CHAPTER III

SENSORY CHARACTERISTICS AND BACTERIAL IDENTIFICATION OF FERMENTED CHILI MASH USING LOCAL MALAYSIAN LACTIC ACID BACTERIAL ISOLATES

3.1 INTRODUCTION

Chilies are agricultural crop belonging to genus *Capsicum* with many species such as *Capsicum annum*, *C. baccatum* and *C. frustescens*. Chilies have been used as condiments in foods since ancient time and have become one of great important crops in Malaysian industry. In recent years, chili is processed as chili powder, chili puree, chili sauce, hot sauce, pasta sauces (Hall & Skaggs, 2003; Hector, 2011) as well as salsa. Furthermore, chili is also used flavoring ingredient in a variety of food preparations.

Product acceptability of chili is based on several characteristics such as the color, viscosity, spiciness, sweetness, sourness and product taste (Kulvadee, 2002). Since chili is hot in taste, it would be difficult to be assessed by sensory taste as its active ingredient capsaicin causes irritation in the mouth a burning fiery sensation that the body perceives as pain. Therefore, assessing of specific attributes in chili products such as chili sauce would be challenging as the specific product uniqueness is masked by the hot and spicy taste. Sniffing is one of the popular methods applied in sensory test due tosense of smell and the first stage of tasting. For example, sniffing in beverages industry such as of coffee provide great deal of information to chemical and physical instrumental analysis (Igor et al., 2012). No instrument has been able to replace a trained tester even though the physiochemical properties fit with laboratory

specification but it could be meaningless if the flavor is not acceptable to the consumers. Due to this reason, sniffing by trained panelist is widely used to assist in quality control (Scott, 1997).

Odor of fermented chili can be determined as volatile components (gases) emanating from the fermented mash stimulate the nerve endings in the nasal cavity at the olfactory bulb region (Hervin, 2008). Humans can distinguish between thousands of odor, which are perceived by neurons in the olfactory epithelium (skin cells) of the upper respiratory passages.The sense of odor or olfaction is even more complex than that of taste. Whether or not a volatile compound produces a stimulus depends on the size, shape, and degree of ionization of the molecule. For example, dry sausages that were evaluated by sniffing method able to detect different trend of odor profile depending on LAB strain inoculated (Mina et al., 2014).

Indeed, LABs are among the most important microorganisms typically associated with fermented food. This bacterium is widely used in fermentation industry as it recognized as beneficial microbes. Manipulating the potential usage of LAB in chilies sauce industry might be beneficial as this bacterium is known as flavor producer microorganisms (Andreja et al., 2012; Di Cagno et al., 2008b). It is well known that, LAB isolated from natural sources has always been the most powerful means for industrially fermented product such as sauerkraut, kimchi, table olives, pickles product and mixed vegetable drinks (Carmen et al., 2012; Sureeet al., 2012). Two types of starter culture that were commonly used were autochthonous and allochthonous. Many researchers focus on developing of autochthonous starter culture as they proposed that autochthonous isolate has better adaptability to fermentation system. An example of

autochthonous starter culture they able to ferment chili under room temperature at 30°Cwas successfully developed by Raffaella et al. (2008).

In many studies, LABs were found to produce nice fermented flavor in yoghurt, cheese and sour cream products (Carmen et al., 2012; Smitt et al., 2005; Vinderola et al., 2002). For example in wine fermentation, the accepted odor that most preferred by consumers were sour, sweet, perfume-like, and fruity odor while non-accepted odor were yeasty, moldy, stinky and flat smell (Mariana et al., 2014; Loureiro & Malfeito-Ferreira, 2003). The idea of producing a fermented chili with unique odor characteristics may be achieved by utilizing the allochthonous and/or the autochthonous starter that may modify the flavor and odor attributes of chilies mash. This would later produce thefermented chili sauce with unique fermented odor characteristics. It is therefore purposeful to initiate fermentation of chili mash via introduction of starter culture either autochthonous and allochthonous source. Thus, the aim of this study was to evaluate the effect of pre-pasteurization on Cilibangi substrate as fermented chili mash as well as choosing potential LAB isolates from allochthonous (milk, yoghurt and raw cow milk) and autochthonous starter (pre-fermented chilies) that would generate desirable odor that mostly preferred by panelists.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of Lactic Acid Bacteria from Food Sources

The LAB from autochthonous and allochthonous sources were used. The autochthonous LAB was from two months pre-fermented Cilibangi while allochthonous LAB were isolated from home-made yoghurt, raw cow milk and commercial yoghurt. The food samples (10 g) were diluted in 90 mL phosphate buffer saline (PBS) solution (Oxoid). Appropriate serial dilution was made until 10^{-5} with PBS solution and 0.1 mL was spread plated on modified MRS agar (Oxoid) added with 0.1% (w/v) calcium carbonate, and incubated anaerobically at 37°C for 48 h. The isolates that developed clear zone on the MRS modified agar plate were presumptive LAB and were further checked with Gram staining and catalase test. Six selected LAB strains were purified, subculture in MRS broth with 10% (v/v) glycerol and kept frozen -80°C (Asma et al., 2012).

3.2.2 Preparation of Lacto-Fermented Chili Mash

Cilibangi fruits were obtained from Tanjong Karang, Selangor and selected to remove their pericarps, free from blemishes, defects, and insect damage. The chilies were washed with tap water to remove any impurities and were ground using a food blender (Panasonic MX337) with 6% (w/w) rock salt added. The mashed chili samples were either pre-pasteurized in steam at 80°C, 10 min or non-pasteurized. The mashed chili samples (20 g) wereweighed into 30 mL universal bottlesinoculated with 1% (v/w) of

24 h LAB cultures and was incubated at 35°C for 14 days. At pre-determined time intervals (0, 3, and 14 days) the samples were evaluated for changes in pH and sensory characteristic. Non-inoculated pasteurized chili mash was served as control sample.

