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Research

Low Heating Effects on The Total Microbial Activity and Physico-Chemical Properties of Stingless Bee (*Heterotrigona itama*) Honey

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ABSTRACT

The high moisture content of stingless bee honey (SBH) is a worrisome problem and heat treatment is used to reduce the moisture and maintain the honey's quality by destroying the microorganisms that affect the physico-chemical properties of honey during storage. Low heat treatment (45 °C) for 0, 30, 60, 90, and 120 min were conducted to determine the total microbial activity using fluorescein diacetate hydrolysis (FDA). The total microbial population that subsequently affected the physico-chemical properties was also analyzed. The total microbial activities of SBH were significantly reduced after thermal treatment at 45 °C for 90 min (63.76 µg FDA/g/h) and 120 min (62.43 µg FDA/g/h) compared with control (67.127 µg FDA/g/h). Also, the moisture content, electrical conductivity (EC), pH, and free acidity of the low heat-treated SBH at all durations were significantly reduced compared with the control. The total microbial activity was detected as significantly correlated to bacterial and fungal populations, moisture content, EC, pH, and free acidity of low heat-treated SBH. Low heat treatment at 45 °C for 120 min was efficient to reduce the total microbial activity, total acidity, and increasing the pH of SBH. Prolonging the heating duration is suggested to further reduce the water content, total microbial activity and further increase the shelf life of SBH.

Key words: Microbiological; thermal treatment; microbial activity; apicultural industry

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INTRODUCTION

Stingless bee honey (SBH) consists of high medicinal value, making it a high-priced honey and widely kept by beekeepers. Also, SBH produced from Malaysia consists of high moisture ranging from 27 - 31% (Lim *et al*., 2019). High water content is recognized as the key factor contributing to the fermentation process in Honey (Akbulut *et al*., 2009). There is various microbiota associated with stingless bee, including virus, bacteria, yeast, and fungus. Bacteria are important in the biochemical transformation of nectar and pollen that benefits the bees (Ngalimat *et al*., 2019). The most abundant bacterial phyla in SBH of *H. itama* were reported as Firmicutes and followed by Proteobacteria (Rosli *et al*., 2020). High water content subsequently induces possible high microbiological activity that can further hasten the spoilage of honey (Oddo *et al*., 2008).

Various methods have been employed to remove the water content in honey. Thermal treatment of honey is a practical method for preventing or postponing crystallization, destroying microorganisms, and facilitating filling by viscosity reduction (Lucero *et al*., 2002). Commercial heating processes can alter the nutritional quality inside, therefore the identification of the optimum condition to maintain shelf life is essential (Saric *et al*., 2013). Also, pasteurization of honey using high temperatures up to 60 – 70 °C to kill the microorganism before storage in the refrigerator temperatures was reported effective to maintain the quality of honey during storage (Contrera et al., 2011). Besides, heating incorrectly can favor hydroxymethylfurfural (HMF)

whereby decreasing levels of diastase activity of honey can destroy the honey quality and be detrimental to human health.

In addition, the heating temperature can directly affect the microbial activity and population distribution in the honey and subsequently change the enzymatic activity and physico-chemical properties. For instance, honey heating at 63, 65, and 68 °C for 35, 25, and 7.5 min, respectively can destroy the yeast cells completely (Kuplulu, 2006). Up to date, there is no study reported on the low heating temperature (45 °C) affecting the total microbial activity based on the determination of total enzymatic activity using fluorescein diacetate hydrolysis (FDA) in correlation to the physico-chemical properties of heat-treated stingless bee honey (*Heterotrigona itama*). FDA is a simple, sensitive, and rapid method to determine the total microbial activity mainly in compost (Schnürer & Rosswall, 1982), soil (Ng *et al*., 2015, Nguyen *et al*., 2018), and aquatic studies (Fontvieille *et al*., 1991).

To extend the knowledge of heat treatment on stingless bee honey, this study aimed to determine the microbiological properties of heat-treated stingless bee honey based on the total microbial activity using FDA and population associated with the physico-chemical properties. The outcomes of the study are an essential aid to develop the quality of stingless bee honey using low heat treatment to prolong the shelf life.

