Changes in quality and bacterial profiles of Tualang and Kelulut honeys preserved by post-harvest maturation

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Changes in Quality and Bacterial Profiles of Tualang and Kelulut Honeys Preserved by Post-harvest Maturation

(Running Title: Post-harvest Maturation of Honey)

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ABSTRACT

Post-harvest maturation of two Malaysian honeys, the Tualang and Kelulut was studied by measuring changes in physicochemical and antioxidant properties, hydroxymethylfurfural (HMF) contents and bacterial profiles at room temperature of 23-26 ^{o}C. After maturation at the recommended period of 26 weeks, water activity of both honeys increased between 0.89-2.34% while free acidity increased between 2.05-2.24%. Results suggested the prominence of fermentation reactions in honey during post-harvest maturation as fructose concentration reduced by 10.6 and 1.05% for the Tualang and Kelulut honey respectively while HMF concentrations were kept at a safe limit of 48.00 and 61.23 mg/kg honey. The total phenolic content of Tualang and Kelulut honey increased significantly by 12.61 and 54.66% respectively. The highlight of this post-harvest maturation process for Kelulut honey was the improvement found in antioxidant properties of DPPH radical scavenging activity by 10.01% to 54.74% and also the probiotic-like potential in terms significant increase in relative abundance of the *Bacillus* genera to 2.6% and *Lactobacillus* to 6.25% at 26 weeks. The prolonged maturation process up to one year however revealed continuous accumulation of HMF to values above 80 mg/kg honey, surpassing the limits by the Codex Alimentarius Commission despite improvements of antioxidant properties and bacterial profiles.

***Keywords**:* Malaysian Honey; Post-harvest Maturation; Probiotic; Honey Preservation

1.0 Introduction

Heat treatment is known to be the simplest and most convenient method to process honey to improve its shelf stability (Subramanian *et al.*, 2007; Turhan *et al.*, 2008; Scepankova *et al.*, 2021). Conventionally, raw honey is processed at temperature of 45-80 ^{o}C for 1-45 min (Subramanian *et al.*, 2007) to delay crystallisation, to reduce viscosity, to remove contaminating microbes and to prevent fermentation (Subramanian *et al.*, 2007; Scepankova *et al.*, 2021). However, heat treatment is a processing method that is often accompanied with significant degradation of food quality, particularly raw honey (Wang *et al.*, 2004; Soni *et al.*, 2016; Tarapoulouzi *et al.*, 2023). Samborska and Czelejewska (2014) reported that multifloral honey processed at 90 ^{o}C for 30 min contained a high hydroxymethylfurfural (HMF) concentration of 67.8 mg/kg. Similarly, study of Zarei et al. (2019) showed that antioxidant DPPH radical scavenging activity and total phenolic content (TPC) of honey reduced by 12.55-20.85% after being processed at 63 ^{o}C for 10-30 min. Other studies have also shown that heat treatments at temperature of between 45 to 90 ^{o}C can reduce nutrients, enzymatic (Cianciosi *et al.*, 2018) and antimicrobial activities of honey (Mat Ramlan *et al.*, 2021) significantly.

Increasing concerns and awareness on nutritional quality of processed honey have prompted studies on various other alternative honey processing methods. The advanced technologies and techniques offer better preservation of honey quality (Chong *et al.*, 2017; Scepankova *et al.*, 2021). Thermosonification was reported to be effective in killing microorganisms in honey and enhance DPPH radical scavenging activity and TPC of Kelulut honey by 63.0 and 58.1% respectively (Chong *et al.*, 2017). The high-pressure processing (HPP) technique used on Manuka honey recorded an increment of TPC by 47.16% (Akhmazillah *et al.*, 2013). The more recent microfludization technique produced a shelf-stable multifloral honey with enhanced antioxidant activity by 37.2% while maintaining a low HMF concentration (Leyva-Daniel *et al.*, 2020). Although these techniques are

beneficial, they are costly and may not be feasible for the smaller scale farmers and honey producers from both the economical and technical aspects.

The post-harvest maturation of honey is a honey preservation technique developed by the Native Bee Rural Community Project in Northeast Brazil for rural honey producers for a more viable honey processing (Drummond, 2013). Unlike other honey processing techniques requiring high-end equipment, post-harvest maturation is simple and does not require use of heat which destroys microbial activity of honey. In post-harvest maturation, honey is allowed to age and ferment naturally for about 26 weeks (around 180 d) in a hygienic and controlled condition (Drummond, 2013; Silva *et al.*, 2023). Processed honey using the post-harvest maturation method is noted to have significant fermented acidic aroma, higher acidity and lower reducing sugars (Ribeiro *et al.*, 2018). The physicochemical and bacteriological properties of processed honey via post-harvest maturation have also confirmed its compliances and standards for safe consumption (Drummond, 2013). The sensory acceptance test of post-harvest matured Tiuba honey suggested acceptance by the consumers (Ribeiro *et al.*, 2018). The research on post-maturation process of honeys are however still limited to Brazilian honeys, *i.e.* native stingless bee (Drummond, 2013), Tiuba (Ribeiro *et al.*, 2018) and Uruçú-Amarela honey (Silva et al., 2023).

