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Yeast Diversity in Honey Produced by Wild Honeybees at Different Elevations

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Abstract

Yeasts inhabit diverse natural environments, including the honeycombs of wild honeybee colonies residing at different elevations. This study investigates yeast populations in honeycombs located at two sampling sites, Bligo and Sukomulyo, Central Java Province, Indonesia, situated in a montane region at elevations of 131.1 and 676 meters above sea level, respectively. Out of the 40 yeast isolates collected, 12 distinct fingerprints were identified, representing ten strains across four species. *Debaryomyces hansenii* emerged as the predominant species, accounting for seven of the total strains, and being found at both sampling sites. The remaining species included *Priceomyces melissophilus*, *Cystobasidium minutum*, and *Meyerozyma guillermondii*. Overall, yeast abundance in this study was relatively low. The index of abundances (H') was higher in Bligo (0.796) compared to Sukomulyo (0.636). These findings suggest that elevation may influence yeast populations within honeycombs, with potential interactions with other factors affecting diversity measures.

Keywords: Diversity, Elevations, Honeycomb, Honey, Yeast.

Introduction

Raw honey derived from wild honeybees is widely regarded as superior to honey produced by farmed honeybees. The unprocessed nature of wild honey preserves its natural nutritional content and antioxidants, making it a coveted product among consumers. Wild or forest honey is also prized for its purity, devoid of additives such as colorants and preservatives. It is generally considered safer, given the absence of chemical contaminants like pesticides commonly associated with cultivated crops during their blooming periods[1].

While wild honey from honeybees has many merits, it is essential to acknowledge that honeybees are not confined to a single habitat. They thrive in diverse environments, ranging from coastal regions to elevated terrains, with their mobility stemming from migratory behavior. These colonies frequently move between lower and higher-elevation nesting sites in response to changing seasons[2]. Honeybees, known for their impressive flight range spanning four to 12 kilometers, are resilient to geographical gradients

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and encounter many environmental factors during their journeys[3–5].

During foraging, honeybees come into contact with various microorganisms from sources like flower nectar, soil, air, water, and phylloplane[6,7]. These microorganisms can inadvertently adhere to the honeybee's external surface or enter their bodies, ultimately becoming part of the honey produced by the bees[8,9]. Among these microorganisms, certain yeast strains from genera such as *Debaryomyces*, *Zygosaccharomyces*, *Candida*, *Meyerozyma*, *Rhodotorula*, *Starmerrela*, and *Saccharomyces* are known to thrive in raw honey. However, as the demand for biotechnological applications of yeast species grows, the discovery of novel yeast species and their diversity has accelerated[10].

Many places and ecosystems remain unexplored despite geographical assessments of yeast biodiversity spanning various regions[11]. Large biomes such as tropical rainforests, montane, and sub-montane ecosystems have been understudied[12]. To our knowledge, no previous research has examined yeast diversity across elevational gradients in Indonesia, particularly in the context of yeast present in honey from wild honeybees. In Indonesia, wild honeybees, such as *Apis cerana* and *A. dorsata*, thrive across elevations ranging from sea level up to 1000 meters above sea level[1]. Their foraging activities encompass both proximate and distant locations, influenced by the characteristics of their surroundings. Microorganisms found in the honeybee's environment, including bees, hives, pollen, flowers, and soil, are likely reflected in the honey they produce. While the physicochemical characteristics of wild or forest honeybee honey in Indonesia have been explored elsewhere[13–15], there is a notable gap in our understanding concerning yeast abundance in such honey, mainly when produced by honeybees residing at varying altitudes. Hence, the main aim of this research was to explore the presence of yeast in wild honey gathered across different altitudes.

Materials and Methods

Sampling Sites and Yeast Isolation

For this study, raw honey was obtained from honeycombs inhabited by wild honeybees in montane regions at varying elevations. The research encompassed elevated areas within DI Yogyakarta and Magelang, Central Java Province, Indonesia, where honeycombs were found at two distinct locations. The first site was Sukomulyo Village (Kecamatan Kajoran, Kabupaten Magelang), situated on Mount Sumbing's slope, approximately 676 meters above sea level (*local government data, unpublished*). The honeycombs were discovered within the inner parts of mahogany tree trunks. The second site was Bligo Village (Kecamatan Ngluwar, Kabupaten Magelang), located in the lower part of Mount Merapi, about 131.1 meters above sea level (*local government data, unpublished*). The honeycombs were found attached to rocks within a cave (Fig. 1A and 1B).

