Aggregation Activity 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 adhere on intestinal epithelial cells. It is shown as aggregation activity of LAB. However, the probiotic properties of LAB naturally occurring in Malaysian fermented foods still remains unexplored. The aims of this study were to investigate the aggregation activity of isolated LAB from Pekasam (fermented fish), jeruk maman (fermented vegetable), tapai (fermented glutinuous rice) and tempoyak (fermented durian). Twenty isolates were identified their colony morphology, physiological and biochemical test to confirm their genus. All isolates 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 salt concentration. Aggregation activity of isolates was determined. Aggregation activity was increased when the incubation time increased. All isolated LAB showed best potential as probiotic. It was 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 (Hotel & Cordoba, 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 aggregate and adhere with intestines epithelial cells for successful colonization.

There are two types of aggregation; autoaggregation and coaggregation. Aggregation means the process of bacterial clumping and precipitate in the medium which they are suspended (Janković et al., 2012). Autoaggregation activity has been correlated with the adhesion capacity of the strain to the intestinal epithelial cells. The adhesion ability prolongs the time of probiotics in gastrointestinal, thus can influence the micro biota host and upgrade the gastrointestinal immune system. Meanwhile, coaggregation ability is the cells clumping between two different strains. The probiotic strain will prevent colonization of pathogenic bacteria in gastrointestinal tract (Janković et al., 2012) through competition of binding sites and nutrients (M. C. Collado et al., 2007).

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 species of LAB has unique characteristics and thus can be used for different purposes. Therefore, the present study aims to investigate the auto aggregation activity of LAB isolated from *Tapai*, *Tempoyak*, *Pekasam* and *Jeruk Maman* as well as their capability to coaggregate with different types of pathogenic bacteria. 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

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 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 with 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 Phenotypic Identification of LAB isolates

All isolated strains were confirmed their genus by morphological, physiological and biochemical examination. The standard procedure of Gram staining, catalase test, gas production test , sugar test with 0.5 and 1.0% glucose (w/v) and salt tolerance test with 1.5, 2.5, 5, 7.5 and 10% NaCl (w/v) concentration were performed.

2.4 Autoaggregation activity

Autoaggregation assay was performed according to the method described by (Del Re et al., 2000) and with some modification as refer to (Kumar et al., 2012) and (Maria Carmen Collado, Meriluoto, et al., 2008). Nine milliliters of an overnight culture was 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 the suspension was mixed by vigorous vortex for 10 s and then incubated at room temperature for 24 hours. At 3 h and 24 h, 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 according to (Janković et al., 2012) as presented in equation 1.

$$(A_0-A_t)/ A_0 \times 100 \ldots\ldots\ldots\ldots (1)$$

Where A_t represents the absorbance at any time (3 and 24 h), A_0 represents the absorbance at time t = 0 h.

2.5 Coaggregation activity

In the coaggregation test bacterial suspensions were prepared as above. 2 mL cell suspension was mixed with the same volume of other cell suspension by vortex 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 determined according to (Maria Carmen Collado, Isolauri, et al., 2008) as presented in equation 2.

$$[(A_{pat} + A_{probio})/(A_{mix})]/(A_{pat} + A_{probio})/*100\text{……..}(2)$$

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.

3. Results and discussion

3.1 Enumeration and Isolation of lactic acid bacteria

Total colony count was observed for all four samples represented in (Table 1). High number of LAB count in *Tempoyak* showed a similar result with the study reported by (Leisner et al., 2001) that LAB was dominant micro flora in *tempoyak*. Total LAB count in Tempoyak is 9.42 log CFU/g followed by *Pekasam* with the total LAB count 8.3 log CFU/g. It has been reported that Plasom (Thailand fermented fish) which has the same fermentation process also showed a high number of isolated LAB (Hwanhlem et al., 2011). Jeruk maman could also be a good source of LAB with a total colony count 8.03 log CFU/g. However, there were no previous reports found on the total LAB count in *jeruk maman*. There was a similar finding on fermented leafy vegetables showed LAB count at the same level as *jeruk maman* (Tamang et al., 2005). The fermentation process involved in tempoyak, pekasam and jeruk maman is a spontaneous fermentation without adding bacteria into it. The LAB present is originated from the raw material itself and the growth is favoured by an addition of salt. Meanwhile, yeast was added in tapai processing. Two simultaneously fermentation occur in tapai which are alcoholic fermentation by yeast and lactic acid fermentation by LAB. Tapai has the lowest total LAB count. However, *tapai* could also be a potential source of LAB.

