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Exploration of natural microflora from stingless bee honey harvested from Limau Manis area, Padang, West Sumatra, Indonesia

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Abstract. *Nurmiati N, Herwina H, Periadnadi P, Janra MN, Hidayat R, Edelwis TW. 2024. Exploration of natural microflora from stingless bee honey harvested from Limau Manis area, Padang, West Sumatra, Indonesia. Biodiversitas 25: 2908-2916.* The natural microflora in stingless bee honey has been explored in three stingless bee species: *Tetragonula laeviceps* Smith, 1857; *Heterotrigona itama* Cockerell, 1918; and *Geniotrigona thoracica* Smith, 1857. This study aimed to identify and analyze the proportions of natural microflora in stingless bee honey. The study used a survey method, with data being descriptively analyzed and presented. The focus was on identifying microflora groups of bacteria and yeast in the honey from these three stingless bee species. Proteolytic bacteria Microflora types were identified based on the medium used: GPA+$CaCO_3$ for acidic microbes, SMA for proteolytic bacteria, and ethanol+CaCO3 for acetic acid microbes. The pour plate method was used to count the microbes. *T. laeviceps* honey (pH 3.3, sugar level 45% Brix) contained a total of 163.6×10^4 CFU/mL microflora (161.6×10^4 CFU/mL bacteria and 2×10^4 CFU/mL yeast). In contrast, honey from *H. itama* (pH 3.4, sugar level 57% Brix) harbored a total of 156.7 x 10^4 CFU/mL microflora (155.7×10^4 CFU/mL bacteria and 1×10^4 CFU/mL yeast) and *G. thoracica* honey (pH 3.7, sugar level 70% Brix) contained 129.6×10^4 CFU/mL bacteria only. Acidic (A) and proteolytic (P) bacteria were observed in honey samples from *T. laeviceps* (A= 69.3×10^4 CFU/mL; P= 36×10^4 CFU/mL), *G. thoracica* (A= 53.6×10^4 CFU/mL; P= 40×10^4 CFU/mL), and *H. itama* (A= 67.0×10^4 CFU/mL; P= 38.6×10^4 CFU/mL). Acidic and proteolytic bacteria were present in all three honey samples. In conclusion, the microflora composition in stingless bee honey varies across species, with bacteria being the dominant group in all samples. The pH and sugar content of honey may influence the microbial load, indicating a possible link between honey properties and microbial diversity.

Keywords: *Geniotrigona thoracica*, *Heterotrigona itama*, microflora, stingless bee, *Tetragonula laeviceps*

INTRODUCTION

Honey is a natural substance characterized by its thick, liquid consistency and sugary taste. It is synthesized by bees or stingless bees from flower nectar (Kumar et al. 2024b). The sweetness of honey comes from its monosaccharides, fructose, and glucose content (Sathianarayanan et al. 2024). Besides containing antioxidant compounds, honey is also rich in simple carbohydrates. Bee workers process flower nectar using enzymes such as diastase and invertase (Rajindran et al. 2022). The savor of honey depends on the flowers from which the nectar originates, while its sugar and carbohydrate content supports the existence of microbial communities within. The microbial activity in honey plays a crucial role in its properties. The gases or steams produced during honey storage are by-products of enzymatic activity from the microbes present in honey (Musa and Elnour 2024). Understanding the microbial community is essential to exploring the full potential of honey as a product with unique qualities.

Stingless bees (Apidae: Meliponini) are significant honey producers, primarily distributed in tropical and subtropical regions of South America, Australia, and Southeast Asia (Biscassi et al. 2024). In Indonesia, stingless bees are known by various local names, including *galo-galo* in Sumatra, *klanceng* or *lenceng* in Java, and *teuweul* in West Java. While honey from bees (Apidae: Apinae) has been extensively studied, honey from stingless bees offers many unexplored scientific aspects. Research on stingless bee honey has focused on its organoleptic properties (Gadge et al. 2023; Mello et al. 2024) and its antibacterial nature (Aburayyan et al. 2024). Honey and its derivative products have become regular consumption items in communities worldwide (Kumar et al. 2024b). There is still room for improvement in honey products, especially considering the presence of natural microflora (Tesfaye et al. 2024). For instance, the existence of lactic acid bacteria (LAB), which may indicate probiotic properties in honey, warrants further investigation. Therefore, this research aims to explore the presence and proportion of natural microflora in honey harvested from various stingless bee species in Padang City, West Sumatra, Indonesia. Stingless bee honey (Meliponini) has been recognized for its unique flavor, texture, and medicinal properties (Bakar 2024). Unlike honey from the more widely known Apis species, stingless bee honey, often referred to as *kelulut* honey, has a more liquid consistency, a tangy taste, and higher moisture content (Jamzuri et al. 2023). These characteristics make

stingless bee honey a distinct product, sparking interest among researchers and consumers alike.

