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Microbial load on raw bee pollen and bee bread across mid- and high-elevation areas in Bukidnon, Philippines

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Abstract

The bacterial population on raw bee pollen and bee bread did not vary significantly across the mid- and high-elevation areas planted with Arabica coffee, with values that range from 5.4 x 10^6 to 6.4 x 10^6cfu/g. On the other hand, significant and highly significant differences in the fungal population across the three locations were observed on bee pollen and bee bread, respectively. A low fungal population was recorded on bee pollen collected from the high-elevation area (Site 3-Miarayon, Talakag) with 3.6 x 10^4cfu/g which was comparable to the population on samples collected from the mid-elevation area (Site 1-Imbayao, Malaybalay City) with 5.4 x 10^4cfu/g. Similarly, a low fungal count was recorded on bee bread collected from the high-elevation area (Site 3-Miarayon, Talakag) with 3.0 x 10^4cfu/g. Moreover, seven bacterial isolates were associated with the bee pollen and bee bread samples; three colonies were Gram-positive and four colonies were Gram-negative based on their reaction to Gram stain. On the other hand, yeasts and filamentous fungal species (*Aspergillus*, *Fusarium*, and *Penicillium)* were present in the two honey bee products. This study demonstrates that the fungal population in raw samples of bee pollen and bee bread from Arabica coffee plants grown at high elevation is lower than that in samples from mid-elevation areas.

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Introduction

Bee-collected pollen and bee bread are two important bee products that had gained special attention at present because of their high nutritive and therapeutic values. Bee pollen comprises pollen grains from plants, nectars, and salivary secretions from forager bees (Kevin & Baker, 1983 as cited by Friedle *et al.*, 2021). This bee product is high in protein containing substantial amounts of essential amino acids which are five to seven times higher than those found in traditional high-protein foods. Bee pollen also contains vitamins A, D, E, K, C, bioflavonoids, B-complex (pantothenic acid and niacin), phenolics, phytosterols and phytochemicals (Fratini *et al.*, 2014).

Bee bread, on the other hand, is a substance that is tightly packed inside the honeycomb cells and consists of a mixture of pollen, honey, and other bacteria. This is like silage in that it uses the pollen that has been harvested as raw material, which is then fermented and used to provide the bees within the hive with stored nourishment (Corby-Harris *et al.*, 2014). Typically, nurse bees consume the bee bread to generate protein-rich larval food by mixing it with secretions from their specialized glands (Cridge *et al.*, 2015).

Because of their high nutritional values, bee pollen and bee bread are now referred to as "human functional foods," and both products provide a variety of health benefits, including antimicrobial, antioxidant, anti-radiation, anti-inflammatory, anti-tumor, hepatoprotective, and chemo-preventive/chemo-protective benefits (Pełka *et al.*, 2021). According to Nainu *et al.* (2021), traditional medicine has employed bee products for a very long time to cure a wide range of illnesses, including cancer and microbial-related ailments. In fact, studies on several chemical components present in bee products had shown that they possess anticancer, antibacterial, antiviral, and antiparasitic properties.

Numerous studies were done as regards their nutritional makeup, and medical and pharmaceutical properties, however, there are still significant knowledge gaps about the microbial environment of bee pollen and bee bread. There is limited information about the influence of external factors such as geographical location and botanical origin on the population of microbial species as well as their contribution to the production of high-quality honey bee products. Knowledge of the associated microorganisms such as bacteria and fungi in bee pollen and bee bread is crucial in order to maintain the health status of mature and larval honey bees within the hive and to ensure the production of high-quality bee products for human consumption. Hence, this study was conducted to assess the microbial load on raw bee pollen and bee bread collected from honey bee (*Apis mellifera* L.) colonies which were established on Arabica farms located at mid- and high-elevation areas in Bukidnon. Further, the characterization of aerobic microbial species associated with the bee products was performed using cultural and morphological methods.

