

CHAPTER II

LITERATURE REVIEW

2.1 Stingless bee

Stingless bee known as meliponines are a massive class of honey bee (about 500 species), small in size compared to the sting bees and many of them are poorly known (Rasmussen et al., 2010). Stingless bees (*Hymenoptera: Apidae: Meliponini*), eusocial insects which are vastly spread over all tropical and subtropical areas excluding some oceanic islands, are significant pollinators in Peninsular Malaysia's dipterocarp forests. Such bees groups present in perennial settlement consisted of hundreds to many thousand workers and employ the resins of more than a hundred various plant species for food, nest construction, and protection against invaders. In Malaysian Borneo 91.5% of stingless bee nests are existent in living trees, and at least 34% of nest trees were big enough to be harvested. Colonies are typically long-lived but have low fertility, and harvesting mostly causes the deaths of many bee colonies existing in felled trees. Previous findings proposed that stingless bees could be used as essentially perfect indicators of environmental integrity because these bees are influenced by tree health and local disturbance considerably (Salim et al., 2012). Stingless bees have an ecological value and the plentiful researches of stingless bees have been achieved in Borneo but some experiments of stingless bees have been done in Peninsular Malaysia.

Salim et al. (2012) conducted an inclusive investigation of stingless bee variety in and around six lowland, hill, and upper hill dipterocarp forest reserves existent in four different parts in Peninsular Malaysia, and reported that there are 17 species of stingless bees and were of different genera compared with those recorded by past studies achieved in Borneo. They observed that *Apidae*, exactly stingless bees, were in rainforest places and were more widespread in primary and secondary forests than in more disturbed places. This study provided baseline data on stingless bee variety in forests over Peninsular Malaysia.

Honey collected from stingless bee in Malaysia have active antagonistic substances against some pathogenic bacteria. The lower acidity of the honey pH from 3.2 to 4.5 is probably the principle factor that inhibit the pathogenic bacteria (Ali et al., 2015). The antibacterial activity of honey collected from several stingless bee species *Trigona* spp., *Hetrotringona laeviceap*, *H. thorasica*, *H. itama* and *H. terminata* revealed that Malaysian stingless bee honey has antimicrobial properties against some Gram negative and positive pathogenic bacteria.

2.2 Lactic acid bacteria

Most study on LAB from honey bees have concentrated on stingl bees of *Apis* spp. LABs are reported presence in honeybee, larvae, fresh honey and bee pollen of sting bees *Apis mellifera* (Endo et al., 2013). The sting bees and larvae included *Lactobacillus*-group which is prevailed by *Lactobacillus kunkeei*, while mature bees contained more complicated microorganisms. LAB was reported present and viable in stomach of bees, pollen and fourteen days old bee bread, but not from the 2 months old bee bread (Vásquez et al., 2009). The researchers suggested that bee bread may be fermented by the LAB present in honeybee stomach which has been added to the pollen during the regurgitated nectar in the honeybee stomach and further confirmed that LABs are associated in the production of bee bread. Similarly, Forsgren et al., (2010) isolated *Lactobacillus* spp. and *Bifidobacterium* spp. from honey bee stomach, and eleven LAB phenotypes showed total inhibition against honey bee pathogen *Paenibacillus larvae*; the authors suggested that the LAB contributed to honey bee health.

Honeybees have a plentiful, varied and ancient LAB in their honey product with useful impacts for bee health and protect them against bacterial threats. Vásquez et al., (2012) reported that *L. kunkeei* was the dominant LAB member and showed antimicrobial activity inside the honey crop of the Western honeybee *Apis mellifera*. The crop microorganisms of *A. mellifera* consisted of 13 microbial species in the genera *Lactobacillus* and *Bifidobacterium* and consider having an important function in the

production of honey and beebread for long-term stored nourishment for both mature honeybees and larvae.