Indonesia, as one of the world's biodiversity hotspots, is known as a home for various stingless bee species (Oddie and Dahle 2024). The Limau Manis area in Padang, West Sumatra, provides an ideal environment for these bees, thanks to its rich flora and favorable climatic conditions. The region's diverse plant life, including many endemic species, contributes to the unique qualities of the honey produced there. This rich biodiversity not only influences the honey's flavor and nutritional profile but also its microbial composition.

The microflora present in honey, including bacteria, yeasts, and molds, is critical in determining its quality, shelf life, and therapeutic properties (Ahmed et al. 2024). Certain microorganisms can enhance honey's antioxidant capacity, and antimicrobial activity, and contribute to its fermentation process (Luca et al. 2024). Therefore, understanding the microbial diversity within honey is essential for elucidating these beneficial properties and ensuring high-quality honey production. Despite the growing interest in stingless bee honey, research focusing on the natural microflora of this honey, particularly from regions like Limau Manis, remains limited. This study aimed to address this gap by investigating the natural microflora in stingless bee honey harvested from this unique area. Identifying and characterizing the microorganisms within this honey will provide valuable insights into the factors contributing to its distinct properties.

The exploration of honey's microbial composition extends beyond understanding its quality and therapeutic properties. Beneficial microorganisms can influence honey's fermentative stability, flavor development, and preservation qualities. Additionally, some bacteria and yeasts found in honey have been linked to probiotic effects, promoting gut health when consumed. Therefore, a comprehensive analysis of honey's microflora could provide critical information for the food and health industries.

Investigating the natural microflora in stingless bee honey from the Limau Manis area is not only a scientific endeavor but also an effort to preserve and enhance a traditional and valuable product. Through this study, we hope to unlock new potential uses for stingless bee honey and promote its benefits to a broader audience.

MATERIALS AND METHODS

Study area and sampling methods

This study involved honey samples collected from three stingless bee species: *Heterotrigona itama* Cockerell, 1918, *Geniotrigona thoracica* Smith, 1857, and *Tetragonula laeviceps* Smith, 1857, bred by the Amami Breeding House in the Limau Manis area of Padang, West Sumatra, Indonesia. Each colony was sampled by extracting 50 mL of honey using a standardized harvesting technique. This technique involved carefully puncturing honey pots within the hive using sterile stainless-steel needles and drawing the honey into sterile syringes.

The sampled honey was immediately transferred into pre-sterilized, airtight glass jars, which were labeled with the species name, colony ID, and collection date. The jars were then stored in insulated containers with ice packs to maintain a stable temperature of 4°C during transport. Upon arrival at the laboratory, the honey samples were stored at 5°C until further microbiological and chemical analysis could be conducted.

The microflora in honey samples was cultured using various media to assess different microbial characteristics. Glucose Peptone Agar (GPA) was utilized to isolate general microorganisms, including bacteria and yeasts, that thrive in glucose-rich environments, making it suitable for honey with its high sugar content. To detect acidic microbes, a combined medium of GPA and calcium carbonate (GPA+$CaCO_3$) was employed to indicate acid fermentation (lactic and acetic acid) through clear zones around bacterial colonies. Meanwhile, ethanol+$CaCO_3$ medium was used to indicate acetic acid bacteria, in other words, the absence of acetic acid bacteria confirms the presence of lactic acid bacteria. Proteolytic microorganisms were identified using Skim Milk Agar (SMA), which helps reveal protease activity by forming clear zones around colonies, thereby distinguishing proteolytic bacteria from non-proteolytic ones. Additionally, the sugar level in honey was measured using a refractometer, and the pH was determined with a standardized pH meter (Periadnadi et al. 2024; Nurmiati et al. 2024).

Bacterial identification and counting

The type of microflora is defined according to the medium on which it grows; GPA medium is used for all types of microbes, GPA+$CaCO_3$ is used for acidic microbes, SMA is used for proteolytic bacteria, and ethanol+$CaCO_3$ is used for acetic acid microbes. The pour plate method was used to count the microbes. The colony of microflora was counted using this method and expressed in colony-forming units per milliliter (CFU/mL) as well as in percentage. The sugar level is measured in % Brix, which indicates the concentration of dissolved solids, primarily sugars, in the honey.

Data analysis

Data were statistically analyzed using linear correlation regression and then descriptively presented. The parameters included in the analysis were total microflora, total fermenting bacteria, total proteolytic bacteria, pH value, and sugar level.