Yeast isolation was conducted from the honeycombs. It is important to note that both hives and honeycombs serve as primary sources of microbes in honey[8](Fig. 1C).

The honeycomb samples were processed through a series of sequential steps, first, an aseptic technique was employed to cut the honeycomb and expose the honey carefully. Next, the honey was extracted by squeezing it from the honeycomb. The extracted honey was then placed into a physiological solution and thoroughly mixed to create a homogenous mixture. Serial dilutions of this honey mixture were prepared using sterile distilled water. Following dilution, 100 µl of each dilution were evenly spread onto YM Agar (0,5% peptone; 0,3% yeast extract; 0,3% malt extract; 1% glucose; 2,0% agar), supplemented with 100 mg/L chloramphenicol to suppress bacterial growth[16]. The prepared plates were incubated at 30°C and monitored periodically. On these plates, distinct yeast colonies displaying unique morphologies were carefully chosen for further analysis. These selected yeast colonies underwent purification and were subsequently maintained on YM agar slants for use in later investigations.

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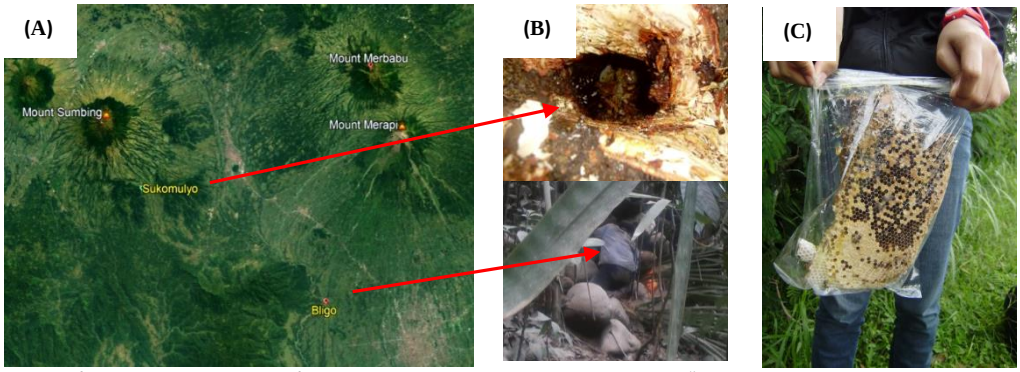

The map is from Google Earth. Coordinates of the villages, Sukomulyo : 7°28'59.12"S , 110°5'13.86"E; Bligo : 7°42'25.45"S , 110°16'19.70"E

Figure 1. Bligo and Sukomulyo Villages (A), Sampling Sites (B) and Honeycomb (C)

Yeast Identification

Genomic DNA extraction was carried out to perform molecular identification. The yeast isolate was cultured in YM broth and incubated at 30°C for 24 hours. Subsequently, the yeast cells were harvested by centrifugation. Genomic DNA was then extracted utilizing a genome extraction kit, specifically the Presto™ Mini gDNA Yeast Kit (Geneaid). The extracted DNA was visualized on a 1% (w/v) agarose gel under a UV transilluminator for quality assessment.

The domain D1/D2 from the LSU rRNA gene was amplified and sequenced using the following primers NL1(5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4(5'-GTCCGTGTTTCAAGACG-3')[17]. Amplification included an initial cycle at 94 ºC for 60 seconds, followed by 35 cycles consisting of 94 ºC for 60 seconds, 55 ºC for 60 seconds and 72 ºC for 2 minutes, and a final extension at 72 ºC for 5 minutes. The amplified PCR products were then subjected to electrophoresis on a 2% agarose gel in 1x TBE buffer for 50 minutes at 100 volts. The gel was stained using FloroSafe DNA stain (1st BASE), and the DNA bands were visualized and photographed under a UV transilluminator.