MATERIAL AND METHODS

Stingless bee honey sample preparation

The healthy stingless beehives of *Heterotrigona itama* were sampled. The beehive was uncapped and small tubing was inserted into the honey pot to drain the stingless bee honey. All the coarse material was manually removed from the collected honey using a sterilized spatula. A stainless-steel sieve of mesh with a diameter of 0.5 mm was used for straining to remove the extraneous matter. The stingless bee honey sample was then homogenized by stirring thoroughly but carefully to prevent air in the surrounding from being stirred into the honey. The stingless bee honey (200 mL) was filled into the blue cap bottle before preparing for heat treatment. This experiment was conducted in a complete randomized design (CRD) with five treatments (control, 30 min, 60 min, 90 min & 120 min) and five replications. All experiments were repeated twice.

Low heat treatment using a water bath

The heat treatment process was started after the water bath achieved the constant temperature of 45 °C. Stingless bees honey samples were placed into the water bath for 30, 60, 90, and 120 min before being cooled in a desiccator and used for analysis. Stingless bee honey samples were sealed hermetically with a thermocouple placed into the geometric center of a tube to monitor sample temperature. After the time reached, the borosilicate glass bottle (DURAN) was taken out and cooled to room temperature.

Determination of the microbiological properties

Fluorescein diacetate hydrolysis (FDA)

FDA method was conducted as described by Adam and Duncan (2001) to determine the microbial activity in honey. One gram of honey sample was weighed in sterile McCartney bottles and 7.5 mL of potassium phosphate buffer (pH 7.6, 60 mL) was allowed to equilibrate at 25 °C on an end-over-end shaker. FDA solution (1000 µg/mL) at 0.1 mL was added to start the reaction then returning the sample's beaker to the shaker. The stingless bee honey sample was left to incubate at 25 °C for 30 min. FDA solution without SBH sample was served as control. After 30 min of incubation, 7.5 mL of chloroform: methanol (ratio 2:1) was added immediately into the samples and blanks respectively to stop the reaction.

The supernatant fraction (1 mL) was decanted to a 1 mL cuvette and measured absorbance at 490 nm on a spectrophotometer against the sample. To make sure that all samples lie within range, a calibration curve was constructed by carrying out a triplicate of 7.5 mL aliquots of each concentration of fluorescein working standards into McCartney bottles, then duplicate was extracted with chloroform: methanol and OD_{490nm} was determined of the clarified upper phase as for the samples.

Total plate count

The stingless bee honey sample (1 mL) was measured and inserted into a sterilized McCartney bottle. Then, 9 mL of sterilized distilled water was added in and shaken. From the homogenous suspension, the serial dilution was carried out up to 10^{-4} dilutions and 0.1 mL aliquots of 10^{-2}, $10^{-3,}$ and 10^{-4} were poured into the Nutrient Agar (NA) and Potato Dextrose Agar (PDA), respectively. Each dilution was cultured in triplicate by the spread plate method. The inoculated PDA plates were incubated at 28 ± 2 °C for 7 days while the inoculated NA plates were incubated for 24 h (Thawai *et al*., 2004). The colony formed on PDA and NA plates were determined and transformed into colony-forming units (CFU).

Determination of the physico-chemical properties

Moisture content (MC)

The moisture content of honey was measured by using the reflectometric method. A moisture analyzer (Model: Madwag MA 50/1. R) was used in this study. Three drops of honey samples were randomly sampled and placed on the light blue area of a refractometer and covered completely. The light gate was closed firmly to spread the honey evenly over the blue plate. The reading of the moisture content was recorded as X and calculated using the following formula (Mohammed Hassan *et* al., 2021).

Moisture content (%) = [−0.2681 – log (X − 1)/0.002243]

Electrical conductivity (EC)

The electrical conductivity of SBH was measured using a conductivity meter (Model: Hanna HI5321-02, USA). Five grams of SBH sample was diluted in 25 mL of deionized water. A conductivity probe was immersed into a stingless bee honey sample. Measurement of electrical conductance (G) was obtained after the temperature was equilibrated to 20 °C. As conductivity meters are direct currents, the measurement time was kept as short as possible to avoid false results due to polarization effects (Bogdanov *et al*., 2002).

pH and free acidity

A stingless bee honey sample (10 g) was dissolved into 75 mL of deionized water in a 250 mL beaker. The solution was stirred with a magnetic stirrer before measuring the pH using a pH meter (Model: Orion910500, USA). The electrode of the pH meter was immersed into the solution and the pH reading was recorded when the constant pH reading was obtained. The solution was titrated with 0.1 M NaOH until it reached pH 8.30 (a steady reading should be obtained within 120 s from the titration). The pH value was reported to two decimal places. The free acidity was expressed as milli equivalents per kg of honey sample calculated (Bogdanov *et al*., 2002):

$$\text{Acidity } \left(\frac{meg}{kg}\right) = \text{volume (mL) of 0.1 M NaOH} \times 10$$

Where 10 indicates the dilution factors of the honey sample used for analysis. The result obtained was recorded in one decimal.

