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Research

Metabolomic Fingerprints: Seasonal and Farm-Specific Differences in *Heterotrigona itama* Bee Bread

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ABSTRACT

Stingless bees' products such as honey and bee bread are beneficial to human health. However, the metabolite composition within the products may differ according to spatial and temporal factors. This study aims to investigate the impact of spatial and temporal factors on the significant metabolite composition present in *Heterotrigona itama* bee bread collected from different seasons and types of bee farms. Thus, tandem mass spectrometry (LCMS/MS) based metabolomics analysis was used to achieve the said goal, focusing on *H. itama* bee bread samples, followed by multivariate analysis using the MetaboAnalyst platform. Our findings revealed significant metabolites that set bee bread samples apart. In terms of temporal factors, the analysis highlights specific metabolites exclusive to the wet season, such as flavones and flavonoids. However, in contrast, during the wet season, there are no significant metabolites in herbal farm samples. The results also underscore the significance of phenolic compounds in wet season samples and as vital metabolites distinguishing the groups. This study highlights a total of 24 annotated significant metabolites identified in different bee bread samples, as well as their capacity to differentiate between seasons and bee farms. Notably, these results suggest a wide variety of potential plant families as a source of bee bread. These findings shed light on the impact of seasons and diverse plant families contributing to bee bread composition, which may impact the growing meliponiculture industry greatly.

Key words: Bee bread, foraging behavior, flavonoid, meliponini, plant families, significant metabolites

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INTRODUCTION

Stingless bee (Meliponini) from the Apidae family, is estimated to have more than 600 species found in tropical and subtropical regions worldwide (Engel *et al.*, 2023). They vary in size, ranging from a few millimeters to a centimeter in length. They depend solely on their modified ovipositor for defense purposes due to the lack of a sting, making stingless bees physically different from other bee species (Kerr & deLello, 1962; Quezada-Euán, 2018).

Similar to honeybees, stingless bee also produces honey, royal jelly, and bee bread, albeit in lower quantities (Chuttong *et al.*, 2016). Although the physicochemical properties of the stingless bee honey have been mostly characterized, the Honey Commission (IHC) has yet to establish a standard for honey (Vit *et al.*, 2004; Souza *et al.*, 2006; Truchado *et al.*, 2011).

The stingless bee bread, on the other hand, did not get much attention as there is no quality standard droughted for bee bread to rank or categorize the quality of bee bread for human consumption (Mohammad *et al*., 2020; Leonora *et al*., 2021). Due to the product's potential as a health and therapeutic supplement (Isidorov *et al*., 2009), the bee bread quality standard has to be developed to ensure the quality and safety of the product for the customer. Thus, identifying the bee bread composition is required to draft a quality standard for meliponini bee bread (Ávila *et al*., 2018).

Bee bread was shown to possess antioxidant and anti-inflammatory properties (Bakour *et al*., 2017). The antioxidant properties of bee bread are not limited to specific stingless bee species. Instead, they can be observed in multiple bee species like *H. itama,* etc. (Shamsudin *et al*., 2019; Fakhlaei *et al*., 2021). In addition to high levels of carbohydrates, minerals, proteins, and fatty acids (Bakour *et al*., 2019), bee bread also contains natural antioxidants such as tocopherols and phenolic compounds, that are capable of removing the overproduced free radicals in the cells (Reuter *et al*., 2010). Phenolic compounds in meliponini bee bread are thought to be the main reason for the bee product's antioxidant effects (Mohammad *et al*., 2020), This powerful antioxidant property of phenolic compounds is also further proven *in vitro* by many researchers around the world, including in Brazil (Duarte *et al*., 2018; Lopes *et al*., 2020) and Malaysia (Harif Fadzilah *et al*., 2017; Mohd & Zin, 2020). In a previous study, Bakour *et al*. (2017) reported that bee bread showed significant antioxidant effects and protective properties in rats during an aluminum toxicity study. It showed anti-inflammatory activity by improving the reduction of hemoglobin levels.

Metabolomics is a field of scientific study involving metabolites, which are small molecules that give insights into the potential chemical or biological processes that occur. The approach is useful in various fields, particularly for identifying the underlying metabolite composition of products, in this case, stingless bee bread (Ardalani *et al*., 2021; Reveglia *et al*, 2022). The detection of metabolomics includes mass spectrometry with gas chromatography (GC), liquid chromatography (LC), and nuclear magnetic resonance (NMR) as the common techniques (Bingol, 2018; Boiteau *et al*., 2018). In terms of bee metabolite identification, the application of liquid chromatography-tandem mass spectrometry (LCMS/MS) is useful in the identifying metabolite composition of bee products such as honey, bee pollen, bee bread, and royal jelly (Bakour *et al*., 2019; Đogo Mračević *et al*., 2020; Sumarlin *et al*., 2021). This study aims to identify the metabolite composition of stingless bee bread under the effect of different environmental settings, namely temporal and spatial factors.

Based on previous literature, the composition of bee bread is generally similar to those found in honey, including flavonoids, polyphenols, carotenoids, sugar, amino acids, organic acids, etc. (Pérez-Pérez *et al*., 2018; Adaškevičiūtė *et al*., 2019; Mohammad *et al*., 2021). However, the diversity of certain compounds found in the products is highly dependent on both the geographical location and botanical origin (Tomás-Barberán *et al*., 2012; Shamsudin *et al*., 2019; Bayram *et al*., 2021). Thakur and Nanda (2020) pointed out in their study that the botanical origin of the bee products, which is the location where it was harvested, will affect the types of beneficial compounds that lie in bee bread. In this study, we tested the hypothesis that geographical factors associated with seasonal variations exert influence on the metabolites of bee bread samples. The term spatial refers to the farm type or geographical factor while the temporal factor in this study refers to the seasonal variations. The application of metabolomics to identifying potential metabolite compositions is not a new technique in the field of stingless bees. For example, Razali *et al*. (2018) successfully identified multiple metabolites within stingless bee honey, including carbohydrates, fatty acids, and multiple types of amino acids. However, in terms of stingless bee bread research, the metabolomics application is lacking. The most important and significant insights provided to this study are that Othman *et al*. (2020) successfully identified bioactive compounds such as isorhamnetin, kaempferol, etc. in stingless bee bread using LCMS/MS, proving some of the potential therapeutic properties of stingless bee bread. In addition, the application of HPLC to stingless bee bread from multiple stingless bee species, such as *Melipona compressipes*, *Melipona favosa*, etc. has also proven successful in identifying the presence of compounds such as kaempferol, quercetin, luteolin, etc. (Vit *et al*., 2018). Similarly, in this study, through LCMS/MS analysis, we successfully identified the distinction between *H. itama* bee bread harvested within different environmental settings that affect the composition of flavones and flavonoids.