3.2.3 pH Measurement

Samples (10 g) of fermented chili mash was mixed with 10 mL distilled water and mixed well before measured with pH-meter (Metler Toledo, Germany). The pH meter was calibrated against standard buffer at pH 4.0 and 7.0 (Merck).

3.2.4 Sensory Analysis

3.2.4.1 Quantitative Descriptive Analysis

A sensory evaluation was performed after 14 days of fermentation (APPENDIX A). A total of 10 untrained panelists were selected form USIM students who have been previously exposed to sensory evaluation of foods. A preparatory session was held prior to testing, so that each panelist could thoroughly understand attribute of fermented chili mash to be evaluated. Sample of (10 g) fermented chili mash was served in closed capped bottle, numerically identified and blind coded. Panelists were required to sniff the sample and rate the intensity of odor characteristic using 5-scale QDA (raw chili odor, sour, sweet, perfume, yeasty, fungi, stinky, fruity and flat). To reduce fatigue, coffee solution was served to cleanse between samples. Each sample was evaluated two times (Wiander & Korhonen, 2011).

3.2.4.2 Hedonic Test

The odor of fermented chili mash was evaluated after 14 days of fermentation by 10 untrained panelists from USIM students (APPENDIX B). This test was conducted parallel with QDA test. The samples were sniffed and rated according to degree of acceptability on a 9 point hedonic scale. Score 9 represent highly acceptable while 1 for highly not acceptable (Virat et al., 2014). Score of hedonic test obtained were then categorized as good when score in the range of 6 to 10, acceptable at 5 and worst in the range of 1 to 4 (Wiander & Korhonen, 2011).

3.2.5 Identification of LAB by API 50 CHL

Lactobacilli isolates grown in 15 mL MRS broth (Oxoid) incubated at 30°C for 24 h were centrifuged at 9800 rpm at 5°C (Eppendorf mini spin, USA) for 10 min. The pellets were washed with PBS then suspended in API 50 CHL medium (API system, BioMérieux, France), then vortex (Mixer Uxusio, Japan). Using sterile pipette, cell suspension was aseptically transferred into each of the 50 wells of the API 50 CH strips. All wells were overlaid with sterile paraffin oil to affect anaerobiosis.

The strips were placed in plastic trays with a little water to maintain humidity and covered as recommended by the manufacturer. The trays were incubated at 30°C for 48 h. Changes in color from violet to yellow were evaluated after 24 h incubation and verified after 48 h. Fermentation of carbohydrates in the carbohydrate medium was indicated by yellow color except for esculin (dark brown); esculin hydrolysis is

indicated by a change to darker or black color. The first strip served as the control well.

Colour reactions were scored as positive or negative in a chart provided by the manufacturer. The results were tabulated and analyzed with API WEB (BioMerieux). This computer program is commercially available and discriminates between species on the basis of a pattern matching principle, but it does not take the basic morphological and physiological issues into account (Asma et al., 2012).

3.2.6 Genotype Identification of Lactic Acid Bacteria

The identities of the LAB isolates were further confirmed using 16S rDNAsequence analysis. Selected LAB was cultured in MRS broth, incubated at 30°C for 24 h, anaerobically. The culture (1 mL) was centrifuged 13,400 rpm (Eppendorf centrifuge 5804 R) 25°C for 2 min, followed by three times washing with PBS buffer.Total genomic DNAs of selected LAB were extracted using BRealGenomics™ Gram-positive DNA purification kit (USA). Cold lysozyme buffer (200 µl) was added and incubated for 10 min at room temperature 25°C. After that 200 µl of gel DNA binding (GB) buffer was added and incubated until clear lysate was obtained.

Ethanol (200 µl) was added to each sample lysate and mixed immediately by gently vortexed for 10 second to concentrate DNA. The GD column was placed on a 2 mL collection tube. All the mixture from the previous step was poured to the GD column, and then centrifuged at 14000 x *g* for 2 min. The flow-through was discarded and the GD column was placed in a new 2 mL collection tube.

Washing steps was done using wash buffer (W1) with (200 µl) added with absolute ethanol poured into the GD column, and then centrifuged at 14000 x *g* for 30 s. The flow-through was discarded and the GD column was placed back in the 2 mL collection tube. This procedure was repeated using W1 at 600 µl. Column was placed back in the 2 mL collection tube, centrifuged at 14000 x *g* for 3 min until the GD column dried.

The dried GD column was transferred into a clean 1.5 mL micro-centrifuge tube, followed by addition of 100 µl of the preheated elution buffer and allowed to stand for 3 to 5 min until elution buffer was absorbed by the matrix. The tubes were centrifuged at 14000 rpm for 30 sec and the eluted purified DNA was collected. The DNA was quantified for its purity and concentration using spectrophotometer Δ260/280 and Δ260/230 nm (Eppendorf, BioPhotometer).

3.2.6.1 PCR Amplification of LAB

One set of oligonucleotide primers specifically for LAB genome was used for PCR amplification. Primers were synthesized at 1st Base, Malaysia. The primers were16S forward: (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3) at 1400 bp. (Magnusson et al., 2003).

