Ministry of Environment of Denmark
Environmental Protection Agency

BumbleBio

Bumblebees as vectors of fungi for biocontrol of insect pests in strawberry flowers

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1. Acknowledgements

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2. Summary

Strawberries are among the most widely produced fruits in Denmark and are cultivated in tunnels, greenhouses, and open fields. Ever-bearing cultivars grown in tunnels enable season-long production but rely on pollination by bumblebees (*Bombus terrestris*) from commercial hives, particularly in early-season when natural pollinators are scarce. Strawberry crops are affected by arthropod pests and plant diseases, including grey mold and several insects, with invasion of thrips being particularly problematic in tunnel-grown flowers during late spring. Although insect nets can reduce pest immigration, they also restrict the entry of natural pollinators. Chemical insecticides are limited for thrips control, but microbial control agents such as the entomopathogenic fungus *Beauveria bassiana* are alternatives. Application of *B. bassiana* may target several arthropod pests, but the fungi may also infect insect pollinators, raising concerns when applied during flowering.

Bumblebees are efficient pollinators due to their foraging behavior, tolerance to cool and wet conditions, and ability to sonicate flowers. They are commercially reared and widely used in Danish strawberry production. Bumblebees can also vector biocontrol agents, so-called api-vectoring, as demonstrated by Flying Doctors® hives delivering fungal spores of *Clonostachys rosea* strain J1446 (previously *Gliocladium catenulatum* strain J1446) against grey mold. Studies have shown that bumblebees can likewise vector *B. bassiana* to target insect pests. Api-vectoring of BotaniGard WP — a commercial product based on *B. bassiana* strain GHA in a wettable powder (WP) formulation — to strawberry flowers via commercial bumblebee hives designed for *C. rosea* vectoring may allow for simultaneous pollination and delivery of *B. bassiana* GHA spores for thrips control, although this may have negative effects on the bumblebees. This project therefore aims (1) to evaluate whether bumblebees in commercial hives can effectively vector BotaniGard WP to strawberry flowers and reduce abundance of Western flower thrips (*Frankliniella occidentalis*), and (2) to assess the effects of BotaniGard WP exposure on the bumblebee longevity and health at both individual and colony levels.

Experimentally, BotaniGard caused dose-dependent mortality in *F. occidentalis* larvae and adults during laboratory bioassays using liquid spore suspensions on strawberry leaf discs. Powder applications (BotaniGard WP mixed with corn flour) on detached strawberry flowers resulted in variable effects on *F. occidentalis* adult mortality and offspring production depending on thrips density, fungal dose, and relative humidity. Bumblebees vectored fungal spores to strawberry flowers in greenhouse settings, where they remained viable for up to one week. However, despite presence in the flowers, api-vectoring of BotaniGard WP did not significantly reduce thrips infestation in the greenhouse, potentially due to suboptimal microclimatic conditions during exposure.

Direct exposure to BotaniGard WP was lethal to bumblebee workers in the laboratory, while exposure at the colony level did not produce detectable effects in the greenhouse over two weeks. When exposing workers to BotaniGard WP in Flying Doctors® hives, fungal spores accumulated inside the hives and altered the gut microbiota of bumblebees, but the exposure did not affect mortality, flight activity or colony weight gain. Longer term effects during commercial hive lifespan, *i.e.*, 6-8 weeks, would be necessary to assess if the negligible effects observed over two weeks would continue.

Api-vectoring of *B. bassiana* to strawberry flowers does occur with the implemented strategy, but biocontrol efficacy must be enhanced by increasing relative humidity to optimize fungal infection. However, such conditions may be conducive for pathogens in developing fruits. Exploring co-vectoring of two microbial control agents for

combined insect pest control and disease management with *B. bassiana* and *C. rosea* may represent a future strategy, if also compatible with application of other natural enemies. Practically, growers would need to adapt greenhouse climatic conditions to support efficacy of api-vectored microbial control agents, *e.g.*, by maintaining relatively high night-time humidity, while avoiding conditions that favor plant pathogen development. Effective api-vectoring also requires managing hive activity and timely refilling of dispensers to ensure consistent inoculum delivery.

