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Original Article

Impacts of metabolites and aflatoxins from *Aspergillus flavus* (Link, 1809) on the health and lifespan of *Apis mellifera* (Linnaeus, 1758)

Bajaree Chuttong[1], Supakhom Klaitanoad[1], Kanokwan Klaithin[1], Sampat Ghosh[2,3], Chuleui Jung[2,4], Ming Cheng Wu[5], Khanchai Danmek[6*]

[1]Meliponini and Apini Research Laboratory, Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University 50200, Thailand

[2]Agricultural Research Institute, Gyeongkuk National University, Andong GB 36729, South Korea

[3]Department of Life Science Sardar Patel University, Balaghat, Madhya Pradesh 481331, India

[4]Department of Plant Medicals, Gyeongkuk National University, Andong GB 36729, Republic of Korea

[5]Department of Entomology, College of Agriculture and Natural Resources, National Chung Hsing University 402202, Taiwan

[6]Biotechnology program, School of Agriculture and Natural Resources, University of Phayao 56000, Thailand

**Corresponding author's email:* khandchai.da@up.ac.th

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Abstract

This study investigates the impact of *Aspergillus flavus*, a mycotoxin-producing fungus, on honeybee health in Thailand, where maize pollen is a key nutritional resource for apiculture but carries a significant risk of fungal contamination. Metabolomic analysis revealed that *A. flavus* produces aflatoxins B1 (AFB_1) at 3,753 ng/mL and B2 (AFB_2) at 327 ng/mL, while *A. nomius* is not toxin-producing. Co-culturing *A. flavus* with *A. nomius* led to a reduction in AFB_1 and AFB_2 to 218 and 26.52 mg/mL, respectively. Newly emerged worker honeybees were fed various diets: 50% sucrose syrup (S, T1), S + semi-synthetic media (T2), S + metabolites of *A. flavus* (T3), S + metabolites of *A. nomius* (T4), and S + metabolites of both *A. flavus* and *A. nomius* (T5). The results indicated that T3 resulted in the shortest lifespan and the smallest hypopharyngeal gland acini size (9.38 ± 1.02 nm compared to 21.07 ± 1.37 nm in T2). However, the lifespan of T4 and T5 was comparable to T1 and T2, indicating that *A. nomius* would not have any harmful impact on honeybee lifespan and may reduce any deleterious impacts of *A. flavus*. Similar inference was possible on the acini size of the hypopharyngeal gland which is an important health parameter of worker honeybees. This study contributes to a deeper understanding of how microbial contamination affects honeybee health and the necessity to reduce the risks associated with toxin exposure under the situation of natural pollen sources declining.

Keywords: Fungal contamination, Honeybee health, Hypopharyngeal glands, Maize pollen, Toxin exposure

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Introduction

The Western honeybee (*Apis mellifera* Linnaeus, 1758) plays crucial roles in supporting global agricultural and natural ecosystems by enhancing crop yields and maintaining biodiversity (Armstrong et al., 2021). The survival of a honeybee colony hinges on its foragers, which collect vital resources such as nectar and pollen for colony growth and reproduction (Ghosh and Jung, 2017; Feketéné Ferenczi et al., 2023). However, the foraging activity also exposes honeybees to a range of environmental contaminants, including fungal spores and microbial metabolites, some of which can adversely impact bee health and colony productivity (Parish et al., 2020; Aynalem et al., 2024). Notably, *Aspergillus* spp., including *A. flavus* (Link, 1809), *A. parasiticus* Speare (1912), and *A. nomius* Kurtzman et al. (1987), are of particular concern due to their potential to contaminate foraged resources and pose significant risks to honeybee health (Foley et al., 2014; Aynalem et al., 2024). These fungi are commonly found in the environment, thriving in soil, decaying plants, and agricultural products (Becchimanzi and Nicoletti, 2022). Spores of these fungi are often present during bee foraging, and the warm and humid conditions within the hive promote fungal growth, which may lead to contamination of hive food sources, such as bee bread and other hive products (Bush et al., 2024; Rutkowski et al., 2023).

Among these, *A. flavus* exhibited a multifaceted relationship with honeybees, encompassing both antagonistic and mutualistic interactions. It is particularly concerning due to its association with stonebrood disease, which causes infected individuals to desiccate, and form hardened masses, severely compromising colony health and productivity. Although less prevalent than chalkbrood, caused by *Ascosphaera apis*, stonebrood can result in significant larval mortality, especially in tropical regions where environmental conditions favor its proliferation (Swanson et al., 2009; Jensen et al., 2013; Jensen et al., 2024). In addition to its effect on larvae, A. *flavus* produces aflatoxins, highly toxic metabolites that pose serious risks to both animal and human health (Kostić et al., 2019). Aflatoxins can contaminate pollen stores, weakening essential nutritional reserves and adversely affecting bee development, lifespan, and the safety of hive products such as honey and propolis (Deveza et al., 2015; Bush et al., 2024). While prior research has investigated the effects of aflatoxins on honeybee health (Niu et al., 2011; Johnson et al., 2012), the specific impacts of aflatoxigenic strains of *A. flavus* remain insufficiently explored. Despite its pathogenic potential, certain strains of *A. flavus* contribute positively by enhancing the nutritional quality of honeybee feed (Bush et al., 2024). The dual role underscores the complexity of its interactions with honeybees and highlights the need for further investigation into its ecological and functional significance.

This research is vital for addressing the escalating challenge to honeybee health, particularly in regions like Chiang Mai in northern Thailand, where such studies are scarce. Agricultural intensification, climate change, and habitat loss have led to a significant decline in natural pollen resources, forcing honeybees to rely heavily on supplemental feeds. Contamination of these feed sources, especially with toxins like aflatoxins, poses an additional threat to their already vulnerable health. By investigating the specific effects of aflatoxigenic strains of *A. flavus*, this study will contribute to understanding how microbial contamination affects honeybee health. The findings could play a crucial role in developing strategies to mitigate these risks through improved nutritional management, ensuring better health outcomes for honeybees as their natural feed sources continue to decline.

