

Effect of storage on viability of lactic acid bacteria and nutritional stability of raw Malaysian *Heterotrigona itama* honey

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Abstract. The nutritional benefits of stingless bee (*Heterotrigona itama*) honey originates from a complex of essential nutrients including carbohydrates, organic acids alongside an assortment of live lactic acid bacteria (LAB). Nonetheless, little is known about the nutritional composition over an extended storage duration. Herein, this study assessed the nutritional contents in Malaysian raw *H. itama* honey in response to changes in viability of LAB over 28 days of storage. LAB total count was monitored against quality parameters *viz.* pH, total flavonoid, total phenolic and antioxidant scavenging activity. Results revealed that LAB population was no longer detected after day 28 days of storage ($p < 0.05$) as compared to initial count of 2.62×10^5 CFU/g. The study recorded minor changes in pH that was reduced from pH 2.96 to pH 2.86. Notably, relatively stable ($p > 0.05$) total flavonoid (36-60 mg QEA/100 g) and total phenolic contents (38-62 mg GAE/100 g), with antioxidant scavenging activity (IC_{50} between 24- 45 mg/mL) were detected for raw *H. itama* honey. It was irrefutably demonstrated that 28 days of storage does little to depreciate the nutritive value of Malaysian raw *H. itama* honey, despite the appreciable decline in live LAB counts.

1. Introduction

Honey of the stingless bee, *Heterotrigona itama* or natively known as Kelulut among the Malaysian, is a highly valued commodity for its therapeutic applications for preventing throat inflammation, gastritis, cataract, as well as supplement to expedite post-birth recovery etc [1,2]. The aforementioned healing properties was possibly contributed by the transfer of a unique array of symbiont microorganisms, i.e. *Lactobacillus* spp and *Fructobacillus* spp. from their stomach into the nectar, during the enzymatic transformation into the end-product, honey [3-6]. For such reasons, many researchers have extensively characterized the plethora of 'good bacteria', particularly those from the lactic acid bacteria (LAB) family and unravelled their potential metabolic function. While the natural human microbiota is stable,



the daily intake of new LAB symbionts in raw *H. itama* honey can populate the intestinal tract and beneficially maintain the overall health of the consumer. Phytochemical compounds derived from nectar sources known for their excellent antioxidant properties, for instance, flavonoids and polyphenols, are also present in raw *H. itama* honey [7,8].

Despite the high nutritive value of raw *H. itama* honey, the question prevails whether the nutritional composition persists or fluctuates over long storage durations. It is somewhat, a rather valid question as most consumers have the tendency to slowly consume the raw honey as it is a rather pricey commodity. The market price of this honey can reach \$100/kg almost twice higher than those produced by *Apis mellifera* (\$20–40/kg) [9,10,1]. Moreover, most raw honey bottles are stored on supermarket shelves for varying durations before they are bought and consumed by consumers. A matter of fact, studies focusing on the changes in nutritional composition of the Malaysian raw *H. itama* honey remains unreported till this day. Therefore, this study believes that monitoring the microbiological and chemical stability of active components in raw Malaysian *H. itama* honey during storage is necessary. This is to ensure that the consumers truly understand and reap the nutritive benefit of the honey, as well as to safeguard their well-being.

Survey of the literature have shown that the impact of extended storage on certain types of honey components i.e. strength and types of antioxidants can vary and it is a complex issue for scientists as well as retailers, since contradictory results have been reported [11,12]. Some studies have shown that the quality and biochemical properties of honey are not only associated with the nectar source, but also with the duration of honey storage [11]. For instance, Wang et al. (2004) reported a decrease in antioxidant activity of several honey samples after 6 months of storage [13]. However, another study by Gheldof and Engeseth (2002) that used the same incubation method, found the antioxidant activity of honey samples unchanged despite an extended storage that spanned over 2 years [14]. With this in mind, this study aimed to investigate the effect of an extended duration over the course of 28 days under ambient conditions ($25 \pm 2^\circ\text{C}$), with regards to viability of LAB *versus* quality parameters of raw *H. itama* honey (pH, total phenolic content, total flavonoid content and antioxidant capacity). Therefore, for the first time, this study profiles the changes in nutritive and physicochemical properties Malaysian raw *H. itama* honey, in relation to LAB viability over a relatively extended storage duration. Furthermore, the findings of this study may contribute to the body of knowledge on the long-term nutritional compositional changes in Malaysian raw *H. itama* honey.

