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Pathogenic Bacterial Communities Isolated and Identified in Stingless Bee (*Kelulut*) Honey from Selected Farms in Terengganu

Noor Aimi Shazana Mohd Yusoff, Fisal Ahmad, Amir Izzwan Zamri, Shamsul Bahri Abdul Razak, Muhammad Fauzi Mahmud and Tuan Zainazor Tuan Chilek*

Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

ABSTRACT

In Malaysia, stingless bees can be categorised into two genera: *Melipona* and *Trigona*, known as "*kelulut*". The high demand for *kelulut* honey boosts the production of the honey industry. Previous studies reported that stingless bee (*kelulut*) honey and its products were contaminated with pathogenic bacteria during harvesting and processing. This research aims to isolate and identify the pathogenic bacteria in *kelulut* honey. Forty-eight samples of *kelulu*t honey (open and closed pot) and propolis were obtained from selected farms in Terengganu by focusing on a major stingless bee species available in Malaysia, *Heterotrigona itama*. In addition, the swabbing technique was done on the wooden beehive of the *kelulut* to evaluate the environmental contamination. The pathogenic bacteria were isolated using specifically selected agar, such as *Bacillus cereus* agar (for *B. cereus*), Baird-Parker agar (for *Staphylococcus aureus*), and MacConkey agar (for other pathogenic bacteria), which were confirmed through a biochemical test. All samples were analysed, and the results showed that *B. cereus* (7/48), *Pseudomonas aeruginosa* (10/48), *Pantoea* spp. (11/48), *Serratia plymuthica* (6/48), and *S. aureus* (9/48) were obtained in the samples. This study indicates that *kelulut* honey was contaminated with *B. cereus*, *P. aeruginosa*, *Pantoea* spp., *S. plymuthica*, and *S. aureus*. Isolated pathogenic bacteria may exist in the *kelulut* honey through food handlers, utensils, and the environment. Hence, the stakeholders should strictly follow good standard operating procedures and guidelines by the *kelulut* honey industry to prevent foodborne illness.

Keywords: Food handlers, foodborne illness, *kelulut,* pathogenic bacteria, stingless bee honey

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E-mail addresses:
p4405@pps.umt.edu.my (Noor Aimi Shazana Mohd Yusoff)
fisal@umt.edu.my (Fisal Ahmad)
amir@umt.edu.my (Amir Izzwan Zamri)
shamsul@umt.edu.my (Samsul Bahri Abdul Razak)
p4809@pps.umt.edu.my (Muhammad Fauzi Mahmud)
t.zainazor@umt.edu.my (Tuan Zainazor Tuan Chilek)
*Corresponding author

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INTRODUCTION

The stingless bee is a small bee from the species of *Trigona* or meliponine and is also known as the '*kelulut*' bee in Malaysia. They are known as stingless bees because these bees are incapable of stinging (Salatino et al., 2019). Two genera that can be used to classify stingless bees are *Melipona* and *Trigona* (Jalil et al., 2017). In Malaysia, two species of stingless bee that are reported to produce honey are *Heterotrigona itama* and *Geniotrigona thoracica*, which have different colours and sizes, and other common species to be found in *H. itama* (Shamsudin et al., 2019). The content of the honey produced by these two species differs depending on its botanical origin, floral source, environmental conditions, geographic location, and methods used to harvest and process the honey (Bakar et al., 2017; Shamsudin et al., 2019).

Kelulut plays an essential role in the economy and culture. Their products, such as honey, pollen, and propolis, have been used for revenue and profit for ages. The Aboriginal people of northern Australia greatly value the stingless bee honey as a food source, which is important to their social customs and ceremonies (Boorn et al., 2010). It is easier to regularly extract honey, pollen, and propolis because the *kelulut* cannot sting. Furthermore, Jalil et al. (2017) reported that stingless bees are more effortless to handle than honeybees, which often abandon their hive and are endangered by disease. *Kelulut* honey also has a unique sweetness assorted with a sour and acidic taste (Jalil et al., 2017).

The pathogenic bacteria cause the most significant national public concern are *Escherichia coli*, *Staphylococcus* spp., *Shigella* spp., *Streptococcus* spp., and *Bacillus* spp. that frequently associated with honeybees (Adadi & Obeng, 2017). The pollen, air, flowers, and digestive tracts of *kelulut* are among the sources of bacteria. Ngalimat et al. (2020) reported that humans, tools, containers, wind, and dust could all be the primary or secondary sources of bacterial contamination in bee products. Most bacterial species associated with *kelulut* colonies are *Bacillus*, *Streptomyces*, and *Lactobacillus* (Ngalimat et al., 2020). The pathogenic bacteria should be concerning because it will show the level of food hygiene, food handlers, and the farm.

