4 DISCUSSIONS

4.1 Characteristics of Fermented Bitter Melon Juice by *Lactobacillus fermentum* LLB3

Fermentation is one of the most ancient method in food processing which can enhance the nutrient value of food through the biosynthesis of vitamin and amino acid by increasing the protein and fiber digestibility. Mostly, the microorganism that is used for fermenting food are yeast and lactic acid bacteria (LAB) (Magala *et al*., 2015). *Lactobacillus fermentum* LLB3 and Instant LAB are microorganisms that were used in this study. *Lactobacillus fermentum* LLB3 was isolated from bamboo shoot pickles and the probiotic characteristics had been analyzed (Lindayani *et al.*, 2018). Instant or commercial LAB that was used in this research is mixture LAB. This instant LAB consist of *Lactobacillus bulgaricus, Streptococcus thermophilus,* and *Lactobacillus acidophilus*. Commercial LAB which is commonly used in beverages fermentation is the mixture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, while *Lactobacillus acidophilus* sometimes are added for additional probiotic value (Bull *et al.*, 2013). Based on Chockchaisawasdee & Stathopoulos (2011), *S. thermophilus* and *L. bulgaricus* have a mutual interaction, since the mixture of *S. thermophilus* can produce formic acid that stimulate the growth of *L. bulgaricus*, whereas *L. bulgaricus* also produces amino acids (glycine, histidine, valine, leucine, isoleucine) that stimulate *S. thermophilus* growth.

Method for analyzing the LAB content in fermented bitter melon juice is total plate count using spread plate method. As reported in the result (Table 1 and Figure 9), fresh bitter melon also has lactic acid bacteria. In accordance with the previous research by (Trias *et al.*, 2008), fresh fruit and vegetable contain lactic acid bacteria. Khemariya *et al.* (2016) also stated that there are 5 isolates of LAB from bitter melon and one of them is identified as *Lactobacillus plantarum*. Meanwhile, fermentation process using *Lactobacillus fermentum* LLB3 and Instant LAB have the same trend line. Based on Table 2 and Figure 10, it showed that pasteurized bitter melon has high antioxidant activity with the amount 84,80%. This result is the same as the previous research conducted by Hamissou *et al.* (2013) who stated that the antioxidant component in bitter gourd was 82,05% as effective as ascorbic acid for inhibiting the free radical DPPH. pH value of bitter melon at 0 hour of fermentation is suitable for the

growth of *Lactobacillus fermentum* LLB3 with the amount around 5 (Table 5 and Figure 15). It has been stated that maximum growth of *Lactobacillus fermentum* is at pH 4 and 5 (Tallapragada *et al.*, 2018).

The optimum viability of both fermented samples are at 24 hours of fermentation. This result is similar to the research conducted by Sivudu *et al.* (2014) about fermentation using *Lactobacillus fermentum* in tomato juice. The increase of LAB content in the fermented bitter melon juice also gives an effect to pH value and sugar content. Table 5 and Figure 15 showed that at 24 hours of fermentation, the pH value is decreasing. Gradual reduction of pH value is due to the formation of lactic acid and acetic acid produced by lactic acid bacteria from sugar that is extracted from bitter melon juice (Silva *et al.*, 2016). While the decrease of sugar content (Table 6 and Figure 16) happened because glucose is a very good carbon source for the growth of lactobacillus (Kumar *et al.*. 2013), especially for producing lactic acid (Mousavi *et al.*, 2011). Therefore, the more amount of LAB content can reduce the pH value and sugar content of fermented bitter melon juice.

Antioxidant activity of fermented bitter melon juice decrease at 24 hours of fermentation and starting to increase at 48 hours of fermentation (Table 2 and Figure 10). The result is quite different from the previous research about antioxidant activity in fermented papaya juice (Chen *et al.*,2018) okra seeds (Adetuyi & Ibrahim, 2014) and bitter melon juice (Hartayani *et al*., 2018). The decreased of antioxidant activity at 24 hours of bitter melon fermentation can be caused by various factors such as microorganism species, pH, temperature, solvent, water content, fermentation time, kind of food, aerobic condition, and other component that can influence antioxidant activity. Antioxidant activity of phenol is affected by their chemical structure. If there is a functional group that attached to basic aglycon, it can decrease the antioxidant activity (Hur *et al.*, 2014).

