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Research Article

Exploring Microbial Diversity in Green Honey from Pulau Banggi Sabah: A Preliminary Study

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ABSTRACT

The microbiological composition of honey can include microorganisms that are beneficial or harmful to human health. Therefore, it is essential to investigate the microbiological quality of different honey types available in the market. However, there is limited information available on the analysis, isolation, and characterization of honey-associated microbes, especially for green honey from Banggi Island. Green honey is sourced from underground areas within the island's forest. This study aimed to assess the microbiological quality of raw (freshly collected) and processed green honey by examining the presence of bacteria, yeast, molds, and pathogens. The results revealed that raw green honey had a slightly higher total plate count (770 ± 0.03 cfu/g) compared to processed green honey (640 ± 0.02 cfu/g). Both raw and processed green honey contained *Lactobacillus* spp. with counts of 350 ± 0.02 cfu/g and 160 ± 0.02 cfu/g, respectively. *Bacillus* count was higher in raw green honey (110 ± 0.01 cfu/g) compared to processed green honey (5 ± 0.01 cfu/g). Molds were only detected in raw green honey, while osmophilic yeast counts were higher in raw green honey (16000 ± 0.03 cfu/g) compared to processed green honey (120 ± 0.02 cfu/g). Mesophilic bacteria, thermophilic bacteria, coliforms, *E. coli*, and *Staphylococcus aureus* were not detected in either raw or processed green honey. Furthermore, green honey was free from pathogenic bacteria such as *Salmonella* spp., *Listeria* spp., and *Shigella* spp. Bacteria isolated from green honey included *Lysinibacillus macrolides*, *Lysinibacillus boronitolerans*, *Paenibacillus cineris*, *Paenibacillus favisp*orus, and *Bacillus oleronius*, none of which were pathogenic. This study identified important microorganisms present in green honey, which have the potential to provide beneficial effects without posing any harm to human health.

Keywords: Bacillus oleronius, Green honey, Lysinibacillus, Microorganisms, Paenibacillus, Sabah honey,

Introduction

The spoilage of honey is a natural occurrence that happens over time due to the growth of microorganisms. To slow down this growth, it is important to control the moisture and temperature levels in honey. Microorganisms commonly found in honey can be categorized into three groups: (i) yeasts and spore-forming bacteria, (ii) microorganisms that serve as indicators of honey's

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sanitary or commercial quality, such as coliforms and yeasts, and (iii) potentially pathogenic microorganisms that require careful attention during honey processing and post-harvest handling to ensure consumer safety [1]. Regulating the growth of microorganisms in honey is crucial to preserve its quality and safety for consumers.

On the other hand, there are microorganisms known as probiotics that provide health benefits to humans. Probiotics, when present in the human gut, have the ability to enhance the absorption of nutrients, such as short-chain fatty acids, ions, amino acids, and vitamins. Additionally, they promote the growth and maintenance of the gut epithelium system and help prevent the invasion of pathogenic microorganisms [2]. As honey's applications expand, it is imperative to conduct comprehensive microbiological investigations of honey to attain a broader understanding of its microbiological characteristics. The microorganisms found in honey may originate from primary or secondary contamination sources. Primary sources relate to honeybee digestive tracts that harbor natural microorganisms and collection sources such as nectar, pollen, propolis, air, flowers, and the hive environment. Secondary sources include humans, equipment, containers, wind, dust, insects, animals, and water [3].

Despite the presence of microorganisms in honey, the levels and diversity of microorganisms are generally very low. This is attributed to the inherent properties of honey, including its acidic pH, high osmotic pressure, and antibacterial properties, as well as industry-wide measures implemented to control microbial growth in honey. Honey's high osmotic pressure is a consequence of its low water activity, averaging 17.2%. At the same time, its acidic pH is maintained by the presence of organic acids like gluconic acid and the enzyme glucose oxidase, which produces hydrogen peroxide. Furthermore, the low protein content in honey inhibits the growth and survival of certain bacteria [2].

