1. INTRODUCTION

1.1 Background of Research

Nowadays, people pay a lot of attention to the relation between food and health. People currently expect something more than delicious food, therefore functional foods with health promoting properties has shown a remarkable growth over the last few years. The growing interest in functional foods with health benefits has led to development of new functional beverages, which can provides a health promotion and disease prevention, by reduce the increasing burden on health care system by a continuous preventive mechanism (Din *et al.*, 2011). The functional beverages should provide taste, refreshment satisfication, and also necessary nutrients to prevent nutrient-related diseases. Beverages are considered as a good medium for the supplementation of nutraceutical compounds for enrichment, such as herbal extract. Vegetable juice is one form of functional beverages that also gaining popularity over the last few years (Din *et al.*, 2011).

According to Fuller (1991), probiotics are viable organisms and supportive substances that improve intestinal microbial balance. Probiotic products can be clarified as microbial cell preparations or processed products containing sufficient quantities of viable probiotic microorganisms which provides beneficial health effects on the host (Tamime, 1999 in Xiao *et al*., 2014). Recent studies showed that *L. pentosus* in fermented green table olives could reduced the growth of different pathogens in the product fermented with the probiotics (Argyri *et al*., 2013). Scientists have suggested that a minimum of $10^6 – 10^7$ CFU of viable probiotic bacteria per milliliter is required in the final products at the time of consumption for probiotics to be effective (FAO, 2002; Khurana & Kanawajia, 2007). Probiotic products are available on the market today and usually in the form of fermented milk and yoghurt. However, there is an increasing of vegetarianism consumers throughout developed countries, therefore a demand for vegetarian probiotic product is also increasing. In current years, a demand for non-dairy-based probiotic products has increased due to many problems, such as lactose intolerance and cholesterol content associated with the consumption of fermented dairy products (Luckow & Delahunty, 2004). These problems lead to the use of vegetables as an alternative raw material for the production of probiotic foods due to their nutritive value and large distribution. Foods such as fruits, grains, and vegetables are reported to contain a large variety of antioxidant components, including phytochemical, such as phenolic compounds, which are considered beneficial for human health and decreasing the risk of degenerative diseases by inhibition of macromolecular oxidation and reduction of oxidative stress (Karovicova & Kohajdova, 2003).

Bitter Melon (*Momordica Charantia L.*) has long been used as a food and medicine. Bitter melon is a powerful nutrient-dense plant consists of a various beneficial compounds. These compounds include vitamins, minerals, antioxidants and bioactive chemicals which all contribute to its capability in treating many kinds of illnesses. Studies by Grover *et al.* (2002) have shown that bitter melon has hypoglycemic activity in laboratory animals. Their hypoglycemic actions are possible due to several constituents in bitter melon, which are alkaloids, insulin like peptides, and a mixture of steroidal sapogenins known as charantin and their hypoglycemic actions are a potential benefit for Diabetes Mellitus (DM) treatment (Anjamma & Bhavani, 2015). Refers to Welihinda *et al* (1986) bitter melon fruit juice has been shown to stimulate glycogen storage by the liver and insulin secretion by isolating cell islets of Langerhans. As a medicinal plant, bitter melon also been reported to possess some health properties, which are antilipolytic, analgesic, antiviral, cytotoxic and antimutagenic (Singh *et al.*, 1998).

Lactic Acid Bacteria (LAB) has several potential applications. These microorganisms are widely used in food fermentation and are also part of intestinal microflora. Research reports showed that LAB has beneficial health effects in humans. They produce some antagonistic compounds able to control pathogenic bacteria and undesirable spoilage microflora, such as bacteriocins (Gourama & Bullerman, 1995). Lactic acid fermentation can help to improve nutrition, safety, shelf-life and also sensory properties of vegetables and fruits, therefore it is also can reduce the use of food additives regarded for its ability to preserve food products from spoilage and improve the organoleptic properties of foods. According to previous study by Mazlan *et al*. (2015), fermentation process resulting in the increase of acidity, which significantly reduce the activities of spoilage bacteria and also contributes to the pleasant taste and desirable aroma. Several *Lactobacillus* species have been proven to develop a range of health promoting activities such as immunomodulation, enhancement of resistance against pathogens, and reduction of blood cholesterol (Karovicova & Kohajdova, 2003). Refers to Panesar *et al.* (2007) during the fermentation process of vegetables, LAB is influenced by several factors including initial sugar concentration, pH, salt concentration, and temperature.