2. Methodology

Two samples of raw *H. itama* honey were collected from Bukit Bilut in Pahang, and Johor, and designated as H1 and H2, respectively. Both honeys were used as the test samples and were analyzed for LAB viability (in colony forming units per gram, CFU/g), pH, phenolic and flavonoid content and antioxidant activity, according to the methods described previously [15, 16]. All analyses were carried out in triplicates and were done immediately after harvesting (day 0), and at every one-week interval of storage (days 7, 14, 21 and 28) under ambient temperature ($\pm 25^\circ\text{C}$). All data were submitted for analysis of variance (ANOVA) to evaluate for statistical significance. The treatment was followed by post-hoc Tukey test to evaluate any significance differences between the tested parameters during the 28 days storage

3. Results and Discussions

3.1. Visibility of Lactic Acid Bacteria in *H. itama* honey

Results for the viability of LAB in the raw *H. itama* honey samples are presented in Table 1. From the findings, both samples showed a significant ($p < 0.05$) monotonic decline in live LAB counts over the 28 days of storage ($p < 0.05$). For sample H1, the LAB counts initially observed at 2.73×10^5 CFU/g on day 0, decreased to 1.77×10^4 CFU/g, 2.33×10^3 CFU/g, 1.47×10^2 CFU/g and finally zero on days 7, 14, 21 and 28, respectively. The raw *H. itama* honey H2 sample, on the contrary, gave a lower initial LAB count (2.5×10^4 CFU/g) on day 0. Consistent with the lower LAB initial count, live LAB decreased

quicker on day 7 at 2.43×10^3 CFU/g, day 14 at 2.03×10^2 CFU/g and no longer detectable by day 21. The live LAB decline profile seen in this study was almost equally similar to a report by Olofsson et al. (2008), except the monitored storage duration was for 2 months [17]. They described that the Fhon2 LAB strain was viable at a concentration of 5×10^4 CFU/g in a fresh raspberry honey, and detectable for up to two months of storage. The strain was isolated from the stomach of the bee and nectar, at the start of the storage and after two months, but not in the honey itself. The findings in this study corroborated the contents in a bee stomach and nectar being in a microaerobic state, filled with nectar sugars and nutrients, under an optimal temperature of 35°C , which environments are optimal niches for survival of LAB [18].

The results also suggest that the high LAB count in early stages of raw honey storage could be due to the higher availability of carbohydrate-rich substances in raw *H. itama* honey, for instance, fructo- and gluco-oligosaccharide. These sugars are known substrates that stimulate growth of LAB, as well as to ensure their viability [19,20]. It is important to note that LAB does not multiply in the honey after a certain duration of storage. This has been linked to two major stresses such as, acid and osmotic stress, that are elevated under extensive storage duration which tend to affect the bee metabolism.

Table 1. Total count of LAB present in honey H1 and H2 throughout 28 days of storage

Honey sample	Total count of LAB (CFU/g)	
	H1	H2
Day 0	2.73×10^5	2.5×10^4
Day 7	1.77×10^4	2.43×10^3
Day 14	2.33×10^3	2.03×10^2
Day 21	1.47×10^2	Nd
Day 28	Nd	Nd

