# ASSESSMENT OF LACTIC ACID BACTERIA ISOLATED FROM FERMENTED *BAMBANGAN* AS PROBIOTIC AND THE ADHESION PROPERTIES OF THE SELECTED STRAINS



**NG SEAH YOUNG**



**FACULTY OF FOOD SCIENCE AND NUTRITION**
**UNIVERSITI MALAYSIA SABAH**
**2018**

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**NG SEAH YOUNG**



**THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

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PN2009-8186

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NAME : **NG SEAH YOUNG**

MATRIC NO. : **PN2009-8186**

TITLE : **ASSESSMENT OF LACTIC ACID BACTERIA ISOLATED FROM FERMENTED *BAMBANGAN* AS PROBIOTIC AND THE ADHESION PROPERTIES OF THE SELECTED STRAINS**

DEGREE : **DOCTOR OF PHILOSOPHY (FOOD SCIENCE)**

VIVA DATE : **19 MAC 2018**



**CERTIFIED BY**

**1. SUPERVISOR**

Assoc. Prof. Dr. Chye Fook Yee

Signature

**2. CO-SUPERVISOR**

Dr. Clarance M. Ongkudon

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## ABSTRACT

Probiotics are continuously gaining attention in the food industry for the development of functional foods due to their ability to confer several health benefits to the hosts. The study aims to elucidate the probiotic properties and the underlying adhesion mechanisms of lactic acid bacteria (LAB) isolated from an indigenous fermentation of *Bambangan* (*Mangifera pajang*). The isolated LAB were confirmed their identities by analyzing their 16S rRNA gene sequences and their probiotic properties were evaluated by a series of *in vitro* assays. The selected probiotic candidates were further investigated for their cell surface proteinaceous components in adhesion to the human CaCo-2 cell line and the corresponding surface proteins were identified. The main species of the LAB isolated from fermented *Bambangan* were identified as *Lactobacillus plantarum* and *Lactobacillus brevis*. Approximately 43% of the identified LAB strains displayed excellent survival at pH 3.0 with at least 6 log CFU/ml for 4 hours and able to withstand 2.0% bile salt. Interestingly, the LAB challenged in simulated intestine juice showed 2 fold increment in viability when pepsin is present at pH 2.0 compared to those without addition of pepsin. Besides, a high aggregation activity (>20%) was found in most of the LAB strains. They also exhibited great antibacterial activity against pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Yersina enterocolitica*. The eight selected probiotic candidates were found comparable to the commercial strain of *Lactobacillus acidophilus* LA05 on probiotic properties. Most of them were tolerable to 60°C for 10 minutes with at least 70% viability and 4 strains were tolerant up to 6% sodium chloride. No probiotic candidate possesses any unusual resistance towards antibiotics; neither produces biogenic amines nor gives cytotoxic effect to the intestinal cells. Among all the tested probiotic candidates, *L. plantarum* 0612 shows the highest adhesion percentage (5.51%) to the human colon epithelial cells (Caco-2). However, the adhesion of *L. plantarum* 0612 to Caco-2 cells has reduced by 70% upon treatment with lithium chloride, indicating the involvement of cell surface proteinaceous components in the adhesion. The cell surface proteins *of L. plantarum* 0612 were fractionated into 8 fractions by using anion exchange chromatography. The fractions were incubated in Caco-2 cells, followed by adhering *L. plantarum* 0612 to the respective Caco-2 cells. Results showed fraction-7 has significantly reduced the adhesion of *L. plantarum* 0612 onto the Caco-2 cells as compared to other protein fractions. The cell surface proteins (fraction-7) that bound to the Caco-2 cells were recovered and the SDS-PAGE revealed that 5 protein bands with molecular weight from 25 kDa to 65 kDa are most likely responsible in the adhesion of *L. plantarum* 0612 to Caco-2 cells. The protein bands have been identified as serine/threonine protein kinase, D-alanine – D-alanine ligase, NADP-dependent malic enzyme, and 2 uncharacterized proteins, which could be novel adhesive proteins. In conclusion, 6 strains of *L. plantarum* (0123, 0140, 0147, 0157, 0611 and 0612) and 2 strains of *L. brevis* (0808, 0871) could be used as probiotic candidates in food and therapeutic applications. *L. plantarum* 0612 is the most promising probiotic bacteria that highly adhesive to the human intestinal cells due to the presence of multiple cell surface associated proteins and novel proteins.