Many LAB have analogous nutritional and growth need, it is not easy to identify these microbiota using conventional technique. Thus, the requirement to effective method of identification is needed; Balcázar et al. (2007) isolated and identified thirteen LAB strains from the intestinal healthy salmonids using polymerase chain reaction amplification of about 500-bp region of the better conserved 16S rRNA gene, V1 and V2 region from this amplicon gave a powerful evidence for the identification of unknown LAB. During the modern years, sequencing of 16S rDNA has been considerably used and counted as the “gold standard” for identification and phylogenetic test of LAB (Tajabadi et al., 2013).

2.3 Probiotic properties of LAB

A probiotic is a culture of living microorganism fundamentally lactic acid bacteria and bifidobacteria that can influence the health when ingested in adequate amounts by the host. The settlement of probiotic bacteria in the digestive tract inhibits the multiplication of hurtful microbiota by competitive exclusion and by the production of organic acids and antibacterial components. Probiotic bacteria have to be capable to survive the acidic positions in the stomach and withstand the bile salts at the beginning of bowels. The capability to resist low pH makes the bacteria survived in gastric juice. The pH of secreted HCl in stomach is 0.9 but, the existence of food increases the acidity rate to the pH 3. Cholesterol in the liver synthesizes bile salts and stores it in the gall bladder then released it into the small intestine after ingestion of the fatty food. This is important as detergent for microorganisms since their cell membranes are consisted of lipids and fatty acids. Meanwhile, some microbiota have the ability to hydrolyse bile salts with bile salts hydrolyse enzyme reducing their solubility, and subsequently weakening the ability of their detergent impact. Erkkilä et al. (2000) determined the probiotic properties of LAB strains isolated from eight commercial meat cultures under condition analogous to those in the gut and found that *Lactobacillus sake* and *Pediococcus acidilactici* had the best

survival capabilities under acidic situations with high concentration of bile acids. Pochart et al. (1992) proved a strain of *Bifidobacterium* has the ability to survive passage over the upper gastrointestinal tract when ingested in fermented milk. Chowdhury et al. (2012) found that four *Lb. plantarum* isolates from eight homemade yoghurt samples showed resistant to bile-acid 0.05-0.3% and maintain good growth in the acidic environment, while the highest growth was observed at acidity around pH 6.0. The isolates were tested for their antimicrobial effect against nine human pathogens belonging to both Gram-positive and Gram negative bacteria such as *Bacillus subtilis*, *B. megaterium*, *B. cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Typhi ATCC 65154, *Salmonella* Paratyphi and *Vibrio parahaemolyticus* ATCC 17802 and found that growth of all targeted bacteria were inhibited to some extent but bigger inhibition zone was observed against *B. cereus* and lower inhibition against *Staphylococcus aureus*.

Mourad et al. (2006) evaluated the probiotic properties of 11 *Lb. plantarum* strains isolated from fermented olives for their resistance to high acidic condition and high bile salt value; the isolates showed high susceptibility to the bile acid concentration 2% oxgall and tolerance at pH 2. Similarly Harun-ur-Rashid et al. (2007) assessed the probiotics traits of LAB strains isolated from traditional fermented milk; some of them showed good resistance to low acidity and high bile salt concentration and found that *Lb. delbrueckii* subsp. *bulgaricus* might be used as probiotic starter culture in fermented milk. Vasiee et al. (2014) identified and characterized the probiotic lactobacilli species from fermented butter milk and wheat flour; the results indicated that three of them have probiotic properties. Similarly, Ashraf et al. (2016) described and distinguished 17 LABs for their ability to survive in bile at concentration 0.5 to 2.0 % and pH 3. All tested LAB showed good growth in bile salt and pH 3, these traits are important in the selection of probably probiotic bacteria.

2.4 Antimicrobial activity of LAB

Honey from the *Apis* spp. has an antimicrobial effect against certain species of honeybee pathogen due to the activity of some strains of bacteria that reside in the bee intestine and/or honey. Sabaté et al. (2009) determined the ability of *Bacillus* spp. strains collected from bee gut and honey and discovered that some strains of *Bacillus* had antimicrobial effect against honey bee pathogens *P. larvae* and *Ascosphaera apis*, *in vitro*. Likewise, Forsgren et al. (2010) reported that lactobacillus and bifidobacterium isolated from honey bee stomach had antagonistic effect against honey bee pathogen, *P. larvae*.