Experimental design and data analysis

To test the significant differences in the total microbial activity, bacterial and fungal populations, and the physico-chemical properties due to the heat treatment at different durations, data collected were subjected to one-way analysis of variance (ANOVA) and further test for significance with Fisher's Protected Least Significant Difference Test (LSD) using SPSS statistics 20 software. A significant difference was considered at $p<0.05$. Pearson's correlation analysis was conducted using the Performance Analytics package in R program 4.1.3.

RESULTS

Total microbial activity

The total microbial activity based on total enzymatic activities using florescent diacetate hydrolysis of SBH indicated a decreasing trend with increasing the duration of heat treatment (45 °C) from 0, 30, 60, 90 and120 min (Figure 1). Heat-treated SBH exhibited significantly low total microbial activities at all treatments compared to the control (67.13 µg FDA/g/h). SBH exposed to 120 min of heat at 45 °C recorded the lowest total microbial activity (62.43 µg FDA/g/ h).

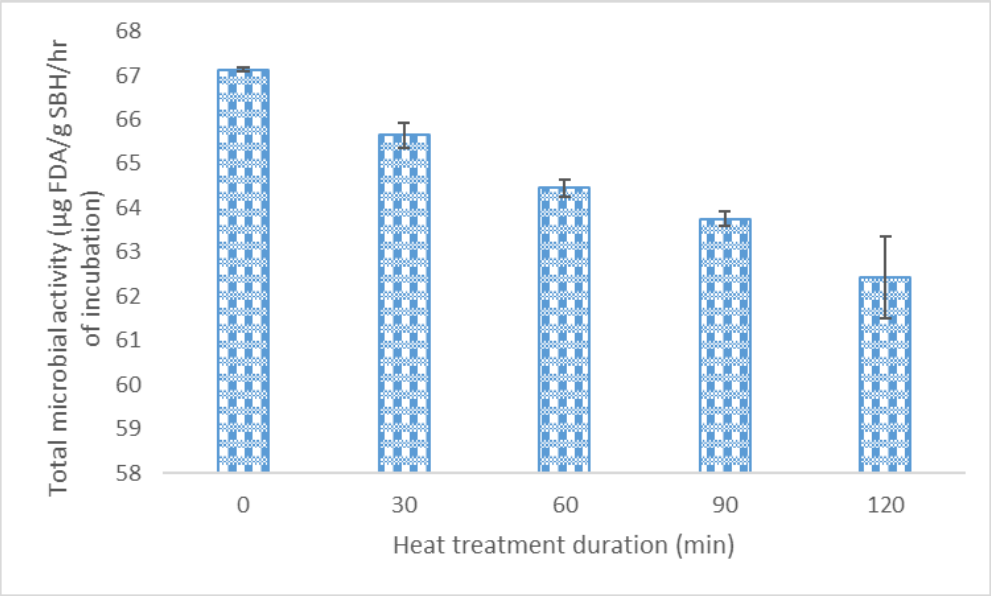

Fig. 1. Total microbial activity of stingless bee honey at different durations of heat treatments (45 °C).

Bacterial and fungal population

The population of both bacterial and fungal were detected in the decreasing trends with prolonged thermal treatment duration (Figures 2 & 3). Bacterial populations were decreased from 5.26 log CFU/mL (1.83 × 10^5 CFU/mL) in the control to log 5.20 CFU/mL (1.6 × 10^5 CFU/mL) in SBH treated with 120 min at 45 °C. SBH exposed to 120 min of heat treatment significantly reduced the total culturable bacteria population on NA compared with control, 30 and 60 min.

The fungal populations of SBH also decreased from 4.78 log CFU/mL to 4.52 log CFU/mL with an increase in thermal exposure duration (Figure 3). Thermal treatment at 45 °C for 120 min significantly reduced the fungal population.