RESULTS AND DISCUSSION

Physical properties of the stingless bees' honey

The honey displayed differences in coloration, with the honey harvested from the *Heterotrigona itama* colony exhibiting the darkest gradient compared to the other two types (Figure 1). This condition is attributed to the phenolic and flavonoid elements in honey. The higher the phenolic content, the darker the honey becomes. A previous study indicated that the dominant phenolic in honey is anthocyanin

(Hernanz et al. 2023). Meanwhile, when honey is dominated by flavonoids, its color turns yellow or lighter (Kang et al. 2023). Aside from primarily containing water and sugar, honey also consists of about 200 different chemical compounds, including vitamins, enzymes, amino acids, and minerals. Therefore, the difference in coloration among stingless bee honey could indicate differences in chemical composition, which in turn may result in different characteristics, advantages, and benefits (Vanderplanck et al. 2023). The honey from the three stingless bees also exhibited differences in sugar level and acidity (Table 1).

Table 1 presents the sugar levels and pH values of honey samples collected from three different stingless bee species in Limau Manis, Padang. The pH value reflects the honey's acidity.

The honey from *H. itama* has a sugar level of 57% Brix and a pH of 3.4. This relatively high sugar content suggests a significant concentration of soluble sugars, which is characteristic of honey, while the pH value indicates a moderately acidic nature.

In contrast, honey from *G. thoracica* shows the highest sugar level at 70% Brix and a pH of 3.7. This elevated sugar concentration indicates a very concentrated honey, with a pH value that, although slightly higher than the other samples, still reflects a moderately acidic environment.

On the other hand, honey from *T. laeviceps* exhibits the lowest sugar level among the three at 45% Brix, accompanied by a pH of 3.3. The lower sugar concentration suggests less concentrated honey, and the pH value is the lowest, indicating a slightly more acidic nature.

In summary, the variation in sugar content and acidity among the honey samples from different stingless bee species demonstrates the typical characteristics of honey, including high sugar concentrations and moderate acidity. These differences could potentially affect the honey's flavor, texture, and health benefits.

Microflora in the honey of stingless bees

The presence of microflora in the stingless bees' honey sampled in this study is shown in Figure 2 and Table 2. The total microflora found in the honey of *T. laeviceps* was 16.36×10^4 CFU/mL, consisting of 161.6×10^4 CFU/mL bacteria (98.77%) and 2×10^4 CFU/mL yeast (1.23%), while in the honey of *H. itama*, total microflora reached 156.7×10^4 CFU/mL, comprising 155.7×10^4 CFU/mL bacteria (99.36%) and 1×10^4 CFU/mL yeast (0.64%). On the other hand, the microflora in *G. thoracica* honey consisted of only bacteria (129.6×10^4 CFU/mL) with no trace of yeast. The condition of microflora can be affected by many factors, such as the length of the honey storage period. Microorganisms in honey mainly derive from nectar and the digestive tracts of worker bees (Tsadila et al. 2023). Yeast and mold are usually present in minuscule amounts; however, some conditions can trigger their multiplication, especially during storage (Gao et al. 2023). Honey can naturally contain osmotolerant yeast, which can cause unwanted fermentation, and the maximum population of yeast should not exceed 5×10^5/10g (Anderson and Mott 2023). As yeast activity depends on water content, honey from moist areas becomes more vulnerable to yeast contamination (Luca et al. 2024).

The existence of microflora in honey is indicated by the appearance of colonies on the appropriate culturing medium (Sami-ul-Haq et al. 2024). Cultures with 30-300 colonies are ideal for population counting. A series of dilutions were then performed on the representative cultures to count the microflora population, with the assumption that the visible colonies represent the overall existing microorganisms in the sampled honey of stingless bees. Variations in microflora counts can be expected depending on the freshness of the honey samples, the harvesting time, or the extraction techniques used to obtain the honey (Abdi et al. 2024). In addition to examining the total microflora present in the honey samples, it is also possible to observe specific bacteria by using appropriate specific mediums.

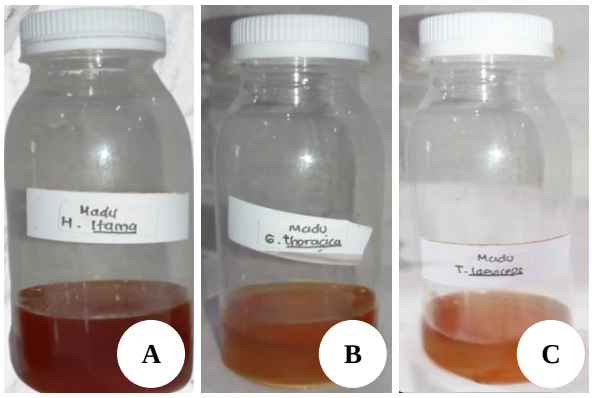


Figure 1. Color of stingless bee's honey: A. *Heterotrigona itama*; B. *Geniotrigona thoracica*; C. *Tetragonula laeviceps*