Table 1

Total lactic acid bacteria count isolated from Malaysian fermented foods on MRS Agar

Food samples	Total Colony Count (log CFU/g)
Tapai	6.1 ± 0.46
Tempoyak	9.42 ± 0.24
Pekasam	8.3 ± 0.47
Jeruk Maman	8.03 ± 0.60

Values represented as mean ± SD

3.2 Identification of LAB

Twenty strains were picked based on colony morphology of lactic acid bacteria on MRS agar. Most colonies on MRS agar plates were small, circle, creamy white or white as shown in (Table 2). 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 (w/v) concentration (Table 3). Among LAB, only Lactobacillus produce CO_2 from glucose. According to (Nuraida, 2015), 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 (homofermentative) or a mixture of lactic acid, carbon dioxide and acetic acid and/or ethanol. It proved that all strains isolated from samples are LAB.

Table 2

Morphology characteristics of LAB isolates

Isolates	Colony morphology	Cell Morphology
P-1	Circle, entire, convex, white, small	rod, purple
P-2	Circle, convex, white, small	rod, purple
P-3	Circle, entire, convex, creamy white, small	long rod, purple
P-4	Circle, entire, convex, creamy white, small	rod, purple
P-5	Circle, convex, creamy white, small	rod, purple
P-6	Circle, entire, convex, creamy white, small	rod, purple
P-7	Circle, entire, convex, creamy white, small	long rod, purple
P-8	Circle, undulate, convex, white, large	long rod, purple
P-9	Circle, entire, convex, white, small	long rod, purple
M-1	Circle, entire, convex, white, small	rod, purple
M-2	Circle, entire, convex, white, small	rod, purple
M-3	Circle, entire, convex, white, small	rod, purple
M-4	Circle, entire, convex, white, small	rod, purple
T-1	Circle, entire, convex, creamy white, small	rod, purple
T-2	Circle, entire, convex, creamy white, small	rod, purple
T-3	Circle, undulate, convex, white, small	rod, purple
Ta-1	Circle, entire, convex, white, small	cocci, purple
Ta-2	Circle, undulate, convex, creamy white, small	rod, purple
Ta-3	Circle, undulate, convex, creamy white, small	rod, purple
Ta-4	Circle, undulate, convex, creamy white, small	rod, purple

P- Strain isolated from *pekasam*
M- Strain isolated from *jeruk maman*
T- Strain isolated from *tempoyak*
Ta- Strain isolated from *tapai*

Table 3

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

3.3 Autoaggregation activity

Autoaggregation 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. In our study, LAB isolates showed wide differences in their auto aggregation activity as shown in (Table 4). The highest auto aggregation was observed in P-8 with aggregation percentage reached to 98.7% after incubated at room temperature for 24 hours which was followed by Ta-2 by 90.2% 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 were also reported by (Dias et al., 2013), that the auto aggregation of *L. plantarum* strains improved with the increase in time of incubation. Increased auto aggregation activity along with the incubation period indicates longer attachment time of strains in epithelial cells. Thus it will provide more benefit on human health. This result indicates that the P-8 strain which is isolated from *pekasam* possesses high potential ability to adhere to epithelial cells and mucosal surfaces. It showed that P-8 is efficacy as a probiotic strain.

Table 4

Autoaggregation ability of Lactobacillus strains

Isolates Code	Auto-aggregation activity (%)[1]	
	3	*24*
P-1	12.24 ± 0.19^{ef}	68.6 ± 2.31^{fg}
P-2	4.74 ± 0.46^{a}	85.9 ± 1.50^{j}
P-3	8.99 ± 0.29^{cd}	34.2 ± 3.20^{a}
P-4	7.63 ± 0.16^{bcd}	41.9 ± 1.35^{b}
P-5	17.04 ± 0.38^{g}	52.6 ± 10.3^{c}
P-6	7.43 ± 0.42^{bc}	60.1 ± 7.90^{de}
P-7	17.45 ± 0.38^{gh}	57.3 ± 1.22^{cd}
P-8	20.30 ± 2.64^{i}	98.7 ± 0.40^{k}
P-9	19.04 ± 3.45^{hi}	74.9 ± 1.05^{hi}
M-1	6.09 ± 0.75^{ab}	64.3 ± 3.28^{ef}
M-2	16.22 ± 0.10^{g}	67.6 ± 3.56^{fg}
M-3	13.27 ± 0.15^{f}	66.1 ± 3.42^{efg}
M-4	6.76 ± 0.48^{b}	57.1 ± 1.73^{cd}
T-1	13.67 ± 0.34^{f}	71.1 ± 1.64^{gh}
T-2	9.38 ± 0.23^{cd}	61.1 ± 2.03^{de}
T-3	9.20 ± 0.93^{cd}	51.8 ± 2.80^{c}
Ta-1	7.8 ± 0.62^{bcd}	44.3 ± 2.24^{b}
Ta-2	7.58 ± 0.35^{bcd}	90.2 ± 0.27^{j}
Ta-3	9.44 ± 0.04^{d}	78.0 ± 1.13^{i}
Ta-4	11.41 ± 0.28^{e}	57.6 ± 1.72^{cd}

