CHAPTER III

MATERIALS AND METHODS

3.1 Sampling

Seven samples of beebread were collected using tweezers from bee hives from three different stingless bees namely, *Itama* spp., *Thorasica* spp. and *Terminata* spp. on April 2016. The bee bread samples were obtained from different sources namely, *Itama* spp. from Sungai Merab, Kajang Selangor (SM), Kedah (K), Terengganu (T) and Negeri Sembilan (NS); *Thorasica* spp. from SM and NS while *Terminata* spp. from NS. The samples were coded and kept in the refrigerator before analysis.

3.2 Isolation of LAB from bee bread

LABs were isolated by two methods (i) by direct plating of the beebread and (ii) by pre-enrichment method. For the first method: one gram of bee bread sample was transferred to 9 ml 0.1% peptone water (Merck, Germany) and vigorously mixes by vortex. Then 0.1 ml of homogenized samples was spread on De Man Rogosa (MRS) agar with 0.8 % $CaCO_3$ (Merck) and tomato juice agar (TJA) with 0.8% $CaCO_3$. All plates were incubated at 30, 37 and 45°C for 48 h in anaerobic jars to exclude growth of undesirable bacteria (Vasiee et al., 2014).

The second method followed the modified method of Chowdhury et al. (2012) in which the samples were pre-enriched in MRS broth for 24 hours at 37°C incubated in shaker incubator, and then streaking on plates with MRS agar with 0.8% $CaCO_3$ and TJA agar with 0.8% $CaCO_3$ added. All plates were incubated in anaerobic jar at 37°C for 48 h.

3.3 Antimicrobial activity

The targeted bacteria used in this study were *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 13311 and *Escherichia coli* ATCC 25922 obtained from the Microbiology Laboratory, Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM). The pathogens were sub-cultured in nutrient agar (Oxoid) and incubated at 37°C for 24 to 36 h then Gram stained to check for purity.

3.3.1 Dual agar overlay method

Antimicrobial activity of LAB isolates was determined against the pathogenic bacteria by dual agar overlay method. LAB were spot inoculated on MRS agar plates and incubated at 37°C for 24 h in anaerobic conditions. The plates were overlaid with 15 ml of nutrient agar containing the overnight culture of target bacteria (10^6 cells per ml). The diameter of inhibition zone was measured after 24 h aerobic incubation at 37°C (Aween et al., 2012).

3.3.2 Antimicrobial activity of LAB supernatant using microtiter plates

Cell free supernatant (CFS) of LAB was obtained by centrifugation (6500× g for 15 min at 4°C) of 24 h culture of LAB in MRS broth incubated anaerobically for 24 h at 37°C by using Eppendorf centrifuge and followed by filtration of the supernatant using 0.02 µm (Minisart). Nutrient broth (Oxoid) was prepared and inoculated with targeted bacteria and incubated for 24 h at 37°C. From 10^7 CFU/ml of these targeted bacteria serial dilution was done to get 10^4 CFU/ml then 100 µL of the supernatant and target bacteria were pipetted into the wells of microtiter plates. A 200 µL of target bacteria in nutrient broth was used as positive control and the analysis was carried out in duplicates. All microtiter plates were incubated at 37°C for 24 h. Bacterial growth was monitored using BioTek EL×800 ELISA reader optical density $(OD)_{560nm}$. The percentage growth of target bacteria was measured using the equation as described by Aween et al. (2012):

Percentage growth of target bacteria = [$OD_{560\ nm}$ with (bacteria/supernatant) after 24 h incubation –$OD_{560\ nm}$ of (bacteria/supernatant) at time 0h] / [$OD_{560\ nm}$ of (bacteria/supernatant) at time 0h] ×100.

3.3.3 Antimicrobial Activity of LAB isolates against targeted bacteria using Agar Well Diffusion Assay

The method of Salleh et al. (2014) was used to determine the antimicrobial activity of pH adjusted cell free supernatant (CFS). The pH of CFS was adjusted to 5, 6, 7 and 8 using NaOH (0.2 N), then tested against the target bacteria. Target bacteria were mixed in nutrient broth and incubated for 24 h at 37°C, then 10^6 CFU/ml was evenly spread onto Muller Hilton Agar (Merck, Germany) using a sterile swab and allowed to rest for about 30 min. An 8-mm diameter well was made into the agar by using a sterilized cork-borer and 20 µl molten agar was poured to the base of each well and allowed to solidify. A 100 µl of pH adjusted CFS of LAB isolates was transferred to each well separately. All plates were aerobically incubated at 37°C for 24 h. The diameter of growth inhibition zones was measured from the edges of the visible microbial growth excluding the diameter of the well. The target bacteria without CFS were used as control.

3.4 Screening for probiotic properties

3.4.1 Acid tolerance

MRS broth was adjusted to pH 2.0, 3.0 and 4.0 using either 0.1 N HCL or 0.1 N NaOH, then autoclaved at 121°C for 15 min (Harun-ur-Rashid et al., 2007). All LAB strains isolated from beebread were inoculated to 10 ml MRS broth (Oxoid) and incubated for 24 h at 37°C. A 0.1 ml of the 24 h culture of the LAB isolates were inoculated into the pH adjusted MRS broth, and then incubated in anaerobic condition for 24 h at 37°C. Growth of the bacterial isolates was measured by using Eppendorf Biophotometer at 560 nm (Chowdhury et al., 2012).

3.4.2 Bile salt tolerance

A 1% (v/v) of 24 h culture of LAB isolates was inoculated to MRS broth containing different bile salt concentration 0.1, 0.3 and 0.5 % (w/v) Oxgall (Difco, Sparks, MD. USA) then incubated at 37°C for 24 h. The survival of LAB in MRS containing ox bile was measured using (Eppendorf Biophotometer) at optical density (OD) 560nm (Chowdhury et al., 2012).

3.5 Antibiotic resistance of lactic acid bacteria

LAB isolates were tested for their resistance towards several antibiotics using disk diffusion method (Aween et al., 2012). The antibiotics tested were vancomycin (5µm), gentamycin (10 µm), streptomycin (10 µm), tetracycline (30 µm), penicillin G (10 µm) and chloramphenicol (30 µm) (Sigma). MRS agar (Merck) was prepared and LAB isolates were evenly spread using a sterile swab and allowed to rest for about 30 min. The antibiotic discs were placed on each plate of MRS agar and incubated at 37°C for 24 h in an anaerobic condition. The inhibition zone around the antibiotic discs was measured. The test was carried out in duplicate.

3.6 Statistical analysis

The Statistical Analysis System (Minitab) was used to perform data analysis. Results were analyzed using ANOVA with Tukey's multiple comparisons test and significant was considered at $P<0.05$ level.