## *ABSTRAK*

### *PENILAIAN BAKTERIA ASID LAKTIK DIPENCILKAN DARIPADA FERMENTASI BAMBANGAN SEBAGAI PROBIOTIK DAN SIFAT LEKATAN PADA STRAIN TERPILIH*

*Probiotik semakin mendapat perhatian di kalangan industri makanan untuk perkembangan makanan berfungsi disebabkan oleh kebolehannya untuk menyumbang kebaikan kesihatan kepada perumahnya. Kajian ini bertujuan untuk menerokai sifat probiotik serta mekanisma lekatan bakteria asid laktik (LAB) yang dipencilkan daripada Bambangan (Mangifera pajang) yang difermentasi. LAB yang dipencil disahkan identitinya dengan penjujukan gen 16S rRNA lalu sifat probiotik dinilaikan dengan satu siri pengujian in vitro. Calon-calon probiotik yang terpilih dikaji dalam penglibatan komponen protin permukaan sel dalam pelekatan dengan menggunakan titisan sel manusia Caco-2 dan protin permukaan sel yang berkenaan telah dikenalpasti. Spesies utama LAB yang dipencilkan daripada fermentasi Bambangan adalah Lactobacillus plantarum dan Lactobacillus brevis. Lebih kurang 43% daripada strain LAB memaparkan kebolehidupan yang tinggi pada pH 3.0 dengan sekurang-kurangnya 6 log CFU/ml selama 4 jam dan dapat bertahan dalam 2.0% garam hempedu. Yang menarik perhatian ialah LAB menunjukkan 2 kali ganda lebih tinggi kebolehhidupan apabila terdapat kehadiran pepsin pada pH 2.0 berbanding dengan yang tiada penambahan pepsin. Selain itu, aktiviti aggregasi yang tinggi (>20%) telah dijumpai pada semua strain LAB. Mereka juga memaparkan aktiviti antibakteria yang baik terhadap bakteria patogenik seperti Listeria monocytogenes, Salmonella* Typhimurium*, Salmonella* Enteritis *dan Yersina enterocolitica. Sifat probiotik pada lapan LAB yang terpilih setanding dengan strain komersial Lactobacillus acidophilus LA05. Kebanyakan LAB bertoleransi terhadap 60°C selama 10 minit dengan sekurang-kurangnya 70% kebolehidupan dan 4 strain telah menunjukkan toleransi sehingga 6% natrium klorida. Tiada calon probiotik yang menunjukkan rintangan antibiotik yang luar biasa, tidak merebes amina biogen dan tidak memberi kesan sitotoksik kepada sel usus. Daripada semua calon probiotik yang dikaji, L. plantarum 0612 menunjukkan peratus pelekatan kepada sel epitelium kolon manusia (Caco-2 sel) yang paling tinggi (5.51%). Namun, pelekatan L. plantarum 0612 kepada sel Caco-2 telah dikurangkan sebanyak 70% setelah dirawat dengan litium klorida, menunjukkan penglibatan komponen protin permukaan sel dalam pelekatan. Protin sel permukaan daripada L. plantarum 0612 telah dipecahkan kepada 8 pecahan dengan penukaran anion kromatografi. Pecahan tersebut diinkubasi dalam sel Caco-2 lalu pelekatan L. plantarum 0612 pada sel Caco-2 dijalankan. Keputusan menunjukkan bahawa pechan-7 telah mengurangkan pelekatan L. plantarum 0612 kepada sel Caco-2 dengan nyata berbanding dengan pecahan lain. Protin sel permukaan (pecahan-7) yang melekat kepada sel Caco-2 telah diambil semula dan SDS-PAGE menunjunkkan bahawa 5 jalur protin daripada 25 kDa hingga 65 kDa berat molekul bertanggungjawab pada pelekatan L. plantarum 0612 kepada sel Caco-2. Jalur-jalur protin tersebut telah dikenal pasti sebagai protin kinase serine / threonine, D-alanine – D-alanine ligase, enzim malik pergantungan-NADP dan 2 protin tidak dicirikan yang mungkin merupakan protin pelekatan novel. Kesimpulannya, 6 strain L. plantarum (0123, 0140, 0147, 0157, 0611 dan 0612) dan 2 strain L. brevis (0808, 0871) merupakan calon probiotik yang boleh digunakan dalam makanan dan aplikasi terapeutik. L. plantarum 0612 merupakan probiotik bakteria paling baik yang dapat melekat pada sel usus manusia dengan baik disebabkan oleh kehadiran beberapa protin berkaitan sel permukaan dan protein novel.*