Honeybees's gut of *A. mellifera* L. is associated with microorganism which consist of yeasts, Gram- positive bacteria and Gram-negative or Gram variable bacteria. The bee's normal bacteria come from pollen ingestion or through contact to older bees. These microorganisms are probably endemic in the gut of mature bees. Audsio et al. (2011) isolated and identified LAB from a worker bee gut of *A. mellifera* in Salta, Argentina and showed that it had an antibacterial impact against the bacterial bee pathogen and human foodborne pathogen.

Malaysian wild honey known as Tualang honey has inhibitory effects towards various pathogens of wound and enteric bacteria. Diverse strains of LAB were isolated from 13 commercially available honey types produced in Malaysia, Libya and Saudi Arabia and, confirmed the antimicrobial effect towards multi antibiotic resistant strains of both Gram negative and positive spoilage bacteria. Aween et al. (2012) reported the strains of *L. acidophilus* isolated from honey produced compounds which may be responsible for the antibacterial properties of honey. Five LAB isolates presented anti-adhesion action against five *Candida* spp, *Lb. plantarum*2 HS isolated from Al-Sedar honey, *L. curvatus* HH isolated from Al-Hanon honey, *P. acidilactici* HC isolated from Tualang honey and *P. pentosaceus* HM isolated from Al-Maray honey (Bulgasem et al., 2015) and reported that cell free supernatant (CFS) of LAB isolated from honey possessed an anti-adhesion effect at pH 3. The CFS of *L. curvatus* isolated from Al-Hanon honey showed more effect at pH 7 particularly towards biofilm formation of *C.*

glabrata ATCC 2001 and *C. albicans* ATCC 14053 with 65.9 % and 58.6 %, respectively.

Microorganisms that exist in honey resist the high acidity and antimicrobial components found in honey. These microorganisms are likely originated from different sources such as pollen, the digestive tracts of honeybees, flowers, dirt, dust and the air. Most species of LAB are dominant in the stomach of honeybees *A. mellifera*, *A. dorsata*, and bumblebees (Tajabadi et al., 2013) as shown in the collected honey samples from forest area in Malaysia. They isolated and identified the dominant lactobacillus strains *Lb. plantarum*, *Lb. pentosus*, and *bL. fermentum* from the stomach of honey bee *A. dorsata*.

Members of the species *Bifidobacterium* have received great attention for use as probiotic bacteria to protect honey bees from infectious illness. The genus *Bifidobacterium* was isolated from the stomach of honeybee *A. mellifera* and showed antagonistic activity against *P. larvae*. Wu et al. (2013) focused their investigation on the Japanese honeybee, *A. cerana japonica*, which is a subspecies of the Asian honeybee and isolated bifidobacteria from the gut of Japanese honeybee and have antimicrobial activity against *Melissococcus plutonius*, *in vitro*.

Olofsson et al. (2014) examined the potential antibacterial effects of LAB isolated from honeybees collected in Sweden against serious wound pathogens like *S. aureus*, *Pseudomonas aeruginosa* and *Enterococcus* spp. Their research proved that LAB has strong antimicrobial activity against all the tested pathogens. These researchers also suggested that LAB materials produced over honey production ought to be present in freshly harvest honey and conserved in mature honey. Salleh et al. (2014) noted that the antibacterial effect of crude bacteriocins from CFS of LAB strains using well diffusion method showed that some LAB from fermented durian flesh had strong inhibitory effect against most of 23 species of *Salmonella* associated with food poisoning cases.

