4. DISCUSSION

4.1. Fermentation of Ampel Bamboo Shoots

Fermentation is one of the most common methods of preserving bamboo shoots. (Pandey *et al*., 2012). In this study, fermentation of Ampel Bamboo Shoots was carried out spontaneously. According to Pederson (1971), spontaneous fermentation occurs by the microorganisms naturally present in raw materials without the addition of starter culture. Fermentation of fruits and vegetables usually occurs "spontaneously", however the starter culture can also be added to provide consistency and reliability of performance (Karovicova *et al*., 1999 in Swain *et al*., 2014). Fermentation of vegetables is mostly carried out in a salted medium. Salting is done by soaking the vegetables in brine solution. Salting is necessary in most kinds of vegetables fermentation which is to promote the growth of LAB over spoilage bacteria. Moreover, salt induces plasmolysis in the plant cells and causes the release of liquid phase, which creates anaerobic conditions around the submerged materials (Swain *et al*., 2014).

The most important factors affecting vegetables fermentation are salt concentration and temperature (Hutkins, 2006). The concentrations of salt used in this bamboo shoots fermentation are 2.5% and 5%. According to Hui *et al*., (2004) the addition of 2.5% salt will restrict the activities of undesirable Gram-negative bacteria. FAO (2007) also stated that fermentation will be carried out by LAB when the concentration of salt used is 2-5%. The temperature used in this fermentation are 15ºC and 30ºC, taking into account that most LAB have an optimal temperature ranges from 20ºC to 30ºC and some LAB can grow well at cooler temperatures (15-20ºC) (Ross *et al*., 2002 in Montet *et al*., 2014).

4.2. Screening, Isolation, and Identification of LAB

Based on Singleton & Sainsbury (2006), isolation is defined as procedure to separate a species presents in particular sample or environment to obtain pure culture. In this study, the isolation of LAB from Ampel Bamboo Shoots Pickle was performed on

MRS-A medium containing 1% of $CaCO_3$. $CaCO_3$ was used as an indicator for acid-producing bacteria since it was dissolved in acid and formed clear zone (Onda *et al*., 2002 in Hwanhlem *et al*., 2011). A total of 35 random single colonies were selected for further assays based on the formation of clear zone around the colonies.

4.2.1. Cell Morphology and Motility Test

Bacteria species can be classified as LAB based on their cell morphology and motility (Salminen *et al*., 2004; Hutkins, 2006). In this study, the cell morphology of bacteria isolated from Ampel Bamboo Shoots Pickle was determined using Gram staining and endospore staining, while the motility of those bacteria was determined by observing the spread of growth in semi-solid agar. Bacteria are mainly grouped according to their shape. The most common shapes of bacteria are cocci (round-shaped), bacilli (rod-shaped), and spirilla (spiral-shaped) (Cambray, 2006 in Tshikhudo *et al*., 2013). LAB are either cocci or rod-shaped bacteria (Salminen *et al*., 2004; Hutkins, 2006). Based on Table 1, it has been found that all isolates were rod-shaped bacteria.

Gram staining is one of the most useful methods in identifying bacteria. It classifies bacteria into two large groups: Gram-positive and Gram-negative. Gram-positive bacteria will retain the crystal violet after decolorization and appear purple (Figure 4a), while Gram-negative bacteria will lose the purple color after decolorization and appear pink to red because they take up the safranin counterstain (Figure 4b). The different reaction between Gram-positive and Gram-negative bacteria is based on structural differences in their cell walls. The cell wall of Gram-positive bacteria consists of many layers of peptidoglycan, forming a thick and rigid structure. In contrast, the cell wall of Gram-negative bacteria contains only a thin layer of peptidoglycan (Tortora *et al*., 2013). Based on Table 1, it has been found that 1 isolate was classified as Gram-negative bacteria and 34 isolates were classified as Gram-positive bacteria.

