1. INTRODUCTION

1.1. Background Research

The growing demand and interest in beverages with health benefits and special characteristics has led to development of new functional beverages in order to increase a healthier lifestyle in society. Nowadays, stress, fatigue, and loss of vitality are just a few examples that happen in society. There are many important role of functional beverages in health promotion and disease prevention, for example can boosting the immunity system, relieving stress, improving intestinal health, and many more. Functional beverage has beneficial effects or more specific body functions and nutrition (Shahidi, 2004)

The functional beverage in market covers many categories, from sports/energy drinks to herbal teas and fruit and vegetable juices, which targeted for all ages category because of that many health benefits. Beverages can be an excellent medium for the supplementation of nutritional components such as soluble fiber or herbal extract. The new formulations of beverages are rapidly changing. Nowadays, the market shelves are full with many different and new formulation of beverages such as soda pop, juices and dairy beverages, but some products taken as functional beverages such as coffees, teas, sports drinks, , probiotic drinks, smoothies and vegetable juices (Din *et al*., 2011). Probiotic drinks are one of functional beverage with added probiotic bacteria. The beneficial effects of food and beverage with added live microbes (probiotics) on human health are being increasingly promoted by health professionals (Schmidl & Labuza, 2000)

Probiotic products that available on the market today are usually in the form of fermented milk and yoghurt. However, with an increase in vegan lifestyle throughout developed countries, there is also a demand for vegetarian probiotic products. In recent years, consumer demand for non-dairy based probiotic products has increased due to the lactose intolerance and cholesterol content problem associated with the consumption of fermented dairy products. An alternative way by developing vegetable based product for the production of probiotic foods due to their large distribution and nutritive aspect (Gibson & Williams, 2000).

Lactic acid fermentation has many benefits such as can help to improve the safety, shelf life, and nutritional and sensory properties of vegetables. Strains of several *Lactobacillus* species have been proven to exert a range of health-promoting activities such as immunomodulation, enhancement of resistance against pathogens and reduction of blood cholesterol levels and are used as probiotics (Goldberg, 1994).Many species of *Lactobacillus* have been found in food industry and produce lactic acid as the end product in their metabolism of lactose and other fermentable carbohydrates (Slattery *et al*., 2010).

The *Lactobacillus* genus are the microbiota that naturally reside in human body and have been used as starter cultures and play a role as probiotic enhancers in fermented foods (Nissen *et al*., 2009; Klingberg *et al*., 2005). Previously, two example of species of *Lactobacillus: Lactobacillus fermentum* and *Lactobacillus pentosus* has been proven that have good probiotic activity and this species has been used as a starter culture in various food fermentation processes, contributing to improve food quality and sensory properties, like flavor, consistency, and texture. Due to the production of lactic acid and other antimicrobial compounds, *L. pentosus* and *L. fermentum* also might contribute for the final products, especially in safety aspect (Hartayanie *et al*., 2016; Tajabadi *et al.*, 2013).

Bitter melon (*Momordica charantia* L.) is an economically important medicinal plant because of the health benefits and belong to the family *Cucurbitaceae* or commonly known as karela. The fruit and leaves of bitter melon produce alkaloid momordicine and responsible of the bitter flavor in bitter melon (Din *et al*., 2011). Natural antioxidants in bitter melon are primarily plant phenolics and polyphenolic compound from fruits and seeds, and can be the alternatives to replace synthetic antioxidants to enhance food quality. Bitter melon fruit contain as many as 14 carotenoids and cryptoxanthin. Charantins in bitter melon are mixture of steroidal saponins in the fruit of bitter melon and have been proposed to contribute to the antihyperglycemic and hypoglycemic activity of bitter melon (Behera *et al.*, 2007)

Bitter melon also contain many beneficial phenolic compounds, including gallic acid, gentisic acid (2, 5dihydroxyl benzoic acid), catechins, epicatechin, and chlorogenic acid The extract of fruit seed and pulp of bitter melon has many various medicinal properties

such as antimutagenic and antitumour activities. Bitter melon fruit juice has also been shown to stimulate, significantly, both glycogen storage by the liver and insulin secretion of Langerhans. Therefore, by a lots of benefit and nutritional content, bitter melon can be use as sources for the growth of beneficial lactic acid bacteria to make a functional probiotic beverage (Din *et al*., 2011).