Although honey possesses natural features that inhibit microbial growth, certain microorganisms have adapted to thrive in extreme conditions, suggesting the presence of specific survival mechanisms. For example, bacteria employ proton-extruding activity to maintain a stable pH level within their cytoplasm, enabling them to survive in acidic environments [4]. Hence, it is crucial to differentiate between pathogenic and beneficial microorganisms present in honey. According to Silva *et al.* [2], fungi, yeast, and bacteria are the most prevalent microorganisms found in honey. *Bacillus* sp. and *Clostridium* sp. have been identified among bacteria. *Clostridium perfringens*, during sporulation, can produce an enterotoxin that is released along with spores when conditions for its development become unfavorable. *Bacillus cereus*, another significant honey pathogen, can produce enterotoxin within pH ranges of 6.0 to 8.0 and temperatures from 6°C to 21°C. However, a high concentration of 107 cells/mL is required for it to pose a significant risk [5].

The investigation of microorganisms in different types of honey is of utmost importance as they can have both beneficial and detrimental effects on human health. This study specifically focuses on the microbial composition of green honey, a unique food product produced by honeybees in the underground regions of the forest on Banggi Island. The distinct green color of this honey is believed to originate from the bees' consumption of algae or the collection of nectar from bamboo trees, which contain chlorophyll pigments [6]. However, our knowledge of the microbial diversity present in green honey is currently limited. Thus, the primary objective of this research is to isolate and identify the microorganisms present in green honey. This investigation is crucial for ensuring the quality and safety of green honey by controlling the growth of the identified microorganisms.

Material and Methods

Honey sample

The study involved the analysis of two types of green honey, namely commercially available processed green honey and freshly collected raw green honey. Both types of honey were obtained from NS Field Sdn. Bhd., Sabah, Malaysia, and were stored in screw-capped bottles at room temperature (approximately 25°C) until analyzed.

Standard plate count in honey

The aerobic plate count analysis was conducted following the protocols provided in the Food and Drug Administration's Bacteriological Analytical Manual [7]. To perform this analysis, a 10 g sample of honey was combined with 90 ml of sterile Butterfield's phosphate buffer (0.25 M KH_2PO_4, pH 7.2 adjusted with 1M NaOH) in a stomacher bag (BagMixer, Interscience, France).

The resulting mixture was then diluted and spread onto standard Plate Count Agar (Oxoid, UK). Subsequently, the agar plates were incubated at 35°C for 24 hours.

Mold count in honey

Mold counting was performed following the established protocols outlined in the Food and Drug Administration's Bacteriological Analytical Manual [7]. A sterile bag containing 50g of honey sample and 450ml (1:10) sterile Butterfield's phosphate-buffered dilution water (0.25 M KH_2PO_4, pH 7.2 adjusted with 1M NaOH) (Butterfield, 1932) was mixed using the Stomacher 400 Circulator for 1 minute. The resulting diluted sample was then spread onto plates containing Dichloran Rose-Bengal Chloramphenicol (DRBC) media (Oxoid, UK), and appropriate dilutions were prepared. Subsequently, the plates were incubated in the dark at 20-25°C for a duration of 5 days.

Bacillus count in honey

The presence of *Bacillus* spp. was determined using a standard method outlined by Merker [8]. A 10 g honey sample was mixed with 90 ml of sterile Butterfield's phosphate-buffered dilution water. The resulting mixture was then spread onto Mannitol Egg Yolk Polymyxin Agar plates (Oxoid, UK) and incubated at 35°C for 24 to 48 hours. It allowed for the detection and enumeration of *Bacillus* spp. in the honey samples.

Lactic acid bacteria count in honey

The identification of Lactic Acid Bacteria (LAB) was performed according to the method outlined by Aween et al. [9]. A 24-hour-old pure culture was subjected to a catalase activity test using 4% hydrogen peroxide. The presence of gas bubbles indicated a positive result, indicating the cells were catalase-positive, a characteristic feature of LAB. Conversely, a negative result indicated the cells were catalase-negative, which is indicative of LAB. This method allowed for the accurate identification of LAB in the honey samples.

Osmophilic yeast count in honey

To detect yeast growth in honey samples, a volume of 1 ml from each sample was carefully pipetted onto Potato Dextrose Agar (PDA40s) plates. The plates were then incubated at a temperature of 30°C for a period of 7 days [10]. This method allowed for the observation and identification of yeast colonies present in the honey samples.