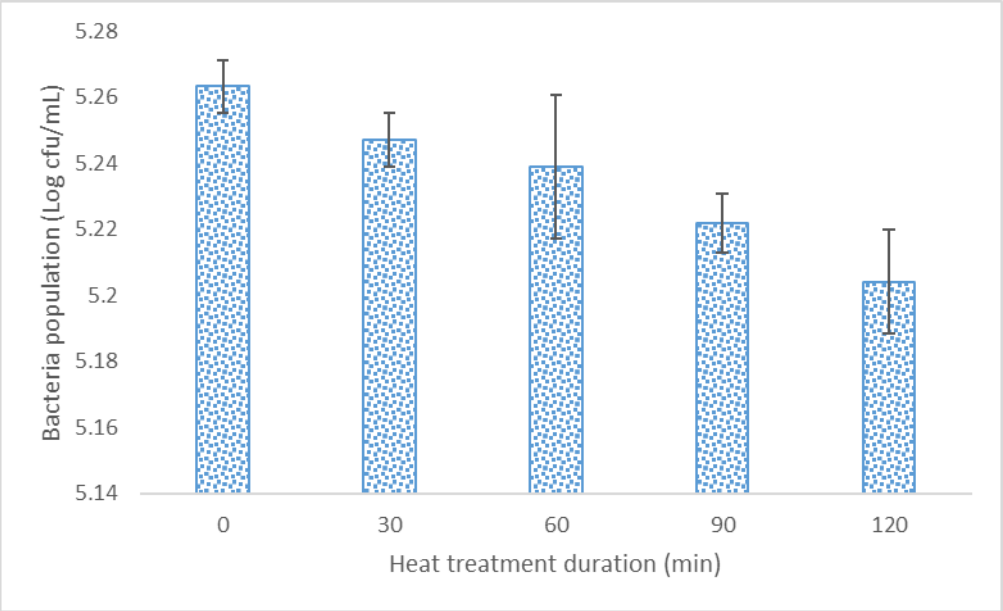

Fig. 2. Bacterial population of stingless bee honey at different durations of heat treatments.

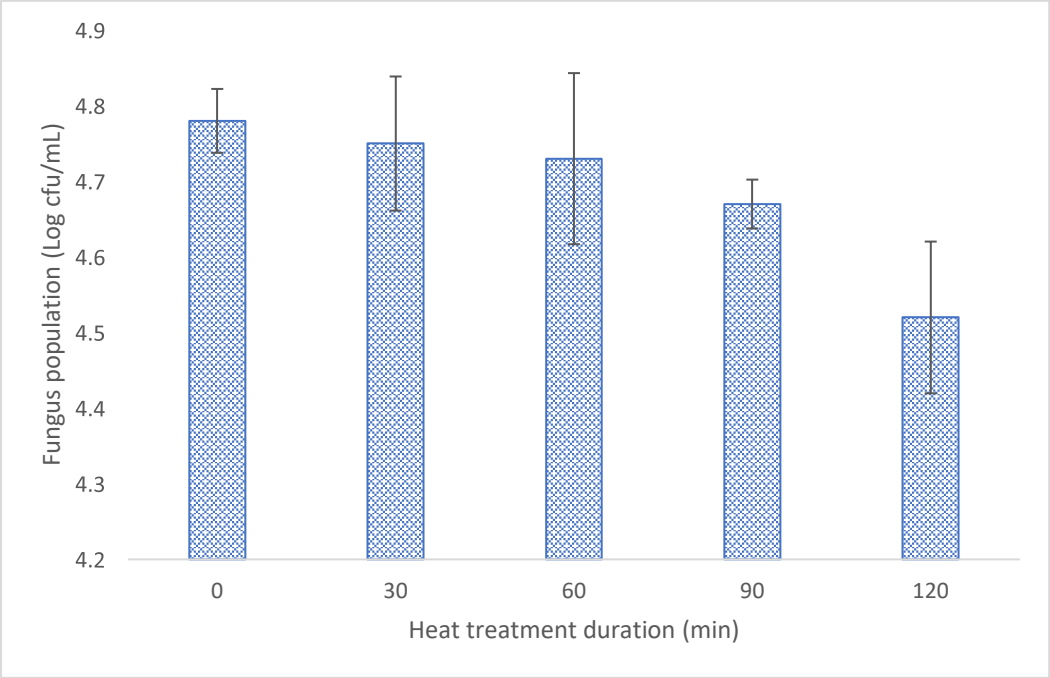

Fig. 3. Fungal population of stingless bee honey at different durations of heat treatments.

Moisture content

Thermal treatment at 45 °C with exposure durations of 30, 60, 90, and 120 min significantly reduced the moisture content in SBH compared with control (without thermal treatment) (Figure 4). The moisture content of SBH after 120 min of heat treatment was reduced to 29% compared with the control (without heat treatment) was 31.85%. However, there were no significant differences between the other thermal treatments' duration.