Table 1. Sugar level and pH of stingless bee honey sampled from Limau Manis, Padang, Indonesia

Species	Sugar level (% Brix)	pH
Heterotrigona itama	57	3.4
Geniotrigona thoracica	70	3.7
Tetragonula laeviceps	45	3.3

Table 2. Natural microflora in stingless bee honey detected with GPA medium

Species	Overall microflora ($\times10^4$ CFU/mL)	%	Total bacteria ($\times10^4$ CFU/mL)	%	Total yeast ($\times10^4$ CFU/mL)	%
Heterotrigona itama	156.7	100	155.7	99.36	1	0.64
Geniotrigona thoracica	129.6	100	129.6	100	0	0
Tetragonula laeviceps	163.6	100	161.6	98.77	2	1.22

The presence of fermenting (acidifying) bacteria was observed in honey from stingless bees (Figure 3). The bacteria formed a clear zone on the GPA+$CaCO_3$ chalky medium (Figure 3) as a result of hydrolysis performed by the microflora, due to the acidity of the medium (GPA) combined with calcium carbonate ($CaCO_3$). This combination neutralizes the lime present in the colony area, resulting in the formation of a clear zone (Periadnadi et al. 2024; Maruška et al. 2024). The assessed population of fermenting bacteria within stingless bee honey can be seen in Table 3.

Table 3 details the population of acidic bacteria in honey samples from various stingless bee species, as determined using the GPA+$CaCO_3$ medium. The total acidic bacteria count, reported in 10^5 CFU/mL, reflects their proportion of the overall bacterial population. The honey from *H. itama* reveals a total acidic bacteria count of 67.0×10^4 CFU/mL, constituting 43.03% of the total bacterial population. This indicates a significant presence of acidic bacteria in this honey sample. In comparison, *G. thoracica* honey has a total acidic bacteria count of 53.6×10^4 CFU/mL, which accounts for 41.36% of the total bacterial population. Although this percentage is slightly lower than that of *H. itama*, it still represents a considerable proportion of acidic bacteria. The highest concentration of acidic bacteria is found in honey from *T. laeviceps*, with a count of 69.3×10^4 CFU/mL, making up 42.88% of the total bacterial population. This suggests a robust presence of acidic bacteria in this sample as well.

Overall, the analysis reveals that all honey samples from the stingless bee species contain a significant amount of acidic bacteria, with some variation in concentration and percentage. These findings emphasize the acidic nature of honey, which may contribute to its preservation and potential health benefits.

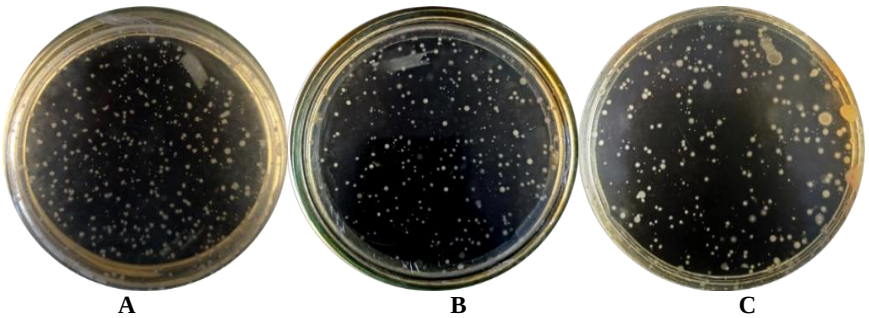
The SMA medium is designed to detect potential bacterial strains capable of breaking down protein in honey (Figure 4). The bacterial population in each honey sample tested can be seen in Table 4. The largest population of proteolytic bacteria was identified in *G. thoracica* honey (40.0×10^4 CFU/mL; 30.86%), followed by *H. itama* (38.6×10^4 CFU/mL; 24.79%) and *T. laeviceps* (36.0×10^4 CFU/mL; 22.28%). The difference in bacterial populations is influenced by the protein levels contained in each honey sample (Li et al. 2024). The clear zone formed in the SMA medium indicated the existence of proteolytic bacteria within the honey, where they hydrolyzed the protein and casein in the SMA medium to produce and activate protease enzymes (Jančič et al. 2024).