*Nd refers to not detected

3.2. pH

pH is an important factor to monitor during the honey storage because the condition is highly related to the stability and shelf life of the product [21]. In present study, pH of raw *H. itama* honey H1 and H2 was assessed over the period of 28 days and the results are illustrated in Table 2. Apparently, the honey sample H1 is marginally acidic than H2 with pH ranging between pH 2.97–2.82 and pH 2.98–2.85 respectively. Notably, honey samples of H1 demonstrated significant declines in pH from day 0 to day 7 ($p < 0.05$), for which the initial pH of 2.97 ± 0.01 , showed a declining trend from days 7 to 14 ($p > 0.05$), from pH of 2.89 ± 0.01 to pH 2.85 ± 0.03 . From days 14 onwards, pH of raw *H. itama* honey stabilized to pH 2.85 ± 0.02 ($p > 0.05$). Honey sample H2 exhibited a similar trend, except the initial pH was higher at 2.97 ± 0.11 . The pH value dropped significantly to pH 2.89 ± 0.05 on day 7, with a slight decrease to pH 2.88 ± 0.02 after day 14, before stabilizing at pH 2.87 ± 0.01 on days 21 and 28.

It was feasible that the rapid pH decline in the honey samples observed between days 0 to 7 were due to an active fermentation activity by existing live LAB, which agreed well with the large number of LAB in raw *H. itama* honey (2.73×10^5 CFU/g of LAB in honey H1, 2.5×10^4 CFU/g of LAB in honey H2). Newly harvested raw *H. itama* honey contains live yeasts that further contributes to the high acidity of the honey. This was due to consistent production of various organic acids that kept the pH of the new raw *H. itama* honey low. Moreover, studies have reported that the inherently high moisture content in raw *H. itama* honey on day 0, tends to favor certain chemical and enzymatic reactions that liberate more acidic compounds into the honey. The reactions include the decomposition of fructose into levulinic or methanoic acids; and the conversion of glucose into gluconic acid by the enzyme glucose oxidase [22]. A matter of fact, glucose oxidase has been reported to be activated immediately after honey harvesting, which subsequently contribute to the modification of the honey composition [23].

Table 2. Quality Parameter of *H. itama* Honey H1 and H2 throughout 28 days of storage

Honey	pH		Phenolic content (TPC) (mg GAE/100 g honey)		Flavonoid Content (TFC) (mg QEA/100 g of honey)		Antioxidant activity (IC ₅₀) (mg/mL)	
	H1	H2	H1	H2	H1	H2	H1	H2
Day								
0	2.95 ± 0.01	2.97 ± 0.11	46.22 ± 2.34	38.26 ± 0.67	57.13 ± 1.22	36.95 ± 0.91	45.01 ± 2.53	32.02 ± 2.36
7	2.89 ± 0.03	2.89 ± 0.05	55.66 ± 1.81	45.00 ± 0.89	60.29 ± 0.76	43.02 ± 0.84	39.25 ± 1.25	30.42 ± 1.83
14	2.85 ± 0.02	2.88 ± 0.02	62.19 ± 1.19	48.93 ± 1.97	58.61 ± 1.19	46.33 ± 0.78	33.82 ± 1.92	24.45 ± 1.25
21	2.85 ± 0.02	2.87 ± 0.01	58.05 ± 1.88	44.19 ± 1.81	57.66 ± 0.91	43.95 ± 1.55	33.84 ± 1.80	25.16 ± 2.44
28	2.85 ± 0.01	2.87 ± 0.01	58.20 ± 1.14	44.12 ± 1.90	58.89 ± 2.13	43.82 ± 1.21	32.24 ± 1.09	25.65 ± 1.24

Stable pH values ($p > 0.05$) were observed from days 7 to 28 corroborates the findings of Jiménez et al. (1994) detailing a practically constant of honey pH with a pH range between 3.8 to 4.0 after 2 years of storage [24]. Similarly, Castro-Vázquez et al. (2008) reported that pH values of citrus honey appeared unchanged before and after 12 months of storage [25]. Gulati and Kumari (2005) also found indistinct variation of pH in stored *Apis mellifera* honey [26]. Contrary, Kędzierska-Matysek et al., (2016) observed a significant reduction of pH from pH 4.20 to pH 3.94 in rapeseed honey for incubations before and after 18 months, respectively [27]. Meanwhile, Qamer et al. (2013) noted that the pH of Nepalian *Apis dorsata* honey remained constant during the initial eight months of storage, but the pH of the honey was significantly increased from pH 4.68 to pH 5.01 (8.54%) in the last eight months of storage [28]. A previously study observed that concentrations of certain compounds such as organic acids, ketones and benzenes such as 2-hydroxy-2-propanone, butanoic acid, benzyl alcohol or 2-phenylethanol found in fresh honey tend to cause a synergistic gradual pH increase with increasing storage time [29].

