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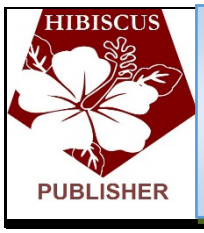

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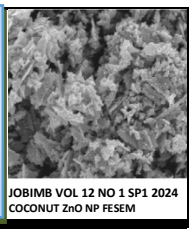

Effects of Pasteurisation on Antioxidant Activities and Microbiological Properties of Stingless Bee Honey Beverage During Storage

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ABSTRACT

Stingless bee honey (*Kelulut*) contains low pH, high antioxidants and moisture content compared to other types of honey with limited study on its product diversification as a honey beverage and its suitable storage conditions. Therefore, this study aims to evaluate the effect of two different pasteurisation conditions on the quality of honey beverages filled in sealed and unsealed High-Density Polyethylene (HDPE) during storage. Microbiological and antioxidant analysis was conducted. Kelulut beverage was pasteurized using High-Temperature Short-Time (HTST) at 73°C for 15 sec and Low-Temperature Long-Time (LTLT) at 65°C for 20 min. Sealed HTST pasteurised honey beverage (HPS), Unsealed HTST pasteurised honey beverage (HPU), Sealed LTLT pasteurised honey beverage (LPS) and Unsealed LTLT pasteurised honey beverage (LPU) stored until 21 days at chill condition. Sealed HTST beverage stored at chill conditions (HPS-CH) showed significantly higher ($p<0.05$) retention of scavenging activity (DPPH inhibitions-39.47±0.2%) and reducing power (82.05±2.6%) than LPU-CH (DPPH value of 37.3±4.9% for unsealed beverage stored at chill) with FRAP value of 60.55±0.0% until 21 days storage. No microbial growth (Total plate and yeast and mould count) was detected in the HPS, HPU, LPS and LPU stored at chill temperature. However, non-pasteurized (NP) kelulut honey beverage spoiled after 7 days of storage based on Kelulut Malaysia Standard. This study may assist the beverage industry to identify optimal storage conditions and packaging of the kelulut honey beverage.

INTRODUCTION

Stingless bees honey such as *Melipona subnitida* honey will appear more fluid than *Apis melifera* due to its high moisture content. High moisture content of the stingless bee honey can cause fermentation to occur after harvest. A functional beverage is the fastest growing segment in new food product development around the world due to its convenience and meeting consumer demand in terms of container contents, size, appearance, as well as ease of distribution and storage for refrigerated conditions and shelf-stable products [1]. Numerous studies on Ready-To-Drink (RTD) beverage include honey as a sweetener. For example, honey acts as a natural sweetener in a lemon ready-to-serve drink. The process of producing RTD may involve pasteurisation to increase the product's shelf life. This method can minimise the quality degradation which combine with temperature (high or low), water activity, redox potential, preservatives, and radiation to preserve food's stability, microbiological safety, and sensory quality [2]. Proper storage study is important in beverage production to maintain its quality, safety, and shelf life over time. Factors like temperature, humidity, light exposure, and packaging can affect the stability, safety and sensory properties such as taste, aroma, and appearance of a beverage [3]. Thermal pasteurization like High-temperature-short-time (HTST) and Low-temperature-long-time (LTLT) are among the safest methods to prolong the shelf life of a product. The HTST and LTLT pasteurisation may reduce the quality and stability of honey beverages, resulting in undesirable changes to the physicochemical properties [4]. However, there is limited studies on the development of RTD honey beverages - and its stability during storage. Therefore, the aim of this study is to evaluate the effects of HTST and LTLT pasteurisation on the physicochemical and microbiology of honey beverages during storage at chill and room temperatures.

MATERIALS AND METHODS

Sample Collection

Kelulut honey from *Heterotrigona itama* species were collected from a farm namely Kelulut Mak Lang Kelubee, located in Jasin, Melaka. Each honey sample was analysed in duplicate.

Honey beverage preparation

For beverage preparation, 16-24% of Kelulut honey was mixed with filtered water. Next, the beverage was packed into a 15 ml volume size of High-Density Polyethylene (HDPE) bottle before being pasteurized (TM6, Thermomix) at different pasteurization treatment of 73°C for 15 sec for High Temperature Short time treatment (HTST) and 65°C for 30 min for low temperature long time (LTHL), respectively [5]. The honey beverages were classified as non-pasteurized sealed (NPS), Non- pasteurized unseal (NPU), HTST pasteurized sealed (HPS), HTST pasteurized unseal (HPU), LTLT pasteurized seal (LPS) and LTLT pasteurized unsealed (LPU). Control samples were identified as non-pasteurized sealed (NPS) and non-pasteurized unsealed (NPU). The beverages were sealed using an induction sealer and the storage study of the beverage was conducted at chill and room temperature. The honey beverages (NPS, NPU, HPS, HPU, LPS, LPU) were analyzed (physiochemical, microbiological and antioxidant analysis) at different time intervals (0 day, 3rd day, 5th day, 7th day, 14th day and 21st during storage.