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# CHAPTER 1

## INTRODUCTION

Lactic acid bacteria (LAB) are generally referred to bacteria that produce lactic acid as the major end product during fermentation of carbohydrates (Waters *et al.*, 2015). They are phylogenetically belong to Gram positive bacteria, possess characteristics of catalase negative, non-spore forming; cocci, cocco-bacilli or rod shaped and have less than 55 mol% G+C content in their DNA (Stiles and Holzapfel, 1997). The lactic acid bacteria cover wide range of genus belonging to *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* genera (Mazzoli *et al*, 2014). However, *Bifidobacteria* is the only genus with exception due to their higher G+C content (55-67 mol%) than other types of LAB (Vasiljevic and Shah, 2008; Lukjancenko *et al.*, 2012). Lactic acid bacteria are widely found in a number of habitats such as human guts, other human body parts, animal guts, plants and fermented products (Dec *et al.*, 2014; Kuda *et al.*, 2014; Stoyancheva *et al.*, 2014; Turroni *et al.*, 2014; Henning *et al.*, 2015; Ladda *et al.*, 2015; Nuobariene *et al.*, 2015). They have been utilized unintentionally by humans for food fermentation and preservation since ancient times. Their role in fermentation process is to produce organic acids that cause acidification of food material, as well as provide flavour compounds that shaped the unique taste of the food (Bull *et al.*, 2013).

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill *et al.*, 2014). The emergence of concept "probiotics" had created robust advancement in the food industry towards development of functional foods. Most commonly used probiotic microorganisms belong to the lactic acid bacteria, especially from *Lactobacillus* and *Bifidobacterium* genus. The commercially available probiotics were mostly originally isolated from fermented dairy products or intestinal tract, thus are generally accepted as safe for consumption (Butel, 2014). However, there are also reports of using non-lactic acid

bacteria as probiotics such as strains from *Bacillus*, *Clostridium*, *Propionibacterium* and *Escherichia coli* (Foligné *et al.*, 2013). Nevertheless, the usage of these strains in food often raise concern on their safety upon consumption, since some of these strains for instance *Bacillus anthracis* and *Bacillus cereus* are known as human pathogens (Cutting, 2011).

LAB are the focus of probiotic research internationally and their health benefits on prevention of diarrhea (Wanke and Szajewska, 2014), reduction of cholesterol level (Jones *et al.,* 2012), pediatric atopic dermatitis prevention (Panduru *et al.*, 2015), relief of irritable bowel syndrome (Yoon *et al.*, 2014) and efficacy in management of lactose intolerance (Almeida *et al.*, 2012) were found effective. Besides, the competitive exclusion properties of probiotics as well as their ability to displace and inhibit pathogens are most important for therapeutic manipulation of the enteric microbiota (Molinaro *et al.*, 2012; Ortiz *et al.*, 2014). Therefore, application of such strategies can contribute to expand the beneficial properties of the selected probiotics on human health against pathogen infection. However, these cannot be extrapolated to other probiotic strains as such effects are strain-specific and considerable work is required to affirm the benefits of each probiotic found (Makinen *et al.*, 2012).



Despite of all the benefits discussed upon ingestion of LAB, it must be remembered that not all LAB exhibit probiotic properties. In spite of the copious amount of available probiotic strains in the market, process of searching new probiotic strain is still ongoing (Leite *et al.*, 2015; Sharma and Trivedi, 2015; Vijayakumar *et al.*, 2015). Thus, numerous conditions have been suggested to standardize the desired characteristics and filter out those cannot fulfil the selection criteria (Tripathi and Giri, 2014). It is suggested that the probiotic microorganisms must be capable to survive through intestinal tract with drastic low pH environment, destructive bile salt as well as gastrointestinal juices (Vandenplas *et al.*, 2015). Besides, the potential probiotic must be able to adhere and colonize the intestinal cell wall. This is necessary to trigger any direct interactions between probiotic and host cells leading to the competitive exclusion of pathogens and modulation of host cell responses (Van Baarlen *et al.*, 2013). Moreover, it must possess significant antimicrobial activity against pathogenic bacteria while remain safe for human

consumption (Bull *et al.*, 2013). In addition, the probiotic selected must be able to survive and retain their functionality upon exposure to harsh industrial processing operations so that their members are sufficient to proliferate in the gut, thus provide the beneficial effects to the host (Sánchez *et al.*, 2012).