MATERIALS AND METHODS

A sampling of *Heterotrigona itama* bee bread

Bee bread of *H. itama* colonies was collected in two different seasons, the dry season (23rd June 2022) and the wet season (15th December 2022) at the stingless bee farms located at MARDI Serdang. The Fruit farm (2.982506, 101.694723) and Herbal farm (2.981052, 101.689034) (Figure 1). The former

consists of around 20 stingless beehives surrounded by fruit trees. The latter consists of around 40 stingless beehives near a vegetable and herbal farm, which includes over 150 different species of herbs and flowering plants. The perpendicular distance between these two farms is around 625 m.

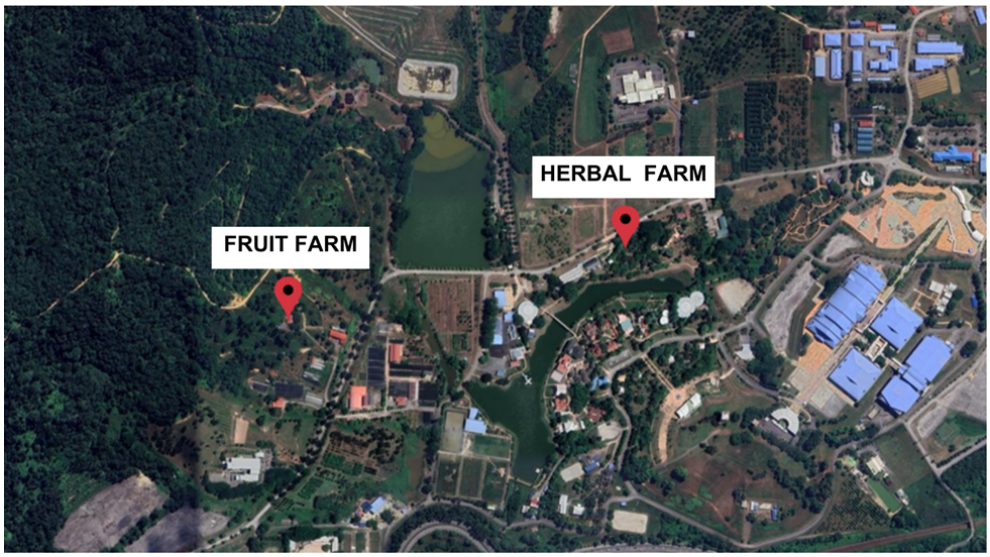

Fig.1. Satellite map location of the fruit farm and herbal farm. Source: Google Earth (Accessed on 10 October 2023)

The wet and dry seasons were determined by the weather and humidity on the World Weather Online website (URL: https://www.worldweatheronline.com). On 23rd June 2022, the rain and humidity were both low for the week and month, ranging from 0.1 mm to 0.3 mm while on 15th December 2022, the rain and humidity ranged from 0.3 mm to 1.9 mm, which is among the highest humidity percentages throughout the year. Bee bread samples were collected on the mentioned date as a representative of the dry and wet seasons, respectively.

Bee bread was collected aseptically by using a sterilized spatula in a sampling tube from three different colonies (Menezes *et al*., 2012; Razali *et al*., 2018; Spulber *et al*., 2018; Dranca *et al*., 2020). C1–C6 indicates the bee bread collected during the dry season while C7–C12 indicates bee bread collected during the wet season. Around 20 g of bee bread were collected with three replicates from each colony, resulting in 36 samples in total, as shown in Figure 2. The samples were then stored at 4°C, until further processing. A total of 2 g from each replicate of each colony was mixed to represent a single colony for further analysis.

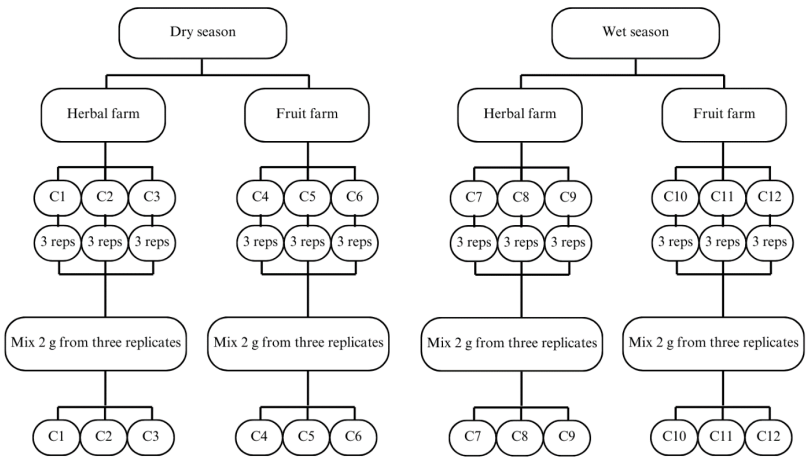

Fig. 2. Total number of bee bread samples collected

Extraction

Metabolite profiling was investigated through the Ultra-High-Performance Liquid Chromatography Tandem Mass Spectrometry (UHPLCMS/MS) technique on the samples (Zawadzka, 2007; Razali *et al*., 2018; Sumarlin *et al*., 2021). Before LCMS/MS profiling, the bee bread samples were extracted according to the protocol conducted by Ardalani *et al*. (2021) and Bayram *et al*. (2021). A total of 6 g of bee bread samples (including 2 g of bee bread from three replicates of the same colony) were allocated in a 15 mL tube. Then, these samples were freeze-dried (Labconco Freezone 4.5 L freeze dryer) for around 40 hour until the samples were fully dessicated and ground into powder form by using a mortar and pestle. Then, 10 mg of the ground powder was mixed with 1.5 mL of extraction solvent (chloroform: methanol: water in a ratio of 2:5:2) in a 1.5 mL tube. The samples were then vortexed for 20 sec followed by a 20 min shaking using an electric shaker at 4℃, followed by 12 min centrifugation at 16,160 × g. After that, 0.6 mL of supernatant was dried in a vacuum (Thermo Scientific Savant DNA SpeedVac Concentrator Kit, DNA120, 115 V, 60 Hz) for 3 hour until dried out. The products were then redissolved in 0.5 mL of an acetonitrile-water mixture at the ratio of 1:1 and proceeded to the UHPLC-MS/MS step. All 12 bee bread samples underwent the same set of steps.