At 48 hours of fermentation, the amount of lactic acid bacteria is decreasing (Table 1, Figure 9). This is due to nutrient contained in media has been reduced or used for the growth of LAB. Therefore, there is no nutrient left for LAB and the LAB is dying. Besides that, because LAB produces lactic acid, it makes the acidity levels are too low to support the living of LAB (Lestariningtyas *et al.*, 2018). The lack of nutrient source also shown in Table 6 and Figure 16 through the decrease of sugar content compare with 24 hours of fermentation. Glucose is a very good carbon source for the growth of lactobacillus (Kumar *et al.*. 2013), but the amount

of sugar content in bitter melon juice is fewer than the amount of lactic acid bacteria after 24 hours of fermentation. In this research, at 24 hours of fermentation using *Lactobacillus fermentum* LLB3, 0,1% glucose could be used by LAB to grow until 5,9 x 10^8 CFU/ml. Another research about fermentation using *Lactobacillus fermentum* also shows the decrease of glucose content during fermentation, but the amount of sugar and LAB content are different. In the previous research stated that 55mM of glucose can be used to grow up to log 8 CFU/ml at 24 hours of fermentation (Vrancken *et al.*, 2008). The decreasing of lactic acid bacteria in the juice happened because of some factors such as strain used, interaction between present species, production of hydrogen peroxide due to bacterial metabolism, culture conditions, final acidity of product, and concentrations of lactic and acetic acids (Guevarra & Barraquio, 2015).

pH value of fermented bitter melon juice using instant LAB is increasing during 48 hours of fermentation (Table 5, Figure 15). According to the previous research by Mousavi *et al.* (2011), the increased of pH value during fermentation happened because lactic acid bacteria used some organic acid inside the media due to the sugar content which already reached a low level. This condition caused the reduction of total acid amount and the increasing of pH value during fermentation.

At 48 hours of fermentation the result showed that the amount of antioxidant was increasing in all samples. The increase of antioxidant activity after 48 hours fermentation of bitter melon juice caused by the releasing of phenolic compound inside the juice. During fermentation, lactic acid bacteria produce some enzyme which can break down plant's cell wall or starch and release the phenolic compound (Huynh *et al.*.2014). Another factor that increase the antioxidant activity is ferulic acid produce by *Lactobacillus fermentum*. Ferulic acid is phenolic compound which able to neutralize free radicals (Tomaro-Duchesneau *et al.*, 2012). The decrease of phenolic compound after 48 hours happened due to the precipitation or oxidation during the fermentation process. The combination of adsorption of phenolic compound with protein and polymerization of this compound caused the losses of phenolic compound (Chen *et al*, 2018). The result (Table 2, Figure 10), showed that antioxidant activity of fermented bitter melon juice using *Lactobacillus fermentum* LLB3 has the same trend line as instant LAB even the antioxidant activity is slightly higher in *Lactobacillus fermentum* LLB3 strain.

Several factors affecting fermentation using lactic acid bacteria are pH, oxygen availability, temperature, salt concentration, water activity, nutrients, and selected starter cultures. LAB can survive in pH 3-4 and for oxygen availability, LAB quite flexible and can grow with or without oxygen. Critical temperature of LAB is around 20ºC to 30ºC and highly tolerant with high salt concentration. LAB requires high amount of water activity (0.9 or higher) and also needs carbohydrate either glucose, fructose or starch and cellulose for their metabolism (Ray and Didier, 2014).

Based on total LAB content's results (Table 1, Figure 9), it showed that LAB content of *Lactobacillus fermentum* LLB3 in bitter melon juice was higher than Instant LAB even they had a same trend line. This was caused by the different of optimum pH and temperature between both of them. *Lactobacillus fermentum* had optimum temperature for their growth at 30ºC and optimum pH at 4 and 5 (Sivudu *et al*, 2014 and Tallapragada *et al*, 2018). Meanwhile, instant LAB (consist of *Lactobacillus bulgaricus, Streptococcus thermophilus*, and *Lactobacillus acidophilus*) had optimum temperature at 35-38ºC and optimum pH at 5-5.6 (Yerlikaya, 2014). Samples with 24 hour fermentation were accepted as probiotic beverages with the criteria total amount of probiotic bacteria $>10^8$ CFU/ml (FAO/WHO, 2002 *cit.* Sandes *et al.*, 2017). Mostly, probiotic food with total amount of probiotic bacteria $>10^6$ CFU/ml already accepted as probiotic food that can give some beneficial effect to human health (Sandes *et al*., 2017; Yerlikaya *et al*., 2013).