Purified DNA of each sample was processed to the PCR amplification using YEAtaq DNA Polymerase (Yeastren Biotech CO., LTD. Taiwan), using 5 µl of 1X reaction buffer ($MgCl_2$) (Yeastren Biotech CO., LTD. Taiwan), 1 µl of 0.2 m MdNTPs mix (Yeastren Biotech CO., LTD. Taiwan), 1 µl of 10 µM 16S forward and 1 µl of 10 µl

16S reverse primers, 1 µl of template DNA, and 0.5 µl of (1.25 U/ µl) YEAtaq DNA polymerase (Yeastren Biotech CO., LTD. Taiwan). Negative control without DNA template was included in parallel. Each reaction sample was added up to 50 µl with ddH_2O.

The PCR (Eppendorf Mastercycler Gradient, USA) protocol were set as follows: Initial at 95°C for 2 min, denaturation at 92°C for 45 sec, annealing at 54°C for 1min and extension at 72°C for 1 min, with 35 cycles for each steps.Final elongation was set at 73°C for 5 min, and then was held at 4°C until further analysis.

From each amplification mixture 10 µl were mixed with 2 µl of 6X DNA loading dye (Fermentans), and then subjected to electrophoresis in 1.0% (w/v) agarose gels (Conda S.A, Spain) in 1x TAE buffer (Bio Basic Canada INC, Canada) for 40 min and 80 V. DNA ladder (6 µl of 1Kb DNA ladder) of 250 to 10000 bp obtained from 1st Base (Malaysia) was used as standard marker. After electrophoresis step the gel was stained in ethidium bromide (Merck, Germany) for 30 min and then washed with dH_2O for another 30 min. The gel was visualized and photographed with UV transilluminator (BIORAD).

3.2.6.2 PCR Product Purification

The PCR products were purified using PCR clean-up kit from (Yeastern Biotech, Taiwan). The PCR product (40 µl) was added into microcentrifuge tube with 200 µl of DF buffer and mixed by vortexing the sample. Next, the sample was applied to DF column with collection tube, and then centrifuged at 6000 x *g* for 30 sec. The flow-

through was discarded and the DF column was placed back to the collection tube. The centrifugation was repeated at 14000 x *g* for 2 min.

The dried DF column was transferred to a new microcentrifuge tube. Elution buffer (15µl) was added into the center of the DF column matrix, allowed to stand for 2 min until the elution buffer was absorbed by the matrix. Purified DNA was eluted by centrifuging at 14000 x *g* for 2 min and was run on agarose gel.

The agarose gel slice containing relevant DNA fragment was excised, then transferred into a new microcentrifuge tube. DF buffer (500 µl) was added to the sample and mixed by vortexing, then incubated at 55°C for 10 to 15 min until the gel slice has been completely dissolved and invert the tubes every 2 to 3 min during incubation.Sample mixture (800 µl) was applied in DF column placed a in a collection tube then was centrifuged at 6000 x *g* for 30 sec. The flow-through was discarded and the DF column was placed back in the collection tube.

A (500 µl) wash buffer (ethanol) was added into the DF column and centrifuged at 6000 x *g* for 30 sec. The flow-through was discarded and the DF column was placed back in the collection tube. The tubes were dried by repeated centrifugation at 14000 x *g* for 2 min.The dried DF column was transferred to a new microcentrifuge tube. Elution buffer (800 µl) was added into the centre of the DF column matrix and allowed to stand for 2 min until the elution buffer absorbed by matrix. Purified DNA was eluted at 14000 x *g* for 2 min.

3.2.6.3 PCR Product Sequencing

The partial 16S-S DNA and 16S rRNA sequencing of PCR product of all the isolates were determined by 1st Base, Malaysia and sequences were analyzed by the BLAST program on the NCBI website databases (http://www.ncbi.nlm.nih.gov/) (Gen- Bank).

3.2.7 Microbial Analysis of During Spontaneous Chili Mash Fermentation

3.2.7.1 Preparation of LAB Inoculated Chili Mash

Cilibangi fruites were obtained from Tanjung Karang, then selected free from blemishes, defects and insect damages. The pericarps were removed and the chilies were washed with tap water to remove any impurities, then ground using a food blender (Panasonic), packed 100g in Scott bottles with 6% rock salt added.Samples were subjected to fermentation at 30°C for 28 day. Samples were evaluated for changes in pH, titratable acidity and microbial count at 7, 14, 21 and 28 days interval.

3.2.7.2 Titratable Acidity Measurement

The total amount of acid present during the 28 days fermentation was evaluated at weekly intervals. Two g of sample was added with 8 mL sterile distilled water. Three drops of phenolphthalein indicator were added and the mixture was thoroughly mixed. The mixture was then titrated against 0.1 N NaOH (Merck) to a pink color last long for 30 second then marked the end point. Volume of NaOH was noted. Total titratable acidity and then expressed as lactic acid

$$\text{Lactic acid (g/l)} = \frac{\text{N x V x ME of lactic acid x 1000}}{\text{Weight of sample (g)}}$$

Where,

N = the normality of the sodium hydroxide

V = sodium hydroxide in ml used to reach the titration point

ME= miliequivalent of lactic acid

$$= \frac{\text{Molecular weight of lactic acid}}{1000}$$

= 0.09008 mg/dL

3.2.7.3 Microbial Count

Sample 10 g of fermented chili mash was aseptically transferred into 90 mL of sterile 0.1% PBS solution and vortexed. For each sample, appropriate serial decimal dilutions were prepared in the PBS solution. and 0.1 mL of the appropriate decimal dilution was spread plated onto suitable agar media. Total plate count (TPC) of all bacteria was determined aerobically using nutrient agar (NA, Oxoid) and total LAB count was determined anaerobically using De Man Rogossa Agar (MRS, Oxoid) incubated at 30°C for 3 days. Total number of mold and yeast count was determined using Potato Dextrose Agar (PDA, Oxoid) incubated aerobically at 30°C for 5 days. All plates were examined for typical colony and morphological characteristic of the microbial isolates.