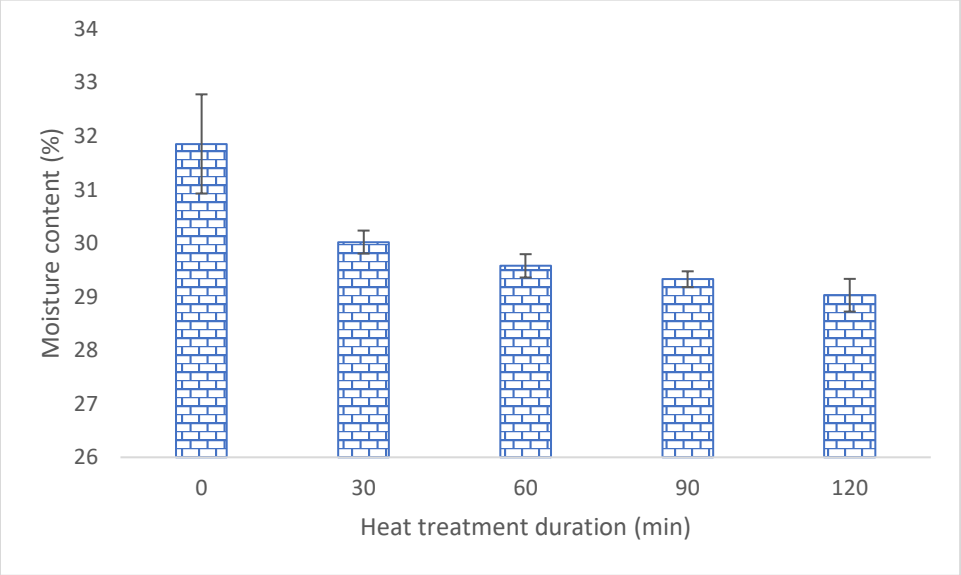

Fig. 4. Moisture content of stingless bee honey at different durations of heat treatments.

Electrical conductivity

The electrical conductivity (EC) was detected as inversely proportional to the moisture content with the increase in thermal treatment duration (Figure 5). Electrical conductivity was detected to increase significantly from 1.25 mS/cm (control) to 1.39 mS/cm (120 min).

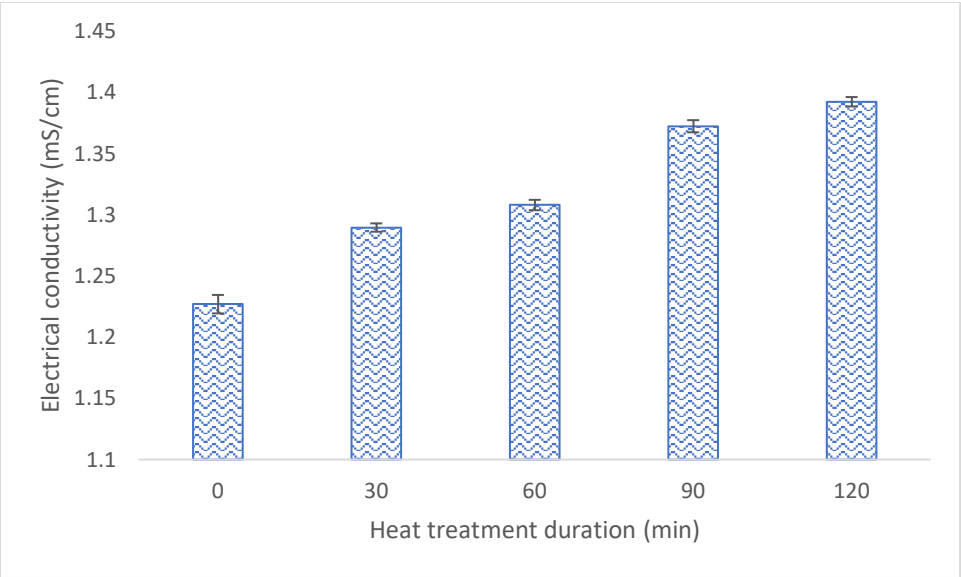

Fig. 5. Electrical conductivity of stingless bee honey at different durations of heat treatments.

pH and free acidity

The least acidity of the SBH, the higher the pH value was detected as illustrated in Figures 6 and 7. The pH and free acidity of SBH exposed to heat treatments were significant differences compared with the control.