Mesophilic spore count in honey

To prepare the honey sample for analysis, a quantity of 10g of the sample was mixed with 90 ml of sterile Butterfield's phosphate-buffer. This buffer solution, composed of 0.25 M KH_2PO_4 adjusted to a pH of 7.2 with NaOH. The mixing process was carried out using a Stomacher 400 Circulator for 1 minute. Subsequently, a bottle containing 100ml of Tryptone Glucose Extract Agar (TGEA) (manufactured by Himedia, India) was prepared. The TGEA was sterilized by subjecting it to a temperature of 121°C for 15 minutes, followed by cooling in a water bath until it reached a temperature of 45°C. Next, 10 of the diluted honey samples were added to the 100 of TGEA, and the mixture was gently agitated in a water bath at 80°C for 30 minutes. Once the process was complete, the entire volume of 100ml was distributed evenly into five sterile plates. The agar was allowed to solidify, and the plates were then incubated at 55°C for 48 hours.

Thermophilic spore count in honey

The method used to determine thermophilic spore count was similar to that of the mesophilic spore count, with the only difference being the heating temperature. The honey sample was subjected to a temperature of 100°C for a duration of 30 minutes. Following the heating process, the sample was divided equally among five sterile plates and allowed to solidify. Subsequently, the plates were placed in an incubator set at a temperature of 55°C and incubated for a period of 48 hours.

Coliform/ E. coli in honey using Most Probable Number (MPN) method

The method for the detection of coliforms in honey samples involved several steps. Firstly, 50g of the honey sample was mixed with 450 ml of sterile Butterfield's phosphate-buffered dilution water, which was adjusted to a pH of 7.2 using NaOH. The mixture was then homogenized for 1 minute using a Stomacher 400 Circulator. Next, a diluted sample of 1ml was transferred into Levine's eosin methylene blue agar (LST) along with a series of serial dilutions. The plates were incubated at 35°C for 48 hours. During incubation, the presence of gas production was observed using

inverted Durham tubes for a duration of 48 hours. Presumptive positive gassing tubes underwent a confirmation test. A loopful suspension from each gassing LST tube was transferred to Brilliant Green Lactose Bile (BGBB) broth, which was then incubated at 35°C for 48 hours for the detection of *E. coli*. A similar procedure was followed, with the confirmation test performed by transferring a loopful suspension from each gassing LST tube to a tube of EC broth. The EC broth was incubated for 48 hours at a temperature of 44.5°C. Furthermore, a loopful of suspension from each gassing tube was streaked onto L-EMB agar plates, which were subsequently incubated at 35°C for 18-24 hours.

Staphylococcus aureus in honey

A 50g honey sample was mixed with 450 ml of sterile Butterfield's phosphate buffer. A 0.1ml dilution of the mixture was then spread on Baird-Parker agar, which contained egg-yolk tellurite emulsion from Oxoid Ltd., Hampshire, England. The agar plates were incubated at 37°C for 24-48 hours.

Checking for pathogenic bacteria in honey

Honey was incubated into selective agar to check the presence of pathogenic bacteria, as described in the following sections.

Salmonella* spp. *in honey

The honey sample weighing 25g was combined with 225ml of lactose broth (LB) and subjected to incubation at 35°C for 24 hours. Then, 0.1ml of the honey-containing LB was inoculated into 10ml of Rappaport Vassiliadis (RV) media and Tretrathionate (TT) broth. The RV media was incubated at 42°C, while the TT broth was set at 37°C for 24 hours. Subsequently, one loopful of each broth was streaked onto Bovine serum albumin (BSA), xylose lysine deoxycholate (XLD), and Hektoen Enteric Agar (HEA) plates. These plates were then incubated at 35°C for 18 to 48 hours to observe the presence of pathogenic bacteria [11].

Listeria* spp. *in honey

A 25g honey sample was aseptically mixed with 225 ml of sterile Buffered Listeria Enrichment Broth Base (BLEB). The mixture was then incubated for 24-48 hours at a temperature of 30°C. Following incubation, the culture was streaked onto Listeria Identification Agar Base (PALCAM) obtained from Himedia, India, and Brilliance Listeria Agar from BIO-RAD, France. The agar plates were incubated at 35°C for 24-48 hours. These procedures were conducted using established methods [12].

Shigella* spp. *in honey

To test for the presence of *Shigella* spp., a modified method suggested by Sadik and Ali was employed [13]. For the enrichment step, a honey sample (25g) was mixed with 225 mL of GN broth (Himedia, India) and incubated at 35 ◦C for 16–18 hours. Following incubation, a 3 mm loopful of enriched GN broth with honey sample was streaked onto Hektoen enteric (HE) agar and further incubated at 35 ◦C for 24- 48 hours.