Table 3. Acidic bacteria population assessed in stingless bee honey using GPA+$CaCO_3$ medium

Honey sample	Total acidic bacteria (10^4 CFU/mL)	%
Heterotrigona itama	67.0	43.03
Geniotrigona thoracica	53.6	41.36
Tetragonula laeviceps	69.3	42.88

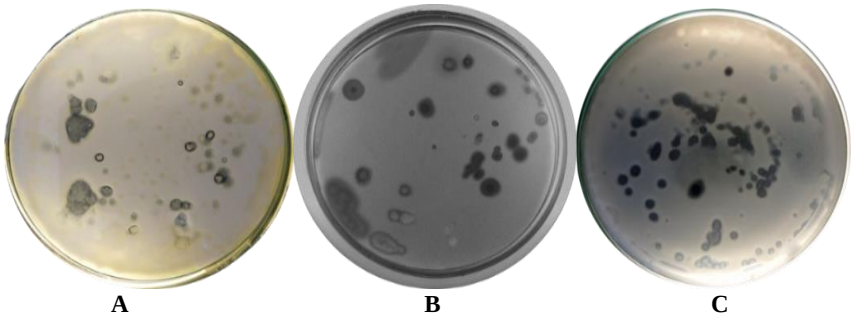
Table 4. Average number of proteolytic bacteria in stingless bee honey in SMA medium

Honey sample	Total proteolytic bacteria (10^4 CFU/mL)	%
Heterotrigona itama	38.6	24.79
Geniotrigona thoracica	40.0	30.86
Tetragonula laeviceps	36.0	22.28



A **B** **C**

Figure 2. Indication of natural microflora in stingless bee honey using Glucose Peptone Agar (GPA) medium: A. *Heterotrigona itama*; B. *Geniotrigona thoracica*; C. *Tetragonula laeviceps*



A **B** **C**

Figure 3. Indication of fermenting bacteria in stingless bee honey using GPA+$CaCO_3$ medium: A. *Heterotrigona itama*; B. *Geniotrigona thoracica*; C. *Tetragonula laeviceps*

The testing for the presence of bacteria using ethanol+$CaCO_3$ medium showed no halo zone formation, indicating no bacteria were detected (Figure 5). Most bacteria are sensitive to ethanol present in the medium, except for acetic acid bacteria, which use ethanol as a source of carbon for their metabolism. Furthermore, the existence of yeast in honey also indicates the possibility of acetic acid bacteria, since yeast produces ethanol by fermenting the hexose in honey (Bauer et al. 2022). In many cases, the higher the presence of yeast in honey, the more likely it is that acetic acid bacteria are present. However, in this study, aside from no acetic acid bacteria being found, yeast was also confirmed to be absent from the honey samples tested.

Knowing the presence and quantity of microflora in stingless bee honey can providing help provide insights regarding the quality of the honey. The presence of total bacteria in honey samples, as well as the specific counts of bacteria according to their nature (as acidifying or proteolytic organisms), is shown in Figure 6. Furthermore, the existence of microflora in honey proportionally affects the sugar level and pH value of the honey. The correlation between microflora and sugar level and/or pH value in the honey samples tested in this study can be seen in Figure 7 below.

To evaluate how sugar content and pH influence the total number of bacteria, we used linear regression analysis. The diagram presents two linear regression lines that illustrate the relationships between these independent variables (sugar content and pH) and the dependent variable (total bacteria). This can be observed in Figure 7.

Discussion

The acidity of the stingless bee honey sampled in this study ranged between 3.3 (in *T. laeviceps* honey) to 3.7 (in *G. thoracica* honey), indicating the acidic nature of honey. This measurement aligns with the previously confirmed acidity range for honey (3.27-3.93) collected from various stingless bee species, including *T. laeviceps* (Ahmed et al. 2024). An increase in honey acidity may result from fermentation carried out by lactic acid bacteria, which causes a sour taste in the honey (Abdi et al. 2024).

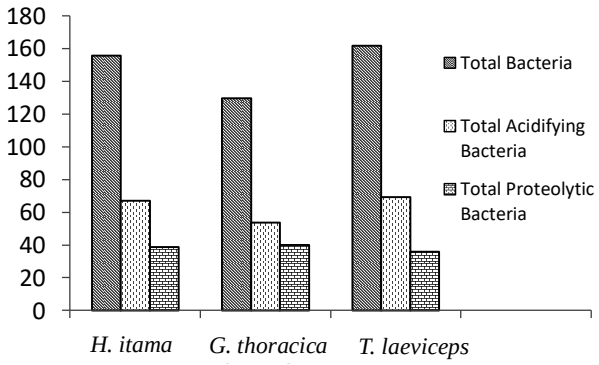


Figure 6. Average number of bacteria types in the honey samples of stingless bees (10^4 CFU/mL)