Material and Methods

Media preparation

Potato dextrose agar (PDA) was obtained from Sigma-Aldrich, St. Louis, MO, USA. Modified *Aspergillus flavus* and *parasiticus* agar (AFPA), as described by Cotty (1994), consisted of 1.0% (w/v) peptic digest of animal tissue, 2.0% (w/v) yeast extract, 0.05% (w/v) ferric ammonium citrate, 1.0 mL of 0.2% (w/v) dichloran in ethanol, and 1.5% (w/v) agar. The semi-synthetic (SS) medium, based on Danmek et al. (2022), was composed of 2.0% (w/v) sucrose, 0.2% (w/v) peptone, 0.1% (w/v) yeast extract, 0.2% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$, 0.01% (w/v) $CaCl_2$, 0.2% (v/v) Tween 80, 0.05% (w/v) $ZnSO_4 \cdot 7H_2O$, 0.05% (w/v) $FeSO_4 \cdot 7H_2O$, and 0.05% (w/v) $MnSO_4 \cdot H_2O$.

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Isolates and culturing

A. flavus was isolated from honeybee bodies collected from an apiary in Chiang Mai, Thailand, (coordinates: N 18.7942141, E 98.9612368). While *A. nomius* had been studied previously, the identification of *A. flavus* represents a novel finding in this research. Initially, fungal identification was conducted by observing growth characteristics on AFPA and PDA under controlled conditions in a growth chamber maintained at 30°C and 60% relative humidity (Danmek et al., 2022). Spore suspensions were prepared by flooding PDA-grown fungal cultures with a 0.85% NaCl solution containing 0.01% Tween 80. The suspension was then agitated to release spores, adjusted to a concentration of 1.0×10^5 conidia/mL and verified microscopically under a laminar flow chamber.

Characterization of *A. flavus*

Macroscopic features such as colony color and texture, along with sclerotia number and size, were observed. After 7 days of incubation, fungal sclerotia were counted and their diameters were measured using a stereo attachment (Leica Microsystems Fluorescence Stereo Microscopes Leica M205 FCA and Leica M205 FA) and a 3D digital microscope camera (Hirox HRX-01 and RX-100, USA). For each isolate, ten sclerotia were randomly selected, and two diameters per sclerotium were measured. To confirm the genus, sequencing was performed using the Internal Transcribed Spacer (ITS) region with primers ITS4 and ITS5, following the method of Romero and Cumagun (2023) with modification. Genomic DNA was extracted from mycelia cultured in PDB, and the PCR protocol was as follows: initial denaturation at 95°C for 5 min, followed by 31 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1.4 min, with a final extension at 72°C for 10 min. The PCR products were sequenced at Central Laboratory (Thailand) Co. Ltd., Chiang Mai, Thailand, using the Big Dye Terminator Cycle Sequencing Kit v.3.1. Sequence comparison was done using BLAST on the NCBI website. A phylogenetic tree was constructed using the IQ-tree program.

Metabolite production

Metabolite production was carried out by culturing *A. flavus*, *A. nomius*, and a 1:1 mixture of both fungi in 100 mL of SS medium in an Erlenmeyer flask. The cultures were incubated on a rotary shaker at 80 rpm and 30°C for one week, followed by a second week of static incubation for fungal sporulation and metabolite production.

Non-targeted LC-MS/MS metabolomics analysis

Metabolomic analysis was conducted by U2Bio (Thailand) Co., Ltd., using the methodology of Fernandez-Cantos et al. (2024). Samples (5 mL) were lyophilized, reconstituted in 70% methanol with sulfadimethoxine (25 ng/mL), and centrifuged. Supernatants were analyzed in triplicate using LC-MS with a Poroshell 120 EC-C18 column and optimized gradient elution. MS analysis was performed in positive/negative modes with high resolution. Data processing in MS-Dial 5.3 included alignment to QC samples and normalization with LOWESS. Features meeting the criteria of correlation (≥0.7/0.5), %CV (≤50%), and identification score (≥0.6) were retained. Statistical analysis in MetaboAnalyst 6.0 included log transformation and imputation of missing values.

Aflatoxin analysis

The characterization of aflatoxins (AFB) was conducted by extracting and quantifying each sample using High-Performance Liquid Chromatography (HPLC), in accordance with the AOAC method (2006). HPLC analysis was performed with a system equipped with a fluorescence detector at 360/450 nm and a C18 column (250 × 4.6 mm, 5 μm) with a guard column at 35 °C. The injection volume was 20 μL. The mobile phase included solvent A (water/methanol/acetonitrile, 6:3:2, v/v/v, with 2% acetic acid) and solvent B (1% acetic acid in water) at 1 mL/min. The gradient program was 0–5 min, 45%A; 5–7 min, 45–100%A; 7–20 min, 100%A; 20–22 min, 100–45%A; and 22–30 min, 45%A.

Experimental syrup incorporating *Aspergillus* metabolites

Honeybee colonies were obtained from the apiary at the Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Thailand, in May 2024. The colonies were maintained according to standard protocols. Sealed brood frames were placed in an insect growth chamber at 33 ± 1°C and 60 ± 1% relative humidity until the pupae metamorphosed into adult honeybees.

The feeding study comprised five treatments: 50% sucrose syrup (S as T1), S+ SS medium at a 1:1 ratio (T2), S+*A. flavus* metabolites (T3), S+*A. nomius* metabolites (T4), and S+ metabolites of both *A. flavus*

and *A. nomius* (T5) at an equal 1:1 ratio. Each treatment was replicated three times, comprising 30 newly emerged adult worker bees housed in polypropylene boxes with a capacity of 600 mL, each featuring 40 ventilation holes (diameter ≤ 5 mm).