3.2.8 Statistical Analysis

All the analysis was carried out in triplicate. Data was analyzed statistically to find out standard deviation and significance. Data was subjected to one-way ANOVA and the

mean degree of treatments was achieved by Tukey's multiple range test at $p<0.05$ using (Minitab 16, Statistical Software, USA).

3.3 RESULTS

3.3.1 Isolation of Lactic Acid Bacteria from Different Food Sources

Six LAB's were isolated from two months pre-fermented chili and yoghurt sources. The isolates AU1, AU2 and AU3 were identified as the autochothonous starter and AlO1, AlO2 and AlO3 were identified as allochothonous. All the isolates produced clear zone in 0.1% modified MRS-calcium carbonate agar. The shapes were short-rod and identified to have positive to gram reaction and negative to catalase reaction (Table 4).

TABLE 4: Characteristics of LAB Isolates

Symbol	Sources	Calcium Carbonate Inhibition Zone	Shape	Gram Reaction	Catalase Reaction
AU1	Pre-fermented chili	+	Short-Rod	+	-
AU2	Pre-fermented chili	+	Short-Rod	+	-
AU3	Pre-fermented chili	+	Short-Rod	+	-
ALO1	Home-made yoghurt	+	Rod	+	-
ALO2	Cow Milk	+	Rod	+	-
ALO3	Commercial Yoghurt	+	Rod	+	-

3.3.2 pH Changes During Fermentation

The initial pH of chili was pH 5.3. The pH of both pre-pasteurized and non-pasteurizedfermented chili mash decreased significantly ($p<0.05$) during the first 3 days fermentation (Figure 2 & 3), and then level off until 14 days fermentation except to non-pasteurized control fermented chili mash (Figure 2).The non-pasteurized control fermented chili mash recorded the lowest pH (3.2). None of LAB inoculated fermented chili mash reached pH lower than 3.5 in non-pasteurized samples. Pre-pasteurized fermented chili mash inoculated with LAB AU2 and ALO2 reached the lower pH (3.3) after 7 days fermentation (Figure 3).

FIGURE 2: Changes in pH of Non-Pasteurized Fermented Chili Mash during 14 Days Fermentation

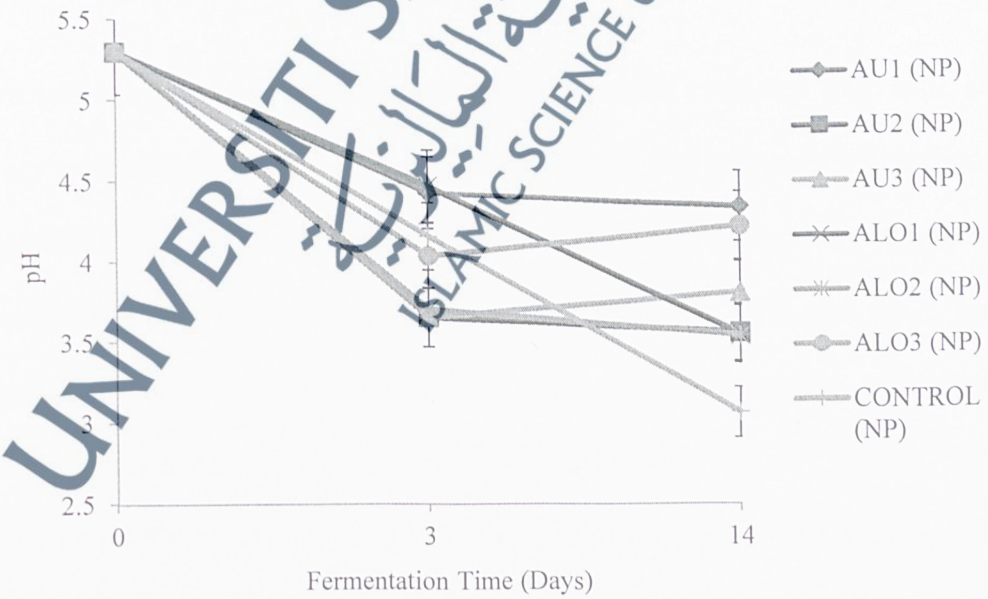
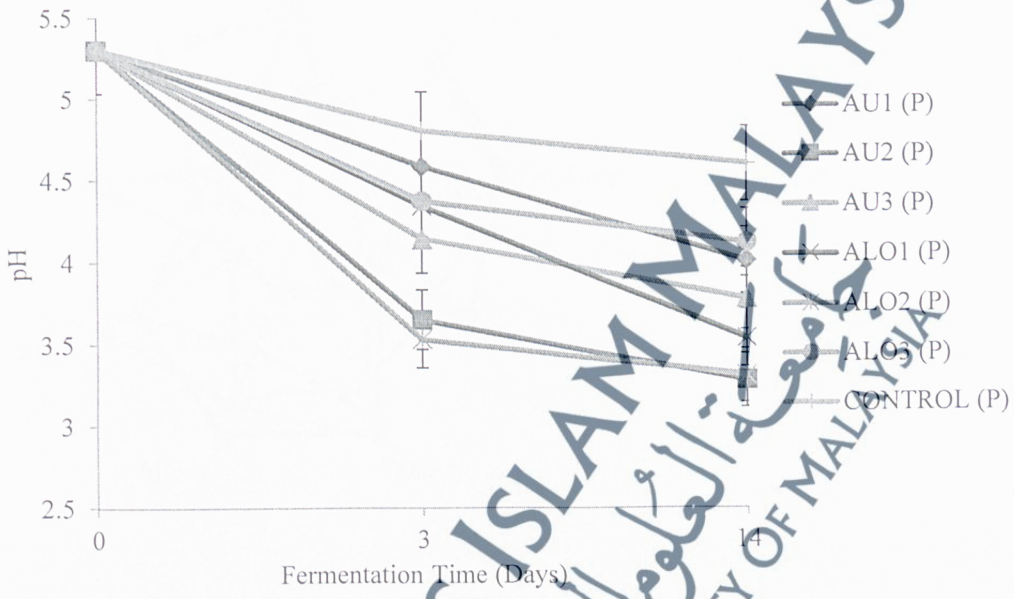