The study noticed that the highly acidic condition of the initial pH of the H1 and H2 samples of raw Malaysian *H. itama* honey may have contributed to the rapid decline in live LAB. Research by Landry et al. (2017) have shown that the survival rate of live LAB inoculated into honey decreases significantly at pH 2 and pH 3, as compared to honey in pH 6.5, which is near the optimal range of between pH 5–9 for LAB survival [30]. In our case, the unusually low pH microenvironment would have interfered with the metabolism of the live LAB, hence justifying the rapid decline in live LAB counts in both H1 and H2 samples.

3.3. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Based on Table 2, H2 honey samples contained lower phenolic (38.26 - 48.93 mg GAE) and flavonoid contents (36.95 - 46.33 mg QEA) as compared to honey H1 (46.22 - 62.19 mg GAE and 57.13- 60.29 mg QEA). Correspondingly, the ANOVA affirmed the significant difference in the contents of phenolic and flavonoid of both honey samples ($p < 0.05$). The outcome seen here is as anticipated as both H1 and H2 honey samples derived from different geographical regions, therefore, correlated with different floral sources of nectar. The TPC of honey samples H1 increased significantly from its initial value of 46.22 ± 2.34 mg GAE/100 g (day 0) to 62.19 ± 1.19 mg GAE/ 100 g on day 14 ($p < 0.05$). The raw H2 *H. itama* honey showed a similar trend, as its initial phenolic content at 38.26 ± 0.67 mg GAE/100 g on day 0 was elevated to 48.93 ± 1.97 mg GAE/100 g on day 14. The study believed the TPC increase seen in both honey samples for days 0–14 was presumptuous of the degradation of higher molecular weight phenolic molecules as a product from enzymatic reactions and/or Maillard reactions. This, consequently, liberated certain chemical substituent groups with reducing power [8]. These compounds then encountered the chemical components used in the respective spectrophotometric analysis and false registered an enhanced phenolic content [8]. Generally, the Folin-Ciocalteu's method is used for determination of the TPC. However, this reagent has its issues related to non-specificity of reaction. The presence of reducing sugars, aromatic amines, sulphur dioxide, ascorbic acid, organic acids and other natural compounds available in honey, are known to affect the readings and leading to often unstable results [8].

A relative stable TPC content from day 14 to day 28 (between 58.05 ± 1.88 mg GAE/100 g to 62.19 ± 1.14 mg GAE/100 g) ($p > 0.05$) was observed in sample H1. However, H2 gave results contrary to that seen in H1. The study noticed a significantly reduced TPC content in honey H2 from days 14 to 28 ($p < 0.05$), which could be due to a few reasons. This instability and consequent degradation of phenolic compounds during storage may have been cause by the susceptibility of certain compounds in raw H2 *H. itama* honey to oxidation and geometric isomerization on the polyene chain [31]. Oxidation is the integral cause of carotenoid loss initiated by a spontaneous free-radical chain reaction in the presence of oxygen, light, metals, enzymes and peroxides [32]. In addition, honey containers are filled with a small gap or commonly known as headspace. Without the correct handling of headspace, the quality of the product may be altered over time, and if the container is not properly closed, more air would enter and further perpetuates the degradation or oxidation of the compounds [8].

The findings seen here corroborates earlier observations on *L. plantarum*, that possesses several phenolic acid decarboxylases capable of degrading containing galloyl groups complex esters of gallic acid and glucose and are esterified directly to the glucose molecule. Several *L. plantarum* strains successfully metabolized six compounds which include p-coumaric, caffeic, ferulic, m-coumaric, gallic, and protocatechuic acids, out of 19 phenolic acids, mediated by the action of one or several decarboxylases and reductases. In this regard, in the decrease in TPC contents in H1 and H2 were also likely due to the same degradation process on such compounds by live LAB. Similarly, Šarić et al. (2012) observed a decreased of TPC by 91.8 % in acacia honey, and by 88.6 % in multiflora honey after one year of storage [33].