Honeybee Lifespan

Feeding was administered using syringes filled with the respective treatments and water. Maize-based pollen patty was prepared by mixing maize pollen with a 50% sucrose solution at a 1:1 ratio and provided to the honeybees. Each container received 2.0 grams of pollen patty placed on a plastic dish inside the cage, covered with plastic wrap containing small perforations. The respective treatment, water, and pollen patties were replaced every three days, and the lifespan of the honeybees was monitored over a period of 21 days, with deceased bees being removed daily. This setup aimed to evaluate the impact of these different fungal treatments on the honeybee lifespan.

Size of hypopharyngeal gland (HPG) acini

On day 7, five honeybee samples from each treatment were selected to assess HPG acini development. The HPG acini were carefully exhibited and placed in plastic petri dishes containing droplets of ice-cold normal saline solution (0.85%, isotonic to hemolymph). Micrographs of the HPG acini were captured using an Olympus BX53 digital upright microscope with an IMTcamCCD5 PLUS camera (IMT i-Solution, Inc.) at 40x magnification. The diameters of 20 randomly selected acini per bee head were measured and analyzed statistically.

Statistical analysis

All data were analyzed using SPSS version 26.0 (IBM Co., Armonk, NY, USA). statistical software package. The mean difference of treatments was compared using Tukey's multiple range test, where $p < 0.05$ was used as an indicator of statistical significance. Kaplan-Meier survival analysis was employed to evaluate the impact of diets on the lifespan of honeybees. The statistical significance of differences in time distributions between groups was assessed.

Results

Morphological and molecular characterization of *A. flavus*

In this study, we isolated a fungal strain, designated L1.1, from deceased honeybees and identified it as *A. flavus*, a member of the *Aspergillus* section *Flavi*, a complex group of green molds characterized by subtle morphological variations among its subspecies. This identification was based on distinct morphological features, including a characteristic yellow-orange reverse colony on AFPA, green conidia, and the formation of spherical sclerotia on PDA. These traits serve as key diagnostic markers for identifying this strain in *Aspergillus* section *Flavi* (Figure 1).

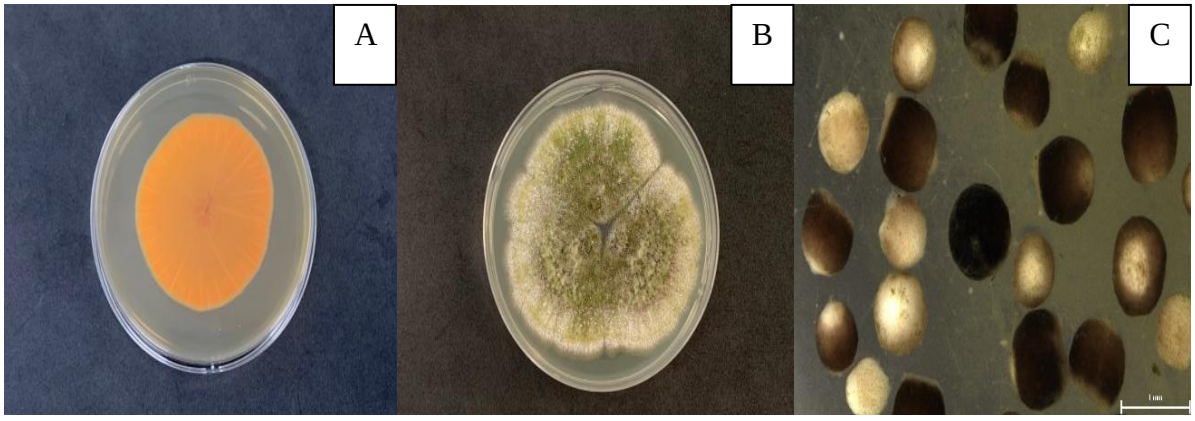

Figure-1. *A. flavus* colonies incubated on AFPA (A), PDA (B) at 30 °C and its sclerotia (C).

Further examination of strain L1.1 on two solid media revealed differences in sporulation. On AFPA, conidia production was relatively sparse, whereas PDA supported abundant conidia formation. Initially, colonies appeared white, but as they matured, the color transitioned from bright green to dark green. After 7 days of incubation at 30°C, the colony diameter on PDA ranged from 67 to 79 mm. Microscopic examination revealed that strain L1.1 had hyaline conidiophores measuring 500–1,000 µm in length, arranged uniseriately, with radiating conidial heads. The vesicles were globose to sub-globose, ranging from 11.3 to 36 µm in diameter (mean 19 µm; n = 20). The phialides varied in shape and size, with an average length of 8.2 µm (range: 4.9–9.2 µm; n = 20). Within 7 days, strain L1.1 also produced smooth-walled, thick, hard sclerotia, ranging in color from light to dark brown, with an average size of 0.81 × 0.80 mm (n = 20). Molecular characterization was carried out using internal transcribed spacer (ITS) gene sequencing, which revealed 100% similarity to a reference *A. flavus* sequence in the GenBank database (accession number PP430412.1) (Figure 2). This molecular evidence conclusively confirmed that strain L1.1 is *A. flavus*.