FIGURE 3: Changes in pH of Pre-Pasteurized Fermented Chili Mash during 14 Days Fermentation



3.3.3 Quantitative Descriptive Analysis

Characteristics of odor profiles of fermented chili mash evaluated were sour, sweetie, flowery as desirable, while yeasty, fungi-like, stinky, flat and raw chilies aroma were undesirable. Five-scale qualitative descriptive analysis (QDA) were conducted and presented as spider web diagram (Figure 4 & Figure 5). AlO1 and AlO2 produced almost similar odor trend ($p>0.05$) in both pasteurization treatments. ALO1 produced fermented chili mash with scores ranged 4.3 to 4.4 sour, 4.0 to 4.2 sweet, 1.7 to 1.8 flowery and 4.5 to 4.6 fruity odor. AlO2 produced fermented chili mash with highest perfume, sweet and fruity odor in pre-pasteurized chili (Figure 4).

FIGURE 4: Spider Web Plot of Odor Attribute of Non-Pasteurized Fermented Chili Mash

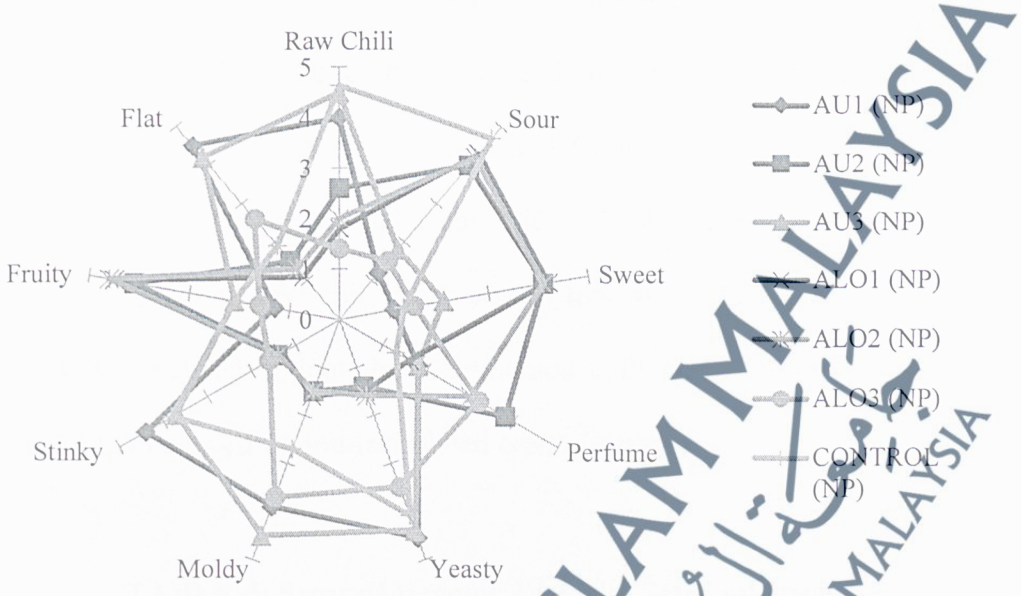
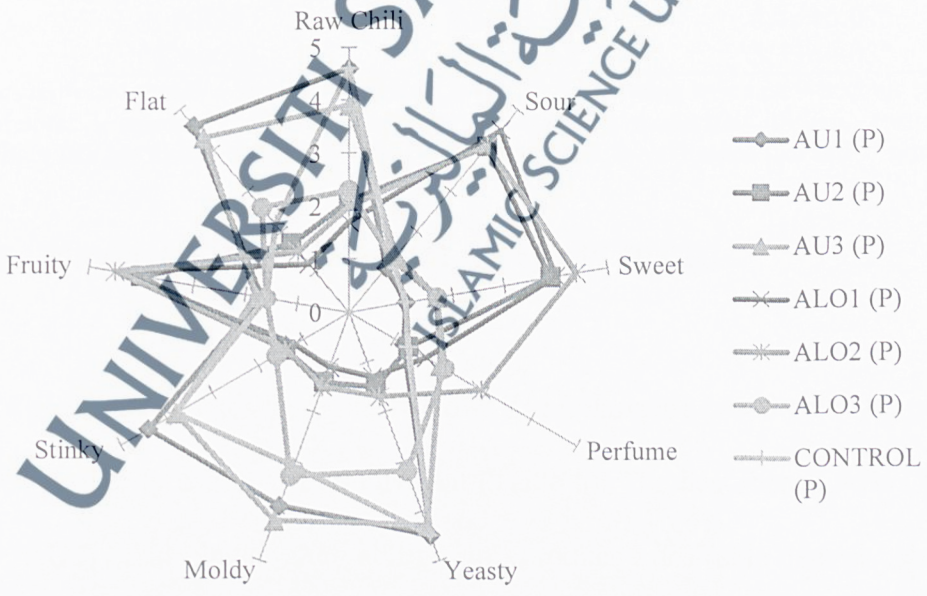


FIGURE 5: Spider Web plot of Odor Attribute of Pre-Pasteurized Fermented Chili Mash



3.3.4 Score of Hedonic Test

Three strains AU2, ALO1 and ALO2 were pre-identified to produce desirable fermented chili smell under both pasteurized and non-pasteurized treatment were concentrated odor of sourness, sweetness and fruitiness. This was supported by the mean hedonic score which ranged from 5.56 to 7.44 as presented in Table 5. Non-desired smell of fermented chili mash scored low in hedonic test with ranged from 3.22 to 4.56 consisted of inoculated fermented chili mash with LAB isolates AU1, AU3 and ALO3 as well as non-inoculated control sample.