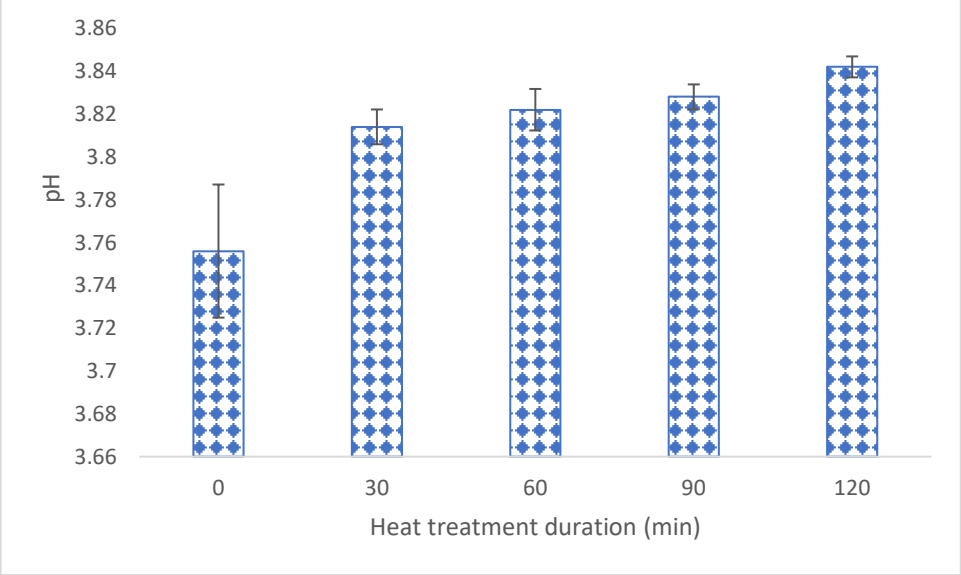

Fig. 6. pH value of stingless bee honey at different durations of heat treatments.

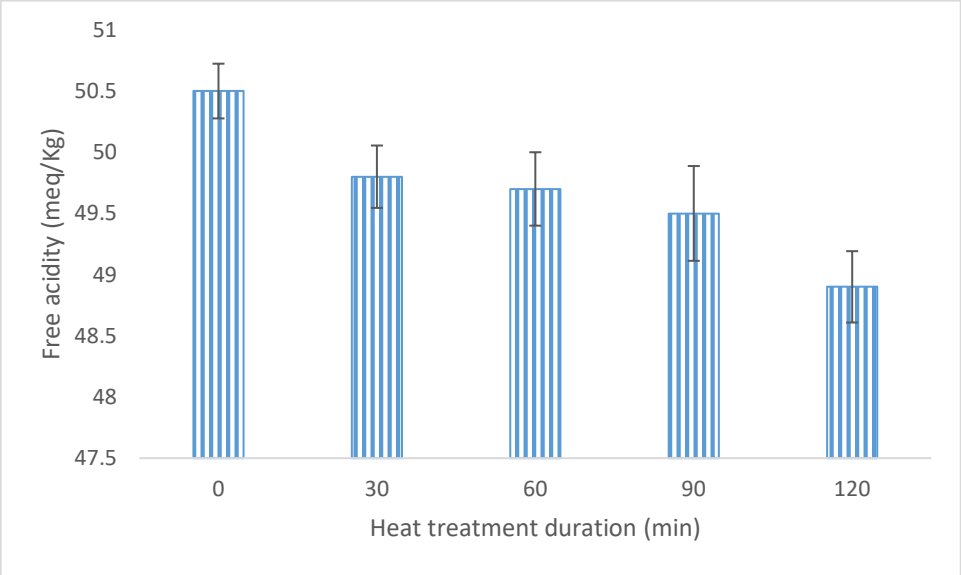

Fig. 7. Free acidity of stingless bee honey at different durations of heat treatments.

Correlation between the total microbial activity and physico-chemical properties

The total microbial activity (TMA) in SBH significantly and positively correlated with fungal (FP) (r = 0.93) and bacterial populations (BP) (r = 0.99), moisture content (MC) (r = 0.93), and free acidity (FA) (r = 0.98) as shown in Figure 8. On the other hand, a significant negative correlation was found between total microbial activity (TMA) and electrical conductivity (EC) (r = -0.97) and pH (r = -0.94).