Bitter melon was used in this research because bitter melon is a medicinal plant which possess various health properties, one of them is their hypoglycemic actions which capable of lowering blood glucose levels. Due to some constituents in bitter melon, such as saponins, momordicosides K and L, as well as momordicines I and II, bitter melon has a bitter taste (Harinantenaina *et al.*, 2006; Yasuda *et al.*, 1984). Due to the bitter taste, so many people refuse to consume bitter melon, even though the health properties of bitter melon are already known well. Fermentation by LAB is capable to improve the sensory properties of vegetables and they also can develop various health promoting activities, in addition vegetables are a good substrate for LAB, due to their natural prebiotic oligosaccharides that may help to protect probiotics LAB against the acidic stress of the stomach (Ranadheera *et al.*, 2010). Previous study by Mazlan *et al.* (2015) mentioned that fermentation of *Momordica charantia L.* fruit juice by *Lactobacillus plantarum* BET 003 could reduced the bitterness, reduced sugar content, production of aglycones and other metabolites, and also increase the inhibitory potential of the anti-diabetic β-glucosidase, compared to the juice without fermentation.

The research about the suitability of "Kambas" bitter melon as a raw material and characteristics of *Lactobacillus pentosus* LLA18 culture as a probiotic for production of probiotic functional beverages are still limited. Furthermore, the research about the effects of lactic acid fermentation on characteristics of fermented "Kambas" bitter melon, including LAB counts, antioxidant activity, changes of pH value and glucose content, bacteriocin inhibitory activity, and antimicrobial activity of probiotics LAB are also still limited. Therefore, the objectives of this study are to investigate the suitability of "Kambas" bitter melon as a raw material, characteristics of *Lactobacillus pentosus*

LLA18 as the LAB culture for production of probiotic functional beverages, and also the effects of lactic acid fermentation on characteristics of fermented "Kambas" bitter melon, including *Lactobacillus pentosus* LLA18 counts, antioxidant activity, changes of pH value and glucose content, bacteriocin inhibitory activity, and antimicrobial activity of *Lactobacillus pentosus* LLA18 as a probiotic bacteria.

1.2 Literature Review

1.2.1 Lactic Acid Bacteria (LAB)

Fermented food can be produced by adding a starter culture to a raw material. The group of Lactic Acid Bacteria (LAB) occupies a main role in these processes and has been used for application and consumption in the production of fermented foods and beverages for a very long time (Caplice & Fitzgerald, 1999). LAB cause acidification of the raw material due to the production of many kinds of organic acids, especially lactic acid. They also produce acetic acid, aroma compounds, ethanol, exopolysaccharides, bacteriocins, and several enzymes. In this way, LAB enhances microbial safety and therefore prolong the shelf life, improve texture, and enhance the sensory profile of the end product. LAB are generally defined as a cluster of lactic acid producing, low %G + C, non-spore forming, Gram-Positive rods and cocci, facultative anaerobes, catalase negative, non-motile, and acid tolerant (Hutkins, 2006).

LAB were believed to have health benefits, such as improving nutrient digestion, reducing symptoms of lactose intolerance and providing functional substances in the gastrointestinal tract (Xiao *et al*., 2014). LAB are mainly divided into four genera, which are *Lactococcus, Lactobacillus, Leuconostoc* and *Pediococcus.* Refers to Magnuss on *et al*. (2003), the antimicrobial efficiency of LAB may be explained by three mechanisms, which are the results of organic acid, nutrients competition, and the production of antagonistic compounds. The classification of LAB into different genera is based on morphology, configuration of the lactic acid produced, mode of glucose fermentation, high salt concentrations, growth ability at different temperature, and acid or alkaline tolerance (Axelsson, 2004). Homofermentative LAB convert sugar mostly into lactic acid via pyruvate to produce energy and to equilibrate the redox balance. Pyruvate can lead to the generation of many other metabolites such as ethanol, acetate, diacetyl, and acetaldehyde (Sharma & Mishra, 2013). Homolactic bacteria ferment glucose (hexose) through the Embden–Meyerhoff–Parnas (EMP) pathway. They have fructose diphosphate aldolase, which can hydrolyze a six-carbon glucose into two molecules of three-carbon fermented lactic acid (Lahtinen *et al*., 2012). Heterofermentative LAB convert glucose to lactic acid, ethanol, acetic acid, and CO_2 through phosphoketolase pathway. This type of LAB has been widely used in food fermentations. These LAB included *Lactobacillus mesentroides* subsp. *cremoris*, *Lactobacillus mesentroides* subsp. *mesentroides*, *Leuconostoc kimchii*, and *Leuconostoc lactis* (Hutkins, 2006). Heterolactic bacteria lack fructose diphosphoaldolase for the EMP pathway, but they can metabolize glucose through the hexose monophosphate shunt (HMS) pathway because they have glucose phosphate dehydrogenase and xylulose phosphoketolase (Ray & Bhunia, 2008 in Lahtinen *et al*., 2012).

