isolated LAB have potential as a probiotic. According to results, all twenty LAB strains were resistant to Vancomycin and showed different degree of susceptibility against various antibiotics used. Studying the potential probiotics properties of traditional Malaysian fermented foods is important as

increasing interest on potential sources of microorganisms, especially LAB. Moreover, this study provides the information on the natural micro biota of Malaysian fermented products. However, complete probiotic properties of isolated LAB have not been fully investigated. The potential and beneficial microorganisms for probiotic in Malaysian fermented foods need to be more explore and extended to microorganisms other than LAB that also presents such as yeasts.

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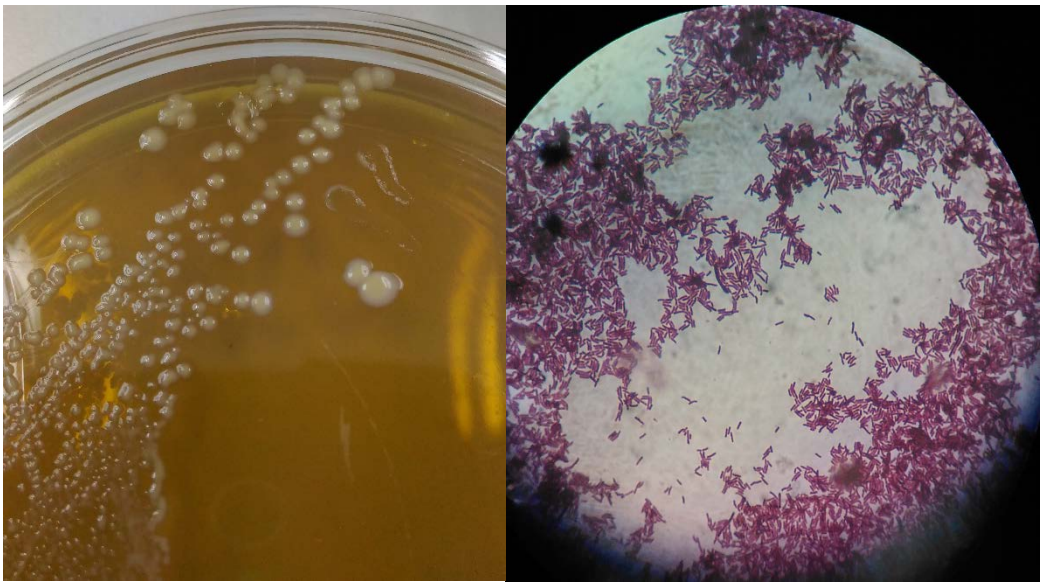
Table 1

Total lactic acid bacteria count on MRS Agar

Samples	Colony Count (log CFU/g)		
	Our findings[a]	Findings from other Authors[b]	
Tapai/ *Khaomak*	8.1 ± 0.46	8.1 to 8.63	(Tanasupawat et al., 1995)
Tempoyak	9.42 ± 0.24	8.88 to 10.42	(L. O. Chuah et al., 2016)
		8.4 to 9.2	(Leisner et al., 2001)
Pekasam	8.3 ± 0.47	8.83	(Ohhira et al., 1991)
		6.05 to 6.20	(Mahyudin et al., 2015)
Jeruk Maman/ Fermented leaves	8.03 ± 0.60	7.5 to 10.72	(Tanasupawat et al., 1995)

[a] Values represented as mean ± SD

[b] A few authors from previous studies



A B

Figure 1: (A: Colony morphology of LAB isolated on MRS Agar; B: Observation of LAB under microscope)

Table 2

Phenotypic Identification of Isolates

Isolates Code	Shape	Catalase[a]	NaCl (w/v) tolerance[b]					Gas[d]
			1.5%	2.5%	5%	7.5%	10.0%	
P-1	rod	-	+	+	+	+	+	+
P-2	rod	-	+	+	+	+	+	-
P-3	rod	-	+	+	+	+	+	-
P-4	rod	-	+	+	+	+	+	-
P-5	rod	-	+	+	+	+	+	+
P-6	rod	-	+	+	+	+	+	-
P-7	rod	-	+	+	+	+	+	-
P-8	rod	-	+	+	+	+	+	+
P-9	rod	-	+	+	+	+	+	+
M-1	rod	-	+	+	+	+	+	-
M-2	rod	-	+	+	+	+	-	-
M-3	rod	-	+	+	+	+	+	-
M-4	rod	-	+	+	+	+	-	-
T-1	rod	-	+	+	+	+	+	-