DISCUSSION

Honey is a natural food produced by bees, and it contains about 200 substances mainly sugar, and other substances such as enzymes, organic acids, vitamins, phenolic compounds, minerals, and aromatic substances (Küçük *et al*., 2007; Silva, *et al*., 2016). These compositions in honey change due to the different chemical reactions that happened during storage and thermal treatment that modify honey constituents (Moreira *et al*., 2010). Honey from stingless bees (*Heterotrigona itama*) was reported to have higher water content than honey from *Apis* (Halwany *et al*., 2020). Hence, the fermentation and crystallization processes are getting active in SBH, especially with high water content (Yap *et al*., 2019). Therefore, thermal treatment of honey is usually applied to reduce the water content and directly inactivate the microorganisms. A heating temperature ranged 30 – 140 °C at various duration had been practiced by honey producers to reduce the water content in the honey below 20% aimed to prolong shelf life (Guo *et al*., 2011).

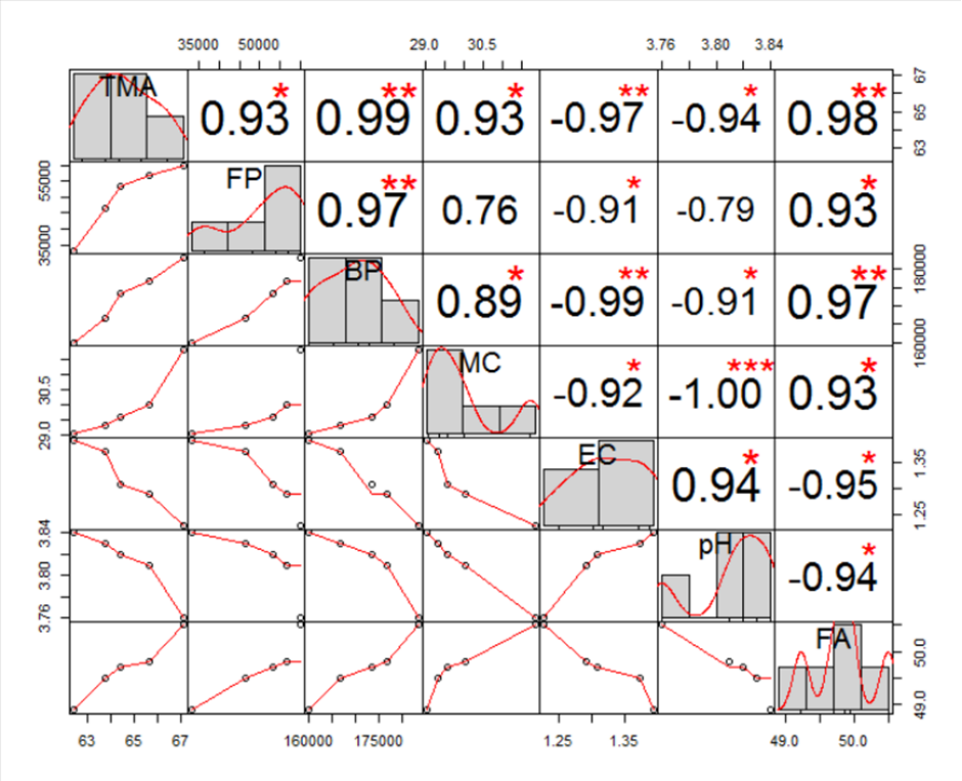

Fig. 8. Correlation matrix plot with significance levels between microbiological and physico-chemical properties. The lower triangular matrix is composed of bivariate scatter plots with a fitted line. The upper triangular matrix shows the Pearson correlation plus significance level (as stars). Each significance level is associated with a symbol: *p*-values 0.01 (**), 0.05 (*). This plot was generated with the Performance Analytics package in R grogram 4.1.3. TMA is the total microbial activity, FP is the fungal population, BP is the bacterial population, MC is the moisture content, EC is the electrical conductivity and FA is the free acidity in the SBH.

Heat treatment of SBH at 45 °C for 120 min significantly reduced the moisture content. Reduce moisture content associated to decelerate the fermentation and crystallization. This was in line with the total microbial activity of SBH after heat treatment at 45 °C significantly and positively correlated to fungal (FP) and bacterial populations (BP), moisture content (MC), and free acidity (FA). The moisture content of the SBH (*H. itama*) before heat treatment was 31.85% and reduced to 29% after 120 min of heat treatment. The moisture contents of SBH ranged from 19 - 45% was also reported by Souza *et al*. (2006). The variation of moisture content in honey is depending on the bee species, the geographical origin (Bijlsma, 2006), the harvesting season (Mohammed Hassan *et al*., 2021), the botanical origin of the honey, the harvesting technique, and storage conditions.