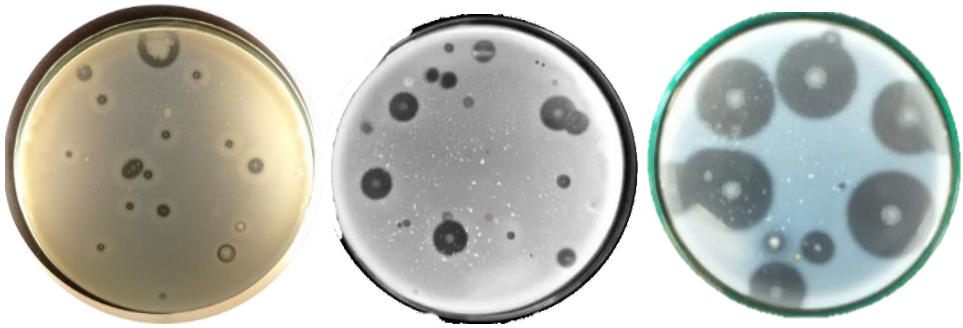


Figure 4. Indication of proteolytic bacteria in stingless bee honey using SMA medium: A. *Heterotrigona itama*; B. *Geniotrigona thoracica*; C. *Tetragonula laeviceps*

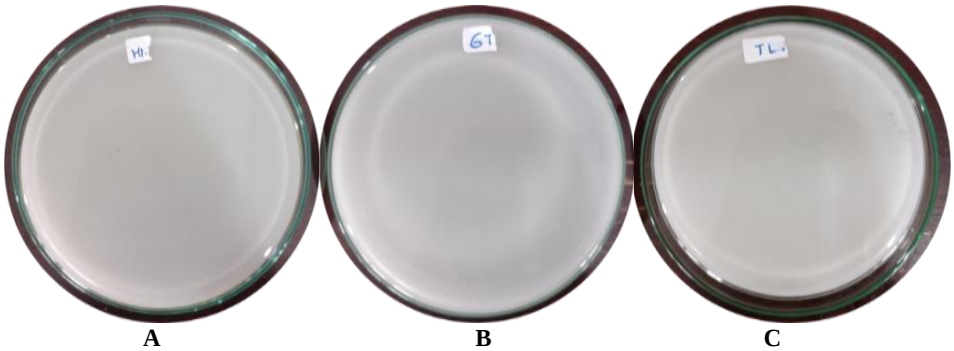


Figure 5. Indication of acetic acid bacteria in stingless bee honey using ethanol+$CaCO_3$ medium: A. *Heterotrigona itama*; B. *Geniotrigona thoracica*; C. *Tetragonula laeviceps*

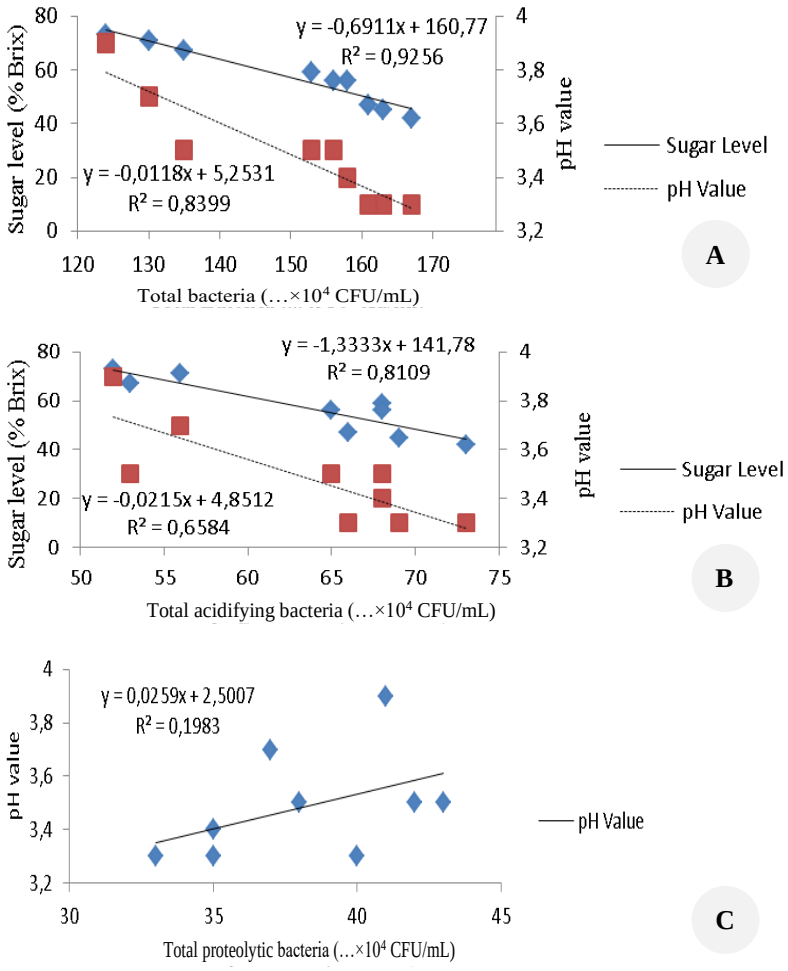


Figure 7. Correlation between microflora and quality of stingless bee honey: A. Bacteria vs sugar level and pH value; B. Fermenting bacteria vs sugar level and pH value; C. Proteolytic bacteria vs pH value

Meanwhile, lactic acid bacteria break down dextrose in honey into gluconic acid, leading to an increase in pH and sourness (Tran et al. 2022). In addition, the color, acidity, and sugar level of honey are determined by bee species, the source of nectar, geographical conditions, and the maturity of bee-worker individuals (Sarioğlu-Bozkurt et al. 2022).