Endospore staining is one of special stains used to isolate specific parts of microorganisms such as endospores. Endospores are relatively uncommon in bacterial cells. However, endospores can be formed by a few genera of bacteria. Endospores are

resistant to stain with most dyes because the dyes do not penetrate the wall of endospores. The most common endospore staining method is Schaeffer-Fulton using malachite green as the primary stain. Endospores will retain the malachite green and appear green, while vegetative cells will lose the stain and appear pink to red because they take up the safranin counterstain (Figure 5) (Tortora *et al*., 2013). Based on Table 1, it has been found that all isolates were classified as non-spore forming bacteria.

Motility is defined as the ability of organism to move by itself. Bacterial motility can be observed on culture medium containing low concentration of agar. The higher concentration of agar will cause the gel too firm to allow the organism to spread. The tubes are stab-inoculated with straight wire, wherein only motile bacteria can migrate throughout the medium (Aygan & Arikan, 2007). Based on Table 1, it has been known that 33 isolates were identified as non-motile since the growth was confined to the stab inoculum (Figure 6), while 2 isolates did not show any growth. As stated in Salminen *et al*. (2004) and Hutkins (2006), LAB are group of Gram-positive, cocci or rod, non-spore forming, and non-motile. In cell morphology and motility test, it could be seen that 32 isolates were identified as LAB and 3 other isolates were eliminated.

4.2.2. Physiological Test

According to current taxonomy, LAB group consists of twelve genera. Seven of the twelve genera of LAB, including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, and *Tetragenococcus*, are used directly in food fermentations. Classification of those genera was based on morphology characteristics, mode of glucose fermentation under standard conditions, growth at certain temperature, salt tolerance, as well as acid and/or alkaline tolerance (Salminen *et al*., 2004; Hutkins, 2006). Therefore, in this study, genus of 32 isolates were identified according to growth capabilities at different temperature (10ºC and 45ºC), pH (4.4 and 9.6), and NaCl concentration (6.5% and 18%).

Based on Table 2, it could be known that only 3 isolates were identified as *Lactobacillus* genus due to their capability to grow at 45ºC, pH 4.4, and 6.5% of NaCl.

According to Salminen *et al*. (2004) and Hutkins (2006), *Lactobacillus* species can grow at 10ºC and 45ºC, pH 4.4, 6.5% of NaCl (response varies between species), but cannot grow at pH 9.6 and 18% of NaCl. Varying results were found in this assay. Result in Table 2 also showed that 6 isolates could not be identified because their characteristics did not match to the characteristics of all genera and 23 isolates were assumed as *Lactobacillus* due to the similarity of their characteristics to *Lactobacillus*. However, some characteristics of these isolates did not match to the characteristics of *Lactobacillus*, such as unable to grow at both 10ºC and 45ºC, unable to grow at 6.5% of NaCl, and able to grow at pH 9.6. Similar result was found by Abbas & Mahasneh (2014) that all *Lactobacillus* species isolated from camel's milk were able to grow well at 37ºC and some of them were unable to grow at 10ºC or 45ºC. It was also found that isolate M1 (*Lactobacillus fermentum*) and isolate M33 (*Lactobacillus brevis*) were unable to grow at 6.5% of NaCl, while some *Lactobacillus* species were found to be able to grow at pH 9.6 and 10% of NaCl. Vasiee *et al*., (2014) also reported that some *Lactobacillus* species isolated from Tarkhineh (traditional Iranian fermented cereal-based product) were unable to grow at 45ºC and 6.5% of NaCl, while some *Lactobacillus* species were able to grow at pH 9.6.

4.3. Screening Probiotic Potentials

Probiotics are known to inhibit the growth of wide range of intestinal pathogens in human. Therefore, probiotics should have the ability to resist the digestion process. It is stated by Salminen *et al*., (1996) in Savadogo *et al*., (2006) that LAB are considered as probiotic due to their resistance to acid and bile, as well as antimicrobial activity against pathogen. In this study, LAB isolated from Ampel Bamboo Shoots Pickle that have been identified their genus according to morphology characteristics, motility, and physiological characteristics were evaluated for their probiotic potentials, *i.e.* acid tolerance and bile salts tolerance. Most probiotics belong to the LAB group such as *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* (Klein *et al*., 1998 in Jamaly *et al*., 2011). Pundir *et al*., (2013) also reported that LAB group including *Lactobacillus* and *Bifidobacterium* are found as predominant members of the intestinal flora and are commonly studied probiotics. In this study, the evaluation of probiotic potentials of 32

isolates was carried out by subjecting the isolates to certain pH values and by exposure to bile salts.