The honey industry in Malaysia is similar to that of Northeast of Brazil where many small scale honey producers exist especially in the suburbs and rural areas (Lim and Baharun, 2009). The Tualang and Kelulut honeys are two more common honey produced in Malaysia. Tualang honey is a multifloral jungle honey produced by *Apis Dorsata* bee (Ahmed and Othman, 2013). It is named after the *Koompassia excelsa* (*Tualang*) tree where the bee hives are built on (Moniruzzaman *et al.*, 2013). Kelulut honey is another common multifloral honey produced mainly by *Heterotrigona itama* bee in Malaysia (Saludin et al., 2018; Kamal et al., 2021). Kelulut honey generally contains a significantly higher acidity (87.0-347.5 meq/kg honey) and moisture content (26.60-33.24%) than other types of honey (Kek *et al.*, 2017; Omar *et al.*, 2019; Yap *et al.*, 2022). The Kelulut honey can be produced

commercially via Meliponiculture practice (Cortopassi-Laurino *et al.,* 2006; Bahri *et al.,* 2016). The source of Tualang honey is relatively limited and inconsistent due to its collection from wild forest and produced by *Apis dorsata* bees which cannot be domesticated commercially due to its highly defensive behavior (Thakar, 1973). Nevertheless, these two Malaysian honeys have gained considerable recognitions in researches due to their significant health beneficial properties.

Published studies reported that the Tualang honey has high level of antioxidants (Kishore *et al.,* 2011; Yap *et al.,* 2022) while the Kelulut honey was found to harbour a significant beneficial probiotic bacteria *i.e. Bacillus* and *Lactobacillus sp.* (Zulkhairi Amin *et al.,* 2019; Yap *et al.,* 2022). Other studies on Kelulut honey include Hasali et al. (2015) who isolated four *Lactobacillus sp.,* Amin et al. (2020) reported two *Bacillus sp.*, Wu et al. (2023) recorded significant abundance of *Lactobacillus* and Goh et al. (2021) isolated lactic acid bacteria in Kelulut honey from Sabah. With the post-harvest maturation technique being capable of preserving honey more naturally for its thermolabile antioxidant compounds and beneficial probiotic bacteria, this research investigated its effects on two honey varieties, the Tualang and Kelulut by measuring their physicochemical and antioxidant properties, hydroxymethylfurfural (HMF) content and bacterial diversities.

2.0 Materials and Methods

2.1 Honey Samples and Post-harvest Maturation

Tualang honey produced by bee species of *Apis dorsata* was collected directly from honey collectors and Kelulut honey from bee species of *Heterotrigona itama* was collected directly from farms with extra practise and care on hygiene. A total of 4.5 kg of honey was collected for each type of honey. The honeys were homogenised and distributed equally into six pasteurised glass jars. The samples were allowed to mature at room temperature of 23-26 ^{o}C in the glass jars under aseptic condition. The glass jars were opened every two weeks to release accumulated gas in the jars (Silva *et al.,* 2023) and for sampling of 50 g of honey for a duration of 52 weeks. The prolonged post-harvest maturation

period beyond the usual practise of maturation for 26 weeks (Drummond, 2013; Silva *et al.*, 2023) was aimed to study honey changes more completely.

2.2 Physicochemical Properties

Water activity at room temperature of honey samples was measured using a water activity meter (Aqualab Pre, Washington, DC, USA). pH value and free acidity of the honey samples were determined following the Association of Official Analytical Chemists (AOAC) Official Method 962.19 (AOAC International, 2005). A honey solution containing 10 g of honey and 75 mL of distilled water was prepared. The pH value of the solution was determined with a pH meter (Mettler Toledo, Greifensee, Switzerland). Prepared honey solution was then titrated with 0.1 mol L^{-1} sodium hydroxide (NaOH) solution and free acidity was calculated from the volume of NaOH needed to achieve pH value of 8.3 and reported in meq/kg following equation (1).

$$\text{Free acidity (meq/kg)} = \text{volume of 0.1M NaOH used} \times 10 \quad (1)$$

Sugar concentration of honey samples, *i.e.* sucrose, fructose, glucose and maltose contents were measured following method suggested by International Honey Commission (Bogdanov, 2002). Honey (5 g) was dissolved in 75 mL of distilled water. The solution was then added with 25 mL methanol, filtered with a membrane filter and analysed with high-performance liquid chromatography (HPLC; Shidmazu, Kyoto, Japan) equipped with refractive index detector (RI-D) and Shidmazu Shim-pack GIST NH2 separation column. The run was done with 10 µL sample injection volume and 1.3 mL min^{-1} acetonitrile-water (75:25) isocratic elution mobile phase. The reading of sucrose for both honey were low and nearing zero thus not reported.