Materials and methods

Selection of study sites

The conduct of this study started on August 2021 to July 2022 in the Province of Bukidnon, Philippines. Highland areas with established Arabica coffee plants were chosen as study sites namely: Imbayao, Malaybalay City (1052 meters above sea level); Songco, Lantapan (1073 meters above sea level); and Miarayon, Talakag (1381.9 meters above sea level). The honey bee colonies used in the study were borrowed from Miel Internacional, Inc. These colonies were distributed to different sites and placed in strategic locations within the established Arabica coffee farms. Regular monitoring and assessments were done on the honeybee colonies to ensure their proper growth and development throughout the duration of the study.

Collection of bee pollen and bee bread

The actual sampling of bee pollen and bee bread from the established honey bee colonies was performed by a bee expert, Mr. Elviro A. Garcines, Jr. Fresh samples were directly collected from the food frames and placed aseptically on sterile glass containers

using a sterile wire loop to minimize contamination on these products (Fig. 1).

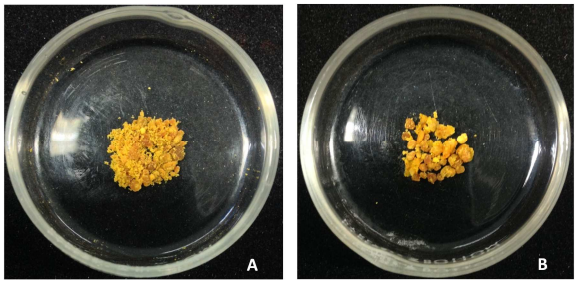

Fig. 1. Freshly collected bee pollen (A) and bee bread (B).

Processing and assay of bee pollen and bee bread samples

The bee pollen and bee bread samples were immediately placed inside the refrigerator after the field collection to preserve microbial species associated with these products and to ensure minimal contamination. Nutrient agar (NA) and acidified potato dextrose agar (PDA) were prepared for the bacterial and fungal assay, respectively. One gram of each sample was serially diluted using 9 ml sterile distilled water up to 10^4 dilution-fold. Initially, the dilution was done up to 10^{10} dilution fold but only few colonies (too few to count - TFTC) were observed starting from 10^5 dilution fold in both samples. Spread plate technique was employed in the assay which considered only the aerobic microbial species associated with the raw bee pollen and bee bread samples. A 0.10 ml of the suspension was pipetted on the surface of the prepared Petri plates and was evenly distributed using a sterile glass rod hockey.

Three replications per location were made and five subsamples each were provided. The culture plates were incubated under room conditions for 48 to 72 hours. The bacterial and fungal population per gram of the sample was determined following the standard plate count method. Individual colonies were counted and the colony-forming units per gram of sample (cfu/g) were computed using the formula:

$$\text{cfu/g of sample} = \frac{\text{Average number of colonies} \times \text{Dilution factor}}{\text{Volume plated (ml)}}$$

Discrete colonies of bacteria and fungi (Fig. 2) were purified separately on NA and PDA slants, respectively, and were kept for use in the characterization of isolates. The data generated from the study were analyzed using Analysis of Variance (ANOVA) and the treatment mean comparison was performed using Tukey's HSD test.

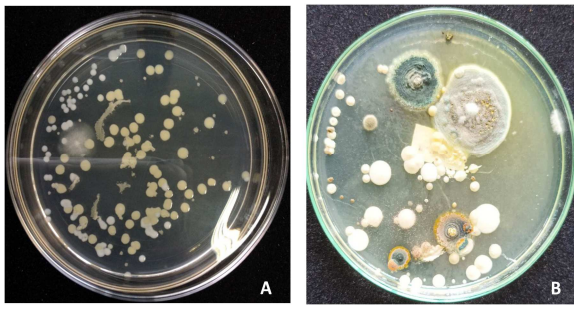

Fig. 2. Bacterial colonies on nutrient agar at 48 hours of incubation (A) and fungal colonies on potato dextrose agar (B) at 72 hours after incubation under room conditions.