Subsequently, the PCR products were submitted to 1st Base in Singapore for DNA sequencing. The sequences were processed using Bioedit 7.2.5[18] and MEGA 11 software[19]. Sequence comparisons were performed using the BLASTn algorithm within the GenBank database. A threshold greater than 97% sequence identity to the closest known yeast species was applied to identify the isolates accurately[20].

MSP-PCR Fingerprinting

To assess potential genetic variation, genomic DNA extracted from each yeast species was subjected to Microsatellite-Primed PCR (MSP-PCR) fingerprinting, employing an M13-core sequence primer (5′GTAAAACGACGGCCAGT-3′). This primer has a well-established track record for distinguishing strains within individual yeast species[21,22].

For the MSP-PCR fingerprinting reaction, a total volume of 25 µl was prepared, consisting of 12.5 µl of PCR mix (MyTaq HS PCR Mix, Bioline), 1 µl of genomic DNA template, 1 µl primer, and 10.5 µl of ultra-pure distilled water. The PCR program was set as follows, pre-denaturation at 95°C for 45 seconds, followed by 40 cycles of denaturation at 93°C for 45 seconds, annealing at 50°C for 60 seconds, extension at 72°C for 60 seconds, and concluding with a final extension step at 72°C for 6 minutes.

The results of the MSP-PCR fingerprinting were subsequently separated by electrophoresis on a 2% agarose gel in 1% TBE buffer. Electrophoresis was conducted for 60 minutes at 90 volts. The agarose gel was examined under a UV illuminator. To validate the existence of several amplicons, the fingerprinting results were additionally observed on an 8% acrylamide gel and subjected to silver staining. Photographs of all the gels were taken and used for fingerprint scoring.

Clustering and Diversity Analysis

The analysis of fingerprinting results involved scoring based on the presence or absence of each amplicon band observed on an agarose gel. Scoring was conducted both manually and with the assistance of the GelJ[23] application for comparative purposes.

Afterward, matrices denoted as *n x t* were constructed using Microsoft Excel 2013 for the clustering algorithm. In this representation, *t* signifies the Operational Taxonomic Unit (OTU),

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which represents the strains, and *n* represents the unit of measurement.

The constructed matrices were subjected to NTSYSpc 2.11[24]. The analysis incorporated the Simple Matching (SM) coefficient, and pair-wise comparisons were conducted using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method.

Yeast Abundance Analysis

The yeast isolation rates were calculated for all samples from Sukomulyo and Bligo. The relative abundance of yeast in each location was further analyzed using Shannon indexing, denoted as H'. All data collected during the study was processed using Microsoft Excel 2013. Visualization and graphical representation of the data were performed using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

Results and Discussion

Yeast Diversity in different Elevations

In total, 40 yeast isolates were obtained from honeycomb samples found in Bligo and Sukomulyo, with each village yielding 20 isolates. The fingerprinting results displayed diverse genotypes among these isolates, resulting in 12 distinct patterns. Genetic variations among strains in Bligo exceeded those observed in Sukomulyo, with nine different fingerprints from Bligo and three from Sukomulyo. This subset of 12 isolates, includes those with nearly similar band patterns (Fig. 2).