The science related to probiotic is recent, thereof the underlying functional mechanisms of probiotics is still not fully understood. It is widely accepted that adhesion and colonization of probiotics to the intestinal mucosa is considered as one of the most important selection criterion for persistent beneficial effects of probiotics (García-Cayuela *et al.*, 2014; Verdenelli *et al.*, 2014). The initial adhesion stage of the probiotic bacteria to intestinal cell wall involves complex physiochemical interactions including hydrophobicity and charges (Ramos *et al.*, 2013; Yadav *et al.*, 2015). Besides, certain probiotics are found to utilize proteinaceous components, especially surface layer proteins (SLPs) to adhere on the intestinal cells (Meng *et al.*, 2014). The SLPs are the outermost structure of cell envelope in bacteria composed of glycoproteins or proteins, which represents up to 15% total protein of the bacterial cell (de sa Peixoto *et al.*, 2015). However, the biological function of SLPs, particularly from *Lactobacillus* are not well understood (Hynönen *et al.*, 2014). Besides acting as cell adhesion mediators, SLPs are also believed to be capable of maintaining the cellular shape as well as playing an important role as immune modulators (Hynönen and Palva, 2013).

To date, surface layer proteins have been discovered on several *Lactobacillus* species but their occurrence in other related probiotic species remain unknown (Hynönen and Palva, 2013). However, they have been generalized to contain from 25 to 71 kDa size without uniformity in types of protein detected even from the same species (Hynönen *et al.*, 2014). For example, SLPs identified from *Lactobacillus helveticus* fb213, *Lactobacillus acidophilus* fb116 and *Lactobacillus acidophilus* fb214 were of molecular masses approximately 49.7, 46.0 and 44.6 kDa respectively (Meng *et al.*, 2014). Moreover, comparison of SLPs from *L. helveticus* reveals slight dissimilarities among different strains in terms of their molecular weight, pI value and amino acid compositions (Waśko *et al.*, 2014a). In addition, complexity occurs when other proteinaceous compounds such as glyceraldehyde-3-phosphate dehydrogenase, elongation factor Tu and mucus adhesion promoting protein are also

involved in *Lactobacillus* adhesion mechanism (Dhanani and Bagchi, 2013; Jensen *et al.*, 2014; Zhang *et al.*, 2015a). Thus, it is worth to explore SLPs more from *Lactobacillus* strains, with the hope to shape clearer picture of their adhesion mechanism to the intestinal cell wall.

Though ethnic fermented foods are widely prepared and consumed in Malaysia, limited studies have been carried out on those predominant microorganisms that are isolated from these traditional indigenous fermented foods. Previous studies showed that LAB are the dominating microorganisms throughout the fermentation of *cincaluk* (fermented shrimp), *budu* (fermented fish sauce), *tapai* (fermented tapioca) and chili boh (chilli puree) (Adnan and Tan, 2007; Liasi *et al.*, 2009; Hajar and Hamid, 2013). In the context of Malaysian fermented fruit, fermented *Bambangan* (*Mangifera pajang*) and *Tempoyak* (fermented durian) are also found to be dominated by LAB during the spontaneous fermentation (Chin *et al.*, 2010; Chuah *et al.*, 2016). However, information on the potential of these isolates as candidates for probiotic is scarce. The only information about probiotic potential of isolates obtained from indigenous Malaysian food is the *Tempoyak*, described by Pato and Surono (2013) whereby only gastrointestinal tolerance was performed. Other efforts from Malaysia to look for new probiotics focused on intestine microbiota or common food source such as milk as their isolation source (Hutari *et al.*, 2011; Ramasamy *et al.*, 2012; Haziyamin *et al.*, 2012; Yap *et al.*, 2015). In addition, the mechanism of bacterial adhesion onto the epithelial intestine which is a prerequisite property of probiotic is not fully understood. Although many studies confirmed the involvement of various proteinaceous components in adhesion mechanism, the type of proteins involved varied among bacterial species. To the best of the knowledge, none of the probiotic strain originated from Malaysian foods had been investigated on their adhesion properties. Hence, there is a need to search for novel probiotic strains from local fermented products. Furthermore, investigation on the adhesion properties of probiotic strains allow the scientific community to fill in the gaps of knowledge in probiotic adhesion mechanisms, especially strains originated from local indigenous food. Thus, the aim of the current study is to determine the probiotic properties of the lactic acid bacteria isolated from indigenous fermentation of *Bambangan*.