Results and discussion

Bacterial population on bee pollen and bee bread

In both honey bee products, the bacterial count did not vary significantly across the three locations of Arabica farms where the colonies were established (Table 1). The bacterial count on raw bee pollen ranges from 5.5 x 10^6cfu/g of sample (Site 3 - Miarayon, Talakag) to 6.0 x 10^6cfu/g of the sample (Site 2- Songco, Lantapan). Similar trend was observed in the bacterial population present on raw bee bread sample. As presented, the bacterial count ranges from 5.4 x 10^6cfu/g of the sample (Site 3 – Miarayon, Talakag) to 6.4 x 10^6cfu/g of the sample (Site 2- Songco, Lantapan).

Table 1. Population of bacteria from raw bee pollen and bee bread samples grown on nutrient agar (NA) at 48 hours after incubation.

Location	Bee Pollen	Bee Bread
Site 1- Imbayao, Malaybalay City	5.7 x 10^6	5.5 x 10^6
Site 2- Songco, Lantapan	6.0 x 10^6	6.4 x 10^6
Site 3- Miarayon, Talakag	5.5 x 10^6	5.4 x 10^6
F-test	ns	ns
CV (%)	16.38	16.44

Ns- non-significant

Fungal population on bee pollen and bee bread

The population of fungal species associated with raw bee pollen and bee bread collected from the three study sites is presented in Table 2. Similar to the bacterial population assay, only the aerobic species were considered in determining the fungal load per sample.

Table 2. Population of fungal species from raw bee pollen and bee bread samples grown on acidified potato dextrose agar (PDA) at 48 hours after incubation.

Location	Bee Pollen	Bee Bread
Site 1- Imbayao, Malaybalay City	5.4 x 10^4 ab	3.7 x 10^4 a
Site 2- Songco, Lantapan	6.3 x 10^4 a	3.8 x 10^4 a
Site 3- Miarayon, Talakag	3.6 x 10^4 b	3.0 x 10^4 b
F-test	*	**
CV (%)	17.24	5.18

Means with the same letter in a column are not significantly different at 5% level of probability based on Tukey's HSD test.

** - highly significant

* - significant

Significant and highly significant differences in the fungal populations across the three locations were observed on raw bee pollen and bee bread, respectively. In raw bee pollen from Arabica coffee, the lowest fungal population was recorded in Site 3 (Miarayon, Talakag) with 3.6 x 10^4cfu/g of the sample which was comparable to the fungal population in Site 1 (Imbayao, Malaybalay City) with 5.4 x 10^4cfu/g. The highest fungal population was observed in Site 2 (Songco, Lantapan) with 6.3 x $10^{4cfu/g}$ of the sample. In raw bee bread, a significantly lower fungal count was recorded in Site 3 (Miarayon, Talakag) with 3.0 x 10^4cfu/g of the sample compared to the fungal population in Sites 1 and 2 with 3.7 x 10^4cfu/g and 3.8 x 10^4cfu/g, respectively.

Characterization of bacterial and fungal isolates

The bacterial and fungal isolates were characterized based on their cultural and morphological features. In both products, seven distinct colonies were observed on nutrient agar (NA) at 48 hours after incubation. The colony morphology and the Gram-stain reaction per isolate are presented in Table 3. As observed, three colonies showed Gram-positive reaction while four colonies exhibited Gram-negative reaction based on the Gram staining technique.

Table 3. Colony morphology and reaction to Gram staining technique of the bacterial isolates from raw bee pollen and bee bread.

Isolate	Colony Morphology			Gram Stain Reaction
	Form	Elevation	Margin	
1	Big-sized, white colonies	Flat	Undulate	(+)
2	Small-sized, white colonies	Raised	Entire	(-)
3	Medium-sized, yellowish colonies	Raised	Entire	(-)
4	Punctiform, white to beige colonies	Raised	Entire	(-)
5	Medium-sized, white colonies	Flat	Lobate	(+)
6	Medium-sized, wrinkled, white colonies	Flat	Undulate	(+)
7	Small-sized, beige colonies	Semi-flat	Entire	(-)

For fungal species, yeast and filamentous fungal species were monitored on acidified potato dextrose agar (PDA). As observed, the yeast population dominated the culture plates after 72 hours of incubation under room conditions. Based on morphological and cultural characteristics, the filamentous fungal genera include *Aspergillus*, *Fusarium*, and *Penicillium*.