UHPLC-MS/MS analysis

UHPLC-MS/MS steps were conducted according to studies done by Ardalani *et al*. (2021) and Bayram *et al*. (2021), respectively. The separation step was performed using a C18 column (AcclaimTM Polar Advantage II, 3 x 150 mm, 3 µm particle size) on an UltiMate 3000 UHPLC system (Dionex). Gradient elution was performed at a flow rate of 0.4 mL/min at 40℃ using H_2O + 0.1% formic acid (A) and 100% acetonitrile (B) with a 22-min total run time. A total of 5 µL sample was injected and eluted using a gradient starting at 5% B (0-3 min); 80% B (3-10 min) and 5% B (15-22 min).

High-resolution mass spectrometry (HRMS) was carried out using a MicroTOF QIII Bruker Daltonic using an ESI positive ionization with the following settings: capillary voltage – 4500 V; nebulizer pressure – 2.0 bar; drying gas – 8 L/min at 300℃. Mass range was at 50 - 1500 m/z. The accurate mass data of the molecular ions, obtained from the TOF analyzer were processed using Compass Data Analysis software (Bruker Daltonik GmbH).

HRMS data preprocessing

The raw mass spectrometry spectra data were processed using the MZMine 3.4 software. MZMine 3.4 is powerful in processing the spectra obtained, providing important functions like normalization, besides providing better visualization of data and a clearer user interface (Chen *et al*., 2022). Raw spectra were converted to .mzXML format file type using the msConvert tool in the ProteoWizard software (Chambers *et al*., 2012) before processing using MZMine 3.4 software. The filter used in msConvert includes peakPicking and msLevel is set to 2, accordingly. Mass detection, chromatogram building, chromatogram resolved, deisotopes, alignment, gap filling, and normalization were carried out in MZMine 3.4, according to their official documentation (URL: https://mzmine.github.io/mzmine_documentation/workflows/lcmsworkflow/lcms-workflow.html), as shown in Supplement 1.

Multivariate data analysis of *H. itama* bee bread

The online platform MetaboAnalyst v6.0 (URL: https://www.metaboanalyst.ca) was utilized to analyze the *H. itama* bee bread metabolome data following data preprocessing from MZMine 3.4. MetaboAnalyst v6.0 is useful in conducting statistical analysis with good data visualization and has been widely used in LCMS spectra analysis (Chong *et al*., 2019; Pang *et al*., 2022). The data were uploaded using the statistical analysis (one-factor) module in CSV format and the filter setting was set according to the variance filter and abundance filter. The former was set at 5% using the interquartile range (IQR) and the latter was set as an abundance filter at 0%. The data was normalized by the median, without any transformation, and scaled using the Pareto scaling method. After that, principal component analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), dendrogram, heatmap and volcano plot were constructed using the MetaboAnalyst v6.0 package. Then, the results obtained are recorded in the form of diagrams and figures.

Meliponini bee bread significant metabolome composition

The annotation of metabolites based on experimental MS/MS data of significant features derived from the volcano plot was achieved using the web-based spectral database KEGG and an in-silico fragmentation tool like MetFrag (URL: https://msbi.ipb-halle.de/MetFrag/ [Accessed on 15 June 2022]) (Ruttkies *et al*., 2016). The statistical technique of using a volcano plot to identify significant metabolites

was reported by Kumar *et al*. (2018), that the study suggested its application under metabolomics study, verifying the significant metabolites present in samples presented as red and blue spots. Identified metabolites were also categorized according to the compound category and potential sources of origin were predicted based on PubChem (URL: https://pubchem.ncbi.nlm.nih.gov/) and Wikidata (URL: https://www.wikidata.org/wiki/Wikidata:Main_Page).

RESULTS AND DISCUSSION

The impact of spatial and temporal factors on the *H. itama* bee bread composition was investigated through metabolomic analysis using LCMS/MS. The *H. itama* bee bread samples were investigated from two different farms during dry (March-July) and wet (August-February) seasons using LCMS/MS analysis. A total of 12 samples were analyzed, representing three colonies during each season at each farm. A total of 324 MS features were detected from the *H. itama* bee breads LCMS chromatogram, where the number of MS features varied throughout the samples. The variation of the metabolite composition was previously suggested due to different plant sources and geographical locations (Karabagias *et al*., 2020; Mayda *et al*., 2020; Mohammad *et al*., 2020). However, a comparison of metabolite composition during different seasons of the same origin (farm) for stingless bees, has yet to be investigated (Koethe *et al*., 2020).

Multivariate analysis of spatiotemporal effect on *Heterotrigona itama* bee bread metabolome

The effect of spatiotemporal on metabolite distribution in *H. itama* bee bread was analyzed using Principal Component Analysis (PCA) to understand the potential underlying relationship according to their metabolites, without considering the spatial and temporal factors grouping. As illustrated in Figure 3a, PC1 (34.1%) and PC2 (19.4%) accounted for over 53% of the total variance. However, the PCA result shows no discrimination between temporal and spatial factors that differentiate bee bread samples between groups. As the PCA result showed lower predictive ability with the majority of the samples clustered in the same cluster (Figure 3b), the metabolites were then analyzed using PLS-DA which classifies samples according to distinct groups that yield a better separation as compared to PCA. As shown in Figure 3c, 45.4% of the cumulative variance was represented in Component 1 (C1) (27.8%) and Component 2 (C2) (17.6%). The predictive ability and fitness of the model were better than PCA as the Q2 and R2 values were 0.28 and 0.93, respectively. The significant contribution from PC1 suggests a dominant trend in metabolite composition due to season (temporal factor) compared to the type of farm (spatial factor), where distinct separation can be observed that separates bee bread samples from the wet and dry seasons. The second component, on the other hand, contributed 17.6% of the total variance, which suggested a minimal contribution of the spatial factors.

The first component separated the wet and dry seasons into two major clusters (Figure 3d), suggesting that the metabolites differ due to season. During the wet season, the metabolites from each farm also differ significantly (Figure 3d). The inseparable cluster between the herbal and fruit farms during the dry season suggested that their metabolite composition was similar during the dry season, despite the different locations and types of farms. Therefore, temporal factors have a greater impact on the metabolite composition of bee bread than farm types, with a significant separation observed according to the season factor in the score plot of the first component.