[1]Values are represented as means ± SD

[a-k] SR with different manuscripts within a column are significantly different ($p < 0.05$)

3.4 Coaggregation activity

Nine selected strains that have high autoaggregation activity were then tested their co aggregation activity with selected food borne pathogens as shown in (Table 5). The coaggregation ability shows the close interaction between tested strains and pathogenic bacteria which are *Escherichia coli*, *Staphylococcus aureus, Vibrio parahaemolyticus, Salmonella typhimurium* and *Bacillus cereus*. Result shows that all nine strains were able to co aggregate with all pathogenic bacteria used. The coaggregation ability of all selected strains was increased at 5 h incubation than 3 h. T-2 showed the best co aggregation ability with *S. aureus*, where 24.6% of bacteria co aggregated after 5 h incubation. P-7 showed the lowest coaggreagation ability with *V. parahaemolyticus*, where 18.1% of bacteria coaggregated after 5 h incubation. Longer ability to aggregate with pathogens could allow them to prevent colonization by pathogen in gastrointestinal by release an antimicrobial substances close to pathogenic bacteria, compete binding sites and nutrients. Result obtained is in agreement with previous report of (Janković et al., 2012) that *Lactobacillus* strains showed coaggregation ability with food pathogenic bacteria.

Table 5

Coaggregation percentage of nine strains of lactic acid bacteria against five pathogens (n=2, x ± SD) after 3 h and 5 h incubation

Strains	*Escherichia coli*		*Staphylococcus aureus*		*Vibrio parahaemolyticus*		*Salmonella typhimurium*		*Bacillus cereus*	
	3 h	5 h	3 h	5 h	3 h	5 h	3 h	5 h	3 h	5 h
T-1	18.5 ± 0.000	20.9 ± 0.012	17.7 ± 0.003	21.2 ± 0.012	15.9 ± 0.003	18.3 ± 0.005	19.6 ± 0.016	23.6 ± 0.044	18.2 ± 0.017	22.1 ± 0.001
T-2	17.7 ± 0.000	18.3 ± 0.022	18.0 ± 0.010	24.6 ± 0.063	17.9 ± 0.003	19.0 ± 0.003	17.1 ± 0.002	20.1 ± 0.000	16.6 ± 0.000	19.6 ± 0.030
T-3	18.6 ± 0.020	21.2 ± 0.002	17.1 ± 0.002	19.6 ± 0.001	16.7 ± 0.001	17.5 ± 0.002	18.6 ± 0.020	20.4 ± 0.003	16.1 ± 0.005	20.5 ± 0.007
M-2	18.8 ± 0.011	19.1 ± 0.002	17.4 ± 0.000	21.2 ± 0.016	15.9 ± 0.006	16.7 ± 0.008	18.0 ± 0.018	18.8 ± 0.000	19.9 ± 0.000	19.8 ± 0.002
P-2	17.4 ± 0.010	20.0 ± 0.003	16.0 ± 0.005	19.9 ± 0.000	14.9 ± 0.008	18.8 ± 0.001	20.0 ± 0.008	21.2 ± 0.001	18.1 ± 0.000	22.7 ± 0.003
P-8	17.7 ± 0.009	18.3 ± 0.009	18.3 ± 0.011	19.0 ± 0.007	14.8 ± 0.004	17.5 ± 0.017	18.9 ± 0.008	24.2 ± 0.001	17.2 ± 0.003	20.2 ± 0.009
P-7	17.1 ± 0.016	19.7 ± 0.006	19.9 ± 0.001	20.2 ± 0.010	17.7 ± 0.000	18.1 ± 0.002	15.7 ± 0.002	19.9 ± 0.005	16.1 ± 0.004	20.5 ± 0.002
Ta-4	18.1 ± 0.002	21.4 ± 0.007	17.4 ± 0.002	21.5 ± 0.009	15.6 ± 0.000	18.5 ± 0.000	14.6 ± 0.005	20.9 ± 0.005	15.6 ± 0.006	20.8 ± 0.002
Ta-1	19.6 ± 0.012	20.4 ± 0.002	17.6 ± 0.011	21.0 ± 0.007	18.5 ± 0.003	20.0 ± 0.010	19.6 ± 0.012	20.3 ± 0.004	17.6 ± 0.014	22.9 ± 0.002

Presented values are mean of duplicate determinations.

± indicates standard deviation from the mean.

4. Conclusion

In this study, all isolated LAB have potential as a probiotic. According to results, all twenty isolates able to adhere on epithelial cell with different degree of aggregation activity. 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. Studies are needed 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 need to be more explore and extended to microorganisms other than LAB that also presents such as yeasts.

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