16S rDNA gene analysis

The bacterial DNA was extracted from an overnight culture grown in Tryptic Soy Agar (TSA) at 35°C using the Bacterial Genomic Extraction Kit (Wizard, USA). The universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5-GGTTACCTTGTTACGACTT-3) were used to amplify regions of the 16S rDNA. The PCR product was purified using the PCR Purification Kit (Qiagen, USA) and sequenced using the Sanger dideoxy method with the same 27F and 1492R primers. The chromatograms of the 16S rDNA gene sequences were analyzed with Chromas ver. 2.6.2 software (Technelysium Pty Ltd, Australia). The final 16S rDNA gene sequence was compared to lines available in the National Center for Biotechnology Information (NCBI) database using BLASTn [14].

Results and Discussion

Microbes in green honey

Microbes present in honey can be classified into three categories: (a) microorganisms commonly found in honey, (b) microorganisms that indicate sanitary or commercial quality, and (c) microorganisms that are pathogenic to human health [1]. *Lactobacillus* spp., *Bacillus* spp., molds, and osmophilic yeasts are some microorganisms commonly found in raw and processed honey. Table 1 shows four types of commonly found microorganisms in green honey.

The Total Plate Count (TPC) estimates the total microbial population in green honey. The TPC values for raw and processed green honey were

Table 1. The commonly found microorganisms in green honey

Parameter	Total Plate Count (cfu/g)	*Lactobacillus spp* (cfu/g)	*Bacillus spp.* (cfu/g)	Mold (cfu/g)	Osmophilic yeast (cfu/g)
Raw green honey	770 ± 0.03	350 ± 0.02	110 ± 0.01	520 ± 0.03	16,000 ± 0.03
Processed green honey	640 ± 0.02	160 ± 0.02	5 ± 0.01	0	120 ± 0.02

Table 2. Identification of bacterial species isolated from green honey based on 16S rDNA analysis

Selected bacteria	Gram staining	Spore staining	% Identity to 16S rRNA sequence in database	Accession number of bacteria species in database
Lysinibacillus macroides	Gram positive	motile bacteria that generate spores	100	NR_114920.1
Lysinibacillus boronitolerans	Gram positive	spore forming bacteria	99	NR_114207.1
Paenibacillus cineris	Gram negative	endospore forming bacteria	99	NR_042189.1
Paenibacillus favisporus	Gram negative	endospore forming bacteria	100	NR_029071.1
Bacillus oleronius	Gram negative	Spore forming bacteria	100	NR_043325.1

statistically indistinguishable. However, raw green honey exhibited slightly elevated TPC values, exceeding processed green honey by 130 cfu/g, ascribed to the higher moisture content of the former. The dehydration process, applied during the production of commercial green honey, creates an unfavorable growth environment for microorganisms, thereby reducing the plate counts in processed honey. Raw green honey has a higher colony count of *Lactobacillus* spp. (350 ± 0.02 cfu/g) compared to processed honey. The *Lactobacillus* spp. could originate from plant or plant-derived materials. *Lactobacillus* spp. is a beneficial microorganism that enhances immunity, reduces fecal enzyme activity, prevents intestinal disorders, and mitigates viral diarrhea [1].

The count of *Bacillus* spp. in raw green honey was 110 ± 0.01 cfu/g, which was higher than that in processed green honey with 5 ± 0.01 cfu/g. *Bacillus* spp. present in green honey might exist as spores as they are spore-forming bacteria. *B. cereus*, among the *Bacillus* species, is known to produce enterotoxins that can harm humans, while the other *Bacillus* species are considered safe. Due to their ability to produce bacteriocins, they hold the potential to investigate antimicrobial properties [15]. Raw green honey contained 520 ± 0.01 cfu/g of mold, while no mold was detected in processed green honey. Most of the mold present in honey may result from common contaminants of bee products [16].