The specific objectives of this study are:

1. To identify the lactic acid bacteria isolated from fermented *Bambangan* based on their genotypic profiles
2. To determine the probiotic properties of the identified lactic acid bacteria via *in vitro* methods.
3. To investigate the ability of the selected probiotic candidates to withstand harsh processing conditions
4. To examine the adhesion properties of the selected probiotic candidates on intestinal cell wall.
5. To determine the proteins that mediates probiotic adhesion to the intestinal cell wall.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Sources of Lactic Acid Bacteria in Various Microbiota

Lactic acid bacteria (LAB) are a group of phylogenetically related microorganisms which are gram positive, catalase negative, non-sporulating, non-motile, low G+C content and contain small genomes of 2 to 3 Mb (Mazzoli *et al.*, 2014). They have been used extensively in food biotechnology for fermentation and preservation purposes. They excrete organic acids especially lactic acids for acidification of the food products and produces other metabolites to achieve the desired organoleptic properties and qualities of food produced (Patel *et al.*, 2013). Thus, understanding their metabolic activity and their roles in these processes would ease the management and improvement of such industrial applications. LAB are generally nutritional fastidious which require various sugars, vitamins, minerals and peptides to support their continuous growth (de Vos and Hugenholtz, 2004; Peres *et al.*, 2012; Arakawa *et al.*, 2015). Therefore, it is not surprising that traces of LAB can be found on plant phyllosphere, fermented food as well as human and animal guts because these nutrient rich habitats are able to sustain the growth of these microorganisms (Williams *et al.*, 2013; Douillard and de Vos, 2014; Carafa *et al.*, 2015). In Malaysia's context, LAB were being isolated and identified from honey comb of the honey bee (Tajabadi *et al.*, 2013), *tempoyak* (fermented durian) (Leisner *et al.*, 2002), chicken intestines (Shokryazdan *et al.*, 2014), *pekasam* (fermented fish) (Ida Muryany *et al.*, 2017) and dairy products produced in Malaysia (Tham et al., 2012).

### 2.1.1 Animal origin

There is no single animal which does not carry microorganisms. These microorganisms, regardless friends or foes to the host, occupy mostly at the gastrointestinal tract. Their population often outnumber the total host cells, where animals are estimated to contain about $10^9$ bacteria per g wet weight, and up to $10^{14}$ microorganisms colonizing the mammalian gastrointestinal tract (Rosenberg and

Zilber-Rosenberg, 2013a). This profuse amount of microorganisms in mammalian gastrointestinal tract comprises of 500 to 1000 bacterial species that constantly interact with the host and other members of the microbial community (Kim and Isaacson, 2015). In addition, the dominating phyla were reported to be *Bacteroidetes* and *Firmicutes*, which accounts approximately 98% of total mammalian gut bacteria known to date (Yoon *et al.*, 2015). Nevertheless, it is well known that animal microbiota are prone to change, which a shift in density and composition of microorganisms can be influenced by age, diet, environment, host genetics, stress, presence of disease (de Theije *et al.*, 2014; Lees *et al.*, 2014; Carmody *et al.*, 2015; Moussaoui *et al.*, 2017). Moreover, variation in microbiome compositions were also detected in each individual, which further complicates the situation (Bolnick *et al.*, 2014). Although it is impossible to provide a definite answer for the microbiota of a particular animal, but efforts had been done to provide a generalized idea on the microbial community of all animal species.

The rodents are the key animal in laboratories for many biological and medical experiments used for a better understanding in the effect of external stimulants on the humans (Wang *et al.*, 2015). Thus, information on their gut microbiota is crucial especially for works involving interactions between the host, nutrients and compounds. The most abundant phyla in the intestine of rodents were reported as *Bacteroidetes* and *Firmicutes*, which accounts approximately 16.3% and 65.7% among total 17 phyla detected respectively (Ley *et al.*, 2008; Hodin *et al.*, 2012). However, other phyla such as *Verrucomicobia*, *Proteobacteria* and *Tenericutes* represent less than 5% of total microbial composition (Hildebrand *et al.*, 2013). At the genus context, the most copious taxonomy were *Bacteroides*, *Helicobacter* and *Robinsoniella* (Linnenbrink *et al.*, 2013). However, within the *Firmicutes*, the dominant genus was found to be *Lactobacillus,* which constitutes one-third of the community (Maurice *et al.*, 2015). Interestingly, the same report had found fewer *Lactobacillus* on late summer and more *Lactobacillus* on spring. Thus leading to an assumption that the abundance of *Lactobacillus* were closely related to the immune status of rodents tested. Therefore, the population of lactic acid bacteria in rats are never static and is subjected to the change. Apart from seasonal variations, reports had shown that amount of lactic acid bacteria in the rodent intestine could also be