TABLE 5: Score of Hedonic Test with Standard Deviation

Sample	Pasteurized	Non-pasteurized
AU1	4.22±5.12[a]	3.67±3.67[a]
AU2	5.56±8.16[a]	6.00±8.42[a]
AU3	4.67±4.75[a]	3.78±4.38[a]
AlO1	6.44±7.96[a]	5.44±5.57[a]
AlO2	5.78±8.21[a]	7.44±8.62[a]
AlO3	4.56±4.75[a]	4.33±5.07[a]
Control	3.22±2.68[a]	3.33±4.03[a]

Values represent means ± standard deviation; n=30; Hedonic rating based on 9-hedonic scales with the descriptors: 1=extremely dislike, 2=very much dislike, 3=moderately dislike, 4=slightly dislike, 5=neither like nor dislike, 6=slightly like, 7=moderately like, 8=very much like and 9=extremely like.

3.3.5 Phenotypic Identification of LAB by API 50 CHL.

The three LAB isolates were identified as *L. plantarum*1 based on sugar profiles with 99.9% similarity using API 50 CHL kit (Table 6). The best three LABs (AlO1, AlO2 and AU2) that have the ability to produce desirable smell characteristic. Phenotypically the isolates were pre-identified as *L. plantarum1* with T. index ranged between 0.69 to 0.73 by API CHL method. The main difference between the three

LABs were the ability to ferment α-Methyl-D- mannoside, α-Methyl-D-glucoside, cellobiose, melezitose, amidon, β-gentiobiose, D-lyxose and gluconate.

TABLE 6: Carbohydrates Fermentation by Lactic Acid Bacteria Isolates using API 50 CHL Identification System.

Carbon source	LAB isolates		
	LAB ALO1	LAB ALO2	LAB AU2
Control	-	-	-
(1) Glycerol	-	-	-
(2) Erythritol	-	-	-
(3) D-Arabinose	-	-	-
(4) L-Arabinose	+	+	+
(5) Ribose	+	+	+
(6) D-Xylose	-	-	-
(7) L-Xylose	-	-	-
(8) Adonitol	-	-	-
(9) β-Methyl-xyloside	-	-	-
(10) Galactose	+	+	+
(11) D-Glucose	+	+	+
(12) D-Fructose	+	+	+
(13) D-Mannose	+	+	+
(14) L-Sorbose	-	-	+
(15) Rhamnose	-	-	+
(16) Dulcitol	-	-	-
(17) Inositol	-	-	-
(18) Mannitol	+	+	+
(19) Sorbitol	+	+	+
(20)α-Methyl-D- mannoside	+	-	+

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(21) α-Methyl-D-Glucoside	-	-	+
(23) Amygdaline	+	+	+
(24) Arbutine	+	+	+
(25) Esculine	+	+	+
(26) Salicine	+	+	+
(27) Cellobiose	-	+	+
(28) Maltose	+	+	+
(29) Lactose	+	+	+
(30) Melibiose	+	+	+
(31) Saccharose	+	+	+
(32) Trehalose	+	+	+
(33) Inulin	+	+	+
(34) Melezitose	-	+	-
(35) D-Raffinose	+	+	+
(36) Amidon	+	-	+
(37) Glycogène	-	-	-
(38) Xylitol	-	-	-
(39) β-Gentiobiose	-	+	-
(40) D-Turanose	+	+	+
(41) D-Lyxose	-	-	+
(42) D-Tagatose	-	-	-
(43) D-Fucose	-	-	-
(44) L-Fucose	-	-	-
(45) D-Arabitol	-	-	-
(46) L-Arabitol	-	-	-
(47) Gluconate	-	+	+
(48) 2 Ceto-gluconate	-	-	-
(49) 5 Ceto-gluconate	-	-	-

3.3.6 Genotypic Identification of LAB

The phenotyphic identification was further confirmed utilizing genotypic identification. After PCR amplificationresulted to clear bands of all the isolates with approximate molecular weight 1400 bp. BLASTING analysis sequence in the NCBI confirmed that allochothonous isolates AlO1 (home-made yoghurt) was identified as *L. plantarum,* allochothonous ALO2 (raw milk) was identified as *L. pentosus,* autochthonous AU2 (pre-fermented chili mash) isolates was identified as *L. plantarum* (Figure 6). Partial sequences of the isolates are listed in APPENDIX C.

FIGURE 6: DNA bands of LAB on the 1.5% (w/v) agarose gel using primers 16S.S:(5 AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3). DNA ladder, ALO1 (Lane 1), ALO2 (Lane 2) and AU2 (Lane 3).