Raw green honey has a significantly higher count of osmophilic yeast, with 16,000 ± 0.03 cfu/g, compared to processed green honey, with a count of 120 ± 0.02 cfu/g of osmophilic yeast. Osmophilic yeast, owing to its ability to grow under acidic conditions even at a limited level of water content, can readily ferment honey. Consequently, the fermentation rate in raw green honey is higher than in processed green honey, resulting in the formation of carbon dioxide gas. However, this high count of osmophilic yeast in honey is a major concern in the industry, as it leads to a shortened product shelf life. To control the yeast population, honey can be exposed to ultraviolet rays for a few minutes during processing, as yeasts are highly sensitive to ultraviolet radiation.

In addition, the green honey was subjected to analysis to determine the presence of microorganisms that serve as indicators of its sanitary or commercial quality. Common microorganisms used to assess the sanitary quality of honey include

mesophilic and thermophilic bacteria, coliforms, *E. coli*, and *Staphylococcus aureus*. The findings indicate the absence of these types of bacteria in both raw and processed green honey. Moreover, the green honey was evaluated for the presence of pathogenic bacteria, namely *Salmonella* spp., *Listeria* spp., and *Shigella* spp. The results demonstrate the absence of these pathogenic species in the green honey. Thus, it can be inferred that harmful bacteria are not present in the analyzed honey.

The 16S rDNA identification of randomly selected bacterial isolates from the green honey

Five bacterial colonies were selected at random from TSA plates and subjected to further identification using 16S rDNA. Based on the BLASTn analysis, the bacteria were identified as *Lysinibacillus macrolides*, *Lysinibacillus boronitolerans*, *Paenibacillus cineris*, *Paenibacillus favisporus*, and *Bacillus oleronius* (Table 2). No pathogenic microorganisms were detected using the 16S rDNA analysis.

Lysinibacillus species are Gram-positive and motile bacteria that exhibit insecticidal properties against various insects, including mosquitoes. Furthermore, these bacteria possess potential for heavy metal remediation, making them a promising candidate for bioremediation purposes. Additionally, *Lysinibacillus* species have garnered the attention of researchers as a potential agent for promoting plant growth and controlling plant diseases, potentially serving as an alternative to agrochemicals [17]. Four *Lysinibacillus* species, namely *L. sphaericus*, *L. fusiformis*, *L. xylanilyticus*, and *L. macrolides*, have been documented to possess bioremediation potential [18]. For instance, *L. fusiformis* can convert the toxic $HgCl_2$ into HgCl, while *L. macrolides*, which were identified in green honey, may be capable of converting hazardous Se oxyanions into elemental Se nanoparticles [19].

P. cineris and *P. favisporus*, both rod-shaped Gram-positive or Gram-variable endospore-forming aerobic or facultatively anaerobic bacteria, were identified in green honey. As they are commonly found in soil, particularly associated with plant roots, it is likely that these bacteria originated from the soil, considering that the green honey is harvested from the underground nests of wild bees. *Paenibacillus* species are known to enhance plant growth and provide protection against insect herbivores and phytopathogens, including bacteria, fungi, nematodes, and viruses, by producing antimicrobials and insecticides. *Paenibacillus favisporus* sp. nov., the species identified in green honey, has been found to synthesize a wide range of hydrolytic enzymes, including xylanases, cellulases, amylases, gelatinase, urease, and β-galactosidase [20].

The results of bacterial identification in green honey were compared with those of previous studies [21-22], where honey samples from various countries were collected, and bacterial species were identified using two different diagnostic approaches. All the bacterial species isolated from green honey, including *L. macrolides*, *L. boronitolerans*, *P. cineris*, and *P. favisporus*, were also detected in the previous study. This finding indicates that *Lysinibacillus* spp. and *Paenibacillus* spp. are common bacterial species found in honey samples.

Conclusion

The findings of this study suggest that green honey has a low microbial load and lacks human pathogenic microorganisms. *Lactobacillus* spp. was identified as probiotic bacteria in green honey, and osmophilic yeast present in green honey could be of potential interest to beverage industries. The absence of mesophilic bacteria, thermophilic bacteria, coliforms, *E. coli*, and *Staphylococcus aureus* suggests that green honey is uncontaminated and safe for consumption despite being harvested from underground soil. Additionally, the isolation of unique bacterial species such as *Lysinibacillus macrolides*, *Lysinibacillus boronitolerans*, *Paenibacillus cineris*, *Paenibacillus favisporus*, and *Bacillus oleronius* is noteworthy due to their bioremediation potential in converting hazardous elements into non-hazardous compounds and synthesizing a diverse range of hydrolytic enzymes.

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