In acid tolerance assay, 2 pH values of the growth medium were used, pH 3 and pH 7, taking into account that the pH of stomach generally ranges from pH 2.5 to 3.5 (Holzapfel *et al*., 1998 in Boke *et al*., 2010). Moreover, probiotics should have the ability to survive in different parts of gastrointestinal tract (GIT), such as stomach (pH values lower than 2), small intestine where the pH is varying from slightly acid (pH 4.0 to 7.0 in jejunum/proximal part) to alkaline one (pH 7.8 to 9.0 in ileum/distal part), and large intestine where the pH is slightly acid (pH 4.0 to 6.0) (Dedlovskya, 1968 in Pelinescu *et al*., 2011). The typical transit time of food in the stomach is between 20 minutes to 3 hours (Goldin *et al*., 1992; Hyronimus *et al*., 2000 in Grosu-Tudor & Zamfir, 2012) and therefore the growth of LAB cultures on agar plates was observed at 0, 1.5, and 3 hours. Based on Table 3, it could be known that 22 isolates that have been identified as *Lactobacillus* and 5 isolates that could not be identified were able to tolerate 3 hours of exposure with pH 3. Therefore, 27 isolates were categorized as acid tolerance bacteria.

Apart from acid tolerance, bile salts tolerance is also considered as important factor affecting LAB viability. According to Tambekar & Bhutada (2010) in Pundir *et al*., (2013) the ability to tolerate bile salts will help LAB to reach the small intestine and colon as well as contribute in balancing the intestinal microflora. Bile, as a result from digestive secretion, plays an important role in lipids emulsification and has ability to affect the phospholipids, cell membranes proteins, and disrupt cellular homeostasis (Burns *et al*., 2008 in Jamaly *et al*., 2011). In bile salts tolerance assay, 2 concentrations of bile salts were used, 0.3% and 0.5%, given that the relevant physiological concentrations of human bile salts range from 0.3 to 0.5% (Zavaglia *et al*., 1998; Dunne *et al*., 2001 in Jamaly *et al*., 2011) and the staying time is suggested to be 4 hours (Prasad *et al*., 1998 in Yavuzdurmaz, 2007). Moreover, Goldin & Gorbach (1992) in Boke *et al*., (2010) reported that the suitable concentration of bile salts used in the selection of probiotic bacteria for human applications is 0.15-0.3%. Based on Table 3, it could be known that 9 isolates that have been identified as *Lactobacillus* and 1 isolate

that could not be identified were able to tolerate 4 hours of exposure with 0.3% and 0.5% of bile salts. Therefore, 10 isolates were categorized as high bile salts tolerance bacteria. Moreover, 12 isolates that have been identified as *Lactobacillus* and 4 isolates that could not be identified could survive only at 0.3% of bile salts, hence were categorized as bile salts tolerance bacteria. Result also showed that 23 isolates were considered as probiotics due to their resistance to pH 3 and 0.3% of bile salts.

4.4. Determination of Antimicrobial Activity

Antimicrobial activity against pathogen is also considered as important factor in probiotic selection (Salminen *et al*., 1996 in Savadogo *et al*., 2006). The antimicrobial activity of 32 LAB isolates were determined using agar well difussion method and the clear zone formed around the wells were measured. In this study, 3 important pathogenic bacteria were used, *i.e. E. coli* (FNCC 0091), *L. monocytogenes* (FNCC 0156), and *S. aureus* (FNCC 0047). Based on Table 4, it could be seen that all isolates had inhibitory effect against pathogenic bacteria since there was clear zone formed around the wells. Although each isolate had different inhibitory effect depending on species. These inhibitory effects are associated with the production of several metabolites by LAB such as organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde, low molecular mass antimicrobial substances, and bacteriocins (Vanderbergh, 1993; Brkic *et al*., 1995 in Suskovic *et al*., 2010).