However, when referring to the cross-validation result (Figure 4) and permutation test (Supplement 2), the p-value of the permutation validates the PLS-DA cluster at p=0.078, indicating that the data suffered from overfitting. In this case, the PLS-DA model represents the data so well that the outliers might be missed, and the predictive ability is also relatively low due to its low Q2 value.

Despite the overfitting issue in the PLS-DA result, PLS-DA suggested that the temporal factor acts as the key factor in determining significant metabolites. Thus, OPLS-DA analysis was conducted with the bee bread samples grouped according to the temporal factor. As depicted in Figure 5a, two distinct clusters were observed in the OPLS-DA score plot with 30 metabolites scored >1.0 in the VIP scores (Figure 5b). Compared to PLS-DA, OPLS-DA includes a paired structure while PLS-DA does not (Worley *et al*., 2013; Ruiz-Perez *et al*., 2020). Thus, OPLS-DA hypothetically provides more reliable results of a more specific and detailed relationship between bee bread samples differentiated based on temporal factors. OPLS-DA was more suitable as it could select the correct hyperplane despite having a small sample size and low clustering separation (Ruiz-Perez *et al*., 2020). The T-score and orthogonal T-score for the OPLS-DA score plots are 13.5% and 15% respectively (Figure 5a), where we can observe that the bee bread samples separated very well in the first component, confirming the hypothesis earlier. However, the second separation did not provide any insight into how to separate the bee bread samples based on different farm types. To confirm the validity of the OPLS-DA model built from MetaboAnalyst, a permutation test was done and validate OPLS-DA clusters at p=0.005, indicating

the significance of the model built (Supplement 3). In terms of data fitness, OPLS-DA does not suffer from data overfitting compared to PLS-DA analysis, resulting in a much more reliable model with higher predictive ability.

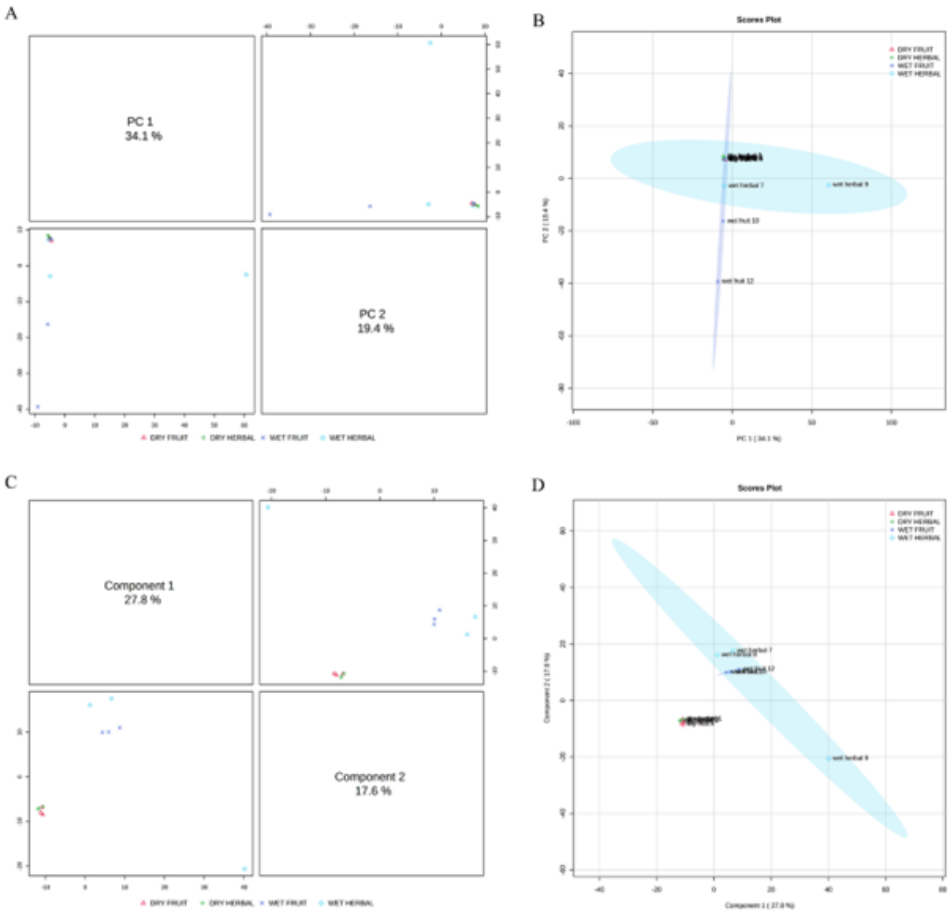

Fig. 3. Multivariate analysis using PCA and PLS-DA through MetaboAnalyst v6.0. PCA overview (a) was obtained with the first two components showing little to non-significant separation; PCA score plot (b); PLS-DA overview (c) with first two components and PLS-DA score plot (d), separation can be observed with stingless bee bread harvested during dry season in the left side and wet season in the right side of the score plot

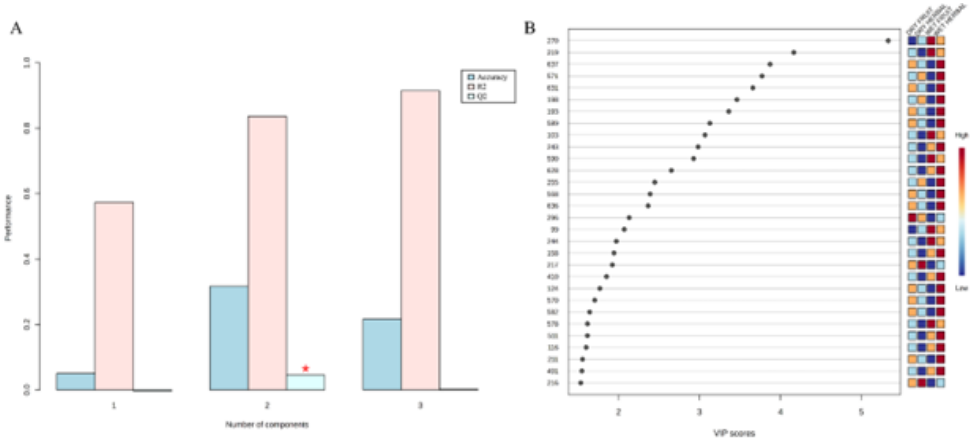

Fig.4. Validation test on PLS-DA through MetaboAnalyst v6.0. PLS-DA cross-validation test (A) was obtained with the first two components showing decent separation and VIP score (> 1.0) for metabolites representing higher importance in influencing the scores in VIP scores (B)