2.3 Antioxidant Properties

Antioxidant properties of honey was measured as the total phenolic content (TPC) (Singleton *et al.*, 1999) and 2,2-diphenyl-1-picrylhy-drazyl (DPPH) radical scavenging activity (Chong *et al.*, 2017). For TPC, the Folin-Ciocalteu spectrophotometric method was used. Honey sample solution was prepared by dissolving 1 g of honey in 20 mL of distilled water. The sample solution (1 mL) was then

added with 5 mL of 0.2 N Folin-Ciocalteu reagent solution and 4 mL of 7.5% (w/v) aqueous sodium carbonate solution. The absorbance of the sample solution at 765 nm was measured using a UV/Visible spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, USA) after 2 h of incubation in dark. TPC was calculated and expressed in milligrams of gallic acid equivalent per kg of honey (mgGAE/ kg honey).

For DPPH, a stock solution (0.1 mM) was prepared by dissolving DPPH powder (Sigma-Aldrich, St Louis, MO, USA) in methanol. Honey solution containing 0.5 g honey and 10 mL of methanol was prepared. The solution was then centrifuged for 15 min at 5700 g (Universal 320, Hettich, USA). Two millilitres of supernatant was collected and then added with 2 mL of DPPH solution. The absorbance of the sample solution was measured at 17 nm using a UV/Visible spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, USA) after 30 min of incubation in dark. The DPPH radical scavenging activity (RSA) of honey was then calculated using equation (2):

$$\text{DPPH (\%RSA)} = \left[1 - \left(\frac{A_S}{A_C}\right)\right] \times 100\% \quad (2)$$

where A_s and A_c are the absorbance values for the sample and control, respectively.

2.4 Hydroxymethylfurfural (HMF) Content

The HMF content of honey sample was determined following the widely recognised White's spectrophotometric method (White, 1979). A solution was prepared with diluted honey solution, Carrez I, Carrez II and ethanol. The absorbance values of the solution were measured using a UV/Visible spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, USA). The HMF concentration of the honey was calculated following equation (3):

$$\text{HMF (mg/kg honey)} = (A_{284} - A_{336}) \times 149.7 \times 5 \times \frac{D}{W} \quad (3)$$

where A_{284} and A_{336} indicate the absorbances values of the solution at 284 and 336 nm respectively. D is the dilution factor and W is the weight of the sample.

2.5 Bacterial Profile Analysis

The bacterial profile study was conducted using the next-generation targeted amplicon sequencing method *i.e.* sequencing the 16S rRNA gene amplified from extracted gDNA. The DNA extraction was done by modifying method suggested by Yap et al. (2022). Honey solution was prepared with 15 mL honey and 135 mL of sterile water. It was incubated in a water bath at 65 ^{o}C for 30 min with occasional shaking to dissolve the honey completely. The honey solution was then filtered on filter with pore size > 100 µM. The solution was filtered again with a membrane filter with a smaller pore size of 0.22 µM to retain the microbes. The membrane was transferred into a tube. One millilitre of lysis buffer containing lysozyme was then added to the tube. The tube was incubated in a rotating incubator at 37 ^{o}C for 3 h. The sample was homogenised by bead beating. The gDNA was extracted by using the spin column method following manufacturer's protocol and eluted with 100 µL of buffer solution.

The V3-V4 region of the 16s rRNA gene was amplified from the extracted gDNA with the primer pair Illumina V3V4F (5'-CCTACGGGNGGCWGCAG-3') and Illumina V3V4R (5'-GACTACHV-GGGTATCTAATCC-3') appended with overhang adapters (5'-TCGTCGGCAGCGTCAGATGTG-TATAAGAGACAG-3') and (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'). The PCR amplification was performed with 3 min of initial denaturation at 95 ^{o}C; 25 cycles of 30 s denaturation at 95 ^{o}C, 30 s annealing at 55 ^{o}C, 30 s elongation at 70 ^{o}C and a final extension at 72 ^{o}C for 5 min. The quality of the amplified PCR products was verified by electrophoresis in a 1% agarose gel after purified with purification kit.

An equal quantity (100 ng) of each PCR amplicon tagged with the sample-specific barcode sequences was pooled and the quantity and quality of DNA was further assessed on a Illumina MiSeq system (Illumina, San Diego, CA, USA). The sequencing of the pooled library was done with the run configuration of 2 x 300 base pairs according to the manufacturer's instructions.