LAB isolates had relatively broader inhibitory activity against *L. monocytogenes* (FNCC 0156) and *S. aureus* (FNCC 0047) than those against *E. coli* (FNCC 0091). This may be due to structural differences in their cell walls. *L. monocytogenes* (FNCC 0156) and *S. aureus* (FNCC 0047) are classified as Gram-positive, while *E. coli* (FNCC 0091) is classified as Gram-negative. The cell walls of Gram-negative bacteria consist of a thin layer of peptidoglycan and an outer membrane which consists of lipopolysaccharides (LPS), lipoproteins, and phospholipids. It provides a barrier to certain substances including antibiotics (Tortora *et al*., 2013). These explain why antimicrobial substances tend to be more effective against Gram-positive bacteria.

4.5. Determination of Bacteriocin Activity

Bacteriocin activity assay was carried out to analyze the antimicrobial potential of the crude bacteriocin to inhibit some pathogenic bacteria. Crude bacteriocin was obtained by adjusting the pH of cell-free supernatant to 6.0. Adjusting the cell-free supernatant to pH 6.0 was aimed to exclude the inhibitory effect of organic acids, especially lactic and acetic acids (Alemu *et al*., 2003; Todorov & Dicks, 2005[b]). Nilsen *et al*., (1998) in Noordiana *et al*., (2013) also reported that high bacteriocin activity was recorded in the range of pH values from 5.8 to 6.5. Neutralized cell-free supernatant for each sample were inoculated in agar wells that had been inoculated with pathogenic bacteria (*E. coli* (FNCC 0091), *L. monocytogenes* (FNCC 0156), and *S. aureus* (FNCC 0047)). Bacteriocin inhibitory activity was indicated by the formation of clear zone around the wells.

Result (Table 5) showed that only 9 of 32 isolates could produce neutralized cell-free supernatant with antimicrobial activity against pathogenic bacteria. Supplemented whey medium was able to support bacteriocin production on some LAB isolates (A8, A10, A17, B3, B11, and B15), which had no bacteriocin inhibitory activity when cultured in MRS-B without supplementation. However, there were also some isolates (A12, A13, A16) that could only produce bacteriocin when cultured in MRS-B without supplementation. This may be due to lack of some essential nutrients in the whey medium for bacteriocin production (Guerra *et al*., 2001 in Khay *et al*., 2012). However, result (Table 5) indicated that supplementation used in this study was ineffective to enhance the bacteriocin production of some isolates on whey medium.

Based on Table 5, it could be known that the optimal bacteriocin inhibitory activity was found in isolate A8 (produced by *Lactobacillus pentosus*) which showed inhibitory activity against all pathogenic bacteria. Isolate A8 produced bacteriocin if only induced by carbon. In this study, glucose was used as supplementary carbon source to stimulate the bacteriocin production on whey medium. Previous studies have been reported that the presence of glucose in the medium accelerates the bacteriocin production (Mulders *et al*., 1991; Matsusaki *et al*., 1996; Kanmani *et al*., 2011 in Shayesteh *et al*., 2014),

while the absence of glucose limits the bacteriocin production (Russell & Mantovani, 2002 in Shayesteh *et al*., 2014). In addition, Eddleman (1999) also reported that glucose is the ideal carbon source for many bacteria including LAB. Most bacteria can grow well when glucose is provided as the primary energy source because they may not be able to digest other carbohydrates. Similar results were reported in Todorov & Dicks (2005[b]) for production of sakacin P (Aasen *et al*., 2000), enterocin 1146 (Parente *et al*., 1997), plantaricin UG1 (Enan *et al*., 1996), plantaricin 149 (Kato *et al*., 1994), plantaricin KW30 (Kelly *et al*., 1996), and plantaricin ST 31 (Todorov *et al*., 2000).