The selection of 21 days is based on the preliminary analysis where the Kelulut honey beverage was spoilt after 21 days of pasteurization. Seal and unseal conditions were selected to further investigate the effect of different types of packaging on the honey beverage during storage.

Microbiological analysis

About 10 g of honey beverages (NPS, NPU, HPS, HPU, LPS, LPU) were weighed into 90 mL bacterial peptone solution before shaken for 30s using stomacher machine (BagMixer 400, France) to homogenize. Inverts solidify petri dish and incubate for 48 h ± 2h at 37. Yeast and mould count were conducted with slight modification [6]. The modification was on the use of potato dextrose agar (PDA) instead of malt extract agar (MEA).

Determination of Antioxidant Activity

The antioxidant activity of DPPH and the absorbance of the complex TPTZ-Fe (II) of honey beverages (NPS, NPU, HPS, HPU, LPS, LPU) were determined according to Majid et al [7].

Statistical analysis

Minitab V.21.4 software (Minitab Inc., State College, PA, USA) was used to analyze the collected data, and each analysis was implemented in duplicate. Data was represented as a mean value±standard deviation (n=3) and analyzed using analysis of variance (one-way ANOVA) with Tukey's test at a significance level of $p<0.05$ for each analysis.

RESULTS AND DISCUSSION

Microbiological Analysis

Microbiological quality (Total plate count/ TPC and Yeast and mould/ YM) of Kelulut honey beverage was investigated. As shown in **Table 1**, the total plate count of honey beverage increased as the day of storage increased at room temperature. At room temperature, the unpasteurized sealed and unsealed beverage (NPU and NPS) started to spoil at day 7.

The microbial count of LTLT-treated honey beverage increased to 4.5 log cfu/mL and 4.79 log cfu/mL of LPS and LPU, respectively after 21 days of storage at room temperature. Other than that, microbial growth in HTST-treated honey beverage is 2.55 log cfu/ml and 3.95 log cfu/ml for HPS and HPU respectively under the same storage conditions. In contrast, no microbial growth was found in both HTST and LTLT-treated beverage under chill storage conditions. This finding is similar to a study on cloudy pomegranate juice subjected to heat treatment at 65 °C for 30s (low-temperature pasteurization) and 90 °C for 5s (high-temperature pasteurization in a semi-tubular pasteuriser) stored at chill conditions. The TPC limit for Kelulut honey is 1 x $10^{3,}$ while the limit for yeast and mould count is less than 1 x 10^{1} (6). Meanwhile, the US Food and Drug Administration (FDA) stated that total plate count should not exceed 50 CFU/mL and the coliform count should be nil in 100mL of beverage sample.

At room temperature, the NPU and NPS show the beverages spoiled at day 7. Microbial count of LTLT-treated honey beverages (LPS and LPU) increased to 3.80l log cfu/ml and 3.82 log cfu/ml respectively. The microbial growth of HTST-treated honey beverage is 3.80 log cfu/ml and 3.83 log cfu/ml for HPS and HPU, respectively under the same storage conditions and time. In contrast, no microbial growth of yeast and mould found in both HTST and LTLT treated beverage stored at chill conditions. This is because microbial development in chilled conditions is slow due to the low temperature, and quick temperature reduction on the food surface thus limit microbial growth and extend the shelf life of a product.

This study also shows that the total plate count of HPS (2.55 $_{log}$ cfu/mL), HPU (3.95 $_{log}$ cfu/mL) for 21 days have higher log cfu/ml count compared with sealed beverages HPS (2.55). This might be due to the unsealed beverages is more exposed to the external environment. Deterioration of packaged food mostly depends on the transfer between the external environment, like moisture vapor from the humid atmosphere and if the package is not an effective oxygen (O_2) barrier, oxidative rancidity will develop. The presence of gases in the environment can influence the growth of microorganisms in the packaged product. However, the unsealed samples (HPU and LPU) for both HTST and LTLT stored at room temperature show presence of growth where TPC count exceeding the standard limit. Therefore, antioxidant analysis was not conducted for PU and PS samples stored at room temperature.