The sugar content of the stingless bee honey samples ranged from 45% Brix in *T. leaviceps* up to 70% Brix in *G. thoracica*; this value is significantly higher than the 55% Brix stated by the Indonesian National Standard (The National Standardization Agency of Indonesia 2018). The sugar content in stingless bee honey is primarily influenced by storage time, as prolonged storage of honey can significantly reduce its sugar content (Mello et al. 2024).

Honey from the Indonesian region is usually high in water content due to its hygroscopic property, which makes it prone to absorbing moisture from the surroundings (Musa and Elnour 2024). High temperatures will affect less humid and highly sugary honey, while in contrast, low temperatures affect highly humid and less sugary nectar. The humidity of nectar correlates with the hygroscopic nature of sugars contained in nectar, as these sugars can absorb water particles from moist air better than from dry air. Furthermore, the humidity of honey is influenced by the season, climate, and type of plant, which then manifests in the physical properties of honey (Li et al. 2024). High water content can trigger the natural microbes to carry out fermentation in honey. Reducing the water content in honey is effective in suppressing microbial activity.

The presence of acidic bacteria in honey samples was calculated as follows (i) 69.3×10⁴ CFU/mL (42.88%) in *T. laeviceps* honey, (ii) 53.6×10⁴ CFU/mL (41.36%) in *G. thoracica* honey; and (iii) 67.0×10⁴ CFU/mL (43.03%) in *H. itama* honey. The quantification of acidifying or fermenting bacteria in stingless bee honey is useful for indicating the fermentation rate that may occur. Fermentation can be impacted by honey ingredients or external factors such as storage conditions or environmental conditions during harvesting and storing. Fermentation in honey usually takes place following crystallization, in which the liquid part of honey (i.e., concentrated mixture of fructose, acid, and water) triggers the existence of yeasts, which then increases the water content in honey (Bakar 2024). The low pH of honey itself, as observed in this study, is favorable for the presence of fermenting bacteria. Hence, the decrease in pH is inversely proportional to the increase in acidity level that

promotes the growth of acidifying bacteria (Anderson and Mott 2023).

The halo zone that formed on the SMA medium confirms the existence of proteolytic bacteria in honey samples (Figure 4). Subsequent counting found that there were 36×10^4 CFU/mL (22.28%) proteolytic bacteria in *T. laeviceps* honey, 40×10^4 CFU/mL (30.86%) in *G. thoracica* honey, and 38.6×10^4 CFU/mL (24.79%) in *H. itama* honey. The population of proteolytic bacteria is closely related to the protein contained in honey (Anderson and Mott 2023). The halo zone results from the activity of proteolytic bacteria in hydrolyzing protein and casein in the SMA medium by activating protease (Achouri et al. 2020). Protein in honey, on the other hand, derives mainly from the contact between honey and bee larvae raised in adjacent cells in the same hive. Bee larvae are most commonly mixed into honey during harvesting and enrich the protein in honey (Oddie and Dahle 2024). The range of proteolytic bacteria contained in each honey sample in this study depends on their ability to ferment protein and produce lactic acid at the same time. Lactic acid also acts as an inhibitor for the growth of spoilage microorganisms such as proteolytic bacteria.

The GPA medium, with its sugar and peptone contents, functions to detect the presence of total microflora in honey samples. This medium is viable for growing and detecting fermenting, proteolytic, and other types of bacteria (Nurmiati et al. 2018). The GPA+$CaCO_3$ medium is specifically used for detecting fermenting bacteria that are potential acid-producers. Adding calcium carbonate to the medium helps to isolate bacteria that produce acid (Rathakrishnan and Gopalan 2022). Naturally occurring fermenting bacteria in honey are present due to various stages of the honey-making process, including nectar harvesting from flowers, transport within the stingless bee workers' abdomen, regurgitation and possible mastication of processed nectar within the hive, and eventual storage in honey pots where fermentation occurs (Stefanski et al. 2020). Furthermore, the SMA medium identifies proteolytic bacteria, especially those that are capable of breaking down proteins in honey.