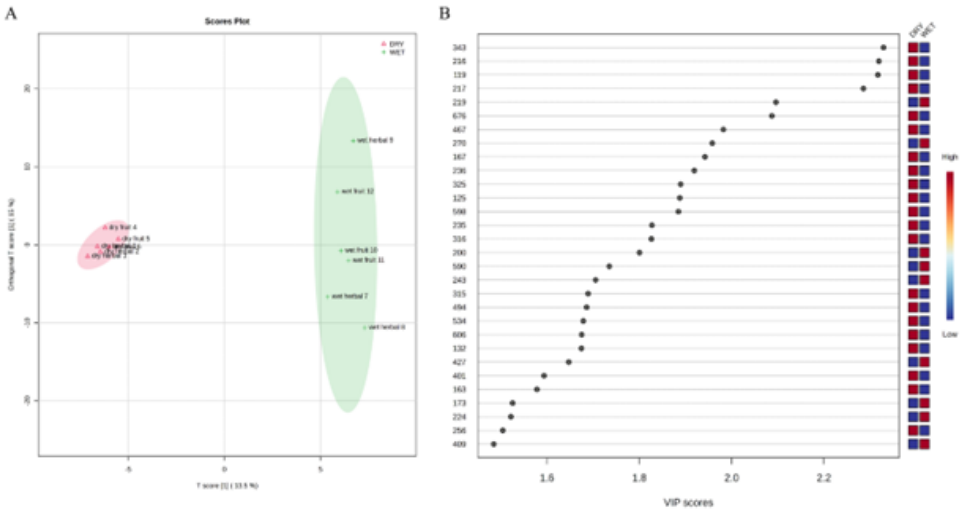

Fig. 5. OPLS-DA validation test through MetaboAnalyst v6.0. OPLS-DA score plot (a) was obtained with the first two components showing decent separation with dry season clustered on the left, wet season clustered on the right, and the variable importance in projection (VIP) scores of the top 30 metabolite features (VIP scores≥1) highlighted their significant contribution to sample segregation (b)

Aside from multivariate analysis of PCA, PLS-DA, and OPLS-DA, a dendrogram was also generated by MetaboAnalyst v6.0 (Figure 6). The dendrogram also suggested that the temporal factor plays a distinct and significant role in determining the metabolite composition of bee bread samples as compared with the farm types, where no clear separation can be seen between bee bread samples obtained from herbal and fruit farms. As depicted in Figure 6, a distinct separation at the first branch between bee bread samples collected during dry and wet seasons, supports the previous multivariate analysis.

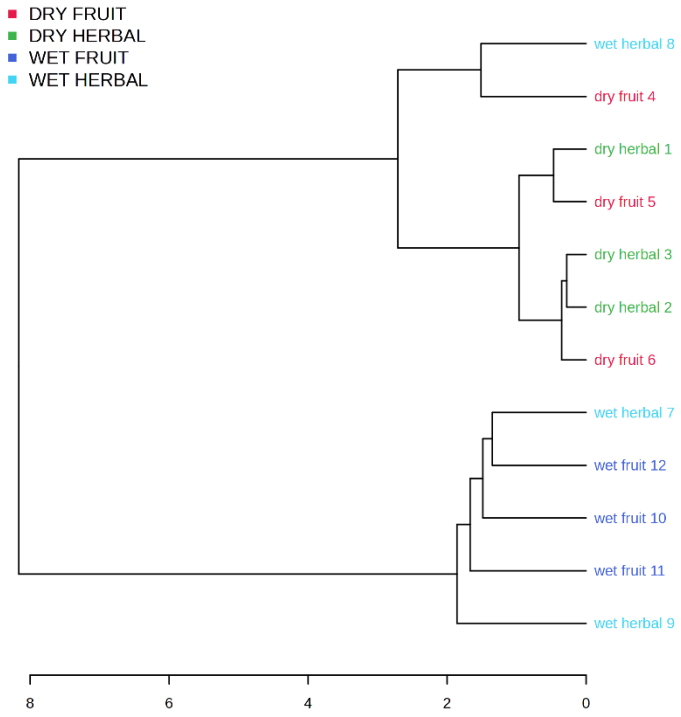

Fig. 6. Dendrogram plot obtained through Pearson correlation

Annotation of significant metabolites present in bee bread samples

The distribution of metabolites present in all samples was investigated through a univariate volcano plot in Metaboanalyst v6.0. The plot displays non-discriminatory variables in the center, while discriminant variables are positioned outside the central rectangle. The total number of significant metabolites found in different bee bread samples varies from each other. From the 39 MS features detected, an annotation step was taken to narrow down the significant metabolites. Hits with a similar m/z value and retention time are omitted from the significant metabolites list as they represent the same metabolite. After annotation, a total of 23 significant metabolites can be found that play an important role in differentiating bee bread samples from different temporal settings, dry and wet seasons (Table 1). As shown in Figure 7, volcano plots based on temporal (dry and wet season) factors demonstrated a significant metabolite profile. Further annotation of the metabolites from MetFrag suggested that they potentially originate from the group of flavones, flavonoids, alkaloids, terpene, antibiotics and fatty acids (Table 1).