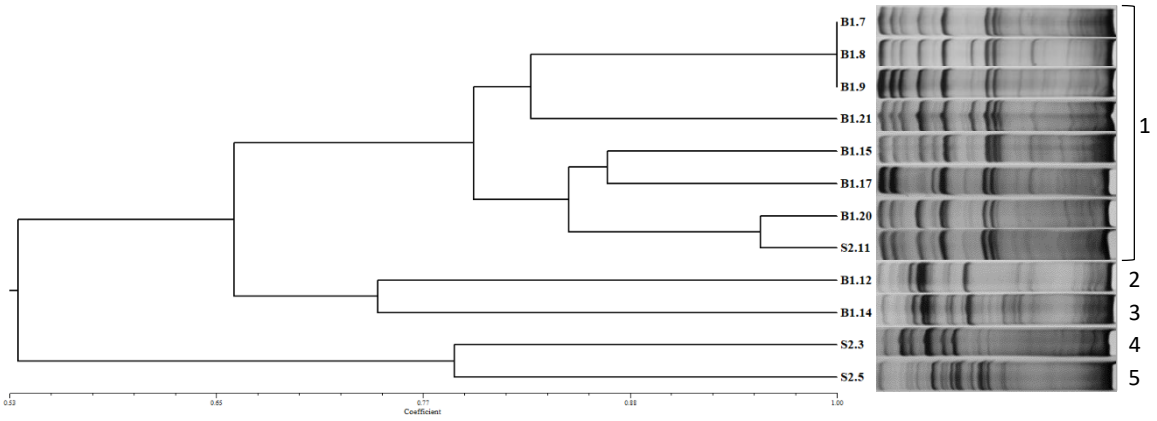

Figure 2. Fingerprints of 12 Yeast Strains found in Honey from Bligo (B) and Sukomulyo (S)

Yeast Species Composition

Among all isolates, the ascomycetes were the predominant group, constituting 39 isolates across nine strains and three species. These species were most closely related to *D. hansenii*, *P. melissophilus*, and *M. guilliermondii*, all of which can be recovered from honey[8]. In contrast, only one basidiomycete was identified, displaying a 99% correspondence with *C. minutum*. It is worth noting that basidiomycetes are present in honey but are less abundant than ascomycetes due to the low water content and the inhibitory effect of high sugar content on their growth. Ascomycetes group being more adapted to the conditions than the basidiomycete, and can reach high densities in nectar[25] (Table 1).

Differences between Bligo and Sukomulyo

This study revealed varying outcomes in yeast isolation rates between the two sampled locations. The abundance of species and strains was more pronounced in Bligo compared to Sukomulyo. Bligo village lies at the lower part of Mount Merapi, with an annual precipitation of approximately 2384 mm/year and an average temperature of about 25.5°C (*local government data, unpublished*). Plants surround the sampling site in Bligo, including *Gnetum gnemon*, coconut trees, bamboo, and rambutan (*Nephelium lappaceum*). Meanwhile, Sukomulyo located at a higher elevation than Bligo, experienced an average annual precipitation of approximately 3445 mm/year and an average temperature of around 23.3°C (*local government data, unpublished*), it featured a different plant environment, with a nursery for mahogany trees and

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mahogany dominating the surroundings, along with banana trees and *Leucaena leucocephala* (Fig. 3A).

Table 1. Yeast Species found in Honey from Sukomulyo and Bligo Village

Isolates	Closest Strains	Accession No.	Homology (%)	Total Isolates	Number of Isolates	
					Sukomulyo	Bligo
B1.7	*D. hansenii* Y3	KC534842.1	87	8		8
B1.8	*D. hansenii* Y3	KC534842.1	95	4		4
B1.9	*D. hansenii* AA-Y20	JQ965891.1	98	1		1
B1.12	*P. melissophilus* C5_1	LC434079.1	85	1		1
B1.14	*C. minutum*	AB025996.2	99	1		1
B1.15	*D. hansenii* TJY 24	EU326128.1	85	1		1
B1.17	*D. hansenii* DYG1	JQ680469.1	86	1		1
B1.20	*D. hansenii* TJY 24	EU326128.1	84	2		2
B1.21	*D. hansenii* CBS 789	MK394103.1	85	1		1
S2.3	*M. guilliermondii* 34	EU285513.1	86	7	7	
S2.5	*M. guilliermondii* SYHZS-1	EU809436.1	85	12	12	
S2.11	*D. hansenii* LL2	EU131182.1	89	1	1	
	Total			40	20	20

Debaryomyces hansenii Dominance

The yeast *D. hansenii*, identified as the predominant species in this study, has been found in various ecological niches, including soil, seas, plants, fruits, and clinical samples[26]. The abundance of this species in honey can be explained by its ability to thrive in high-sugar environments, making it a frequent yeast species in food products and an important non-*Saccharomyces* yeast in winemaking. The species has also been observed during coffee fermentation at different altitudes, however, its abundance decreasing as elevation increases[27]. This aligns with the study's findings, where strains belonging to *D. hansenii* were more prevalent in Bligo than Sukomulyo.