The LTLT shows a significantly lower count of TPC and YM than HTST. Similar to report on watermelon juice treated at 60°C for 30 min (LTLT) and 100°C for 5 min (HTST). Based on **Table 1**, the chill conditions of the honey beverage are the most appropriate condition to store as the microbial growth is slower when compared to the room temperature. Heat treatment which is LTLT pasteurization 63 °C for no less than 30 min and 72°C for 15s and above combined with chill refrigeration could prevent microbial growth without adversely affecting its nutritional properties in pomegranate juice [8].

Antioxidant Analysis

DPPH Radical Scavenging Activity

Table 2 shows value of DPPH inhibition of honey beverage treated using HTST and LTLT. The HPS (39.47±0.198%), HPU (38.86±7.40%), LPS (39.07±2.07%) and LPU (39.8±7.40%) stored in a chiller have a higher amount of DPPH scavenging value compared to HPS (39.71±0.899%), and LPS (39.0±2.07%) stored at room temperature.

The effect of storage duration (21 days) on DPPH scavenging ability for HPS-CH (39.47±0.2%), although pasteurised at higher temperature was similar with LPS-CH (39.07±2.07%) after they were stored at chill temperature implying that lower storage temperature could reduce the decline of DPPH scavenging activity. This finding is similar to DPPH scavenging activity of blanched bitter gourd which decreased further at higher storage temperature from 536 µmol Trolox to 355 µmol Trolox in -18°C compared to 536.1 µmol Trolox to 559.5 µmol Trolox in -40 °C [9].

Table 1. Total Plate and Yeast and mould count of honey beverages at different processing conditions (non-pasteurized and pasteurized at high and low temperature with sealing and unsealed conditions) during 21 days of storage.

Microbiological Analysis														
Type of beverage		Storage condition	Total plate count log x 10^{-3} (cfu/mL)						Yeast and mould log x 10^{-3} (cfu/mL)					
			Storage time (days)											
			0	3	5	7	14	21	0	3	5	7	14	21
NPS		RT	1.26	1.65	2.10	2.45	-	-	4.00	4.13	4.15	4.19	-	-
		CH	ND	ND	ND	ND	-	-	ND	ND	ND	ND	-	-
NPU		RT	1.26	1.68	1.95	2.28	-	-	4.00	4.13	4.20	4.18	-	-
		CH	ND	ND	ND	ND	-	-	ND	ND	ND	ND	-	-
HTST		RT	1.22	1.39	1.56	1.61	2.15	2.55	<1.00	<1.00	3.40	3.45	3.80	TNTC
	PS	CH	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	PU	RT	1.22	1.37	1.85	2.10	2.20	3.95	<1.00	<1.00	3.68	3.50	3.83	TNTC
		CH	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
LTLT		RT	1.21	1.34	1.86	1.90	2.23	4.50	<1.00	<1.00	3.20	3.40	3.82	TNTC
	PS	CH	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	PU	RT	1.21	1.28	1.70	1.95	2.50	4.79	<1.00	<1.00	3.33	3.45	3.80	TNTC
		CH	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Abbreviation= ND- Not detected, TNTC- Too numerous to count, NP-Non-pasteurized, P- Pasteurized, S- Sealed, U – Unsealed, CH-chiller, HTST- High temperature short time, LTLT- Low temperature long time, CH- Chiller, RT-room temperature
Symbol (–) day 14-21-refers to deteriorated samples.

Table 2. DPPH scavenging assay of honey beverages at different processing conditions (non-pasteurized and pasteurized at high and low temperature with seal and unsealed conditions) during 21 days of storage.