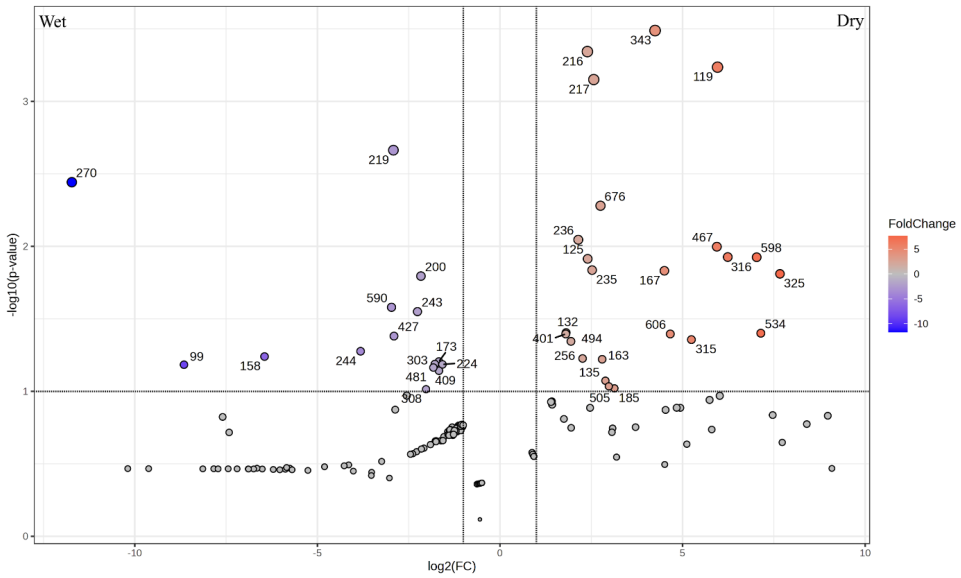

Fig. 7. Volcano plot based on the temporal factor, indicating bee bread samples obtained from the wet season (blue plots) and dry season (red plots). A total of 39 potential significant metabolites were identified in the volcano plot which yields a total of 23 significant metabolites after annotation. Several significant metabolites are higher in the dry season (right) compared to the wet season (left)

In addition, a heatmap was generated to visually explore the patterns within these clusters. The ungrouped heatmap was generated and it is hard to spot its significance (Supplement 4). Thus, the grouped heat map revealed a pattern of differences between multiple parameters including temporal and spatial, representing the normalized abundance of key metabolites across the groups (Figure 8). As shown in the heatmap, a total of nine flavones and flavonoids (within the range of retention time between 8 – 12 min) were clustered into their specific groups, further supporting the impact of seasonal factors on available metabolites.

As mentioned earlier, the composition of metabolites in meliponini bee bread is mostly affected by the season and the type of pollen sources during meliponini's foraging habit. Previously, Aleixo *et al*. (2017) reported that the amount of pollen collected by *Scaptotrigona aff. depilis* increased during the rainy season but with lower pollen diversity. Despite the fixed foraging area of melliponini within 0.8 km, Macías-Macías *et al*. (2016) reported that external factors might affect it. The change in foraging behavior of *H. itama* later has been proven by the study done by Jaapar *et al*. (2018), where *H. itama* favors the temperature range between 29℃ to 32℃ by actively bringing nectar and pollen back to the nest. Rainfall, however, limits the foraging process as the foraging worker bee stays in the nest until the rain stops (Jaapar *et al*., 2018). In this study, we can observe that the significant metabolites identified during the dry season are greater than those identified in the wet season, suggesting that stingless bee forages are less common during the wet season. Thus, to hypothesize, there is a change in the *H. itama* behavior during the wet season, possibly resulting in a reduction in a foraging coverage area that makes the types of metabolites smaller. Increased rainfall during the wet season might limit the foraging of *H. itama*, resulting in the reduction of the foraging area in a smaller radius. Previous studies done by Lawson and Rands (2019) and Maeda *et al*. (2023) have shown the effects of weather conditions on

Table 1. Potential metabolites in bee bread samples collected from different groups. ("/" indicates the presence of the said potential metabolites in the bee bread samples)

ID	Category	Potential metabolites	m/z value	rt	Dry season herbal farm			Dry season fruit farm			Wet season herbal farm			Wet season fruit farm		
					C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
135	Alkaloid	Lunarine	438.2378423	8.083802		/	/			/						
270	Alkaloid	N1,N5,N10-Tricoumaroyl spermidine	584.2759072	10.569354			/				/		/	/	/	/
185	Alkaloid	N1,N5,N10-Tricaffeoyl spermidine	625.1745755	9.220863	/	/	/									
224	Alkaloid	Dicaffeoyl Coumaroyl Spermidine	616.2651515	9.877187								/		/		/
125	Antibiotic	Kanamycin B	484.2420878	7.8719687		/	/		/	/						
676	Fatty acid	Crepenynate	279.23026	17.214016	/	/	/		/	/						
316	Flavone	Isoscutellarein	287.0547252	11.541687	/	/		/	/	/						
315	Flavone	Isoetin	303.0495874	11.514983	/	/	/	/		/						
158	Flavone	Isoetin	303.049778	8.761297	/		/		/	/	/	/				/
173	Flavone	Isorhamnetin	317.0644794	8.9366									/		/	/
325	Flavone	Isorhamnetin	317.0648595	12.004657	/	/	/	/		/						
467	Flavone	Xanthohumol	355.1522513	14.147514	/	/	/	/		/						
167	Flavone	Rutin	611.1582627	8.892119	/	/	/	/		/						
343	Flavonoid	Kaempferol	287.0545312	12.360247	/	/	/	/	/	/						
308	Flavonoid	Luteolin	287.0550871	11.328821							/	/		/		
606	Terpene	Fusaproliferin	445.291927	16.017801		/	/		/	/						
244	Carboxamide	N-(2-fluorophenyl)-4-[5-(1-methoxycyclopropyl)-4-[3-[2-(trifluoromethyl)phenyl]pyrrolidine-1-carbonyl]pyrazol-1-yl]piperidine-1-carboxamide	600.2697214	10.188956							/		/	/		/
534	Carboxylate	Methyl (3"aS,4"R,5"R,7"aS)-3"a,7"a-dimethyl-6"-oxo-4"-[(2E)-2-(2,6,6-trimethylcyclohex-2-en-1-ylidene)ethyl]spiro[1,3-dioxolane-2,1"-3,4,5,7-tetrahydro-2H-indene]-5"-carboxylate	431.2754665	15.15509	/	/	/		/	/						
119	Carboxamide	4-amino-N-[1-(3,4-dipropoxyphenyl)ethyl]-6,8-dihydro-5H-pyrido[4,5]thieno[1,2-c]pyrimidine-7-carboxamide	470.2267435	7.7084126	/	/	/	/	/	/						
132	Ester	2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethyl 3-[[2-[(3-guanidinobenzoyl)amino]acetyl]amino]butanoate	498.2577764	8.031212		/	/			/						
243	Amide	(2S)-N-[(1S,2R)-1-benzyl-2-hydroxy-3-[isobutyl-(1-methylimidazol-4-yl)sulfonyl-amino]propyl]-3-methyl-2-[3-[(2-methylthiazol-4-yl)methyl]-2-oxo-imidazolidin-1-yl]butanamide	660.2903768	10.18115							/		/	/	/	
216	Unknown	Unknown	316.6348512	9.788088	/	/	/		/	/						
236	Unknown	Unknown	323.6428487	10.062147	/		/		/	/						

flower-pollinator interactions, where extreme weather such as higher wind speeds, increased rainfall, and low temperatures will make the pollinator less active. Therefore, in this study, this heavy rainfall will increase the difficulty of stingless bees to fly out of the hives, limiting the foraging route and duration. The situation has become even worse due to the smaller body size of *H. itama* (Engel *et al*., 2023).