3.3.7 Changes in Microbial Profile, pH and Titratable Acidity of Spontaneous Fermentation

Generally, the TPC for aerobic bacteria, LAB, yeast and mold count of spontaneous fermented chili mash increased during 28 day fermentation (Figure 7). From the figure, LAB increased significantly ($p<0.05$) from $\log_{10}$ 2.01 to 6.10 after 7days fermentation and remained at $\log_{10}$ 7.47 during 28 days fermentation. TPC and mold and yeast count increased significantly ($p<0.05$) from $\log_{10}$ 4.19 to 6.06 after 14 days and became the dominant microorganism after 28 days. It was also observed that a slight decreased in pH of fermented chili mash from 4.56 to 4.5 was recorded during 7 days fermentation, remained the same until 21 days then decreased significantly ($p<0.05$) to pH 3.8 after 28 days fermentation (Figure 8). Titratable acidity as lactic acid (g/L) of fermented chili mash increased slightly from 0.3 to 0.5 after 7 days and remained at this concentration until 21days fermentation then increased significantly ($p<0.05$) to 0.9 after 28 days fermentation.

FIGURE 7: Microbial Changes in Spontaneous Fermentation

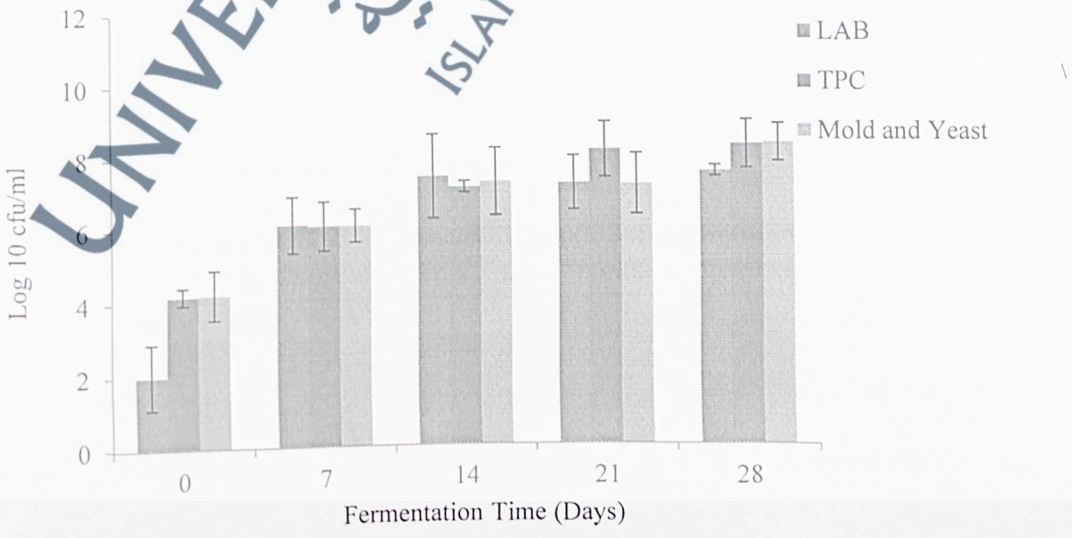
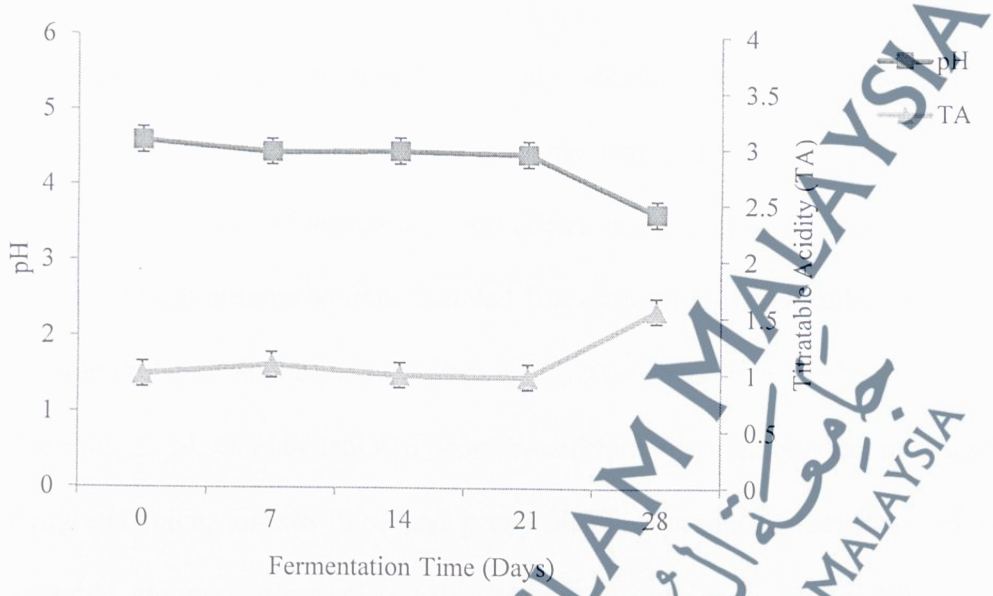


FIGURE 8: pH and Titratable Acidity of Spontaneous Fermentation.



3.4 DISCUSSION

Lactic acid bacteria is known as flavor producer microorganism. It has the ability to modify the flavor of any fermentable substrate such as cheese, sausage, fermented sauces, and fermented vegetables (Chao et al., 2008; Smitt et al., 2005). This study showed that three LAB isolates namely ALO1, ALO2 and AU2 produced acceptable flavor of fermented chili mash with hedonic score between 5.44 and 6.44, 5.78 and 7.44 and 5.56 and 6.99, respectively for both pasteurized and non-pasteurized condition. Indeed fermented chili mash with intense sourness, sweet, flower and fruity flavor was rated high in QDA test. However, fermented chili mash inoculated with AU1, AU3 and ALO3 were evaluated as yeasty, moldy, stinky and raw chili flavor and were rated low by the panelists (Figure 4 and 5; Table 5). The results showed flavor of fermented chili mash was affected by LAB used as similarly observed by

Andreja et al. (2012); Di Cagno et al. (2008) flavor of fermented foods are dependent on the type microbes used to initiate the fermentation.