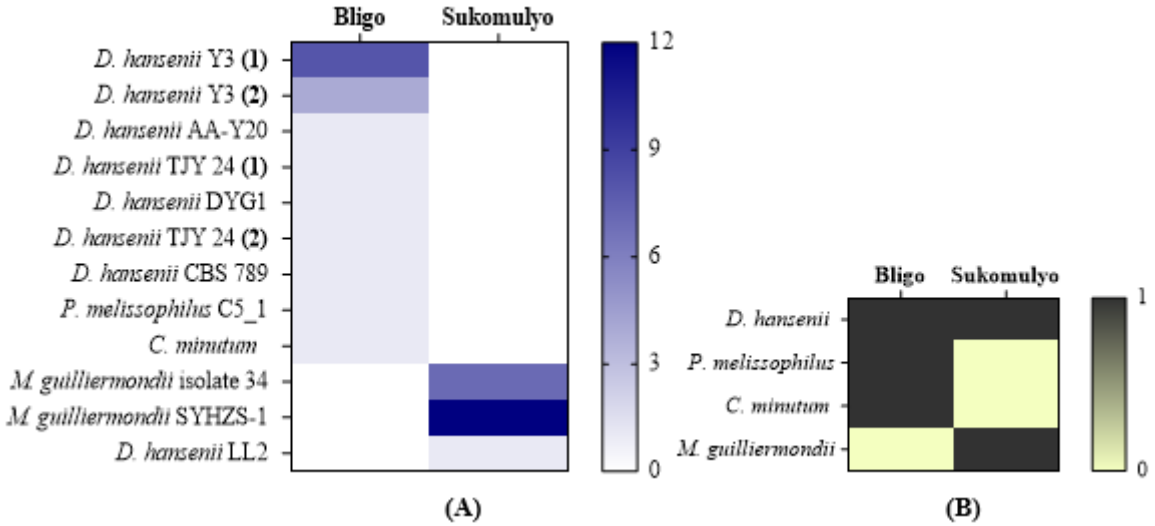

Figure 3. Yeast in Honey from Bligo and Sukomulyo. (A) Isolates distribution and abundance, (B) Species found in both villages

Priceomyces melissophilus and other Species

Species that are now categorized under the name *Priceomyces* were formerly classified under the names *Debaryomyces*, *Meyerozyma*, *Torulaspora*, and *Yamadazyma*. The type strain of *P. melissophilus* was found in the honeybee's gut (*Apis mellifera* var. adansonii). Two other isolates were found in South African soil, lichen, and trees[28]. According to some studies, the species is endophytic. In view of strain sources, the habitat of this species is uncertain, however, since this species can be found in tea

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flowers cultivated in high elevations [29], *P.melissophilus* is most likely can survive in a similar habitat.

A single member of basidiomycetes, *C. minutum* (previously *Rhodotorula minuta*), was identified in this study. The *Cystobasidium* genus includes mycoparasitic species that are commonly found in various environments, including the phylloplane, soil, freshwater, saltwater, sediments, and regions spanning temperate to cold climates[30]. This species is known not only as a pathogenic species but also as a potential biological control agent for plant diseases and a phytase producer[31–33]. While its presence in honey and other food sources is documented, it is important to be aware of the potential for opportunistic infections caused by *C. minutum*.

The species *M. guilliermondii*, previously classified as *Pichia guilliermondii* and *Candida guilliermondii*[34,35], is commonly found in crops and the digestive systems of nectar- and pollen-collecting bees. It has also been frequently observed in untreated honey[36]. This study identified two strains resembling *M. guilliermondii*, both isolated from Sukomulyo. The ubiquitous species *M. guilliermondii* has demonstrated its ability to endure high altitudes during coffee fermentation, and can be found at elevations ranging from 300 to higher than1800 meters above sea level[16,27].