Bacteriocin production of some isolates could only be stimulated by combination of carbon and nitrogen supplementation. Some isolates could be stimulated by tryptone (A10, A17, B11, B15) or combination of tryptone and yeast extract (B3). Similar result has been examined by Todorov & Dicks (2006) for *Lb. plantarum* strain ST23LD and ST341LD isolated from spoiled olive brine. Todorov & Dicks (2006) found that optimal bacteriocin ST341LD production were obtained in the presence of tryptone as sole nitrogen source, while optimal bacteriocin ST23LD production were obtained in the presence of combination of tryptone and yeast extract. According to Shayesteh *et al*., (2014) tryptone is the most effective nitrogen source because it contains high amount of free amino acids and short peptides that accelerate bacteriocin production. In addition, tryptone is usually selected as bacterial culture media due to its availability of nitrogen, energy, and carbon (Eddleman, 1999).

The presence of yeast extract as sole nitrogen supplementation did not yield any bacteriocin inhibitory activity on all isolates. Similarly, Todorov & Dicks (2006) has been reported that the presence of yeast extract as the only nitrogen source resulted low level of bacteriocin ST23LD production and reduction of bacteriocin ST341LD production from *Lb. plantarum*. In contrast, previous studies have been examined that yeast extract may take a part in deactivation an inhibitor of bacteriocin synthesis (Mahrous *et al*., 2013). Thus, there should be a positive correlation between the addition of yeast extract and the production of bacteriocin. Yeast extract is a rich source of amino acids, vitamins, and trace minerals (Sommer, 1998). Chae *et al*., (2001) also stated that yeast extract consists primarily of amino acids, peptides, nucleotides, and

other soluble components of yeast cells. Moreover, yeast extract is mostly used by microorganisms which come from plant and animal origin (Eddleman, 1999). Varying results can occur due to various environmental factors, such as temperature, pH, and nutrient sources which influence bacteriocin production (Nilsen *et al*., 1998; Qiuju *et al*., 2010 in Anacarso *et al*., 2014). According to Todorov & Dicks (2006), specific nutrients are required for bacteriocin production. Based on the results, it could be known that different type of supplementation had different effect on each isolate.

Many bacteriocins only inhibited Gram-positive pathogenic bacteria, while some bacteriocins were able to inhibit both of Gram-positive and Gram-negative pathogenic bacteria. Generally, bacteriocins have narrow killing spectrum which inhibit only closely related strains (Cleveland *et al*., 2001). Furthermore, it has been reported that Gram-negative bacteria were mostly resistant to bacteriocins of LAB (Zhou *et al*., 2014). According to Smaoui *et al*., (2010) only few bacteriocins have been described to possess antagonist activities against Gram-negative bacteria, *e.g.* plantaricin 35d produced by *Lb. plantarum* (Messi *et al*., 2001), bacteriocin ST34BR produced by *Lb. lactis* subsp. *lactis* (Todorov & Dicks, 2004), bacteriocin ST26MS and ST28MS produced by *Lb. plantarum* (Todorov & Dicks, 2005), and bacteriocin AMA-K produced by *Lb. plantarum* AMA-K (Todorov *et al*., 2007). Smaoui *et al*., (2010) found that bacteriocin BacTN635 produced by *Lb. plantarum* strain TN635 was able to inhibit Gram-negative bacteria. Zhou *et al*., (2014) also found that bacteriocin C8 isolated from Chinese pickle showed high inhibitory activity against not only Gram-positive but also Gram-negative bacteria.