Organic acids produced in foods are end products or additives of carbohydrate degradation by LAB. The main products of the fermentation of carbohydrates by LAB are lactic and acetic acids. Refers to Dalié *et al.* (2010), these acids are agents for the preservation of foods that Generally Recognised As Safe (GRAS). These acids diffuse through the membrane of target organisms, reduce cytoplasmic pH and stop the metabolic activities. The death of susceptible organisms can be explained by the action of organic acids on the plastic membrane by neutralizing its electrochemical potential, increasing its permeability, therefore leading to bacteriostatis. According to Wu & Lu (2014), some LAB can also produce antimicrobial fatty acids that enhance the sensory quality of fermented products, such as caproic, propionic, butyric, and valeric acids.

1.2.2 Lactic Acid Bacteria (LAB) as Probiotics

Probiotics are live microorganism which provide a health benefit on the host when administrated in adequate amounts (Food and Agriculture Organization of the United Nations and World Health Organization, 2002). Moreover, probiotics beneficially affect the host by improving its intestinal microbial balance (Fuller, 1989). The use of probiotics in maintaining and providing health obtaining so much attention, and probiotic products in the market continue to expand. Probiotic products are microbial cell preparations or processed products which contain adequate quantities of viable probiotic microorganisms that provide a beneficial effect on the host (Tamime, 1999 in Xiao *et al.,* 2014). In order to defined as probiotic, microorganisms should be alive when administered, and then taxonomically defined at a species level, evaluted and documented for health benefits in the target host, and the most important is demonstrated to be safe for its intended use (Xiao *et al.*, 2014). Probiotics can grow in very different conditions, *i.e* for psychotropic cultures, the range of growth temperature are 2-30^{o}C, with the optimal growth temperature is 10^{o}C. For mesophilic cultures, the range of growth temperature is 5-60^{o}C, with the optimal growth temperature is 25^{o}C. For thermophilic culture, the range of growth temperature is 30-65^{o}C, with the optimal growth temperature is 40^{o}C. Probiotics in human gut have to generate a small local thermogenesis, because the local human gut temperature is around 37-38^{o}C (Xiao *et al.*, 2014). Probiotics produce various compounds, including lactic acid, acetic acid, hydrogen peroxide, lipopolysaccharides, lactoperoxidase, and also bacteriocin (Lindayani *et al.*, 2018).

Probiotics are known to provide immune response and suppress inflammation (Lomax & Calder, 2009 in Xiao *et al.*, 2014). Probiotics also have been proven to prevent diarrhea, improve constipation, balancing intestinal microbiota, prevent allergic disorders, improve lipid metabolism in order to suppress the development of cancers, and stimulate the immune system. As a result of the evidence of health benefits, probiotics are currently used as treatment of diseases, specifically of the intestinal disorders (Xiao *et al.*, 2014). Generally, the beneficial effects of probiotics can be explained from two aspects, which are direct effects via the cell components and indirect effects via modulation of intestinal microbiota (Xiao *et al.*, 2014). Immunomodulatory effects are obtained when the cell components interact with the intestinal immune system, mediated by intestinal antigen presenting cells, which later resulted in probiotic effects, due to the generation of a balanced gut microbiota. Besides that, the components of dead cells could also exert an immunomodulating effect, one of them is an anti-inflammatory response. The use of non-viable microorganisms could prolong the shelf-life of the products by solving the problem due to stability issues of active constituents in handling and preservation (Adams, 2010). Moreover, according to Taverniti & Guglielmetti (2011), non-viable microorganisms could reduce the risks of microbial invasion, translocation, and production of toxin.

1.2.3 Important Roles of Lactic Acid Bacteria (LAB) in Raw Material Fermentation

Lactic fermentation has traditionally been done to preserve foods and LAB are the main microbes involved in the natural fermentation process. Many strains of the species of LAB and *Bifidobacterium* have been classified as probiotics. These microorganisms are characterized by many health promoting effects, which are balanced of the intestinal microbiota, inhibition of enteric pathogens, adjustment of the intestinal immune system, supply of antioxidants and antimutagens (Xiao *et al*., 2014). Nutraceuticals are food components that contribute to the health of the consumer through specific physiological action (Andlauer & Furst, 2002), thus several Nutraceuticals from bacterial origin have been added to produce foods. The activity of LAB can be arrange to increase the content of Nutraceuticals in fermented foods through strain selection and process optimization. The selection criteria for probiotic LAB include: safety, human origin, resistance to acid and bile, adherence to gut epithelial tissue, production of antimicrobial substances, the viability / activity in delivery vehicles, ability to stimulate a host immune response and the ability to influence metabolic activities such as vitamin production, cholesterol assimilation and lactose activity (Masud & Anwaar, 2002).