Factors Influencing Yeast Diversity

Diversity indices revealed that the diversity index in Bligo was higher than in Sukomulyo. The H' index is 0,796 for Bligo and 0,636 for Sukomulyo. Generally, both villages exhibited relatively low diversity indices in their honeycomb samples. This result aligns with previous research indicating that microorganisms, including yeast, are prevalent in natural habitats such as soil, air, and water but have lower diversity in microhabitats like food and related substrates, including honey[37]. Additionally, plant diversity was greater in Bligo than in Sukomulyo, corresponding to classic biodiversity patterns where plant and animal diversity typically decreases at higher elevations and is more varied at lower elevations in montane regions. However, microorganisms like yeast do not always follow this pattern, as demonstrated by the lower yeast diversity in Sukomulyo[38]. The pattern could be attributed to the unique characteristics of the plant species at higher elevations, which may lead to variations in nectar composition and availability[39]. Another study showed that the moisture content of honey produced at higher altitudes tends to be lower than that from lower regions[40]. Additionally, gradient elevations also play a substantial role in the bee communities[41], which likely explains the difference in yeast diversity between Bligo and Sukomulyo. Further investigations involving a broader range of elevations and plant species are required to elucidate the complex interplay of factors influencing yeast diversity in honeycombs.

Conclusion

In conclusion, this study provides insights into the yeast diversity present in honeycombs collected from different elevations. Various yeast isolates were identified as common inhabitants of honey and other bee-derived substances, primarily belonging to the genera *Debaryomyces*, *Meyerozyma*, *Priceomyces*, and *Cystobasidum*. While our research focused on honeycombs from two distinct elevations, notable differences in yeast diversity were observed between the two sampling sites. However, it is important to note that overall yeast diversity in honeycombs from both villages remained relatively low, with greater diversity observed at lower elevations compared to higher ones.

To gain deeper insights into how elevation affects the yeast populations within honeycombs. Further investigations are warranted that encompass a wider spectrum of elevations and geographic regions. Additionally, applying culture-independent methods may reveal a more comprehensive view of yeast diversity in these habitats. This research contributes to the expanding field of knowledge regarding the microbial communities associated with honeybees and their honeycombs, emphasizing the need for continued exploration in this field. Such studies hold the potential to advance our understanding of honey quality and its broader implications for both ecological and biotechnological applications.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at Universitas Gadjah Mada, Indonesia.

Authors' Contribution Statement

M.M was engaged in generating concepts, data processing, interpretation, and authorship of the work. C.R.P mainly consisted of laboratory work, data analysis, and data interpretation. D.W provided the study concept, proposed strategies for workflow management, and offered suggestions for laboratory activities and the writing process. S.W and I.D.P actively engaged in data interpretation, providing valuable comments throughout the laboratory work and contributing to the revision process.

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تنوع الخميرة في العسل الذي ينتجه نحل العسل البري على ارتفاعات مختلفة

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الخلاصة

تعيش الخمائر في بيئات طبيعية متنوعة، بما في ذلك أقراص العسل الموجودة في مستعمرات نحل العسل البرية التي تتواجد على ارتفاعات مختلفة. تبحث هذه الدراسة في تجمعات الخميرة في أقراص العسل الموجودة في موقعين لأخذ العينات، بليجو وسوكومولیو، بمقاطعة جاوة الوسطى، إندونيسيا، الواقعتين في منطقة جبلية على ارتفاعات 131.1 و676 مترًا فوق مستوى سطح البحر، على التوالي. من بين 40 عزلة من الخميرة تم جمعها، تم التعرف على 12 بصمة مميزة تمثل عشرة سلالات من أربعة أنواع. ظهرت Debaryomyces hansenii باعتبارها الأنواع السائدة، حيث تمثل سبعة من إجمالي السلالات، وتم العثور عليها في كلا موقعي أخذ العينات. وتشمل الأنواع المتبقية Priceomyces melissophilus وCystobasidium minutum و Meyerozyma guillermondii. وبشكل عام، كانت وفرة الخميرة في هذه الدراسة منخفضة نسبيا. كان مؤشر الوفرة (H') أعلى في بليجو (0.796) مقارنة بسوكومولیو (0.636). وتشير هذه النتائج إلى أن الارتفاع قد يؤثر على أعداد الخميرة داخل أقراص العسل، مع وجود تفاعلات محتملة مع عوامل أخرى تؤثر على مقاييس التنوع.

الكلمات المفتاحية: التنوع، الارتفاعات، قرص العسل، العسل، الخميرة.