Antioxidant Activity								
Type of beverages	Storage conditions		DPPH Storage time (days)					
			0	3	5	7	14	21
NPS	RT		41.71±4.27^{Aa}	41.87±4.48^{Aa}	40.6±3.54^{Aa}	40.32±1.66^{Ba}	-	-
	CH		43.02±12.07^{Aa}	42.89a±2.3^{Aa}	41.52±0.0^{Ba}	41.34±2.75^{Ba}	-	-
NPU	RT		41.71±2.18^{Aa}	41.60±3.7^{Aa}	40.43±1.81^{Aa}	39.7±1.8^{Ba}	-	-
	CH		42.78±2.38^{Aa}	42.68± 2.00^{Aa}	41.32±1.98^{Aa}	40.99±5.47^{Ba}	-	-
HTST	HPS	RT	41.45±0.08^{Aa}	41.41±3.7^{Aa}	40.22±.067^{ABa}	39.51±8.88^{ABa}	28.04±0.89^{Ba}	39.71±0.899^{ABa}
		CH	41.31±0.012^{Aa}	41.14±0.237^{Aa}	40.10±0.01^{ABa}	40.11±4.52^{ABa}	29.02±5.35^{Ba}	39.47±0.198^{ABa}
	HPU	CH	41.60±8.98^{Aa}	41.60±8.86^{Aa}	40.34±1.44^{ABa}	40.1±8.8^{ABa}	29.0±2.3^{Cb}	38.86±7.40^{Ba}
LTLT	LPS	CH	40.70±2.54^{Aa}	40.6±2.48^{Aa}	39.59±2.10^{Aa}	39.8±9.55^{Aa}	28.62±1.81^{Ba}	39.07±2.07^{Aa}
	LPU	CH	38.6±15.4^{Aa}	38.5±1.8^{Aa}	37.87±2.77^{Aa}	37.69±1.78^{Aa}	26.15±2.11^{Ba}	37.3±4.9^{Aa}

Values are expressed as mean ± sd. Values with different letters in the same row (A-C) are significantly different at different storage days and letters in the same column (a) are no significantly different at p<0.05 for processing conditions. Abbreviation: NP – Non pasteurized, P- Pasteurized, S- Sealed, U – Unsealed, RT- Room Temperature, CH-chiller, HTST- High temperature short time, LTLT- Low temperature long time, CH- Chiller, RT-room temperature. Symbol (–)- refers to deteriorated samples.

Table 3. FRAP of honey beverages at different processing conditions (non-pasteurized and pasteurized at high and low temperature with seal and unsealed conditions) during 21 days of storage.

Antioxidant Analysis								
Type of beverage		Storage conditions	FRAP Storage time (days)					
			0	3	5	7	14	21
NPS		RT	217.86± 4.95^{Aa}	213.091±1.157^{Aab}	192.59± 1.61^{Bab}	151.46± 0.643^{Ca}	-	-
		CH	214.55± 1.99^{Aa}	214.41± 2.51^{Aa}	210.77± 1.61^{Aa}	154± 0.9^{Ba}	-	-
NPU		RT	210±6.43^{Aa}	205.5±0.0^{Ab}	180.91± 1.41^{Bbc}	130.73±0.51^{Cb}	-	-
		CH	217.87± 0.84^{Aa}	217.36± 1.54^{Aa}	160.77±12.54^{Bcde}	145.68±5.08^{Ba}	-	-
HTST	HPS	RT	185.14±1.61^{Ab}	183.90± 0.514^{Ac}	169.77±0.94^{Bbcd}	131.95±0.193^{Cb}	110.9±0.129^{Dab}	90.18±0.25^{Ea}
		CH	182.05±6.88^{Ab}	181.23±1.22^{Ac}	169.6± 16.3^{ABbcd}	135.73±5.14^{BCb}	127.59±13.44^{Ca}	82.05±2.64^{Dbc}
	HPU	CH	181.36±1.93^{Abc}	182.59±0.193^{Ac}	180.82± 0.257^{Abc}	107.45±2.57^{Bc}	100±6.69^{Babc}	77.045±0.70^{Ccd}
LTLT	LPS	CH	160.05±7.52^{Ade}	155.50± 2.38^{Ad}	150.09± 2.83^{Ade}	101.41±1.221^{Bcd}	85.27±0.9^{Cbc}	60.59±4.44^{De}
	LPU	CH	147.64±1.54^{Ae}	145.45±0.9^{Ae}	144.27± 1.80^{Ae}	98.04±0.064^{Bd}	78.59±10.48^{Cc}	60.55±0.00^{De}

Values are expressed as mean ± sd. Values with different letters in the same row (A-D) are significantly different at different storage days and different letters in the same column (a-e) are significantly different at p<0.05 for each processing conditions.
Abbreviation: NP – Non pasteurized, P- Pasteurized, S- Sealed, U – Unsealed, RT- Room Temperature, CH-chiller, HTST- High temperature short time, LTLT- Low temperature long time, CH- Chiller, RT-room temperature. Symbol (–)-refers to deteriorated samples.