2.5 Antibiotic resistant

Bacteria has the ability to overcome antibiotic stress. These methods fall into “resistance” and “tolerance”. In “resistance” category the microorganisms can multiply in the presence of an antibiotic, taken into account the concentration of the antibiotic is not so much; while “tolerance” refers to when the microorganisms can survive antibiotic therapy at different concentration. Ashraf et al. (2016) evaluated LAB isolated from Australia for their antibiotic resistant and found that all isolates were susceptible to ampicillin and erythromycin and showed rare resistance to gentamycin and tetracycline. Additionally, Muhialdin et al. (2012) evaluated LAB isolated from Malaysian fermented fruits and foods and found that all LAB isolated strains were resistant to vancomycin and all LAB isolates were susceptible to chloramphenicol. Likewise, Mourad et al. (2006) assessed antibiotic resistant of 11 LAB isolated from fermented olive and found that all isolates were sensitive to penicillin G, ampicillin, vancomycin and chloramphenicol, three of them were entirely sensitive to all antibiotics tested.

2.6 Bee bread and its health benefits

Bee bread is deemed as “complete food”; it contains considerable amount of carbohydrates, crude fibers, proteins and lipids and minor components such as amino acids, minerals, trace elements, vitamins, carotenoids, phenolic compounds, flavonoids, sterol, terpenes among others. Additionally, it is reported that bee pollen have a significant effect on human health like antitumor, chemo-preventive/chemo-protective, antimicrobial, antifungal, antioxidant, anti-radiation and anti-inflammatory actions (Kostić et al., 2015). The researchers compiled bee pollen from Serbia and assessed the physicochemical structure and techno-functional properties and concluded that bee bread could be applied as food components in varied food products. Bee pollen has been used for several centuries in conventional medicine and as a food for human because it has nutritional and therapeutic traits. Also it is rich source of polyphenol compounds, especially flavonoids that make it suitable for the human diet. Krastyjan et al. (2015)

reported that bee pollen can be used as a dietary supplement and revealed that it have a distinctive activity on the physical, chemical and health-promoting traits.

Abouda et al. (2011) focused their study on determining the antibacterial action of natural bee-bread and bee-pollen collected from different parts in Morocco from various aromatic and medicinal plants on antibio-resistant microbial strains which were isolated from human disease and detected that most of pathogenic strains were impeded by the dilution 1/2 and 1/4 of bee-bread and bee-pollen samples.

Loper et al. (1980) determined the chemical and microbiological changes occurring in hand- and honey bee-collected pollen collected from California, and then packed wax combs to stimulate natural fermentation into bee bread. They analysed the bee bread after 7, 21, and 42 days for three vitamins, inositol, titratable acidity, and five minerals and revealed that lipoidal (fatty acid) and acids which were added by the bees showed the largest distinction between hand-collected and corbicula pollen. Sitosterol content reduced while titratable acidity increase during conversion of pollen to bee bread. The vitamins that were reduced when the pollen were converted to bee bread were ascorbic acid and pyridoxine. Similarly, Herbert et al. (1978) collected and evaluated fresh pollen and bee-stored pollen extracted from brood combs of free-flying colonies from Beltsville, Maryland for their nutrition value. Bee bread and pollen were tested for moisture, protein, reducing and non-reducing sugars, lipids, sulfated ash, starch, pH, pectins and crude fiber. They observed that there were some differences in nutritive value, there were changes in protein, moisture, and lipids between pollen and bee bread. Also, there was no notable starch in any of the samples of bee bread while starch was present in pollen. In addition, bee bread contained high amounts of reducing sugar and crude fiber than pollen except lower content of ash. The pH of bee bread was at an average 4.1 in comparison with pollen which was 4.8.

Nagai et al. (2004) assessed three extracts namely, hot-water fraction (HWF), water-soluble fraction (WSF), and ethanol-soluble fraction (ESF), prepared from fresh bee bread obtained from Lithuania for its functional characteristic like antioxidative ability and scavenging action of reactive oxygen species. They proved that the protein and all

phenolic contents of these samples were high and the bee bread extracts have a remarkable antioxidant and free radical scavenging properties. Vásquez et al. (2009) suggested that bee bread is possibly fermented by the addition of LAB from the stomach of honey via regurgitated nectar from the honey stomach. Almost all of the LAB was recovered in a viable state from both the two-week old bee bread and the pollen, but not from the two month old bee bread.