Normally, researchers are interested to apply allochthonous starter to carry out fermentation. This is because allocthonous starter especially from dairy sources can modify the flavor profile of fermented food (Chen et al., 2006). *L. pentosus* that was isolated from autochthonous sources isolated from yogurt and cow milk improved the odor of fermented cheese and yoghurt (Pan et al., 2014; Valérieet al., 2003). Similarly, autochthonous *L. plantarum* isolated from sauerkraut then inoculated into cabbage juice, cougrette juice, tomato juice and pumpkin juice produced harmonic odor that are acceptable among the panelists after fermented within 48 h (Kohajdová et al., 2006). Interestingly to note that allochthonous *L. pentosus* ALO2 which has been isolated from raw milk sources has the ability to produce the acceptable odor of fermented chili mash.

It is known that LABs are flavor producer microorganisms and are responsible for the characteristic flavor of fermented products (Chen et al., 2006). *L. plantarum* and *L. brevis* are the main strain that produce lactic acid as indispensible constituent of particular flavor (Shi & Zou, 2011). Chao et al. (2008) observed that flavor development of stinky tofu brines was the combined interactions of 7 different genera that consist of 32 species of indigenous *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weisella.* Marshall (1987) pointed that bother types of LAB such *Leuconostocmesentroides* ssp. *cremoris* and *Leoconostoc lactis* were the aroma producer microorganism. Thus, LABs are beneficial microbes that contribute to food organoleptic attributes.

Indeed LAB inoculated fermented chili mash showed rapid decreased in pH after 3 days fermentation as observed in fermented chili mash inoculated with AU2, and ALO1 and ALO2 isolates (Figure 2 and 3). These LAB inoculated to pasteurized fermented chili mash scored high degree of sourness as evaluted by the panelists. Both volatile and non-volatile acid contributed to the sourness odorand related to the decrease in pH (Marené, 2013). However, non-pasteurized fermented chili mash without LAB inoculation (control) scored higher in sourness odor (Figure 5) compared with pre-pasteurized fermented chili mash (Figure 4). Both pasteurized and non-pasteurized fermented chili mash without LAB scored low in hedonic score that ranged between 3.22 to 3.33 (Table 5). The differences in pH values and sourness may resulted from the types of microorganism present in fermented chili mash during fermentation. Spontaneous fermentation process may happen in non-pasteurised fermented chili mash due to successive fermentation resulted by different types of microorganisms under non-controlable condition (Chen et al., 2006). Heating at 80°C for 15 min caused natural flora reduction in vegetable surfaces (Aamir et al., 2013). This lead to lower competition between inoculated LAB with microorganisms that naturally present in fermented chili mash. Thus, rapid incease in LAB count and pH reduction were obtained.

The naturally occurring microbes are able to initiate spontaneous fermentation (Plengvidhya, 2007) whereby there is interplay between LAB, natural flora and yeast. This study showed that LAB naturally presents in chili mash (around $\log_{10}$ 2 cfu/g) increased gradually through 28 days fermentation (Figure 7). This showed that LAB has the ability to adapt with the chili substrate made the number to increase parallel with fermentation time (Di Cagno et al., 2008). This observation was similar to that

observed with natural black olives fermentation that showed gradual increase of lactic acid bacteria from $\log_{10}$ 2.8 and increased significantly to $\log_{10}$ 8.9 after 14 days under ambient temperature and 6% salt concentration (Tassou et al., 2002). LAB count is normally low in the range of $\log_{10}$ 2 and increased to $\log_{10}$ 7 depending on type of flora present during fermentation (Di Cagno et al., 2011a; Bourdichon et al., 2012). The most predominant species that normally present in vegetable fermentation are *L. plantarum*, *Leuconostoc mesentroides, L. pentosus, L. lactis* and *L. curvatis* (Kim & Chun, 2005). It was reported that in vegetable fermentation *Leuconostoc mesentroides* and *P. pentosaceus* are present at during initial of fermentation, followed by combination of *L. maltaromicus* and *L. bavaricus* and become dominant at later stage (Plengvidhya, 2004).

Phenotypic identification of the LAB isolates producing the desirable flavor characteristics of fermented chili mash using API 50 CHL system as *L. plantarum* 1 with 99.9% similarity. Using API 50 CHL system for phenotypic characterization is sufficient for a rough characterization but not for unequivocal identification purposes (Montel et al., 1991). It is useful to characterize the isolates based on substrate utilization such as carbohydrates. However, for identification of the isolate the genotypic characterization using DNA16S-S and 16S-R sequence is preferred (Maryam et al., 2013), and the isolated ALO1 was identified as *L. plantarum*, ALO2 as *L. pentosus* and AU2 *L. plantarum*.

3.5 CONCLUSIONS

In conclusion, sources of starters either authocthonous or allocthonous can be used to ferment chili mash since both sources of isolates have their own characteristic odor profile and pH reduction. Fermented chili mash can be produced either from pre-pasteurized or non-pasteurized chili sample. QDA test revealed fermented chili mash that is acceptable by consumer's preference have an intense smell in sour, sweet, flowery and fruity smell. Sample that intense in yeasty, fungi, stinky and raw smell were not desirable by consumers' preference. Fermented chili mash with unique odor profile can be generated by inoculating allochthonous *L. plantarum* ALO1, allochthonous *L. pentosus* ALO2 and autochthonous starter *L. plantarum* AU2. These LABs are potentially useful to be inoculated into fermented chili for industrial production.