This research aims to isolate and identify the pathogenic bacteria in the *kelulut* honey. A few research were done on the microbiological contamination of bee honey, or *kelulut* honey, and its other products, including pollen and propolis with *E. coli*, *Staphylococcus* spp., *Shigella* spp., *Streptococcus* spp., and *Bacillus* spp. (Adadi & Obeng, 2017). These bacteria can cause serious food poisoning if contaminated with honey products. In addition, there is limited data on pathogenic bacteria contamination and characterisation in *kelulut* honey available in Malaysia. Malaysian Standard (MS 2683:2017) for *kelulut* honey specification is issued to control the quality and safety of honey produced. Honey entrepreneurs are still poorly implementing the food supply chain concept to avoid contamination by pathogenic bacteria.

MATERIALS AND METHODS

Samples

The samples of *kelulut* honey and propolis were obtained from four selected farms (locations A, B, C, and D) in Kuala Terengganu and Kuala Nerus by focusing on major *kelulut* species available in Malaysia: *H. itama* species. Forty-eight samples of *kelulut* honey consisting of the open pot (OP), close pot (CP), and propolis (PP) were obtained from the selected farms for pathogenic bacteria analysis. The swabbing technique was done on the hive swab (HS) of the *kelulut* to evaluate the environmental contamination. These samples were transported to the laboratory in a chilled condition at 4°C before being analysed. The samples were analysed within 24 hr.

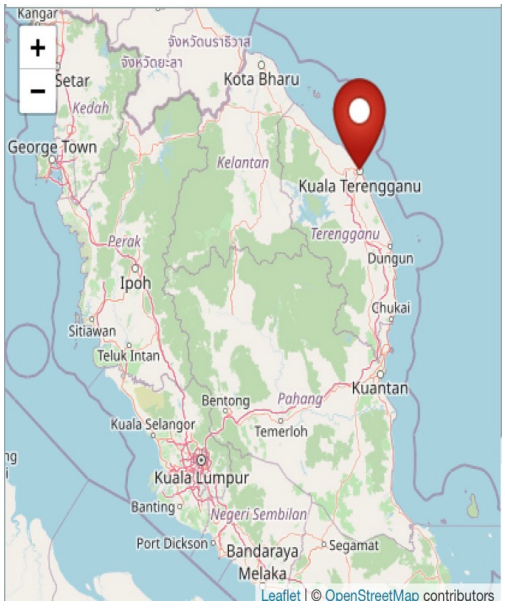

Figure 1. Maps of the region in Terengganu, where the samples were collected

Samples Location

The Google map provided the Global Positioning System (GPS) for the honey locations. Honey from *H. itama*, propolis, and hive swab samples were obtained from Kuala Terengganu, Terengganu (5°19'48.72"N 103°08'26.88"E) and Kuala Nerus, Terengganu (5° 21' 37.2882102"N 103° 1' 37.3284"E). Figure 1 shows the region maps in Terengganu where the samples were collected.

Isolation and Identification of Pathogenic Bacteria

Bacillus cereus agar (BCA, Oxoid International Ltd., United Kingdom), Baird-Parker agar (BPA, Oxoid International Ltd., United Kingdom), and MacConkey agar (non-selective agar, Oxoid International Ltd., United Kingdom) were used to isolate the *B. cereus*, *S. aureus*, and other pathogenic bacteria. Homogenous samples were prepared through a serial dilution, where 10 g of samples were obtained. Then, 90 ml of peptone water (Oxoid International Ltd., United Kingdom) was added as the first dilution. A total of 0.1 ml of each dilution were pipetted out, and then the spread plate method was applied for BCA, BPA, and MacConkey agar. Plates were incubated at 37°C for 24 to 48 hr, and then the morphology of colonies was observed. The bacterial cells are deposited at widely separated points on the surface of the medium and develop into colonies (Sanders, 2012). The positive colonies were confirmed using biochemical tests (Andrews & Hammack, 2022).

Swabbing Technique

The sterile cotton bud-tipped swab was moistened with 0.1% buffered peptone water (BPW) (Oxoid International Ltd., United Kingdom). Then, the swab head was gently pressed to remove the excess BPW. The stick was repeatedly swabbed on the wooden beehive surface area (10 cm x 10 cm). After swabbing, the swab head was immersed in 0.1% of BPW (Willes et al., 2013). The sample will be serially diluted up to 10^2 dilutions. The sample taken was conducted within 24 hr and continued for further analysis.