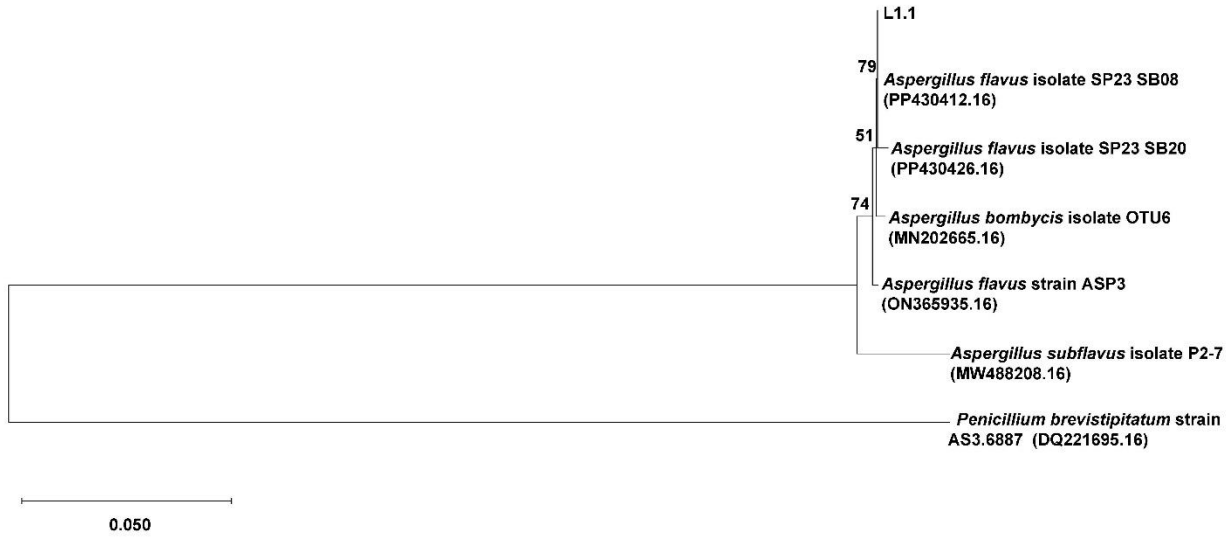

Figure-2. Maximum likelihood phylogenetic analyses based on internal transcribed spacer (ITS) sequence. The isolation of L1.1 formed a monophyletic group with *A. flavus* in the GenBank accession no. PP430412.1. The scale bar represents the number of nucleotide substitutions per site.

Metabolites and aflatoxin production

This study employed non-targeted metabolomics to investigate the secondary metabolites produced by *A. flavus*, *A. nomius*, and their co-culture. Principal component analysis (PCA) of the metabolite profiles revealed distinct separation among the three treatment groups, with PC1 and PC2 accounting for 48.6% and 29.9% of the variance, respectively (Figure 3). These findings confirm that *A. flavus* and *A. nomius* possess distinct secondary metabolite profiles and that co-culture conditions significantly influence metabolite expression, as evidenced by the PCA and heatmap results (Figures 3 and 4). The metabolomic analysis identified 5,840 features in positive ion mode, spanning various metabolite classes, including amino acids, nucleotides, organic acids, and toxins. Key metabolites in *A. flavus*, including aflatoxins, cyclopiazonic acid, tenuazonic acid, citrinin, butyrolactone II, and kojic acid, were further examined.

HPLC-FLD analysis validated the metabolomic findings, confirming that *A. flavus* primarily produce AFB_1 and AFB_2 while failing to synthesize AFG_1 and AFG_2. Specifically, *A. flavus* was identified as a high aflatoxin producer, with AFB_1 and AFB_2 detected at concentrations of 3,753 ng/mL and 327 ng/mL, respectively. In contrast, an atoxigenic strain of *A. nomius* did not produce aflatoxins. Under co-cultural

conditions, AFB_1 and AFB_2 concentrations decline to 218 ng/mL and 26.52 ng/mL, respectively (Figure 5). These findings highlight the significant impact of co-culture on metabolite production and highlight complex interspecies interactions. Notably, cyclopiazonic acid production by *A. flavus* remained unaffected in co-culture, while tenuazonic acid, predominantly produced by *A. nomius*, decreased, suggesting potential inhibitory effects exerted by *A. flavus*.