The forward and reverse reads were merged using FLASH2 (Magoč and Salzberg, 2011) and its quality was screened for sequence length and nucleotide ambiguity. Sequences that are shorter than 150 bp or longer than 600 bp were removed. Chimeric erros were checked by aligning the reads with 16s rRNA database. High quality reads were clustered at 97% similarity into OTUs using QIIME with de novo open reference clustering algorithm (UCLUST) (Venkatavara Prasad *et al.*, 2015). Rare OTUs with only 1 (singleton) or 2 reads (doubleton) were eliminated. All OTUs were annotated to different classification levels (from domain to genus) with the SILVA ribosomal 16s RNA database (Quast *et al.*, 2013). The bacterial profile analysis was conducted only for the Kelulut sample but not the Tualang based on its more consistent, availability and potential to be developed into probiotic matured honey.

2.6 Statistical Analysis

All samples were tested in triplicates and results are reported as means ± standard error of mean. One-way analysis of variance (ANOVA) statistical analysis and Tukey's test at confidence level of 0.05 was performed using Minitab software (version 18, Minitab Inc., State College, PA, USA) to evaluate the significant differences between the data.

3.0 Results and Discussion

3.1 Physicochemical Changes

Figure 1 shows the changes of water activity of honeys during post-harvest maturation. Water activity of both honeys fluctuated in the beginning of post-harvest maturation storage period but increased gradually from weeks 18 onwards. Despite a lower water activity for the Tualang honey, its increase after 26 weeks of post-harvest maturation was higher at 2.34% from 0.710 to 0.727 when compared to Kelulut at 0.89% from 0.783 to 0.790. This increase is consistent with the Tiuba honey at 1.76% from 0.675 to 0.687, also at about 26 weeks (180 d) of post-harvest maturation (Ribeiro *et al.*, 2018). The increase of water activity continued after 26 weeks and reached 0.728 with a total increase of 2.54% for Tualang and 0.803 (2.55%) for Kelulut honey at the end of 52 weeks maturation. The

remarkably high water activity, above threshold value of 0.6 facilitates natural fermentation of honey (Sanz *et al.*, 1995) as Drummond (2013) also reckoned microbial fermentation to occur during post-harvest maturation of honey. These fermentation processes are well complemented to the sensory profile results reported by Ribeiro et al. (2018) where post-harvest matured honey had fermented characteristics and acidic taste.

Figure 2(a) shows that the pH values of both honey decreased with post-harvest maturation. At 26 weeks, pH reduction for Tualang was 11.20% from 3.66 and for Kelulut was 17.26% from 3.07. The reduction continued to 3.13 (14.48%) and 2.58 (15.96%) respectively at the end of post-harvest maturation of one year (52 weeks). Figure 2(b) shows supporting results of pH where free acidity increased steadily over the entire post-harvest maturation period. Previous post-harvest maturation studies on *Melipona quadrifasciata* (da Silva et al., 2022) and Uruçú-Amarela honey (Silva et al., 2023) have similarly reported increase of acidity of post-harvest matured honey. The increase of honey acidity was mainly due to the formation of gluconic acid from enzymatic decomposition of glucose as explained in metabolomics study by Silva et al. (2023). Due to high water activity and the presence of active microbes, fermentative activities which lead to increase of honey acidity are common phenomena in unprocessed honey (Sanz *et al.*, 1995; Subramanian *et al.*, 2007). Yap et al. (2022) supported and showed the presence of *Actinobacteria*, *Firmicutes* and *Proteobacteria* as the core bacterial phyla of Tualang and Kelulut honey. These bacteria are often categorised as facultative anaerobes and possesed significant fermentative ability (Lee *et al.*, 2015). As honey sugars are converted to ethyl alcohol and organic acids, *i.e.* succinic, lactic and acetic acid during honey fermentation, its free acidity increase and pH decrease (Özcelik et al., 2016; Sanz et al., 1995; Silva et al., 2023). The increased acidity of honey during post-harvest maturation is seen beneficial as a natural method of honey preservation that helps inhibiting growth of pathogenic microbes (Silva et al., 2023).

Figure 3 shows the changes of predominant honey sugars, *i.e.* fructose, glucose and maltose during the post-harvest maturation period. The results show that Kelulut honey contains a lower glucose (Figure 3b) and higher maltose concentration (Figure 3c) than Tualang honey. Previous studies have consistently reported a lower reducing sugar concentration in Kelulut (Zawawi *et al.*, 2022), *e.g.* low glucose (140-210 g/kg honey) and high maltose content (33.7-45.2 g/kg honey) in stingless bee honey (Chuttong *et al.*, 2016a; Tuksitha *et al.*, 2018). Braghini et al. (2021) suggested that the high maltose concentration in stingless bee honey could be attributed to its low α-glycosidase activity, an enzyme that catalyses hydrolysis of a bond joining a sugar of a glycoside to another sugar unit or alcohol.