Analytical Profile Index (API) Technique

The API 20E system (Bio-Mérieux, Marcy l'Etoile, France) is a non-fastidious Gram-negative rod to identify Enterobacteriaceae was used in this study. Twenty microtubes make up this apparatus, which is filled with dehydrated substrates. The bacterial suspension used in these experiments was inoculated, reconstituting the media. The incubation process causes colour changes due to metabolism, which can occur naturally or be seen by adding chemicals. The API is used to identify the reactions after reading them in accordance with the table for reaction interpretation (Ashgar & El-Said, 2012).

Other Tests

Coagulase Test. Slide tests were conducted to detect bound coagulase. It causes a fast cell agglutination by immediately reacting with fibrinogen in plasma. The ends of a slide were given a drop of physiological saline. A piece of the isolated colony was emulsified in each drop using the loop straight wire to create two thick suspensions. One of the suspensions was given a drop of human or rabbit plasma, and it was gently mixed in within 10 s, a clumping of the organisms was observed (Leber, 2016).

Oxidase Test. In this study, the filter paper spot method was used. A well-isolated colony was selected using a loop from a new bacterial plate; then rubbed onto a small piece of filter paper. One or two drops of the 1% Kovács oxidase reagent (Sigma-Aldrich, USA) were applied to the organism smear. Then, a colour shift was noticed (Leber, 2016).

Catalase Test. The catalase test was conducted using the slide method. A small amount of colony development was transferred onto the surface of a clear, dry glass slide using a loop or sterilised wooden stick. Then, a drop of 3% hydrogen peroxide (H_2O_2, Sigma-Aldrich, USA) was added to the glass slide. The oxygen bubbles evolved were observed (Leber, 2016).

RESULTS

Selected samples were obtained from locations A, B, C, and D (Tables 1–4). Isolation using selected agar (BCA and BPA) which targeted pathogenic bacteria that commonly cause food poisoning, and non-selected agar (MacConkey agar) for suspected colonies were observed, characterised, and identified. Forty-eight

samples of *kelulut* honey comprised OP, CP, PP, and HS.

There are five strains of pathogenic bacteria obtained in the open pot honey, propolis, and hive swab from this study, which are *P. aeruginosa*, *B. cereus*, *S. aureus*, *Pantoea* spp., and *S. plymuthica*. Surprisingly, no pathogenic bacteria were found in the close-pot honey samples from all locations. The close pot honey was shielded and protected from any contamination by the pot's structure. Jalil et al. (2017) reported that honey is stored in a cerumen pot, which is used to mummify intruders and maintain a sterile environment inside the hive. However, in the open pot honey, 80% (10/12) of samples were contaminated with pathogenic bacteria (Tables 1–4). To our knowledge, no study has been done in open and closed-pot samples. In addition, propolis and hive swab samples were also contaminated with pathogenic bacteria with 100% (12/12) samples and 92% (11/12) samples, respectively (Tables 1–4). A few studies reported that *B. cereus*, *S. aureus*, and *Pantoea* sp. are found in honeybee and *kelulut* honey (Adadi & Obeng, 2017; Amin et al., 2020; Ngalimat et al., 2019; Pucciarelli et al., 2014).

In summary, out of twelve samples of propolis, three samples were contaminated with *S. aureus* (25%), five samples were detected with *P. aeruginosa*, three samples were identified with *B. cereus* and *Pantoea* spp., and two samples were confirmed with *S. plymuthica* strains. In addition, five out of twelve samples of the hive swab were contaminated with *P. aeruginosa*, two samples with *S. aureus*, six with *Pantoea* spp., and three with *S. plymuthica*. Lastly, four samples of open-pot honey contained *Pantoea* spp., six samples (50%) with *B. cereus*, and only two with *S. aureus*. Only *S. plymuhica* and *P. aeruginosa* were not found in the open-pot honey.