An equally increasing pattern of total flavonoid content (TFC) was observed, albeit the increase in TFC was not as high as that of TPC for both raw *H. itama* honey samples. Significant increases were only detected from days 0 to 7 of storage ($P < 0.05$), while the TFC contents were held relatively constant for storage days 7–28 ($p > 0.05$). It is apparent that our observation contradicted a report by Wang *et al.* (2004). These authors found the TFC in clover and buckwheat honeys to decrease after 6 months of storage, and the same was observed for acacia honey that reduced by 45.6 % and in a multiflora honey, which TFC declined by 43.8 % [31].

3.4. Antioxidant Activity

In present study, the IC_{50} value was used to define the antioxidant activity. Particularly, the smaller the IC_{50} value, the greater the antioxidant activity. The initial average IC_{50} value quantified in raw *H. itama* honey H1 was 32.02 ± 2.36 mg/mL, while honey H2 showed a higher average value of 45.01 ± 2.53 mg/mL. The data seen here agreed with the higher TPC and TFC contents in sample H1 compared to sample H2. After 14 days of storage, the IC_{50} of honey H1 showed a significant decrease than the initial values (day 0). The IC_{50} value of sample H1 value was decreased from 32.02 ± 2.36 mg/mL to 24.45 ± 1.25 mg/mL. As for sample H2, a significant decrease in IC_{50} was noticed as early as day 7 of storage, by which the initial value of 45.01 ± 2.53 mg/mL on day 0 was reduced to 39.25 ± 1.25 mg/mL by day 7. The reduced IC_{50} values indicated a general increase in antioxidant activity of raw *H. itama* honey during storage, which is nutritionally beneficial for regular consumers of raw *H. itama* honey. According to Pinelo et al. (2004) the increase in antioxidant activity of in honey may be explained by the strong tendency of polyphenols to undergo polymerization reactions, whereby the resulting oligomers possess larger areas available for charge delocalization [34]. When the degree of polymerization exceeds a critical value, the increased molecular complexity and steric hindrance tends to reduce the availability of hydroxyl groups in reaction with the DPPH radicals. This causes a concomitant increase in the antiradical capacity [35]. Such reactions hence explain the observed increase in antioxidant activity of both honey samples H1 and H2.

Additionally, the noteworthy improvement in antioxidant activities in both samples, H1 and H1 raw *H. itama* honey might be due to a fermentation that occur during storage since the abundant presence of sugars and variety of microorganisms such as LAB and yeasts in the honey. Perez-Perez et al. (2007) also reported a similar observation for a stingless bee *T. angustula* honey that showed a build-up of alcohols and loss of sugars [36].

4. Conclusion

The present study demonstrated that changes in viability of LAB may have somewhat affected certain nutritional parameters of the Malaysian raw *H. itama* honey samples, H1 and H2. The results indicate that viability of LAB in raw *H. itama* honey was the highest when the honey was freshly harvested from the pods and, significantly declined with increase in storage time. While there wasn't any sort of drastic changes to the nutritional composition of the two raw *H. itama* honey samples, it is recommended that the honey is consumed with 3 three weeks of harvest. This is to ensure that the consumers are also able to reap the probiotic benefits of live LAB microbiota into their digestive tract. Although the behaviour of LAB in honey observed for factors pH, phenolic and flavonoid content and total antioxidant capacity of the analyzed honey samples were highly variable, it can be concluded that their compositions

remained relatively stable over the course of 28 days. Despite our findings that contradicted certain studies on nutritional compositions of bee honey, the results clearly demonstrated that any changes in nutritional composition of raw honey under varying storage durations, is dependent on the source of nectar when the bees forage. It is also dependent on the type of gut microbiota that was transferred from the bee gut into the honey.

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