The changes during post-harvest maturation of both honey samples showed similarity. The fructose concentration of both honeys shows a more significant decreasing trend (Figure 3a) than glucose (Figure 3b) and maltose (Figure 3c). After 26 weeks of post-harvest maturation, fructose concentration of Tualang and Kelulut honey recorded a decrease of 10.6 and 1.05% respectively. The values dropped further to 271.35 g/kg honey (15.4%) and 247.20 g/kg (14.92%) respectively at the end of 52 weeks maturation. For Tualang honey, glucose and maltose content reduced by 14.66 and 24.47%, respectively to 217.89 and 36.15 g/kg honey after 52 weeks. In comparison to Kelulut honey, the reduction of glucose and maltose is less pronounced, *i.e.* 6.13 and 17.99% respectively. The reduction of fructose and glucose during post-harvest maturation are also reported by Ribeiro et al. (2018) for Tiuba honey and Silva et al. (2023) for Uruçú-Amarela honey. The results complemented the sensory study reporting significantly lower sweetness in post-harvest matured honey (Ribeiro *et al.*, 2018). The observed minor fluctuations of honey sugars in honey is generally explained by the myriads of complex reactions that occur during post-harvest maturation process such as non-enzymatic transglycosylation which converts complex oligosaccharides to simpler sugar (Silva *et al.*, 2019); glucose oxidase enzymatic activity converts glucose to gluconic acid (Silva *et al.*, 2023); series of non-enzymatic browning reactions that transform fructose and glucose to furan compounds and other by-products (Shapla *et al.*, 2018); isomerization of sucrose and glucosylation of fructose to form

trehalulose (Zhang *et al.*, 2022). Trehalulose is a bioactive dissacharide that was recently proposed to be a characteristic sugar component of stingless bee honey (Fletcher *et al.*, 2020; Zawawi *et al.*, 2022). Silva *et al.* (2023) suggested that fermentation of trehalulose might occur during maturation of Uruçú-Amarela honey. The understanding on formation of trehalulose in honey is limited and it is still not regulated in honey standards (Codex Alimentarius Commission, 2001; Department of Standards Malaysia, 2017). Thus, trehalulose was not measured in the present study.

The similar physicochemical changes of both Tualang and Kelulut honey samples during post-harvest maturation *i.e.* decreased fructose and glucose concentrations (Fig. 3) and increased water activity (Fig. 1) and acidity (Fig. 2) suggest prominence of microbial fermentation activities. According to the findings of Silva et al. (2023), fermentation was more evident after 72 days of post-harvest maturation, leading to remarkable changes on Uruçú-Amarela honey's physicochemical properties, *i.e.* increased acidity and reduced glucose and fructose. Following practice in Brazil, the stabilising period of post-harvest maturation of honey is determined solely based on visual observation on the adhering of honey's foam on the wall of flask (Drummond, 2013). According to Drummond (2013), the end of stabilising period is achieved usually after around 180 d (about 26 weeks) of post-harvest maturation, *i.e.* when the increasing consistency of honey's foam collar does not move when the glass flask is inclined. In this research, the stabilisation period of both Tualang and Kelulut honey properties were evaluated by using not just the physico-chemical changes but also the changes in TPC, DPPH, and HMF concentration. It is known that high HMF concentration in honey can give negative impacts to health due to its potential carcinogenic, mutagenic, genotoxic and organotoxic characteristics (Abraham *et al.*, 2011; Shapla *et al.*, 2018; Choudhary *et al.*, 2020). The Codex honey standards has set a maximum limit of 80 mg/kg honey for tropical honey (Codex Alimentarius Commission, 2001).

3.2 Changes in TPC, DPPH and HMF

The total phenolic content (TPC) and DPPH radical scavenging activity are considered positive health promoting properties while HMF is a negative quality parameter of honey. HMF increase during

honey aging is a natural process and unavoidable (Kesić *et al.*, 2014) thus has to be compensated by the anticipated increase of its antioxidant properties. Figure 4(a) shows that TPC of Tualang and Kelulut honey increased steadily by 12.61 and 54.66% after 26 weeks respectively, from 1286.36 and 483.52 mgGAE/kg honey to 1448.57 and 747.83 mgGAE/kg honey for its first half of maturation period. The increase was less for the second half of maturation period, *i.e.* by a further 8.49% for Tualang and 0.35% for Kelulut honey to 1571.62 and 750.41 mgGAE/kg respectively at the end of post-harvest maturation. The earlier work of da Silva et al. (2020) has shown a similar increasing trend of TPC for *Apis Mellifera* L. honey during storage but the otherwise was reported for Uruçú-Amarela honey, where TPC reduced from 515.11 to 463.5 mgGAE/kg honey after 180 d of post-harvest maturation (Silva *et al.*, 2023). Silva et al. (2023) explained that the increase of honey's acidity during post-harvest maturation may cause the structural changes of phenolic compound. According to Wojtunik-Kulesza *et al.* (2020), phenolic compounds exist commonly in polymerized, glycosylated and esterified forms. In acidic conditions, the compounds can transform to a new phenolic derivatives through ethylation, glycosylation, hydroxylation or dimerization reactions (Wojtunik-Kulesza *et al.*, 2020; Silva *et al.*, 2023). The transformation and stability of the compound structures vary significantly between different phenolic compounds (Šarić *et al.*, 2020). For instance, a previous study have shown that flavonols can transform to hydroxyphenylacetic acids, phenylvalerolactone and hydroxyphenylpropionic acids while flavones and flavanones can degrade to hydroxyphenylpropionic acids (Wojtunik-Kulesza *et al.*, 2020).