T-3	rod	-	+	+	+	+	-	-
T-2	rod	-	+	+	+	-	-	-
Ta-1	rod	-	+	+	+	+	+	-
Ta-4	rod	-	+	+	+	+	+	+
Ta-3	rod	-	+	+	+	+	+	-
Ta-2	rod	-	+	+	+	+	+	+

*[a] (-) = no catalase production
*[b,c] (+) = growth, (-) = no growth
*[d](+)=gas production, (-)=no gas production

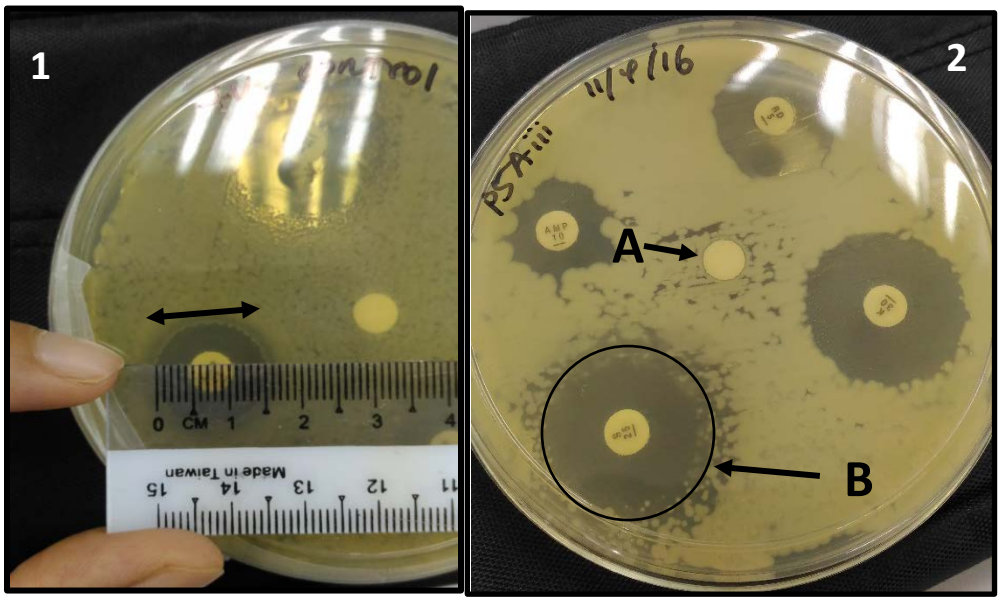


Figure 2: 1= Inhibition zone was determined by measuring the diameter of clear zone

2= Inhibition zone of antibiotic on LAB (A: Blank disc as a control showed no inhibition zone; B: Antibiotic disc showed clear inhibition zone)

Table 3

Assessment of LAB isolated to eight different types of antibiotics

Strains	Inhibition Zone (mm)							
	RD	K	S	AMP	C	P	Va	Te
Ta-1	++	+++	+	+++	+++	+++	+++	+++
Ta-2	+++	+++	+++	+	++	++	+++	+++
Ta-3	++	+++	++	+	+	++	+++	+++
Ta-4	+++	++	++	+++	+	+++	+++	+
P-1	+++	+++	+++	+++	+++	+++	+++	+++
P-2	++	+++	+++	+++	++	+++	+++	+++
P-3	+++	++	++	++	+	++	+++	+++
P-4	+++	+++	+++	+	+++	+++	+++	+++
P-5	+++	+++	+++	+++	++	+++	+++	++
P-6	+++	++	++	+++	+++	++	+++	+++
P-7	++	+++	+++	+++	+++	+++	+++	+++
P-8	+++	+++	+++	+++	+++	+++	+++	+++
P-9	+	+	+	+++	+	+++	+++	+
T-1	+++	+++	+++	++	+++	+++	+++	+++
T-2	+	+++	+++	+++	++	+++	+++	+++
T-3	+++	+++	+++	+++	+++	+++	+++	+++
M-1	+++	+++	+++	++	+	+	+++	+++
M-2	++	++	++	+	+	+	+++	+++
M-3	+++	+++	+++	+++	+++	+++	+++	+++
M-4	+++	+++	+++	+++	+++	+	+++	+++
A1	+++	+	++	+++	+	+++	+++	+

A2	+++	+++	+++	+++	+	+++	++	+++

Values represented as resistant: +++, intermediate: ++, susceptible: +
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.