This shows that the best condition for storage is at chill conditions [8]. However, this study differs with DPPH scavenging activity increased significantly from 71.41% to 89.04% in kelulut honey (*Heterotrigona itama*) from Kuching, Sarawak after applying heat treatment (45–90 °C for 30–120 min). Samples used in this study show significant ($p<0.05$) decreased in DPPH inhibition after 21 days for HPU-CH (38.86±7.40%) and LPU-CH (37.3±4.9%) honey beverages compared to 0 day. Similarly, heat treatment applied to the *Apis melifera* honey causes the DPPH inhibitions in the honey to be reduced except for the fresh honey after heating to 90 °C and storage at 22 and 40 °C [9]. **Table 2** also shows HPS-RT and LPS-CH have a higher value of DPPH of 39.475±0.198% and 39.07±2.07% respectively compared with HPU-CH at 38.86±7.40% after 21 days of storage. This is probably due to exposure of the unseal condition to the oxygen lead to microbial growth that causes honey beverage to have lower DPPH scavenging activity than the PS beverage although it has been pasteurized.

Ferric-Reducing Antioxidant Power (FRAP)

Table 3 shows NPS (214.55 µmol/mL) at chill storage have significantly higher FRAP activity than NPS (217.86 µmol/mL) and NPU (210 µmol/ml) at room temperature. The intense blue colour of ferrous ion with a 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) complex occurs when high antioxidant of stingless bee honey reacted with the pale yellow of a ferric ion with a TPTZ complex. The ferric reducing antioxidant power (FRAP) analysis was performed to measure the ability of phenolic compounds as an antioxidant to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}).

A high level of antioxidant activity is attributed to vitamin C and phenolic compounds in the fresh sample of baobab juice where FRAP value reduced by 2.6% after being pasteurised. Another study reported higher FRAP values of raw stingless bee ranging between 283.80 and 1401.80 µM Fe (II)/100 g [10]. The variation of FRAP could be attributed to the difference in amount and types of phenolic content present in the honey sample. It is also probably due to the different botanical origin. NPS shows no significant different ($p<0.05$) in the FRAP assay at day 0 (214.55±1.99) to day 5 (210.77± 1.61) probably due to the antioxidant in the sample are stable, undegraded or oxidized significantly [7]. However, FRAP assay for pasteurized honey beverage decreased significantly ($p<0.05$) during the 21 days storage. The LTLT treated beverage has significantly ($p<0.05$) higher changes in FRAP assay as LPS-RT and LPS-CH at 62.41± 0.321 and 60.59±4.44 respectively. **Table 3** shows FRAP-assay value significantly differed between the HTST which include HPS-RT, HPS-CH, HPU-CH and LTLT (LPS-RT, LPS-CH, LPU-CH) pasteurized honey beverage. In contrast, an increment of FRAP value after the honey was treated at 90 °C and 95 °C for 15 s and 60 s, respectively [10].

Table 3 shows significant reduction ($p<0.05$) in FRAP assay value during 21 days of storage with the highest value of FRAP at 90.18 µmol/mL (HPS) and 62.41 µmol/ml (LPS) at room temperature compared with 82.05 µmol/ml (HPS) and 60.59 µmol/ml (LPS) at chill conditions, respectively. Another similar study reported a significant decrease for Kelulut honey after pasteurisation at 90°C. The heat treatment at 90°C reduced the ferrous ion in FRAP by 20% compared to the control temperature which is 45°C [9]. The HPU-CH has significantly ($p<0.05$) maintained higher FRAP until day 7 (107.45±2.57) compared to other treated beverages. Meanwhile, LPU-RT shows significantlty higher ($p<0.05$) FRAP at 0 day 147.64±1.54 and decreased significantly during storage in 21 days than LPU-CH at 0 day 143.41±4.31. The high temperature in heated honey is believed to reduce the FRAP activity [10].

CONCLUSION

The antioxidant activities of HTST possessed significantly ($p<0.05$) higher retention of antioxidant value than LTLT as shown by FRAP-assay of HPS-RT (90.18±0.25) and HPS-CH (82.05±2.64) compared with LPS-CH (60.79±4.44). The DPPH inhibitions of HPS-CH (39.47±0.198) were higher than LPS-CH (39.07±2.07). Non-pasteurized honey beverage has a shelf life of 7 days (2.45_{log} CFU/ml for NPS) with count of 2.28_{log} CFU/ml in NPUS for plate count and 4.19_{log} CFU/ml in NPS and 4.18_{log} CFU/ml in NPUS for yeast and mold. Sealed beverage has reduced the number of contaminants by slowing down the growth rate of microorganisms. No microbial growth was detected in all treated honey beverages stored at chill temperature for 21 days. Kelulut honey beverage industry may establish the appropriate shelf life.

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