4.2 Antimicrobial and Bacteriocins Inhibitory Activity Test

4.2.1 Antimicrobial Inhibitory Activity Test

According to Table 3 and Figure 13, it showed that fresh bitter melon juice had an antimicrobial inhibitory activity against *Escherichia coli* FNCC 0091, *Staphylococcus aureus* FNCC 0047, *and Salmonella typhimurium* FNCC 0187. This result was the same as another research that stated about bitter melon extract can inhibit the growth of pathogen bacteria with the different inhibitory activity (Mahmood *et al.*, 2012 ; Jagessar *et al*., 2008). Related to Kight (2003) *cit.* Mahmood *et al* (2012), the antibacterial potential in bitter melon is because they have some antimicrobial components such as alkaloids, charantin, chorine, goya glycosides, goyasaponins, linoleic, lenolinic acid, and other acids.

Based on the result of antimicrobial activity in fermented bitter melon using *Lactobacillus fermentum* LLB3 (Table 3, Figure 13), it was expressed that antimicrobial activity of fermented bitter melon using *Lactobacillus fermentum* LLB3 against *Escherichia coli* FNCC 0091 was gradually decreasing during the longer time of fermentation. Whereas, the antimicrobial activity against *Staphylococcus aureus* FNCC 0047 and *Salmonella typhimurium* FNCC 0187 were decreasing at 24 hours of fermentation and increasing at 48 hours of fermentation. Fermented bitter melon juice using *Lactobacillus fermentum* LLB3 had antimicrobial potential. This is related with research conducted by Bao *et al.*, (2010) about *Lactobacillus fermentum* that shows the inhibitory activity of *Lactobacillus fermentum* can inhibit the growth of gram positive (*L. moocytogenes, S. aureus*) and gram negative (*E. coli, S. flexneri,* and *S. typhimurium*). The production of metabolites like lactic acid, acetic acid, diacetyl, fatty acid, aldehydes bacteriocins, and other compounds by *Lactobacillus* strain are the most powerful antimicrobial in LAB (Maryam & Abubakr, 2018).

According to Table 3 and Figure 13, it showed that antimicrobial activity of fermented bitter melon using instant LAB against *Escherichia coli* FNCC 0091 and *Staphylococcus aureus* FNCC 0047 were gradually decreasing during the longer time of fermentation. Whereas, there was no antimicrobial activity against *Salmonella typhimurium* FNCC 0187 at 24 hours of fermentation and antimicrobial activity starting to inhibit the pathogen at 48 hours of fermentation. LAB had antimicrobial properties which came from the production of metabolites like lactic acid, acetic acid, diacetyl, fatty acid, aldehydes bacteriocins, and other compound. Among that components, lactic acid, acetic acid, and bacteriocins were the most powerful antimicrobial in LAB (Maryam & Abubakr, 2018). The production of lactic acid by LAB reduced the pH of fermenting medium. Therefore, this acid condition was not suitable for the growth of another bacteria especially spoilage bacteria (Ukwuru & CG, 2018). All of the acids that produced by LAB will interact with cell membrane and cause intracellular acidification and protein denaturation. Protein denaturation makes the physiological and morphological of bacteria's membrane change due to the leakage in cytoplasmic contents of bacteria (Sharma *et al.*, 2017).