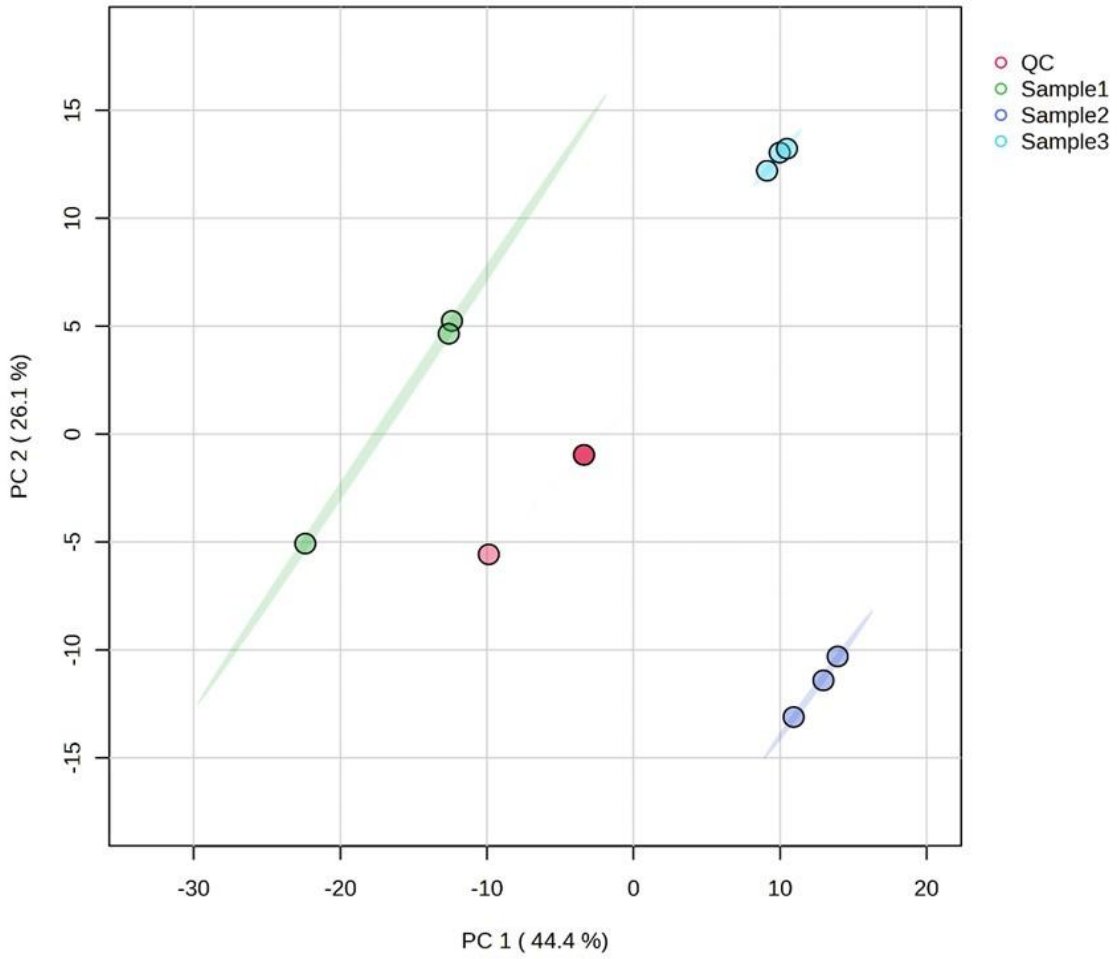

Figure-3. PCA analysis of three groups of *Aspergillus* cultures. The clustering of different sample types is illustrated, with *A. flavus* represented by green (Sample 1), *Aspergillus nomius* by dark blue (Sample 2), and co-culture by light blue (Sample 3).

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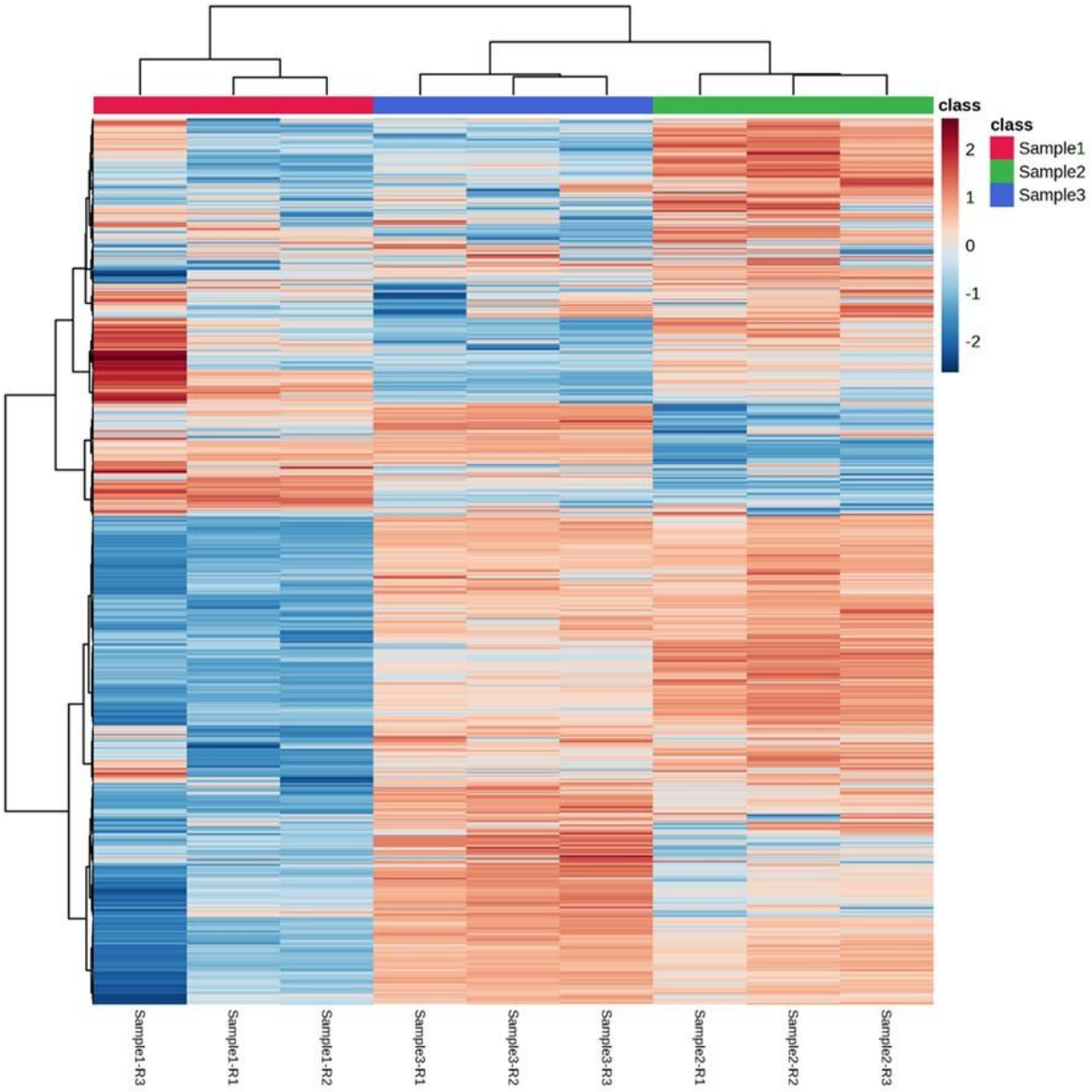

Figure-4. Heatmap representation of the significantly increased metabolites on *Aspergillus* cultures. *A. flavus* represented by red (Sample 1), *A. nomius* by green (Sample 2), and co-culture by blue (Sample 3).