LAB produce several natural antimicrobials, which is organic acids, including acetic acid, lactic acid, formic acid, caproic acid, and phenyllactic acid. They also produce hydrogen peroxide, carbon dioxide, ethanol, diacetyl, bacteriocins, reuterin, and reutericyclin. Acetic acid itself contributes to prevent mold spoilage and also contributes to the aroma development of fermented food products (Messens & De Vuyst, 2002). Bacteriocins from LAB are a low molecular mass peptide or proteins which have an antibacterial mode of action especially related to Gram positive bacteria. LAB that produce bacteriocin can be applied for food preservation because of their physiological, microbiological, and technological advantages. Bacteriocins that produced by in-situ may increase the competitiveness of the producer strain in the food matrix and approach a prevention of food spoilage (Leroy & Vuyst, 2004). According to Van Kranenburg *et al.* (2002) LAB can acidify the food, resulting in a tangy lactic acid taste, they also frequently exert proteolytic and lipolytic activities, produce aromatic compounds, such as amino acids as results of further bioconversion, therefore LAB also contribute to the flavor and aroma of fermented products.

It has been reported by Sharma & Mishra (2013) that LAB which able to utilise bitter melon, bottle melon, and carrot juice for cell synthesis and production of lactic acid without nutrient supplementation are *L. acidophilus*, *L. plantarum* and *P. Pantosaceus*. These LAB cultures grew well in vegetable juice at 30^{o}C, and the viable cell counts could reached nearly 8 log CFU/mL after fermentation within 72 hours at 30^{o}C. These LAB cultures were found capable of surviving in the fermented vegetable juice at 4°C for several weeks. The minimum number of viable probiotic organisms in the final food products should be 6-7 log CFU/g for the maximum health benefits, so a daily probiotic ingestion of 8-9 log CFU can be reached when an average of 100 g or 100 mL of food is consumed/day (Jayamanne & Adams, 2006). From the results of the study, it has been concluded that *L. plantarum* could be used for the production of a healthy beverage from vegetables bitter melon, bottle melon, and carrots for vegetarians or lactose intolerance consumers.

1.2.4 Nutritional Properties of "Kambas" Bitter Melon

Momordica charantia L. usually known as bitter melon is a medicinal plant belong to the family of *the Cucurbitaceae.* Bitter melon are a good source of vitamin C, vitamin A, vitamin B1, B2, B3, as well as B9 (folate). Bitter Melon has a bitter flavor due to the alkaloid momordicine produced in the fruit and leaves. Refers to Harinantenaina *et al.* (2006) charantins which are a combination of steroidal saponins that are contained in the fruit of bitter melon, have been claimed to contribute to the hypoglycemic activity of bitter melon which are a potential benefit in treatment of Diabetes Melitus (DM). Other components contribute to the hypoglycemic activity of bitter melon are alkaloids and insuline like peptides (Anjamma and Bhavani, 2015) and also glycosides, such as the momordicosides S and T, terpenoids, and alkaloids (Harinantenaina *et al*., 2006). In a previous study by Khanna & Jain (1981) reported an insulin-like protein in bitter melon fruit, named as "polypeptide p", was being responsible for lowering blood glucose levels in types I and II DM patients. Moreover, bitter melon is a good source of phenolic compounds, such as gentisic acid (2,5-dihydroxyl benzoic acid), Gallic acid, cathecins, epicatechin and chlorogenic acid (Horax *et al.*, 2005). Medicinal value of bitter melon has been attributed to its high antioxidant properties due in part to flavonoids, phenols, terpenes, isoflavones, anthroquinones, and glucosinolates (Snee *et al.*, 2011).