Thus, the extreme diverse phenolic profiles between honey samples could lead to unpredictable outcomes as different phenolic compounds which reacted differently leading to the contrasting trends reported in different studies (Lawag *et al.*, 2022). Khalil et al. (2011) have identified six phenolic acids, *i.e.* gallic, syringic, caffeic, p-coumaric and trans-cinnmic acids, and five flavonoids, *i.e.* luteolin, kaempferol, catechin, apigenin and naringenin in Malaysian honeys, with each type of honey showing a different profile. A more recent research by Lawag et al. (2022) reported that there are as many as 161 different phenolic compounds that have been reported in honey from around the globe.

In general, phenolic contents in honey show significant therapeutic potential and give rise to its health-beneficial properties, *i.e.* the anti-inflammatory, anti-neoplastic and antimicrobial activities (Uthurry *et al.*, 2011; Cianciosi *et al.*, 2018). Phenolic compounds in honey also contribute to the good antioxidant activities due to its excellent free radicals scavenging ability (da Silva *et al.*, 2016; Cianciosi *et al.*, 2018).

Studies have reported positive correlation between TPC and antioxidant DPPH radical scavenging activity of honey consistently to the extend where TPC presents an estimation on the antioxidant level of honey (Sant'Ana *et al.*, 2014; Yap *et al.*, 2022). The DPPH parameter, however, is known to provide a more specific and direct measurement on antioxidant activity of honey from its radical scavenging capacity (Lewoyehu *et al.*, 2019). The DPPH results in Figure 4(b) shows consistency with TPC indicating Tualang honey has a higher antioxidant level than Kelulut honey. This finding is agreeing with previous studies showing high antioxidant activities in Tualang honey (Kishore *et al.*, 2011; Ahmed and Othman, 2013). However, from Figure 4(b), the high DPPH value of Tualang honey reduced from 85.16 to 63.92% whereas the lower antioxidant Kelulut honey increased from 49.73 to 54.74% after 26 weeks of post-harvest maturation. Thereafter, the DPPH values of Tualang honey rebounced back to 76.54% and Kelulut honey further increased to 63.95% at 44^{th} weeks of post-harvest maturation. The results suggested that the post-harvest maturation process might be benefical in elevating the antioxidant level of honey with lower antioxidant activities. The only other source that reported this similarly is Silva et al. (2023), who mentioned that antioxidant ABTS radical scavenging activity of Uruçú-Amarela honey reduced by 9.3% after 36 d and then rebounced by 6.6% to 8.22 TEAC $mM.g^{-1}$ after 180 d of post-harvest maturation. Previous studies have also reported contradicting antioxidant DPPH radical scavenging activity during honey storage. Zarei et al. (2019a) showed that DPPH value of Thyme honey reduced from 63.8 to 28.4% after one year whereas da Silva et al. (2020) reported a 30-52% DPPH increase for multifloral *Apis Mellifera* L. honey after 450 d of storage. The DPPH radical scavenging activity of a honey is affected by the complex reactions of its antioxidant bioactive compounds including the phenolic compounds (Alvarez-Suarez *et al.*,

2009), amino acids (Pérez *et al.*, 2007) and enzymes (Gheldof *et al.*, 2002). The formation and degradation of these bioactive compounds due to the reactions thereby cause fluctuations of DPPH radical scavenging values in honey (da Silva et al., 2016; da Silva et al., 2020). Braghini et al. (2020) reported six bioactive compounds that were not originally present in honey, *i.e.* vanillin, quercetin, gallic, p-hydroxymethylbenzoic, kaempferol and protocatechuic were detected after 90 d of storage.