The development of functional beverage based on fruit and vegetable juice with probiotics because of taste profiles that are appealing to all age groups and are perceived as healthy and refreshing beverage. These beverages are highly nutritious and the taste also have been develop to be delicious product. Fruits and vegetables contain many bioactive compounds with antioxidant activities, such as vitamins A, C, E, and have a high antioxidant and phenolic compounds. Because of many benefits of antioxidant and probiotic bacteria in probiotic functional beverage, and as it has been known that the number of probiotic and antioxidant activity are the parameter that sensitive to change, thus to guarantee that it still contain the number of antioxidant and probiotic bacteria in the standard number for consumer, maintaining the viability of lactic acid bacteria and stability of antioxidant activity in bitter melon juice are really important to make sure that consumer still get benefit either the juice is already keep in some period of time, and to know how long the juice can be keep in the storage condition. Maintaining the viability and activity of probiotics in beverage during the shelf-life, there are an important criteria to be fulfilled in juices that the number of viable cells must have at least one billion (10^6) viable cells per portion where low pH represents a drawback (Daneshi *et al.*, 2013).

1.2. Literature Review

1.2.1. Bitter Melon

Bitter melon *(Momordica charantia* L.) is a tropical medicinal plant widely found in Asia, West Africa and North America. It is a flowering vine from the family *Cucurbitaceae* and the fruit is oblong in shape with a similarity to cucumber with some ridges on the surface of the fruit. Generally, bitter melon is rich in many nutritive compound such as carbohydrate, protein, vitamin, and mineral. Bitter melon also has some phytochemical such as saponin, glycoside, alkaloid, resin, reducing sugar, phenolic compounds, oil and

free acids (Din *et al*., 2011). Out of 45 species bitter melons reported in Asia and Africa, there are two species that most commonly found in Indonesia, namely “Kambas” bitter melon (long-typed bitter melon) and “Katak” bitter melon (small-typed bitter melon). “Kambas” bitter melon is commonly used in culinary while “Katak” bitter melon is famous for its natural anti-diabetic property (Myojin *et al*., 2008).

Figure 1. Kambas Bitter Melon

Bitter melon contain many beneficial chemical properties, such as saponin, charantin, steroidal glycoside, visine, polypeptide k, alkaloid and terpenoid. Bitter melon also has many benefits for health, and one of examples was identified as one of the most famous natural remedy in order to treat Diabetes Mellitus (DM) because of the cheap cost, easier to manage, less side effect and able to maintain the blood glucose level for long term effect as compared to the other conventional medicine (Tan *et al*., 2015)

Bitter melon extracts traditionally used as vegetable insulin possess hypoglycemic, antioxidative, and antidiabetic agents that are useful in the treatment of diabetes. Charantine (insulinlike peptide) or a mixture of steroidal saponins in bitter melon appears to be responsible for the hypoglycemic actions. There are at least three components (steroidal saponins, insulinlike compounds, and alkaloids) were found in bitter melon plant parts that responsible to bring benefits for people that suffer diabetes mellitus (Anjamma & Bhavani, 2015).

Bitter melon fruit contains as many as 14 carotenoids and this compounds depend on stage of maturity, where cryptoxanthins where turn as the principal chloroplast and chromoplast pigment will found in ripe fruit (Rodriguez *et al*., 1976). The antioxidant

properties of carotenoids in bitter melon can protect plants during photosynthesis, can also protect humans from carcinogens and mitigate free radical effects. Natural antioxidant that formed from primarily plant phenolics and polyphenolic compounds (e.g., in fruits and seeds of bitter melon), are one of the alternatives that can synthetic antioxidants for decreasing oxidative deterioration in fruit. Bitter melon also has many rich source of phenolic compounds, where gallic acid, chlorogenic acid, gentisic acid, catechin and epicatechin are typically abundant (Horax *et al*., 2005). The phenolic compound on these plant are potential to act as an excellent natural sources of food antioxidants, especially to reduce total cholesterol/triglycerides, blood pressure, cancer and cardiovascular diseases (Anjamma & Bhavani, 2015).

1.2.2. Lactic Acid Bacteria (LAB) as Probiotic

Lactic acid bacteria (LAB) are a group of gram-positive, non-spore forming, anaerobic bacteria which excrete lactic acid as the main fermentation product into the medium if supplied with suitable carbohydrates. Lactic acid bacteria are important microorganisms in the body and environment of human beings (Leroy & De Vuyst, 2004). LAB can be divided into two types based on the results of fermentation, which are homofermentative and heterofermentative. Homofermentative LAB convert glucose to lactic acid through glycolytic Embden-Meyerhoff pathway. This type of LAB includes *Lactococcus lactis*, *Pediococcus sp*., *Streptococcus thermophilus*, *Lactobacillus belveticus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*. While, 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. Included are *Lactobacillus mesentroides* subsp. *cremoris*, *Lactobacillus mesentroides* subsp. *mesentroides*, *Leuconostoc lactis*, and *Leuconostoc kimchi* (Hutkins, 2006).