The high moisture content in SBH promotes microbial activity, growth, and propagation that influence the quality due to fermentation and various biochemical activities. This is evident that a high microorganism population can increase the acidity in honey, by fermenting honey by acting on glucose and fructose and converting it into alcohol and carbon dioxide (Finola *et al*., 2007). Alcohol can be broken down into acetic acid and water which makes the fermented honey more acidic when in the presence of oxygen (Prica *et al*., 2014). This scenario closely explained the positive correlation of free acidity to the total microbial activity in heat-treated SBH.

It is common that honey to get contamination by the source of indigenous microflora, during or post-processing and resulting in spoilage or the persistence of some bacteria in honey (Olaitan *et al*., 2007). Microbes such as molds, commonly found in honey may survive, so high activity is often related to recent contamination during processing by the environment or by equipment used (Finola *et al*., 2007, Snowdon & Cliver, 1996). The presence of these microorganisms could imply the hygiene indicators in which the product was processed, handled, and stored adopted by the processor (Ayansola & Banjo, 2002). Results showed that the total microbial activity of SBH decreased with the increasing heating period. Prolong heating was associated with destroying microbes in stingless bee honey as indicated by the significant difference in heating duration. These results agreed with the findings reported by Eshete and Eshete (2019), where thermal treatment at 63 °C for a prolonged period destroyed the yeast cells in SBH. The total microbial activity decreased progressively in this study and was associated with the decreasing microbial population due to thermal treatment.

The electrical conductivity (EC) of honey is associated with mineral content and acidity, revealing the presence of ions, organic acids, and proteins (Yücel & Sultanoglu, 2013). The EC of SBH after heat treatment still falls within the average range of SBH honey collected from Southeast Asia (Chuttong *et al*., 2016). The SBH after heat treatment exhibited a significantly increasing over the heating duration from 1.25 mS/cm

(control) to 1.39 mS/cm (120 min). The rise in EC increased along with thermal treatment duration was in line with Ngoi & Vivian, (2016). Also, the electrical conductivity of SBH was negative and significantly correlated (r = -0.92) with moisture content. This suggested that the viscosity of honey increases although in small changes after heat treatment (Chong *et al*., 2017). Consequently, the sample contains lesser moisture and more dry matter which results in more noticeable electrical conductivity (Sancho *et al*., 1992). The electrical conductivity of the honey is closely related to the concentration of mineral salts, organic acids, and proteins with great variability accordingly to the flora origin (Terrab *et al*., 2003).

The low pH value of SBH affected consumers' preference as tasted sour. The pH values of honey are important and change during honey extraction and storage, due to their influence on texture, stability, and endurance (Terrab *et al*., 2003), most commercial honey generally ranged between 3.5 - 6.0 (Singh & Bath, 1997, Esti *et al*., 1997, Al-Khalifa & Al-Arify, 1999, Anupama *et al*., 2003). The pH value of SBH increased from 3.75 (control) to 3.84 (120 min) after heat treatment at 45 °C. These pH values were still within the acceptance range of stingless bee honey collected from Southeast Asia (Chuttong, 2016). The low pH of SBH was associated with the presence of organic acids such as gluconic, formic, and acetic in the honey (Osmojasola. 2002).

In this study, a negative and significant correlation between the pH of SBH to the total microbial activity, and the fungal and bacterial population was exhibited. This might be associated with heat treatment of SBH significantly inhibiting the total microbial activity, a fungal and bacterial population that indirectly reduces the fermentation process of honey into acetic acid. Also, the pH of the SBH ranged from 3.75 - 3.84 might be an inhibitory factor for the growth of most microorganisms, as the optimum pH for microorganisms is ranged from 7.2 - 7.4 (Suárez-Luque *et al*., 2002). A possible explanation for the increasing pH in SBH with a prolonged heating period may be associated with the volatility of the organic acid that is present in SBH (Osmojasola, 2002).

CONCLUSION

Low thermal treatment at 45 °C significantly improved the microbiological and physiochemical properties of SBH. The reduction in moisture content of SBH helps to reduce the growth of the microorganism population and the total microbial activity. Results reveal that low heat treatment at 45 °C for 120 min can be considered as a possible treatment to maintain the quality of SBH. Further increasing the heating duration of SBH is also suggested to reduce the water content in SBH below 20% to extend the shelf life through limiting the microbiological activity and growth in the SBH.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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