4. DISCUSSION

4.1. Viability of Lactic Acid Bacteria during Storage Condition

There were three types of lactic acid bacteria that used in this study and found capable of rapidly utilising bitter melon fermented juice: *Lactobacillus pentosus* LLA8, *Lactobacillus fermentum* LLB3, and instant lactic acid bacteria,. Viable counts (CFU ml^{-1}) of LLA8, LLB3, and instant lactic acid bacteria in fermented bitter melon juice drink during storage at 4ºC over 30 days are presented in Table 1 and Figure 9. For probiotics to be effective, scientists have suggested that there be a minimum of 10^6 –10^7 CFU of probiotic bacteria per milliliter of product at the time of consumption which has also been achieved by these two strains (Khurana & Kanawajia, 2007). Based on existing standards and from a health view-point, it is very important that probiotic strains retain their viability and functional activity throughout the fermentation of the product. Some probiotic strains do not grow well in milk. In such cases the presence of plant-based ingredients may improve the growth of probiotic cultures in milk such as tomato juice, peanut milk, soy milk, bitter melon, carrot and cabbage juice (Perricone *et al*., 2015).

Based on Table 1, it has been known that *L. pentosus* LLA8, *L. fermentum* LLB3, and instant lactic acid bacteria showed a decreasing level of viable cells in bitter melon fermented juice drink during storage condition. For *L. pentosus* LLA8 and *L. fermentum* LLB3 remained viable above the critical level of 10^6 CFU ml^{-1} in bitter melon drink for 12 days during 30 days storage condition. For instant lactic acid bacteria remained viable above the critical level of 10^6 CFU ml^{-1} in bitter melon drink for 15 days during 30 days storage condition.

The viable cell counts of *L. pentosus* LLA8 in the fermented bitter melon juice drink from Table 1 are 10.00–127.00 x 10^6 CFU ml^{-1} on day 0, 20.00–73.00 x 10^6 CFU ml^{-1} on day 3, 0.00–18.00 x 10^6 CFU ml^{-1} on day 6, 0.00–5.00 x 10^6 CFU ml^{-1} on day 9, 0.00–16.00 x 10^6 CFU ml^{-1} on day 12, and there's not have a viability of probiotic bacteria until 30 days storage condition during 48 hours incubation time. The viable cell counts of *L. fermentum* LLB3 in the fermented bitter melon juice also showed the viability of probiotic bacteria until 12th day which is same with the viability of *L. pentosus* LLA8. The viable cell counts of *L. fermentum* LLB3 in the fermented bitter melon juice drink from Table 1 are 2.00–475.00 x 10^6 CFU ml^{-1} on day 0, 42.00–93.00 x 10^6 CFU ml^{-1} on day 3, 10.00–45.00 x 10^6 CFU ml^{-1} on day 6, 1.00–45.00 x 10^6 CFU ml^{-1} on day 9, 0.00–7.00 x 10^6 CFU ml^{-1} on day 12, and don't have a viability of probiotic bacteria until 30 days storage condition during 48 hours incubation time.

Different with *L. pentosus* LLA8 and *L. fermentum* LLB3, from Table 1, the viability of instant lactic bacteria grew until 15 days storage condition, which are 0.00–34.00 x 10^6 CFU ml^{-1} on day 0, 5.00–42.00 x 10^6 CFU ml^{-1} on day 3, 2.00–35.00 x 10^6 CFU ml^{-1} on day 6, 3.00–15.00 x 10^6 CFU ml^{-1} on day 9, 0.00–3.00 x 10^6 CFU ml^{-1} on day 12, 0.00–2.00 x 10^6 CFU ml^{-1} on day 15 and don't has a viability of probiotic bacteria until 30 days storage condition during 48 hours incubation time. After 30 days of refrigerated storage (4^{o}C), bitter melon juice which has the highest viability of probiotic bacteria was bitter melon juice fermented with *L. fermentum* LLB3 which contain until 475.00 x 10^6 CFU ml^{-1} on day 0 and also has a gradually decline on day 15th compared to another strain. While, bitter melon juice which fermented with instant lactic acid bacteria has the longer time of viability of probiotic bacteria compare to the other strain.