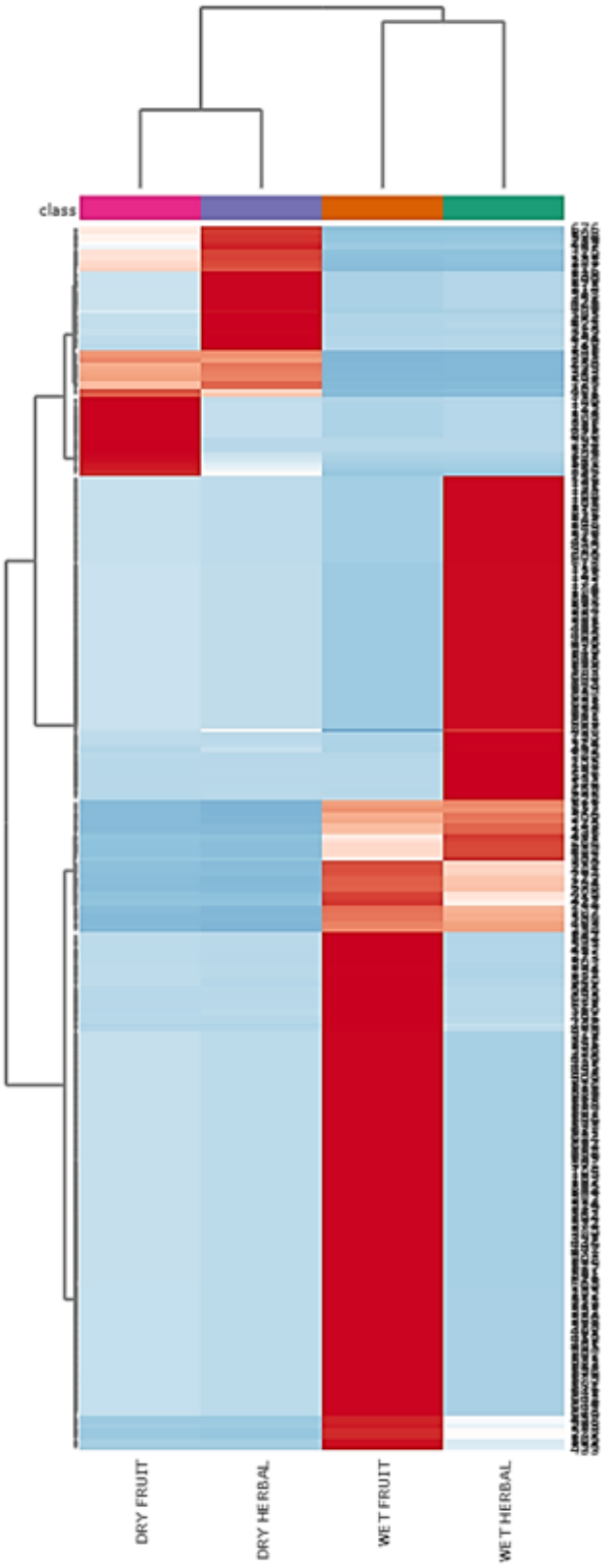

Fig. 8. Heatmap of metabolites found in bee bread samples generated from Pearson algorithm in MetaboAnalyst v6.0

The group flavone and flavonoid make up the most among all the significant metabolites identified in this study (Table 1). Flavonoids are a diverse group of polyphenolic compounds found in plants, with over 8000 individual compounds known (Pietta, 2000). They are extremely vital to the plant and contribute much to various functions, such as antioxidant, anti-inflammatory, anticancer, and UV protection (Anulika Nwokeji *et al*., 2016; Velu *et al*., 2018; Archoo *et al*., 2022). Plants are exposed to various environmental stresses such as excessive water, infection by pathogens, and lower amounts of sunlight. These conditions could induce the plants to produce more flavones, flavonoids, and alkaloids for defense purposes (Panche *et al*., 2016; Mathesius, 2018). When the stingless bees visit the plants to forage (Benedick *et al*., 2021), they also bring back pollens to their hives and end up in the bee metabolites. According to the heatmap built in Figure 8, we can observe that the differences between potential metabolites present more significantly between seasons. Metabolite groups such as antibiotics are present in both wet and dry seasons, not giving any clues on how these metabolites differentiate the bee bread samples, aside from different antibiotic types found in different sample groups.

Flavone, however, is a specific subgroup of flavonoids characterized by a 15-carbon backbone with two aromatic rings connected by a three-carbon bridge (Çetinkaya *et al*., 2022). As the structures of flavone and flavonoid are similar, they have similar functions in plants. Among the most important functions of these phenolic compounds is their antioxidant activity. Previous studies have shown interest in flavonoids' antioxidant activity, with their ability to scavenge and reduce the formation of free radicals (Pietta, 2000; Panche *et al*., 2016). Kaempferol, also possesses antioxidant properties (Vellosa *et al*., 2011; Shahbaz *et al*., 2023). In addition, Sharma *et al*. (2021) reported that the antioxidant properties of kaempferol also possess potent and effective prevention against liver cancer by reducing oxidative stress and endoplasmic reticulum (ER) stress that eventually leads to hepatocellular carcinoma.

Kaempferol is known to be produced by various plant families including herbal, fruits, and vegetables (Archoo *et al*., 2022). This supports our findings that the potential source of *H. itama* bee bread comes from Fabaceae, commonly known as the pea, bean, and legume family. Among the species that are identified under the Fabaceae family are *Glycine max* (soybean)*, Hedysarum caucasicum*, *Lotus tenuis*, *Lotus corniculatus*, *Melilotus albus*, *Melilotus sulcatus*, *Trifolium polyphyllum*, Phaseolus and *Pisum sativum*. In terms of distribution, Fabaceae is a relatively large plant family and widespread in all locations around the world (Wink, 2013; Xu & Deng, 2017). Fabaceae have a wide variety of growth forms, including trees, and vines but the majority of them exist in the form of herbaceous perennials (Xu & Deng, 2017). The Fabaceae family is also well-known for its ability to produce secondary metabolites with a high nitrogen composition, thanks to their vital role in nitrogen-fixing microorganisms (Wink, 2013; EL Sabagh *et al*., 2020; Raza *et al*., 2020; Zhao *et al*., 2021).