All mediums used in this study, except the ethanol+$CaCO_3$ medium, reveal variability in physical features in all honey tested, including sugar level and pH. They also indicate the existence of several microflora groups in each honey sample and variability in their proportions. Honey can be contaminated by different microorganisms through contact with pollen, workers' digestive tract, soil, water, air, and nectar (Kumar et al. 2024a). Additionally, microflora can grow in honey once introduced during processing, handling, and storage by humans. Physicochemical properties of honey, i.e., low water activity, low pH, high sugar concentration, and different enzyme activities theoretically influence the survival of different bacteria within it.

The population of bacteria in stingless bee honey negatively correlated to pH value (Y=-0.0118x+5.2531), which means that every 10,000 increase in bacterial count will bring a decrease in pH by 0.0118. In other words, the increase in the bacterial population in honey will have an inverse effect on pH value (Figure 7.A; red rectangles). The bacterial population also negatively correlated to the sugar level in honey (Y=-0.6911x+160.77), where an increase in 10,000 bacteria is associated with a decrease in the sugar of 0.6911 (Figure 7.A, blue rectangles). The population of fermenting bacteria in stingless bee honey negatively correlated to pH (Y=-0.0215x+4.8512), where the growth in 10,000 bacteria is associated with a decrease acid by 0.0215 (Figure 7.B; red rectangles). Similarly, the relationship between fermenting bacteria and sugar level was a negative correlation (Y=-1.3333x + 141.78), where an increase of 10,000 bacteria was associated with a decrease in sugar of 1.3333 (Figure 7.B; blue rectangles). A positive correlation was observed between the population of proteolytic bacteria and pH (Y=0.0259x+2.5007), where the increase in the 10,000 bacterial population is associated with an increase in the acid of 0.0259 (Figure 7.C).

The inverse was relation was observed between the total bacterial population and fermenting bacteria concerning sugar level and pH. Previous studies have indicated that bacteria can alter sugar levels and acidity in honey, including honey harvested from hives in natural forests (Abdi et al. 2024). Acidic bacteria trigger fermentation, which subsequently increases the pH of honey. When pH decreases in honey, it creates an acidic environment that promotes the growth of acidifying bacteria (Kumar et al. 2024a). As bacteria utilize carbon from sugar molecules as their energy sources, this results in a decrease in sugar levels in honey (Kang et al. 2023). Meanwhile, a positive relationship between proteolytic bacteria and pH occurs because low pH triggers the growth of fermenting bacteria in honey and produces lactic acid, which in turn inhibits the development of spoilage bacteria such as proteolytic bacteria (Zhuang et al. 2023). The more lactic acid is produced by fermenting bacteria, the greater the inhibition of proteolytic bacteria. Lactic acid bacteria are known for their ability to inhibit pathogenic bacteria and improve hygiene and health in humans (Tran et al. 2022).

In conclusion, this study confirmed the presence of natural microflora, including bacteria and yeast, in honey from various stingless bee species in West Sumatra, Indonesia, specifically *H. itama*, *G. thoracica*, and *T. laeviceps*. It identified both acidifying and proteolytic bacteria, with lactic acid bacteria emerging as a key group of acidifying bacteria. The microflora composition varied significantly among the species: honey from *T. laeviceps* (pH 3.3, sugar level 45% Brix) contained 163.610^4 CFU/mL of microflora (161.6×10^4 CFU/mL bacteria and 2×10^4 CFU/mL yeast), while *H. itama* (pH 3.4, sugar level 57% Brix) had 156.7×10^4 CFU/mL of microflora (155.7×10^4 CFU/mL bacteria and 1×10^4 CFU/mL yeast), and *G. thoracica* (pH 3.7, sugar level 70% Brix) contained 129.6×10^4 CFU/mL of bacteria alone. The concentrations of acidic and proteolytic bacteria were as follows: *T. laeviceps* (A= 69.3×10^4 CFU/mL; P= 36×10^4 CFU/mL), *G. thoracica* (A= 53.6×10^4 CFU/mL; P= 40×10^4 CFU/mL), and *H. itama* (A= 67.0×10^4 CFU/mL; P= 38.6×10^4 CFU/mL). Factors such as bee species, food sources, honey storage duration, and geographical conditions influenced bacterial populations. Further research is needed to isolate acidifying bacteria from stingless bee honey to explore their potential as probiotic candidates. This research confirmed the existence

of natural microflora (bacteria and yeast) in honey harvested from several species of stingless bees in West Sumatra. The acidifying bacteria and proteolytic bacteria were recognized from the honey of stingless bees *H. itama*, *G. thoracica*, and *T. laeviceps*. Furthermore, this study confirmed that the lactic acid bacteria group is representative of acidifying bacteria found in the honey of stingless bees tested. Some factors influenced the population of bacteria observed in this study, which included stingless bee species, food sources, storage time of honey, and some geographical-related conditions. Further studies to isolate the acidifying bacteria from stingless bee honey with potential as probiotic candidates are needed.

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