LAB has a wide range of antimicrobial activities. Besides the production of bacteriocins, some LAB are able to synthesize other antimicrobial peptides that may also contribute to food preservation and safety. The group of lactic acid bacteria (LAB) also play a role for application and consumption in the production of fermented foods and beverages (Caplice & Fitzgerald, 1999). LAB are found in milk, meat, and fermented products (vegetables

and beverages). In this way they enhance shelf life and microbial safety, improve texture, and contribute to the pleasant sensory profile of the end product (Ross *et al*., 2000). LAB are able to produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, useful enzymes, or nutraceuticals, or LAB with health-promoting properties, so called probiotic strains. This represents a way of replacing chemical additives by natural compounds, at the same time providing the consumer with new, attractive food products. It also leads to a wider application area and higher flexibility of starter cultures (Chandan, 1999).

The genus *Lactobacillus* is the largest of genera included in LAB and widespread in nature. Many species of *Lactobacillus* have been found in food industry and produce lactic acid as the major end-product because of their metabolism of lactose and other fermentable carbohydrates (Slattery *et al*., 2010). In addition to a carbohydrate carbon source, lactobacilli also need another nutrient compound such as amino acids, vitamin, fatty acid, peptides, salts, esters, and nucleic acids derivatives to sustain their growth. The *Lactobacillus* genus are the beneficial qualities microbiota that naturally reside in human body and have been used as starter cultures and play a role as probiotic enhancers in fermented foods such as fermented drinks, yoghurts and cheese (Nissen *et al*., 2009; Klingberg *et al*., 2005). Therefore, a lot of *Lactobacillus* species have been found in many spontaneous lactic fermentations such as vegetable fermentations (Lindayani *et al*., 2018; Axelsson, 2004).

Two example of species of Lactobacillus are *Lactobacillus fermentum* and *Lactobacillus pentosus*. *Lactobacillus fermentum* has characteristic that short, single and paired square bacilli in MRS broth after 24 h of incubation at 37°C in anaerobiosis. This strain was microaerophilic, it needed reduction of oxygen grade, probably on account of the sensibility of yours enzyme in strong conditions of oxidation. The strain of *Lactobacillus fermentum* has antimicrobial activity and tolerance to bile. It could be a suitable strain for probiotic (Reque *et al*., 2000). *Lactobacillus fermentum* is commonly found in fermented plant and animal based food, and it has been long used in food processing and has a well-characterized as a probiotic strain because of efficacy in the prevention and treatment of infections in the human being (Tajabadi *et al*, 2013). In the other hand, *Lactobacillus*

pentosus that isolated from plants has proven to produce high level of bacteriocin in environmental conditions. *Lactobacillus pentosus* also has been used as a starter culture in many food fermentation processes, and has a contribution to improve food quality and sensory properties, especially flavor, consistency, and texture. *L. pentosus* also might contribute for the safety of the final products because of the production of lactic acid and other antimicrobial compounds (Hartayanie *et al*., 2016; Tajabadi *et al*, 2013).

1.2.3. Free Radical and Antioxidant

Antioxidant properties in natural compounds are very important because of their uses in medicine, food and cosmetics (Liu, 2003; Halliwell, 1996). In living systems various metabolic processes and environmental stresses generate various reactive species. These are free radicals and mainly reactive oxygen species (ROS). Increased level of ROS can damage structure of biomolecules, modify their functions, lead to cellular dysfunction and even cell death. The cumulative effect of increased ROS can increase oxidative stress in systemic level and it is manifested in the form of a variety of health problems such as cancer, age related disease and cardiovascular diseases (Noguchi & Niki, 2000). Antioxidants are believed to play a very important role in the body defense system against reactive oxygen species (ROS), which are the harmful byproducts generated during normal cell aerobic respiration. Increasing intake of dietary antioxidants may help to maintain an adequate antioxidant status and, therefore, the normal physiological function of a living system. Some functional foods and vegetables are the important sources of exogenous antioxidants (Pokorny *et al*., 2001).