Table 1

Isolation and characterisation of selected samples obtained from location A

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
A	BCA	AOP3*	Blue green colonies	*Bacillus cereus*	Oxidase (+) Catalase (+)	Positive *B. cereus*
	BPA	APP1, APP2,	Black colonies with a clear zone	*Staphylococcus aureus*	Oxidase (+) Catalase (+) Coagulase (+)	Positive *S. aureus*
	MacConkey	AOP2	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Table 1 *(Continue)*

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
		APP1	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
		APP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		AHS1	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
A	MacConkey	AHS2	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
			Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
		AHS3	Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*

Note. API = Analytical Profile Index; BCA = *Bacillus cereus* agar; BPA = Baird-Parker agar; OP = Open pot; CP = Close pot; PP = Propolis; HS = Hive swab

Table 2

Isolation and characterisation of selected samples obtained from location B

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
	BCA	BOP1	Blue, green colonies	*Bacillus cereus*	Oxidase (+) Catalase (+)	Positive *B. cereus*
	BPA	BOP2, BPP2	Black colonies with a clear zone	*Staphylococcus aureus*	Oxidase (+) Catalase (+) Coagulase (+)	Positive *S. aureus*
B	MacConkey	BPP1	Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		BPP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Table 2 *(Continue)*

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
B	MacConkey	BHS1	Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		BHS3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Note. API = Analytical Profile Index; BCA = *Bacillus cereus* agar; BPA = Baird-Parker agar; OP = Open pot; CP = Close pot; PP = Propolis; HS = Hive swab

Table 3

Isolation and characterisation of selected samples obtained from location C

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
C	BCA	COP1, COP3, CPP1, CPP3	Blue, green colonies	*Bacillus cereus*	Oxidase (+) Catalase (+)	Positive *B. cereus*
	BPA	CPP2, CHS2	Black colonies with a clear zone	*Staphylococcus aureus*	Oxidase (+) Catalase (+) Coagulase (+)	Positive *S. aureus*
	MacConkey	COP2	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		COP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		CPP1	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
			Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*

Table 3 *(Continue)*

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
C	MacConkey	CHS1	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
			Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		CHS2	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		CHS3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		CHS4	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
			Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		CHS5	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Note. API = Analytical Profile Index; BCA = *Bacillus cereus* agar; BPA = Baird-Parker agar; OP = Open pot; CP = Close pot; PP = Propolis; HS = Hive swab

Table 4

Isolation and characterisation of selected samples obtained from location D

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
D	BCA	DOP1, DOP2, DPP2	Blue, green colonies	*Bacillus cereus*	Oxidase (+) Catalase (+)	Positive *B. cereus*
	BPA	DOP2, DPP3, DHS3	Black colonies with a clear zone	*Staphylococcus aureus*	Oxidase (+) Catalase (+) Coagulase (+)	Positive *S. aureus*
	MacConkey	DOP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Table 4 *(Continue)*

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
		DPP1	Colourless, flat, and smooth colonies	*Pseudomonas aeruginos*a	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		DPP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		DHS1	Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
D	MacConkey	DHS2	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
			Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		DHS3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Note. API = Analytical Profile Index; BCA = *Bacillus cereus* agar; BPA = Baird-Parker agar; OP = Open pot; CP = Close pot; PP = Propolis; HS = Hive swab

DISCUSSION

Pathogenic bacteria are usually found in the environment, such as soil, water, and air (Cavicchioli et al., 2019; Pandey et al., 2014). Abusing techniques from handling and the storage pattern before consumption can contribute to cross-contamination (Augustin et al., 2020). This statement is always true while handling food commodities, from the farm level to the customer throughout the supply chain. In this case, propolis and hive swab samples are the most contaminated with pathogenic bacteria at the farm level. According to Putri and Susanna (2021), the pollen, honeybee's digestive systems, and the environment (soil, dust, and air) are the primary sources of contamination, whereas food handlers, cross-contamination, harvesting equipment, buildings, and the environment are secondary sources.

Additionally, the presence of insects such as lizards, cockroaches, and ants might result in pathogenic bacteria that may cause the contamination of hive swab samples. Most food poisoning cases are caused by bacteria, which are from animal sources, and it has been discovered that pathogens can enter food supply chains through animal hosts, transporters, or inappropriate handling techniques (Kordiyeh, 2018).

Microorganisms in honey may affect the product's stability and hygienic quality (Erkan et al., 2017). However, it is quite challenging to eliminate the contamination of honey throughout the food supply chain. In contrast, good farming practices (GFP) and good manufacturing practices (GMP) can limit the secondary causes of honey contamination (Rivera-Gomis et al., 2019).

Naturally, open containers are exposed to bacteriological contamination. In this case, the open-pot honey was commonly exposed to environmental contamination. However, in this study, the open-pot honey samples were less contaminated with pathogenic bacteria than propolis and hive swab samples. The open-pot honey samples were less contaminated because the chemical transformation process of sugar by the bees is not complete yet. Hydrolyse the sucrose in the nectar into fructose and glucose by enzymes secreted by the worker bees is needed to maintain the sterile environment in the cerumen pot (Jalil et al., 2017). After collecting nectar, the bees released the enzyme invertase to break down the sucrose into a mixture of glucose and fructose that contribute to the function of antimicrobial properties (Hongu et al., 2017). Hence, the pathogenic bacteria were not found in all close-pot honey compared to open-pot honey because the process of breaking down sucrose is completed, the antimicrobial properties are well functioning, and the pot's structure protects against contamination.