Inhibition mechanism of antimicrobial compounds is done by disrupting the bacterial cell wall. The resistivity of Gram-negative bacteria towards bacteriocin is explained by the presence of a protective outer membrane that consists of lipopolysaccharides (LPS) with diglucosamine molecules, proteins, and phospholipids (Gyawali & Ibrahim, 2014; Nikdorff *et al*., 1978 in Pattanayaiying *et al*., 2014). However, some exceptions were found, bacteriocin A8 had a broader inhibitory activity against *E. coli* (FNCC 0091) than *L. monocytogenes* (FNCC 0156) and *S. aureus* (FNCC 0047), while bacteriocin A16 only inhibited *E. coli* (FNCC 0091). These exceptions could occur due to the

differences in the growth conditions of pathogenic bacteria and the diffusion of bacteriocin on agar media. In this case, *E. coli* (FNCC 0091) had a longer lag phase than *L. monocytogenes* (FNCC 0156) and *S. aureus* (FNCC 0047). During 3 hours of incubation at 4ºC, bacteriocins diffused on agar media while pathogenic bacteria were inactivated. According to Augustin & Carlier (2000), the pre-incubation conditions such as temperature can influence the duration of lag phase. Hudson (1993) in Augustin & Carlier (2000) observed that the lag phase duration of *Aeromonas hydrophila* was the shortest when the pre-incubation temperature equals to the incubation temperature.

Fermentation of Ampel Bamboo Shoots in condition B (5% of salt concentration at 30ºC for 4 days) yielded less bacteriocin-producing LAB strains compared to fermentation in condition A (2.5% of salt concentration at 15ºC for 5 days). Based on the previous studies, it has been found that growth at low temperature resulted in low specific growth rates and high specific bacteriocin productivities because slow growth causes more energy available for bacteriocin production. Bacteriocin production was stimulated by less favorable growth conditions, such as low temperature, low specific growth rate, and the presence of potentially toxic compounds (sodium chloride, ethanol, oxygen), as well as the presence of competing microflora (De Vuyst *et al*., 1996). Results (Table 5 – Table 6) also showed that same LAB species from different fermentation conditions had different bacteriocin inhibitory activity, such as bacteriocin A17 and B3 which were produced by *Lactobacillus fermentum 1*. This may be because of the initial fermentation conditions (salt concentration and temperature) which affected the characteristics of LAB produced (Fleming *et al*., 1985), particularly in the potential to produce bacteriocin.

Bacteriocin inhibitory activity was not found on some isolates. Moreover, only low levels of bacteriocin inhibitory activity were recorded on some isolates. This may occur because each isolate has different optimal temperature and time for bacteriocin production. It has been reported by Mahrous *et al*., (2013) that *Lactobacillus fermentum* M1 and *Lactobacillus pentosus* CH2 showed highest bacteriocin inhibitory activity when incubated at 30ºC. Similar finding has been found by Asha & Gayathri (2012) that the optimal bacteriocin production of *Lactobacillus fermentum* was obtained when

incubated at 30ºC for 18 hours. Incubation time was associated with the growth phase of bacteria. Bacteriocin production started in the log phase through early stationary phase (Hoover & Steenson, 1993). In this case, some isolates tested had entered the stationary phase before 24 hours which led to the decrease of bacteriocin production. Therefore, it could be known that the incubation temperature and time used in this study (37ºC for 24 hours) may not be optimal for bacteriocin production of some isolates.

Each species has different bacteriocin types. According to Riley (2011), kinds of bacteriocin will differ for different species. Within a species, ten or even hundred different kinds of bacteriocin are produced depending on environmental pressures. Therefore, the same incubation temperature and time may result different response for each isolate. Apart from incubation temperature and time, pH values also affect bacteriocin production. It has been reported by Van Reenen *et al*., (1998) in Zhou *et al*., (2014) that the bacteriocin inhibitory activity had a narrow pH range and if the pH values were above 6.0, all inhibitory activity would lost. Moreover, each bacteriocin has different pH range to inhibit pathogenic bacteria. The differences in bacteriocin inhibitory activity could be influenced by the characteristics of bacteriocin itself (Ennahar *et al*., 1999). Todorov *et al*., (2000) in Todorov *et al*., (2012) also reported that higher bacteriocin levels are often produced at lower temperature and pH than those required for growth. The optimal condition for growth was different from the optimal condition for bacteriocin production (Juarez Tomas *et al*., 2002).