Many natural antioxidants also can help to improve the biological effects, including antibacterial, antiallergic, antithrombotic, antiviral, anti-inflammatory, and vasodilatory actions. Antioxidant activity has some important properties for life because of many of the biological functions, such as antiaging, antimutagenicity, and anticarcinogenicity (Velioglu *et al*., 1998). The antioxidants can protect from the potentially damaging oxidative stress because of an imbalance between the formation of reactive oxygen species and the body antioxidant defense. Antioxidants also have been used in food industry because of many benefits, such as to prevent deterioration, off-flavoring, and

nutritional losses in various foods, especially those containing polyunsaturated fatty acids. Recently, interest in finding natural antioxidants in foods because of their potential in health promotion and disease prevention, high safety and consumer acceptability (Gorinstein *et al*., 2004).

The presence of antioxidant activity in food can be influenced by many factor, such as the microorganism species, pH, temperature, solvent, fermentation time, water content, kind of food and aerobic conditions. The decreasing level of antioxidant activity which may be caused by the degradation of phenolic compounds, thus this phenolic loss is responsible for the decreasing level of antioxidant activity. High temperature and exposure to light were also the main factors that decreased antioxidant activity during storage condition (Hur *et al.*, 2014). Mostly, the antioxidant activity in food can be maintained at pH 4.5-5.5 and degradation can be minimize at low temperature (Hur *et al*., 2014).

1.2.4. Fermentation using Lactic Acid Bacteria

Fermentation is a relatively cost effective, low energy preservation process, which is essential in ensuring the shelf-life and microbiological safety of the product (Liu *et al*., 2011). The biosynthesis of essential amino acids, proteins, and vitamins can enhance the nutritional value of food during fermentation by improving digestibility of protein and fiber, enhancing micronutrient bioavailability, and degrade anti-nutritional factors. Fermentation can influence the food safety aspect by reducing toxic compounds, such as aflatoxins and produce antimicrobial factors, such as lactic acid, bacteriocins, CO_2, H_2O_2, and ethanol, which can inhibit or eliminate food-borne pathogens. Because of fermentation can increase the nutritive, safety and preservative effects, fermentation can enhance the food because of the production of a diversity of flavours, textures, and aromas (Hutkins, 2006; Giraffa, 2004).

Recently, functional starter cultures often use in the food fermentation industry. One of the functional starter cultures in the food fermentation industry is by using lactic acid bacteria (LAB). LAB has many benefits for food and beverage industry such as food

preservation and safety, improvement of texture, and production of aroma and flavor. LAB can produce several natural antimicrobials such as organic acids (lactic acid, phenyllactic acid, acetic acid, formic acid, and caproic acid), carbon dioxide, ethanol, bacteriocins, hydrogen peroxide, diacetyl, reuterin, and reutericyclin. Acetic acid contributes to the aroma and prevents mould spoilage, and can be applied as food preservative (Messens & De Vuyst, 2002). In the other hand, bacteriocin that produced by LAB can be applied as a food preservation because of their microbiological, physiological and technological advantages (Goldberg, 1994; Nettles & Barefoot, 1993).

LAB also contribute to the aroma and flavour of fermented products. LAB can make the food more acid, produce tangy lactic acid taste, and aromatic compounds from, for instance, amino acids upon further bioconversion (van Kranenburg *et al*., 2002; Williams *et al.*, 2001). LAB also produce volatile substances that contribute to the typical flavor of certain fermented products, such as sourdough (determined by the lactate/acetate ratio), kefir and koumiss (ethanol), butter and buttermilk (diacetyl), and yoghurt (acetaldehyde) (Yvon & Rijnen, 2001).

The presence of lactic acid bacteria also can influence antioxidant activity during the fermentation of plant-based foods because of lactic acid bacteria have antioxidant activity. Fermentation with LAB has a positive influence on the total phenolic content and antioxidant activity of food. However, the degree of influence depends on the species of microorganism. Many lactic acid bacteria have an enzymatic and non-enzymatic antioxidative mechanisms and minimize the generation of reactive oxygen species to levels that are not harmful to cells (Hur *et al*., 2014)

1.2.5. Probiotic Beverage

Probiotics are a live microbial food ingredients that are beneficial to health. Probiotics are live microbes that usually use to formulate many different types of product, including foods, drugs, and dietary supplements (Schmidl & Labuza, 2000). There are two species that most commonly used as probiotics which are *Lactobacillus* and *Bifidobacterium* are. Lactic acid bacteria, including *Lactobacillus* species, which have been used for

preservation of food by fermentation for a long time and can serve a dual function by acting as agents for food fermentation and potentially imparting health benefits (WHO & FAO, 2006; Shahani & Ayebo, 1980).