The decrease of antimicrobial activity can related to the production of antimicrobial compound by LAB. According to Saranraj *et al.* (2013) and Reis *et al.* (2012), each antimicrobial compound has their own optimum temperature and pH condition to produce maximum antimicrobial component. The inhibition zone of *Escherichia coli* FNCC 0091 are

wider than *Staphylococcus aureus* FNCC 0047. It is due to *Escherichia coli* FNCC 0091 is gram negative bacteria whereas *Staphylococcus aureus* FNCC 0047 is gram positive bacteria. This result was not same as another research by McKane & Kandel (1996) *cit.* Hartayanie et al., (2016) who stated that Gram Positive bacteria's cell wall have single-layered with low lipid content (1-4%), while Gram Negative bacteria had more complex structure consisting of three-layered outer layer and high lipid content (11-12%). Therefore, Gram negative bacteria was more resistant than Gram Positive bacteria.

4.2.2 Bacteriocins Inhibitory Activity Test

Based on Table 4 and Figure 14, it showed that fresh bitter melon had bacteriocins activity against *Escherichia coli* FNCC 0091, *Staphylococcus aureus* FNCC 0047, *and Salmonella typhyimurium* FNCC 0187. This result was similar to the research conducted by Jagessar *et al.* (2008). Bacteriocins activity of fermented bitter melon juice using *Lactobacillus fermentum* LLB3 and Instant LAB against *Escherichia coli* FNCC 0091 and *Staphylococcus aureus* FNCC 0047 get decreasing due to the longer time of fermentation. Whereas, in the inhibition against *Salmonella typhimurium* FNCC 0187, *Lactobacillus fermentum* LLB3 showed inhibitory activity at 24 hours of fermentation and decreased after 48 hours of fermentation. Meanwhile, instant LAB only has bacteriocins activity at 0 hour and 48 hours of fermentation. The decrease of bacteriocins production can be caused by many factors such as medium composition, surfactant, temperature, and pH (Abbasiliasi *et al.*, 2017). According to Abbasiliasi *et al.* (2017), the production of bacteriocins does not only depend on the type of carbon and nitrogen contained in media, but also the ratio and concentration of them. In this case, the nutrient inside the media mostly be used for the growth of lactic acid bacteria and only a few amount of them used to produce bacteriocins. The addition of surfactant can improve the production of bacteriocins because it can increase the sensitivity of the indicator strain and micelles using proteinaceous compound to stabilize the production of bacteriocins. In this research, surfactant is not added. Besides that, the optimum temperature and pH for producing bacteriocins may not be the same as the optimal temperature and pH for bacterial growth. Another research by L. De. Vuyst (1996) *cit.* Abbasiliasi (2017) stated that bacteriocins can be produced at pH 5-5.6 with the temperature slightly lower than the optimal growth temperature.

Bacteriocins are group of antimicrobial peptides produced by some microorganisms including LAB. Bacteriocins produced by LAB can be used to preserve food against pathogen or another spoilage bacteria (Mokoena, 2017). *Lactobacillus fermentum* also one of LAB strains that has a good ability for inhibiting the growth of pathogen such as *Escherichia coli* (El *et al.*, 2016) and *Staphylococcus aureus* (Riaz *et al.*,2010). According to previous research by Bao *et al.* (2010), *Lactobacillus fermentum* showed inhibitory activity against gram positive (*L. moocytogenes, S. aureus*) and gram negative (*E. coli, S. flexneri*, and *S. typhimurium*) pathogen, even though each strain has different antimicrobial activity due to the production of bacteriocins that are not same. *L. bulgaricus* and *L.acidophilus* inside instant LAB also have an ability as bacteriocins producer (Sarvari *et al.*,2014).

Based on the result (Table 4, Figure 14), the inhibition zone against *Staphylococcus aureus* FNCC 0047 (Gram Positive bacteria) is wider than *Salmonella typhimurium* FNCC 0187 (Gram Negative bacteria). This is due to the different structure and composition of the cell wall between Gram Positive and Gram Negative bacteria. Gram Positive bacteria's cell wall have single-layered with low lipid content (1-4%), while Gram Negative bacteria have more complex structure consisting of three-layered outer layer and high lipid content (11-12%). Therefore, Gram negative bacteria is more resistant than Gram Positive bacteria (McKane & Kandel, 1996 *cit.* Hartayanie *et al.*, 2016). Bacteriocins activity that shows from both of the LAB strains were different against different type of pathogens bacteria. This is caused by the different target of bacteriocins, type and concentration of bacteriocins produced by each LAB strain (Sari *et al*, 2018).