Discussion

The assessment of the microbial load on raw honey bee products is crucial in determining their quality considering that these are food sources within the colony and also consumed by human for nutritional and medicinal purposes. Based on the results of the study, both raw honey bee products from Site 3 (Miarayon, Talakag) exhibited lower fungal counts compared to the samples obtained from Sites 1 (Imbayao, Malaybalay City) and 2 (Songco, Lantapan). The lower population may be attributed to the prevailing conditions present in the area since this site has an elevation of 1381.9 meters above sea level (asl). The elevation of the area with established Arabica coffee plants may have directly influenced the population count of fungal species associated with

these products. This result conforms with the findings of Hani *et al.* (2012) who revealed that the variations in the fungal population across different elevations depend on several factors. They disclosed that the microbiological quality of bee pollen depends strongly on its geographic and botanical origin, the weather at the time of collection, as well as on the postharvest processing procedure by the beekeeper. Moreover, this result supported the findings of Nogueira *et al.* (2012) who revealed that the microbial composition in the collected bee pollen was greatly affected by plant source or botanical origin, geographical origin, and beekeeper activities. Moreover, they found that the major changes in the microorganism composition of bee pollen had occurred during storage under simulated "warm" conditions.

In terms of microbial load, the bacterial population reached as high as 6.4 x $10^{6cfu/g}$ on raw bee bread sample compared to the fungal population with only 6.3 x 10^{4}cfu/g on raw bee pollen. Bacterial species were also found dominant organisms associated with bee bread and bee-collected pollen samples from Japan as reported by Asama *et al.* (2015). Based on the results of their study, *Lactobacillus* was the most dominant genus associated with the two products, 83.9% in bee bread and 74.6% in bee pollen.

L. kunkeei was the most abundant species in bee bread and pollen, honey, royal jelly, and even in the honey bee stomach. In addition, *Burkholderia* (2.1%), *Gluconobacter* (3.4%), and *Paenibacillus* (2.1%) were found in bee bread and bee-collected pollen samples, respectively. According to Donkersley *et al.* (2018), bee bread contains, on average, 13 different bacterial phyla, with Bacteroidetes, Firmicutes, Alpha-proteobacteria, Beta-proteobacteria, and Gammaproteobacteria being the five most prevalent. In terms of genera, *Pseudomonas*, *Arsenophonus*, *Lactobacillus*, *Erwinia*, and *Acinetobacter* were the most prevalent. Additionally, they linked environmental factors to the bacterial diversity found in bee bread, indicating that a change in land use can have an indirect negative impact on the microbiome of this honey bee product.

Aspergillus and *Penicillium* species, two potential mycotoxin-producing fungi, are confirmed to be associated with bee bread and bee pollen collected from Arabica coffee plants. Given that bee bread was the primary food source in the hive, it might have had an impact on honey bee health. Moreover, the contamination of these fungal species poses risks to people if the bee pollen from these sources is intended for human consumption.

This result, however, conforms with the findings of Gonzalez *et al.* (2005) who assayed 90 ready-to-eat bee pollen samples and revealed that the samples contained yeasts, *Penicillium,* and mycotoxin-producing fungi such as *Penicillium verrucosum, Aspergillus niger aggregate, Aspergillus carbonarius, Aspergillus ochraceus, Aspergillus flavus, Aspergillus parasiticus* and *Alternaria* spp. Friedle *et al.* (2021) also reported that bee pollen under warm and humid conditions contained *Aspergillus* spp. and *Zygosaccharomyces*, both are considered notorious spoilage fungal organisms.

Conclusion

The study, therefore, confirms that elevation affects the fungal population on bee bread and bee pollen collected from Arabica coffee plants, but it has no effect on the bacterial population. This finding is useful for beekeepers to further improve the quality of honey bee products, particularly bee bread and bee pollen.

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