Fermentation of food makes some characteristics taste profile and lower the pH. Fermentation also can prevent food contamination by potential pathogens. Fermentation commonly applied in the preservation of a range of raw agricultural materials (fruit and vegetables, cereals, roots, tubers, milk, meat, fish) (Hutkins, 2006). Probiotic bacteria should be able to survive in gastric transit and reach the small intestine in sufficient numbers to be effective to be beneficial in the human body. Most probiotics are group of organisms known as lactic acid-producing bacteria and are normally consumed in the form of yogurt, fermented milks or other fermented foods (Rolfe, 2000). Lactic acid bacteria has some of the beneficial effect which are improving intestinal tract health, enhancing the immune system, reducing symptoms of lactose intolerance, synthesizing and enhancing the bioavailability of nutrients, decreasing the prevalence of allergy in susceptible individual, and reducing risk of certain cancers (Benchimol & Mack, 2004).

Dairy products such as yogurts, cheese, and fermented sour milk are some product that commonly consumed as probiotic food and beverage and being develop at present. Although fermented dairy foods can be considered as one of the most common as well delivering as probiotics to humans, at present, many non-dairy as well delivering as probiotic products inherently contain essential nutrients, they are good-looking and have good taste. Fruit and vegetable juices, soy products, and cereal based products can be considered as main non-dairy probiotic foods available in the market at present. The type of carrier foods could affect on the viability of probiotics during processing and storage and functional properties, such as susceptibility to adverse conditions in the gut (acidity, bile and various enzymes) (Marco *et al*., 2013). The nondairy product also effective in enhance the bile acid tolerance and survivability of probiotics (Ranadheera *et al*., 2013). However, the physical structure of non-dairy probiotic carrier foods such as vegetables might provide protective environment for probiotics and reduce their exposure to harsh gastrointestinal conditions as well (Perricone *et al*., 2015).

The interest in the development of fruit and vegetable juice/milk based functional beverages with probiotics because of the taste profiles that are acceptable to all age groups and perceive as healthy and refreshing foods (Yoon *et al*., 2004; Tuorila & Cardello, 2002). The beverages have a highly nutritious besides being delicious. Fruits and vegetables contain various bioactive compounds with antioxidant activities, such as vitamins A, C, E, high antioxidant capacity, and phenolic compounds, which are good contributors to the total antioxidant capacity of the foods that contain them (Zulueta *et al*., 2007; Sánchez-Moreno *et al*., 2006).

1.2.6. Stability of Probiotics in Fermented Beverage

Many health benefits of probiotic mainly depends upon their concentration in foods, especially on their ability to survive in many condition of the gastrointestinal tract. Hence, even if the probiotic viability is to be strain-dependent (Corbo *et al*., 2014), they should be at least 10^6 CFU ml^{-1} in the product at the end of the shelf life (Nualkaekul & Charalampopoulos, 2011; WHO & FAO, 2006). Although juices contain some essential nutrients (vitamins, mineral, dietary fibers, antioxidants), there are another mainly factors that could limit probiotic survival in juices. That factors are food parameters (pH, water activity, titratable acidity, molecular oxygen, presence of salt, sugar and chemicals, like bacteriocins, hydrogen peroxide, artificial flavoring and coloring agents), processing parameters (heat treatment, cooling rate, packaging materials and storage methods, oxygen levels, incubation temperature, volume), and microbiological parameters (rate and proportion of inoculation, strains of probiotics) (Tripathi & Giri, 2014).

One of the most important factors that affect the survival of probiotics is pH. Juices contain a high level of organic acids and the low pH increases the concentration of undissociated form. In juice, the existence of a combined action of acidic conditions and the intrinsic antimicrobial effect of acids. In general, *Lactobacilli* resistant and survive in juices with pH ranging from 4.0 to 5.5. On the other hand, *Bifidobacteria* has a less acid tolerant, and resistant in pH of about 4.6 for their survival (Tripathi & Giri, 2014).

The international standard describe that probiotic products should be contained minimum of 10^6 viable probiotic bacteria per gram of product at the time of consumption for health and functional claiming (Daneshi *et al*., 2013; WHO & FAO, 2006; Roy, 2005). Vegetable juice drinks still can be claimed as probiotic product after 20 days keeping in cold storage. The initial cell counts in the product should be increased to 10^7-10^8 CFU ml^{-1}. Some strain of probiotic bacteria can prolong the shelf life of vegetable juice drinks compare with non-probiotic drinks. The fermentation was limited by keeping the samples in refrigerator, but some bacteria cells have some fermentative activity even during storage at 6ºC (Daneshi *et al*., 2013).

1.3. Objectives

The objectives of this study are to determine the viability of lactic acid bacteria and to investigate the stability of antioxidant activities and storage parameter (pH and sugar concentration) in the bitter melon fermented drink during storage condition.