Figure 5 shows that HMF of both honeys accumulated steadily during post-harvest maturation, from 0.3 and 0.25 mg/kg honey to 48 and 61.23 mg/kg honey for Tualang and Kelulut honey respectively at 26 weeks and reached a high value of 121.54 and 162.25 mg/kg honey after 52 weeks. The rate of HMF accumulation is higher in Kelulut than Tualang honey. In other honey studies, Ribeiro et al. (2018) showed no detection on HMF in Tiuba honey, Silva et al. (2023) recorded a low amount of HMF of 18.81 mg/kg honey in Uruçú-Amarela honey after 180 d of post-harvest maturation, Mouhoubi-Tafinine et al. (2018) reported a high concentration of 100.84-353.09 mg/kg honey after 9 months and Khalil et al. (2010) also reported a high content of 128.19 and 206.06 mg/kg honey for Tualang honey stored for one year. Hydroxymethylfurfural (HMF) is a widely recognised quality parameter of honey (Codex Alimentarius Commission, 2001). It is a potentially carcinogenic compound (Capuano and Fogliano, 2011) formed through Maillard reaction and hexose dehydration that occur during processing and ageing of honey (Choudhary *et al.*, 2020). Studies have consistently recorded low concentration of HMF in fresh honey (0-27 mg/kg honey) and it spiked high in heat-processed and aged honey (43-1426 mg/kg honey) (Khalil *et al.*, 2010; Braghini *et al.*, 2020; Sabireen *et al.*, 2020). Thus, it is generally accepted that a higher HMF indicates a lower quality of honey. A maximum concentration limit of 80 mg/kg honey is stated in Codex STAN 12-1981 for tropical honey (Codex Alimentarius Commission, 2001).

The rate of HMF formation in honey is affected by its physicochemical properties, *i.e.* water activity, acidity, amino acids, sugars, minerals and concentration of vitamin E as explained by Choudhary et al. (2020). The higher water activity (Figure 1) and acidity (Figure 2(b)) of Kelulut honey could have

facilitated the formation of HMF (Chuttong *et al.*, 2016b; Shapla *et al.*, 2018) thus resulting a higher HMF accumulation (Figure 5). Referring to Figure 5, at the 80 mg/kg honey HMF cut-off, the maximum post-harvest maturation period is around 44 weeks for Tualang and 34 weeks for Kelulut. This is recommended as the stabilisation period for Tualang and Kelulut honey with a safe increase of TPC by 12.61 and 54.66% at least as recorded at 26 weeks.

3.3 Bacterial Profile Changes

The results of next-generation sequencing yielded a total of 898608 16s high quality rRNA sequences. The sequences were assigned to 11032 bacterial operational taxonomic units (OTUs) respectively at 97% sequence similarity. The bacterial OTUs were successfully assigned to 26 phyla and 308 genera of bacteria. Figure 6 shows the simplified bacterial profiles of Kelulut honey at three stages of maturation, *i.e.* in the beginning, middle (26 weeks) and at the end of maturation of 52 weeks. Three bacterial phyla identified as the main and dominating bacterial that undergo substantial changes during the post-harvest maturation process are the *Proteobacteria*, *Firmicutes* and *Actinobacteria.* Previous studies by Hroncová et al. (2018) and Yap et al. (2022) have consistently reported the prevalence of *Proteobacteria*, *Firmicutes* and *Actinobacteria* phyla in honey. The relative abundance of *Proteobacteria* reduced from 71.7 to 70.7% while *Firmicutes* increased from 2.4 to 6.4% after 26 weeks of post-harvest maturation. At the end of 52 weeks, *Proteobacteria* reduced further to 53.9% while *Firmicutes* increased dramatically to 21%. The least changes was the *Actinobacteria* where it fluctuated with slight decrease from 7.2 to 4% at the end of 52 weeks. The phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria* are categorised as fermentative bacteria which possess the ability to breakdown saccharides to form lactic or acetic acid (Thierry *et al.*, 2011; Lee *et al.*, 2015). They are highly responsible for the fermentative activity and this is proven from the significant changes and shifting of their relative abundance during post-harvest maturation process.

Zooming into bacterial profiles of honey at genus level (Table 1), the *Proteobacteria* phyla of Kelulut had the most diverse genera, mainly environmental bacteria where bees may have acquired during

foraging activities. It was dominated by genera *Acinetobacteri* (2.25%), *Mesorhizobium* (4.95%), *Comamonadaceae* (5.65%), *Rhizobium* (5.9%), *Burkhoderia* (10.22%) and *Ralstonia* (24.22%). *Burkhoderia* genera was detected in honeybees and bumble bee specimens (Martinson *et al.*, 2011) while *Ralstonia* genera was isolated from Australian stingless bees, *i.e. Austroplebeia australis*, *Tetragonula carbonaria* and *Tetragonula hockingsii* (Leonhardt and Kaltenpoth, 2014). The relative abundance of *Burkhoderia* and *Ralstonia* genera reduced by 0.84 and 5.21% respectively at the end of 52 weeks of post-harvest maturation. *Burkhoderia sp.* is a common environmental bacteria that can stimulate growth of plants, form an antagonistic interactions with fungi and establish a symbiosis with insects (Eberl and Vandamme, 2016). However, despite exhibiting some functional effects, there are also some bacterial species within the genus *Burkhoderia* that possess pathogenic potential. According to Eberl and Vandamme (2016), *Burkholderia pseudomallei* and *Burkholderia mallei* were catogorised as animal pathogens while *Burkholderia cepacia*, *Burkholderia caryophylli*, and *Burkholderia gladioli* were considered as plant pathogens. Likewise, *Ralstonia* is a non-fermenting Gram-negative bacteria genus that also consisted of some pathogenic species, *i.e. Ralstonia pickettii*, *Ralstonia insidiosa* and *Ralstonia mannitolilytica* (Ryan and Adley, 2014). The reduction of *Burkhoderia* and *Ralstonia* genera during post-harvest maturation sugggests the benefits of maturation process which have reduced potentially pathogenic bacteria genera in Kelulut honey.