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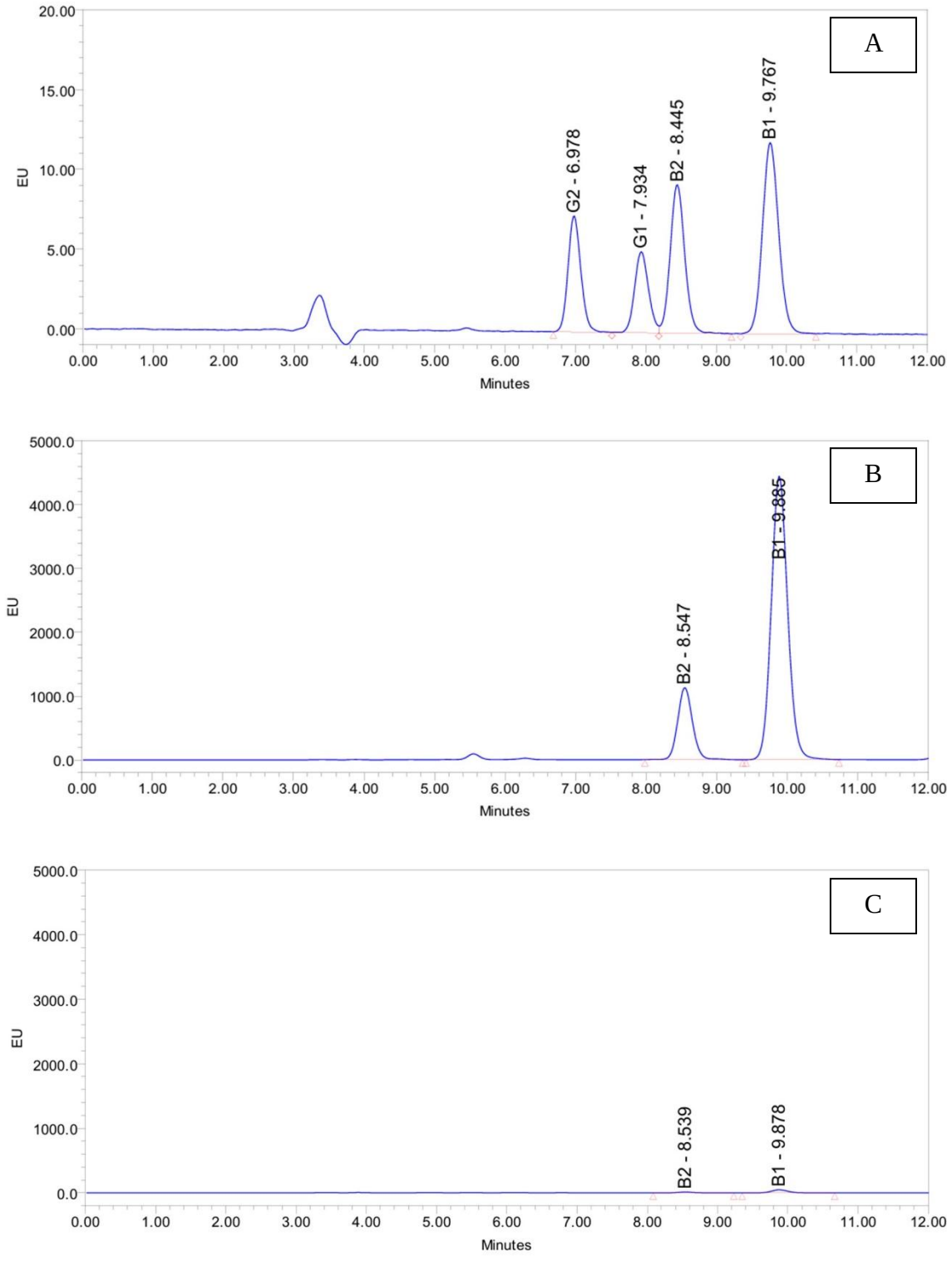

Figure-5. HPLC chromatogram showing the chromatogram and retention times from aflatoxin standard (A), *A. flavus* (B) and when co-cultured *A. flavus* with *A. nomius* (C).

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Impacts of fungal metabolites on health and lifespan

This study examined the effects of metabolites produced by *Aspergillus* spp. on honeybee health and lifespan, with a particular focus on aflatoxins and other metabolites. The results showed a significant increase in mortality rates among honeybees fed syrup containing *Aspergillus* metabolites. Worker honeybees exposed to metabolites from *A. flavus* (T4), known for its high aflatoxin (AFB_1 and AFB_2) production, exhibited severe mortality, with all 30 bees perishing within 12 days (Figure 6). In comparison, bees exposed to lower aflatoxin levels, such as those fed metabolites from *A. nomius* (T3) or a combination of *A. nomius* and *A. flavus* (T5), showed significantly ($p < 0.05$) lower mortality rates. These findings highlight *A. flavus* as not only a direct cause of honeybee mortality due to stonebrood but also as a significant factor in honeybee death through aflatoxins. Other metabolites, including cyclopiazonic acid and citrinin produced by *A. flavus* and tenuazonic acid produced by *A. nomius*, were also evaluated. While these metabolites caused stress and increased mortality, their effects were less severe than those of aflatoxins, particularly in bees fed syrup containing *A. flavus* metabolites. Bees in control groups (T1 and T2) that were not exposed to fungal metabolites exhibited the lowest mortality rate, underscoring the need to prevent fungal contamination and aflatoxin exposure to support honeybee health and survival.

Honeybees fed syrup supplemented with SS medium (T2) displayed the largest HPG acini size (21.07±1.37 nm), significantly ($p < 0.05$) larger than those in other treatment groups. Honeybees fed syrup with *A. flavus* metabolites (T4) had the smallest acini (9.38±1.02 nm), while those exposed to metabolites from both fungal species (T5) had moderately sized acini (13.92±0.27 nm). However, honeybees receiving metabolites from *A. nomius* (T3) showed less impact, with no significant difference ($p \geq 0.05$) in acini size (19.38±1.02 nm) when compared to the control (T1) (16.47±0.38 nm).