Spore-forming bacteria from the genus *Bacillus* are commonly associated with stingless bee species (Ngalimat et al., 2019; Pucciarelli et al., 2014; Yaacob et al., 2018). A few studies reported that *B. cereus*, *S. aureus*, and *Pantoea* sp. were found in honeybee and *kelulut* honey in Malaysia (Adadi & Obeng, 2017; Amin et al., 2020; Ngalimat et al., 2019; Puciarealli et al., 2014). It is in line with this study; the pathogenic bacteria in the *kelulut* honey are *B. cereus*, *S. aureus*, and *Pantoea* species. Furthermore, pathogenic bacteria such as *P. aeruginosa*, *Serratia* sp., and *Pantoea* sp. were found in the hive swab samples.

A previous study by Akkaya et al. (2020), Feng and Hartman (1982), and Loir et al. (2003) discovered that *Clostridium botulinum*, *E. coli*, *P. aeruginosa*, and *S. aureus* spores contaminated the propolis. The contamination may come from dust in the air, the bees' gastrointestinal systems, pollens, bees' legs, and contaminated bee foods (Akkaya et al., 2020). Our results are similar to the studies mentioned above, where *P. aeruginosa* and *S. aureus* were found in the propolis samples.

These pathogenic bacteria can cause food poisoning. For example, *S. aureus* produces a fatal enterotoxin that frequently can cause pneumonia, wound infections, and nosomial bacteremia (Dinges et al., 2000; Tiemersma et al., 2004). Staphylococcal enterotoxins found in contaminated food can cause severe symptoms like vomiting, diarrhoea, and a high temperature with or without nausea and vomiting (Colombari et al., 2007). Moreover, *B. cereus* that invades the human body can cause bacteremia, pneumonia, and infection in the eye, central nervous system (CNS), and soft tissue

(Avashia et al., 2007; Gaur et al., 2001). Additionally, *Serratia* spp. are frequently found to be the source of illnesses such as meningitis, sepsis, and infections in the urinary tract, skin, bloodstream, and respiratory (Engelhart et al., 2003; Wu et al., 2013).

Good farming practices should be followed accordingly to prevent contamination at the farm level. The utensils used for harvesting and storage are expected to be free from contamination. Good practice by food handlers is compulsory to be followed. Personal hygiene is the key to preventing bacterial contamination through food handlers to avoid foodborne illness. The possibilities of contamination throughout the food supply chain of *kelulut* honey are shown in Figure 2.

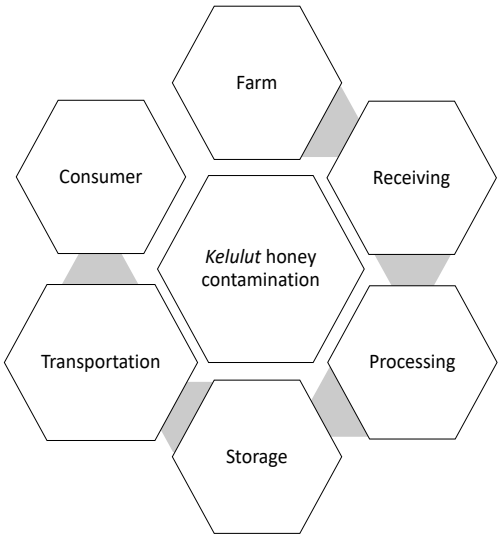

Figure 2. The possibilities of contamination throughout the food supply chain of *kelulut* honey

CONCLUSION

Pathogenic bacteria namely *B. cereus*, *S. aureus*, *Pantoea* sp., *P. aeruginosa* and *S. plymuthica* were isolated and identified in the *kelulut* honey, propolis and the wooden beehive samples. These pathogenic bacteria were confirmed with a few confirmation tests such as API 20E test, catalase test, oxidase test and coagulase test. *Kelulut* honey is a potential source of *B. cereus*, *S. aureus*, *Pantoea* sp., *P. aeruginosa* and *S. plymuthica* which may cause foodborne outbreaks. Every stakeholder should be responsible for the production of good quality *kelulut* honey to ensure that the industry is growing rapidly in the future.

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