According to Table 1 and Figure 9, it showed that the viability of probiotic bacteria were decreasing during storage condition. Although the bitter melon juices contain some essential nutrients (minerals, vitamins, dietary fibers, antioxidants), there are some strong factors that could limit probiotic survival and make the number of viable cell count were decreasing in juices which are food parameters (pH, molecular oxygen, water activity, presence of sugar and chemicals), processing parameters (heat treatment, incubation temperature, cooling rate, packaging materials and storage methods, oxygen levels, volume), and microbiological parameters (strains of probiotics, rate and proportion of inoculation). pH is one of the most important factors affecting the survival of probiotics. which *Lactobacilli* are generally resistant and survive in juices with pH ranging from 4.0 to 5.5 (Tripathi & Giri 2014).

It was shown that *Lactobacillus* is a slow-growing organism and quite survives well in milk and dairy products (Ostlie *et al*., 2003). The *L. pentosus* LLA8 and *L. fermentum* LLB3 strain ferments lactose to lactic acid and it is very stable and has a high resistance towards acids in fermented dairy products. The optimum pH for its growth is between 4.0 to 5.5 (Tripathi & Giri, 2014). In this research, during the storage time, pH values of the three types of probiotic drinks were almost constant, whereas the initial pH value was about 4.3, and it has changed significantly after 12 days of cold storage at 4ºC.

The main factors for loss of viability of probiotic organisms have been attributed to the decrease in the pH of the medium and accumulation of organic acid as a result of growth and fermentation (Yoon *et al*., 2004). Thus, high viability of the probiotics in this study might be due to constant pH throughout the 4 week storage period and/or nutrient content of bitter melon probiotic drink, but the decreasing of viable cell count can be affected by the changes of pH. Mortazavian *et al*. (2007) investigated the effect of cold storage temperature on the viability of probiotics in yogurt and reported that the highest viability of *Lactobacillus* after 20 days, was observed in less cold storage temperature (2ºC). Saccaro *et al*. (2009) noted a reduction of more than 2 log 10 cycles per ml in the counts of *Lactobacillus* at the end of the storage period in yogurt.

The international standard describe that the probiotic products should be contained at least 10^6 CFU ml^{-1} in the product at the end of the shelf life when it consume for health and functional claiming (WHO & FAO, 2006; Roy, 2005), thus the bitter melon probiotic drinks in this research could be probiotic after 12 days keeping in cold storage if the provided initial cell counts in the product should be increased to 10^6-10^8 CFU ml^{-1} compared with the bitter melon non-probiotic drinks. The present results (Daneshi *et al*., 2013) showed that some strain of probiotic bacteria can prolong the shelf life of bitter melon probiotic drinks in comparison with non-probiotic drinks (Nighswonger *et al*. 1996).

4.2. Antioxidant Activity of Fermented Bitter Melon Juice during Storage Condition

The results of antioxidant activity of fermented bitter melon juice with different lactic acid bacteria (*L. pentosus LLA8, L. fermentum LLB3*, and Instant LAB) and different storage time are shown on Figure 10. Fermentation technology can prolong the shelf-life and enhance nutritional and organoleptic qualities of food. During fermentation, many biochemical changes, leading to an altered ratio of nutritive and anti-nutritive compounds, therefore, products properties such as bioactivity will affect significantly (Hur *et al.*, 2014). 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 (Din *et al*., 2011).

As reported by Hur *et al*, (2014) during fermentation there was a bioconversion of the conjugated forms of phenolic compounds into their free forms, thus enchancing their antioxidant activity. Recently, this bioprocess has been applied to the production and extraction of bioactive compounds in the food. Fermentation has been applied to increase the content of bioactive phenolic compounds in vegetables, thus enhancing their antioxidant activity. For the reasons discussed above, the fermentation of food materials is a useful tool to improve the antioxidative activity of food products (Hur *et al*, 2014).

The results of antioxidant activity determined as free radical scavenging activity were decreasing after 30 days of storage condition, which has been shown in Table 2. In this research, at the beginning of the experiment using the DPPH method, the bitter melon probiotic drink presented highest antioxidant activity (Figure 10). During 30 days storage condition, the antioxidant activity of *L. pentosus* LLA8 in the fermented bitter melon juice drink are 89.42±0.99 on the initial day (day 0) and decreasing significantly to 70.92±1.30 (day 30). In the other hand, the antioxidant activity of *L. fermentum* LLB3 in the fermented bitter melon juice drink are 86.16±1.23 on the initial day (day 0) and decreasing significantly to 70.94±1.20 (day 30). On the other side, the antioxidant activity of instant lactic acid bacteria in the fermented bitter melon juice drink are 88.86±1.44 on the initial day (day 0) and decreasing significantly to 70.86±1.16 (day 30). The changes of antioxidant activity of all the strains during cold storage are significant ($p < 0.05$). In the other hand, based on Table 3, the bitter melon probiotic drink presented highest antioxidant activity on the day 0. At the 15th storage day the antioxidant activity of fermented bitter melon juice are decreased around 6.84-10.34% while the there's no viability of LLA8 and LLB3. At the last storage day the antioxidant activity of fermented bitter melon juice are decreased around 17.66-20.69% compared to day 0.