The two beneficial bacteria genera of *Bacillus* and *Lactobacillus* that have been reported regularly in honey studies are the dominant genera of phylum *Firmicutes*, constituting to 0.22 and 1.29% of Kelulut honey's bacterial profile (Alberoni *et al.*, 2016; Audisio, 2017; Anjum *et al.*, 2018). The relative abundance of *Bacillus* and *Lactobacillus* increased to 2.6 and 6.25% respectively after 26 weeks of post-harvest maturation. The values continue to increase as the post-harvest maturation progressed, reaching a higher value of 4.85 and 18.46% respectively for *Bacillus* and *Lactobacillus* genera after 52 weeks. Both *Bacillus* and *Lactobacillus* are recognised as probiotic bacteria (Esawy *et al.*, 2012; Zulkhairi Amin *et al.*, 2019) due to its various reported health-beneficial factors. The *Bacillus sp.* possess good antioxidant potential and is able to produce lipase and cholesterol oxidase

enzyme that help in reducing the low-density lipoprotein cholesterol (Abdelsamad *et al.*, 2022). Likewise, the *Lactobacillus spp.* is also promising for its antimicrobial effects against some foodborne pathogens, *i.e. E. coli*, *Salmonella enteritidis*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Shigella flexneri* (Lashani *et al.*, 2020). The increased prevalence of *Bacillus* and *Lactobacillus* genera during post-harvest maturation of honey is potentially positive in enhancing honey's probiotic qualities as suggested by earlier work of Yap et al. (2022).

The reduction of phylum *Actinobacteria* is mostly attributed to the *Propionibacterium* genus where relative abundance reduced from 4.21 to 1.03%. Although *Propionibacteria* is more commonly detected in dairy products, sourdough and fermented vegetables (Thierry *et al.*, 2011; Gautier, 2014), it has also been detected in the gut of honeybees (Callegari *et al.*, 2021). It is a fermentative bacteria that produces propionic acid, the major end product that effectively prevent food spoilage caused by yeast and also 1,4-dihydroxy-2-naphthoic acid that gives benefit of stimulating growth of probiotic bacteria (Thierry *et al.*, 2011). The activities of *Propionibacterium* genus is said to be inhibited by the increasing acidity during fermentation (Gautier, 2014; Piwowarek *et al.*, 2018), which explains its reduction during post-harvest maturation.

4.0 Conclusions

Post-harvest maturation is a simple honey preservation technique that allows honey to age naturally in a controlled condition. Natural fermentation occured during post-harvest maturation process of honey due to its consistently high water activity (> 0.6) and the presence of fermentative bacteria, *i.e. Proteobacteria*, *Firmicutes* and *Actinobacteria* phyla resulting increase in free acidity by 2.05 and 2.24% for Tualang and Kelulut honey respectively while its fructose and glucose content decreased by 1.05-10.62% and 12.11-15.77% after the recommended maturation period of 26 weeks. At this recommended maturation period, the total phenolic content of Tualang and Kelulut honey increased by 12.61 and 54.66% respectively with HMF concentrations maintaining between 48.00-61.23 mg/kg honey, well within the permissible limit. The results suggest that post-harvest maturation is a good

preservation technique for honey, particularly for Kelulut honey because of improved bioactivity from increased DPPH radical scavenging activity by 10.07% and improved probiotic bacteria profile of the *Bacillus* and *Lactobacillus* genera in terms of relative abundance increase between 1.51 and 8.85%.

Conflict of Interest

Authors declare there is no conflicts of interest in this research.

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Ethical Guidelines

Ethics approval was not required for this research.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Legends to Figures

Figure 1: The changes of water activity in honey during post-harvest maturation

Figure 2: The changes of (a) pH and (b) free acidity in honey during post-harvest maturation

Figure 3: The changes of (a) fructose, (b) glucose and (c) maltose concentration in honey during post-harvest maturation

Figure 4: The changes of (a) total phenolic content and (b) DPPH radical scavenging activity in honey during post-harvest maturation.

Figure 5: The changes of hydroxymethylfurfural (HMF) in honey during post-harvest maturation

Figure 6: The bacterial profiles of Kelulut honey during post-harvest maturation at phyla level