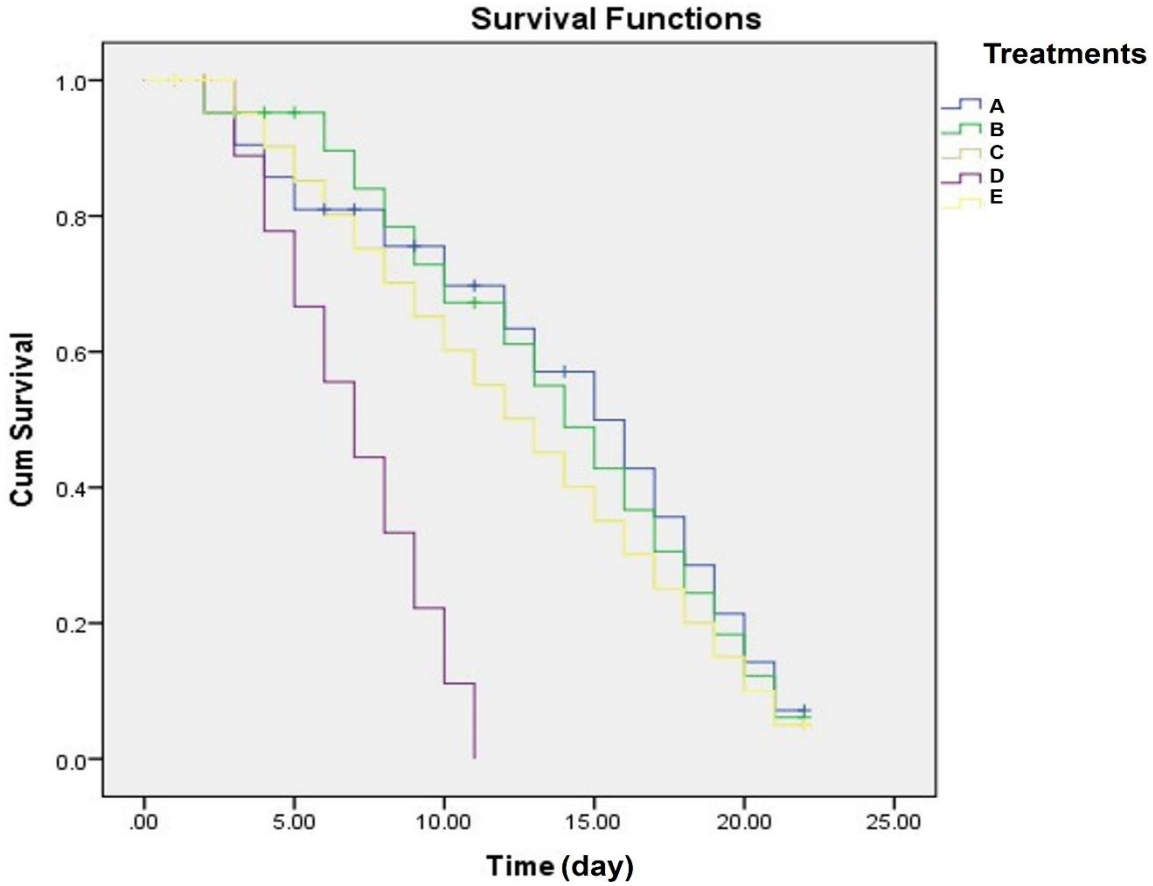

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Figure-6. Lifespan (days) of honeybees fed a 50% syrup solution prepared with various diluents, including water (A), SS medium (B), *A. flavus* metabolite (C), *A. nomius* metabolite (D), and a metabolite derived from the co-culturing of *A. flavus* and *A. nomius* (E), under laboratory conditions of 30°C and 60% relative humidity.

Discussion

The fungus *Aspergillus* spp. isolate L1.1, obtained from a deceased honeybee in Thailand, exhibited a characteristic yellow-orange coloration on the reverse side of colonies cultured on AFPA, a medium designed for detecting fungal strains in the *Aspergillus* spp. This observation aligns with findings reported in previous scientific studies (Cotty, 1994) (Figure 1). The observed yellow to yellow-orange pigmentation is attributed to the production of secondary metabolites, which are influenced by specific components of AFPA, such as ferric ammonium citrate. These compounds may stimulate the biosynthesis of pigments or unique byproducts in the fungus. Microscopic morphological examination revealed that the L1.1 isolate produces abundant conidia and spherical sclerotia, exhibiting features consistent with other species in the *Aspergillus* section *Flavi*. However, morphological similarities among species within this section can lead to misidentification (Samson et al., 2014; Frisvad et al., 2019). To enhance the accuracy of species identification, the sequencing of the internal transcribed spacer (ITS) region was employed following initial morphological assessments (Tam et al., 2014). The ITS region has been widely recognized as a reliable molecular marker for differentiating *A. flavus* from closely related species (Figure 2). The experimental results confirmed that the L1.1 isolate is identified as *A. flavus*, underscoring the utility of the ITS region as a robust molecular tool for species identification, particularly in cases where traditional methods may be insufficient (Tam et al., 2014; Samson et al., 2014; Frisvad et al., 2019). These findings align with the study by Romero and Cumagun (2023), which employed ITS sequencing to identify multiple *Aspergillus* spp. isolated from maize in Isabela Province, Philippines.