1.2.5 Characteristics of *Lactobacillus pentosus*

Lactobacillus pentosus is one of six species and subspecies from the *Lactobacillus plantarum* group. According to Hammes & Hertel (2009) in Sun *et al.* (2014) this group is very homogenous in terms of metabolic features since all species are FHE (Facultative Heterofermentative). *Lactobacillus plantarum* is an oxygen tolerant and can grow in high density (Wu & Lu, 2014). Accordings to Lahtinen *et al.* (2012) *Lb. plantarum* ferments glucose homofermentatively, but they also ferment other carbohydrates in a heterofermentative manner as well. Recent studies showed that *L. pentosus* in fermented green table olives could reduce the growth of different pathogens in the product fermented with the probiotics (Argyri *et al.*, 2013). During the storage of fermented green table olives in brine with a potential probiotic strain of *L. pentosus* B281, the growth of inoculated pathogenic strains such as *E. Coli* O157:H7, *Salmonella enteriditis* and *Listeria monocytogenes* were suppressed, but they may survive for a long period in such a stressful environment in the fermented product with low pH value (4.2) and high salt concentration (6.0%) (Argyri *et al.*, 2013).

Refers to Panagou *et al*. (2003) the addition of *Lactobacillus pentosus* as a lactic starter culture was done in order to decrease the possibility of spoilage, particularly during the first stage of fermentation when the risk of gas pockets and butyric acid spoilage are very high. In the previous work, the most important factors that could inhibit the adaptation of *Lactobacillus pentosus* to the brine environment were the initial salt concentration, nutrient availability and natural inhibitory compounds, since the fruits were not subjected to lye treatment (Panagou *et al*., 2003). *Lactobacillus pentosus* also claimed to have the ability to hydrolyze phenolic compounds and contributing partially to the debittering process, because the concentration of phenolic compounds in brines inoculated with *Lactobacillus pentosus* were lower than in the brines without inoculation in the previous research by Panagou *et al.* (2003).

1.2.6 Antimicrobial and Bacteriocin Activity of Lactic Acid Bacteria (LAB)

Bacteriocins are peptide compounds which are synthesized by ribosom of bacteria to inhibit other bacteria growth. Many bacteriocins have a narrow killing spectrum, which inhibits only closely related strains (Cleveland *et al*., 2001). Bacteriocin-producing LAB can be applied for food preservation because of their microbiological, physiological and technological advantage (De Vuyst & Vandamme, 1994). Bacteriocins are also considered as safety additives or food grade. LAB produce several natural antimicrobials, including organic acids which are lactic acid, acetic acid, phenyllactic acid, caproic acid, and formic acid. LAB also produce bacteriocins, carbon dioxide, hydrogen peroxide, diacetyl, reuterin, ethanol, and reutericyclin (Messend & De Vuyst, 2002). The antimicrobial activity obtained by LAB strains may help to combat microbial contamination, thus it could be a replacement for chemical food additives which are commonly applied in food preservation technology (Leroy & Vuyst, 2004).

Lactic acid bacteria that have been isolated from boza, a beverage from a combination of different cereals produced in Bulgaria, which was belong to the genera *Lactobacillus* spp. revealed an antibacterial activity against a number of Gram-positive bacteria, and some Gram-negative bacteria, including *Escherichia coli* (Ivanova *et al*., 2000 in Todorov & Dicks, 2007). In most of these studies, the antimicrobial properties of the strains have been regarded to the production of bacteriocins (Matsusaki *et al*., 1996 in Todorov & Dicks, 2007). Several studies have indicated that LAB starter strains are able to produce bacteriocin compounds in food matrices and consequently display inhibitory activity towards sensitive food spoilage or pathogenic bacteria, which had been observed in fermented sausages (Calleawaert *et al.*, 2000), fermented vegetables and olives, and dairy products (Benkerroum *et al.*, 2002). Many bacteriocins are active against many foodborne pathogens such as *Clostridium botulinum, Staphylococcus aureus, and Listeria monocytogenes* (Nettles & Barefoot, 1993). Each bacteriocin has different inhibition activity against food-borne pathogens. It has been reported that bacteriocin production is influenced by several factors, which are carbon and nitrogen sources, fermentation conditions, such as pH, temperature, and agitation (Kumar *et al*., 2015). Bacteriocin is closely related with the growth of bacterial culture, because it is released during the growth of bacteriocin-producing cultures and at the end of bacterial growth its efficiency decreases due to protease degradation (Hur *et al*., 2000 in Thirumurugan *et al*., 2015).

1.3 Objectives

The objectives of this study were to investigate the suitability of "Kambas" bitter melon as a raw material for the growth of *Lactobacillus pentosus* LLA18, and also the effects of lactic acid fermentation on characteristics of fermented "Kambas" bitter melon juice, including amount of *Lactobacillus pentosus* LLA18, antioxidant, antimicrobial and bacteriocin inhibitory activity against 3 pathogenic bacteria, *i.e Escherichia coli* (FNCC 0091), *Staphylococcus aureus* (FNCC 47) and *Salmonella typhyimurium* (FNCC 0187) in two different fermentation time (24 and 48 hours).