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). After 30 days of refrigerated storage (4^{o}C), bitter melon juice which has the highest antioxidant activity was bitter melon juice that fermented with *L. pentosus* LLA8 which contain until 89.42±0.99 (%) on day 0, but in the 30th day, bitter melon juice which fermented with *L. fermentum* LLB3 showed the highest antioxidant activity compared another strain which was 70.94±1.20 (%).

The result showed that number of antioxidant activity were affected by the different LAB strain. Hur *et al.* (2014) mentioned that the antioxidant activity can be affected by microbial strains present during the fermentation of plant-based foods, thus the effects of fermentation on the antioxidant activity depends on the species of microorganism. A different effects of lactic acid bacteria strain on antioxidant activity could be influenced by different configuration of the lactic acid produced, mode of glucose fermentation, high salt concentrations, growth ability at different temperature, and acid or alkaline tolerance (Hur *et al*., 2014)

4.3. Sugar Concentration of Fermented Bitter Melon Juice during Storage Condition

Based on Figure 11, there was a changes of total soluble solid expressed as sucrose concentration in all of bitter melon juice fermented by *Lactobacillus pentosus* LLA8, *Lactobacillus fermentum* LLB3, and also Instant LAB. After adding of 3 types of lactic acid bacteria to bitter melon juice, there was significant changes in total soluble solid of bitter melon juice during cold storage of control samples at 4ºC. The total soluble solid showed a significant changes ranging from 3.10±0.12 to 3.33±0.12 for *L. pentosus* LLA8, 3.13±0.05 to 3.43±0.08 for *L. fermentum* LLB3, and 3.17±0.12 to 3.40±0.10 for instant lactic acid bacteria.

According to this results, it can be known that sugar concentration is one of parameter that indicate metabolism of strain during storage condition. The metabolism of the strain means that LAB use sugar as a substrate of fermentation. Thus, the decreasing of sugar concentration indicated that all the sucrose were metabolized by all the strains, and indicate fermentation during storage condition. This results also shown that metabolism of carbohydrates can be different for each strain, which depends on the substrate and even on the fermentation time (Mousavi *et al.*, 2011). In the order hand, the changes of total soluble solid changes for bitter melon probiotic drink not have a wide range and relatively stable. It also has been report by Stanbury *et al* (2016), during storage condition there was just a little fermentation activity of probiotic bacteria that using sugar for substrate of fermentation which made the total soluble solid hasn't changes gradually.

4.4. pH Changes of Fermented Bitter Melon Juice during Storage Condition

The changes of pH of the different bitter melon probiotic drinks studied in this work were shown in Figure 12. After adding of 3 types of lactic acid bacteria to bitter melon juice, there was significant changes in pH of juice during cold storage of control samples at 4ºC. The pH showed a significant changes ranging from 4.27±0.01 to 4.35±0.07 for *L. pentosus* LLA8, 4.26±0.05 to 4.36±0.08 for *L. fermentum* LLB3, and 4.24±0.02 to

4.35±0.03 for instant lactic acid bacteria. This result indicated that lactic acid, which is the main metabolite produced by lactic acid bacteria, was produced by all the strains and kept increasing during the fermentation, thus resulted in the decreased of pH value of fermented bitter melon juice (Mousavi *et al*., 2011). In the order hand, the changes of pH for bitter melon probiotic drink not have a wide range and relatively stable. It also has been report by Stanbury *et al* (2016), during storage condition, there was just a little fermentation activity of probiotic bacteria during storage which made a little bit changes on the pH.

During storage, the changes in pH and acidity also might be due to degradation of artificial sweetener and carbohydrate present in the bitter melon extract by the action of microorganisms which causes production of acids in beverage. It also has been report by Miguel *et al*. (2004) that shown a decreasing trend of pH in beverages during storage. High acid and low pH may be due to production of acetic acid and lactic acid during storage.