Other phenolic compounds such as luteolin and isorhamnetin were also found in this study. Previous studies by Dranca *et al*. (2020) and Sumarlin *et al*. (2021) have also suggested the presence of these compounds in meliponini bee bread. Besides possessing significant antioxidant activity as the other phenolic compounds, isorhamnetin also possesses antimicrobial activity towards *Escherichia coli*-induced Sepsis (Chauhan *et al*., 2019). The potential of isorhamnetin was also revealed by Seo *et al*. (2014) that the introduction of isorhamnetin leads to a reduction in ROS activity, contributing to the inhibitory effect on inflammation. Similar to other flavonoids, luteolin also possesses great therapeutic potential such as anti-inflammatory, anti-allergic, antioxidant, and anticancer properties. Lin *et al*. (2008) reported that the anticancer property of luteolin through the sensitizing of carcinoma to therapeutic-induced cytotoxicity by suppressing cell survival pathways such as X-linked inhibitor of apoptosis protein (XIAP) which then induces stimulation of tumor suppressor p53. In terms of the sources of luteolin and isorhamnetin, they are commonly found in herbal plants, carrots, peas, berries, and citrus fruits (Calderón-Oliver & Ponce-Alquicira, 2018). Our finding suggested the presence of plant families such as Fabaceae, Asteraceae, Ranunculaceae, and Lamiaceae. that contribute to the source of luteolin and isorhamnetin. Among the potential sources of these flavones are *Parkinsonia florida*, *Inula salicina*, *Mangifera indica*, etc.

On the other hand, xanthohumol was also identified as the significant metabolite that causes the difference in bee bread samples between seasons. This compound can be found in the dry season *H. itama* bee bread sample and is known to originate from *Hulus lupulumus*, which is commonly known as hop, a type of flowering plant under the family Cannabinaceae. This compound possesses anticancer properties where it exerts multiple inhibitory effects on the proliferation of cancer cells via signaling pathway modulation, in addition to other therapeutic effects such as anti-aging, antidiabetic, and anti-inflammation (Lee *et al*., 2011; Girisa *et al*., 2021).

On top of that, antibiotics can be observed in the bee bread sample group harvested during the dry season. Kanamycin B can be found in only dry season bee bread samples. Previously, Ngalimat *et*

al. (2019) identified 41 microbes from Firmicutes, Proteobacteria, and Actinobacteria in the meliponini bee products, such as honey, propolis, and bee bread. The bacteria from genera such as *Bacillus* and *Streptomyces* were known to produce antibiotics (Tenebro *et al*., 2021). Antibiotics such as kanamycin B and streptomycin originate from microbes such as *Streptomyces*, *Streptosporangium*, *Saccharopolyspora*, *Micromonospora*, *Streptoalloteichus* and *Dactylosporangium* (Piepersberg & Wehmeier, 2009; Grosset & Singer, 2013). We understand that bee bread is produced as the result of fermentation that happened in meliponini beehives (de Paula *et al*., 2021). Thus, the presence of antibiotics in bee bread is potentially produced by the same microorganisms that aid the bee bread fermentation, granting powerful antibiotic properties to bee bread (Martínez-Puc *et al*., 2021). However, the reason behind having antibiotics present only in dry-season bee bread samples remain unknown.

Moreover, alkaloids account for a significant portion of the significant metabolites identified in melliponini bee bread, which are isolated from different seasons. Among the four potential alkaloids that have been successfully identified in this study are lunarine, decaline, N1,N5,N10-tricaffeoyl spermidine, and N1,N5,N10-tricoumaroyl spermidine. In this study, the presence of alkaloids in plants is very diverse. All types of bee bread samples contain alkaloids. For example, lunarine can only be found in dry season bee bread samples and originates from *Lunaria annua*, commonly known as honesty, categorized under the family Brassicaceae (Sagner *et al*., 1998). Lunarine is an important inhibitor of trypanothione reductase, that catalyzes the reaction converting trypanothione and NADP to trypanothione disulfide and NADPH (Hamilton *et al*., 2002). On the other hand, N1,N5,N10-tricaffeoyl spermidine can be found in *Quercus dentata*, which is also known as the oak tree (Bokern *et al*., 1995). These spermidine alkaloids are often related to numerous pharmacological effects such as anti-inflammatory, antibiotics, etc. (Zhang *et al*., 2022).

Even though there are plenty of unidentified significant metabolites based on the library used for metabolite annotation, terpene such as fusaproliferin can be identified in dry season *H. itama* bee bread. Microbes like *Fusarium subglutinans* and *Fusarium fujikuroi* primarily produce this compound. It is a type of mycotoxin that can cause harmful effects on plants and insects (Logrieco *et al*., 1996). Jestoi (2008) reported that this mycotoxin is often related to the food contamination known to be produced by fungi which is often neglected by humans. In addition, Jajić *et al*. (2019) reported that the presence of fusaproliferin will negatively impact the production yield of important crops such as maize. However, the presence of fungus and mycotoxin in bee bread may signal a more serious concern following a recent report by Carrera *et al*. (2023), which reported the presence of mycotoxin from 80 samples of bee bread products collected from 28 different countries.

CONCLUSION

The metabolomics study of *H. itama*'s bee bread reveals a significant difference in metabolite distribution between seasons compared to farms. Flavones and flavonoids are the two major groups of metabolites that differentiate bee bread between seasons besides other metabolites such as antibiotics and alkaloids. Although the study only covers two farms, the results are highly interesting for bee farmers, who might have to consider the importance of seasonal variation that affects the composition of metabolites which may affect the product quality or colony health indirectly. The bee bread metabolites were also identified putatively from both plants and microorganisms which varied according to different seasonal settings. Even though bee bread is presented as a secondary product, analyzing the metabolites is still important to expand the knowledge and ensure the safety of meliponini bee bread as a healthy and safe food source. The study will also provide initial data to develop a quality standard for bee bread product monitoring, especially in the presence of pesticides.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

All authors have seen and agree with the contents of the manuscript and there is no conflict of interest, including specific financial interest and relationships and affiliations relevant to the subject of the manuscript.

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