Two *Aspergillus* spp., namely *A. flavus* and *A. nomius*, were selected for this study due to their well-documented ability to produce mycotoxins, particularly aflatoxins (Foley et al., 2014). *A. flavus* generally produces only AFB, whereas *A. nomius* can synthesize both AFB and AFG, with AFG production typically exceeding that of AFB (Kurtzman et al., 1987; Pickova et al., 2021). However, in this study, a strain of *A. nomius* incapable of aflatoxin production was identified and selected. The metabolite profiles of this *A. nomius*, as analyzed by PCA, heatmaps, and AF chromatograms, differed significantly from those of *A. flavus* (Figures 3, 4, and 5). Furthermore, the co-cultivation of the two fungal species resulted in significant changes in metabolite levels, including a substantial reduction in aflatoxins. Additionally, information on the metabolites classified as mycotoxins produced by *Aspergillus* spp. was provided in accordance with the guidelines outlined by Varga et al. (2011) (Table 1). These results indicate that the *A. nomius* has potential as an atoxigenic strain, comparable to established biocontrol strains like *A. flavus* AF36 and *A. flavus* NRRL21882 (Xu et al., 2021), as well as certain strains of *A. oryzae* (Alshannaq et al., 2018).

Honeybees have specific nutritional requirements, and the addition of fungal metabolites to their diet necessitates careful investigation to assess safety. Numerous studies highlight the negative effects of fungal spores and metabolites, particularly aflatoxins produced by *A. flavus*, on the health and development of various insects, both directly and indirectly (Niu et al., 2011; Foley et al., 2014; Becchimanzi and Nicoletti, 2022). Direct supplementation of *A. flavus* spores into honeybee diets is neither recommended nor appropriate. Consequently, we cultured *A. flavus* in liquid media to facilitate metabolite production prior to its incorporation into syrup, followed by an assessment of its impacts on honeybee health and safety through both ingestion and contact exposure. The findings reveal that *A. flavus* is a significant pathogenic fungus for honeybees, as previously reported to cause stonebrood disease (Foley et al., 2014; Miller et al., 2021). This study further confirms the adverse effects of fungal metabolites, particularly aflatoxins, on honeybee health. Non-target metabolomics and aflatoxin analysis demonstrated that *A. flavus* produced substantial quantities of aflatoxin, specifically AFB_1. Survival analysis indicated that honeybees exposed to syrup containing high concentrations of AFB_1 exhibited rapid mortality, with complete colony loss within 12 days. Research has demonstrated that AFB_1 can impair the immune system and disrupt foraging behavior and reproductive functions in honeybees, particularly *A. mellifera* (Johnson et al., 2012; Foley et al., 2014). We indicated to focus on the discussion of AFB_1 for the longevity assay, as it is a naturally occurring fungal toxin found

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in the honeybee environment. Additionally, the lifespan of adult worker honeybees exposed to AFB_1 decreases, which is consistent with the findings of Niu et al. (2011) and Johnson et al. (2012). These studies provide further insight, suggesting that the mortality observed in bees exposed to AFB_1 may be attributed to a reduction in enzymatic activities of cytochrome P450, leading to a decreased ability to tolerate the toxin. In addition, the mortality rate in this group was significantly higher ($p < 0.05$) compared to control groups and other treatments. For example, honeybees exposed to syrup derived from *A. nomius*, a fungus that does not produce aflatoxins but generates other metabolites such as tenuazonic acid, citrinin, butyrolactone II, and kojic acid, had the same report as Varga et al. (2011) and showed significantly lower mortality rates. Similarly, treatments combining *A. flavus* with atoxigenic *A. nomius* resulted in reduced aflatoxin concentrations and mitigated negative impacts. These findings suggest that the presence of *A. nomius* may regulate or inhibit aflatoxin production by *A. flavus*. Moreover, feeding honeybees syrup containing low levels of AFB_1 did not result in detrimental effects on their health or lifespan. This underscores the importance of precise fungal metabolite management to mitigate risks associated with aflatoxin exposure to honeybees.

The impact of fungal toxins, such as aflatoxins, on honeybee health has been studied, including the lethal dose of aflatoxin to *A. mellifera* exposed to varying concentrations, using standard aflatoxins (Niu et al., 2011). This study extends previous research by investigating the effects of metabolites from *A. flavus* on the health of the same honeybee species, with a particular focus on HPG. The HPG serves as a reliable physiological indicator of bee health, as it is influenced by factors such as age, nutrition, and environmental conditions (Danmek et al., 2024). The findings indicate that honeybees fed syrup contaminated with metabolites from an aflatoxin-producing strain of *A. flavus* experienced detrimental effects on HPG development, resulting in increased mortality. These results are consistent with previous studies by Niu et al. (2011) and Johnson et al. (2012). Further evidence supporting these conclusions comes from the use of SS medium, a complex nutrient substitute for distilled water in syrup production. This method seemed to offer benefits to the honeybees, suggesting potential directions for future research, especially considering the declining availability of naturally nutritious pollen, which is frequently contaminated with fungal spores and chemicals (Parish et al., 2020; Aynalem et al., 2024).

Conclusions

This study emphasizes the critical impact of *Aspergillus* spp., particularly *A. flavus*, on honeybee health due to aflatoxin production and other harmful metabolites. Elevated aflatoxin levels were linked to increased mortality and impaired hypopharyngeal gland development, underscoring the importance of preventing fungal contamination in bee feed. Ensuring proper nutrition and implementing effective fungal control measures are essential for safeguarding honeybee health and survival. These findings shed light on the significant threats posed by fungal metabolites and reinforce the need for sustainable beekeeping practices to support health and productivity.

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Contribution of Authors

Chuttong B: Conceptualized the study, developed the methodology, conducted the investigation, and prepared the original draft.

Jung C: Conceptualized the study, developed the methodology, performed validation, contributed to

writing, reviewing, and editing, and provided supervision.
Ghosh S & Wu MC: Assisted in developing the methodology and contributed to writing, reviewing, and editing the manuscript.
Klaithin K: Assisted in preparing honeybees for testing.
Klaitanoad S. Participated in the investigation.
Danmek K: Contributed to conceptualization, validation, writing the original draft, reviewing and editing the manuscript, and secured funding.

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