3. Sammendrag

Jordbær er blandt de mest udbredt producerede bær i Danmark, hvor de dyrkes i tunneler, drivhuse og på friland. Remonterende sorter dyrket i tunneler muliggør produktion gennem hele sæsonen, men denne dyrkningsform er afhængig af bestøvning af humlebier (*Bombus terrestris*) fra kommercielle stader, særligt tidligt på sæsonen, hvor naturlige bestøvere er få. Produktionen af jordbær påvirkes af skadedyr og plantesygdomme, herunder gråskimmel og forskellige insekter, hvor især indflyvning af trips er problematisk i tunnel-dyrkede jordbær i det sene forår. Selvom insektnet kan reducere indvandring af skadedyr, begrænser de også adgangen for naturlige bestøvere. Kemiske insekticider til bekæmpelse af trips er begrænsede, men mikrobielle bekæmpelsesmidler, som den insektpatogene svamp *Beauveria bassiana*, udgør et alternativ. Anvendelse af *B. bassiana* kan virke mod flere skadelige insekter, men svampen kan også inficere bestøvende insekter, hvilket giver anledning til bekymring ved anvendelse under blomstring.

Humlebier er effektive bestøvere på grund af deres fourageringsadfærd, tolerance over for kølige betingelser samt evne til at vibrere blomster (sonikering). De opdrættes kommercielt og anvendes i vid udstrækning i dansk jordbærproduktion. Humlebier kan også fungere som vektorer for biologiske bekæmpelsesmidler, som det er brugt med Flying Doctors®-stader, der leverer svampesporer af *Clonostachys rosea* stamme J1446 (tidligere *Gliocladium catenulatum* stamme J1446) mod gråskimmel. Studier har også vist, at humlebier kan udbringe *B. bassiana* til bekæmpelse af insektskadegørere. Transport af BotaniGard (et kommercielt produkt baseret på *B. bassiana* stamme GHA) til jordbærblomster via humlebier fra kommercielle humlebistader designet til udbringning af *C. rosea* kan muliggøre samtidig bestøvning og levering af *B. bassiana* GHA-sporer til bekæmpelse af trips i jordbærblomsterne, men dette kan potentielt have negative effekter på humlebierne.

Dette projekt har derfor til formål (1) at evaluere, om humlebier i kommercielle stader effektivt kan udbringe BotaniGard til jordbærblomster og reducere forekomsten af Saintpaulitrips (*Frankliniella occidentalis*), og (2) at vurdere effekterne af eksponering for BotaniGard på humlebiernes overlevelse og sundhed på både individ- og koloniniveau.

Eksponering for BotaniGard medførte dosisafhængig dødelighed hos larver og voksne af *F. occidentalis* i laboratorieforsøg med sporesuspensioner på afskårne jordbærblade. Tilførsel af svampesporer i pulverform (BotaniGard blandet med majsmel) i afskårne jordbærblomster resulterede i varierende effekter på voksendødelighed og produktion af afkom hos *F. occidentalis* afhængigt af tætheden af trips, svampedosis og relativ luftfugtighed. Humlebier udbragte svampesporer til jordbærblomster i drivhusforsøg, hvor sporernes levedygtighed blev opretholdt i op til en uge. På trods af tilstedeværelsen af svampesporer i blomsterne reducerede BotaniGard dog ikke signifikant forekomsten af trips under væksthusbetingelser, muligvis på grund af suboptimale mikroklimatiske forhold under eksponeringen.

Direkte eksponering for BotaniGard var dødelig for humlebier i laboratoriet, mens eksponering på koloniniveau ikke gav målbare effekter på overlevelse i drivhuset over en periode på to uger. Ved eksponering af humlebiarbejdere for BotaniGard i Flying Doctors®-stader akkumuleredes svampesporer inde i staderne, og eksponeringen ændrede humlebiernes tarmmikroflora, men eksponeringen påvirkede ikke dødelighed, flyveaktivitet eller koloniernes vægtøgning. Observation af langtidseffekter over den forventede stadelevetid, dvs. 6–8 uger, er nødvendige at gennemføre for at vurdere, om de ubetydelige effekter observeret over to uger vil fortsætte.

Transport af *B. bassiana* til jordbærblomster ved humlebier finder sted med den anvendte strategi, men den biologiske bekæmpelseseffekt skal forbedres ved at øge den relative luftfugtighed for at optimere svampeinfektionen i trips. Øget luftfugtighed kan dog også fremme udviklingen af sygdomme i jordbærrene. Undersøgelse af samtidig udbringning af to mikrobielle bekæmpelsesmidler til kombineret insekt- og sygdomsbekæmpelse med både *B. bassiana* og *C. rosea* kan udgøre en fremtidig strategi, hvis den også er forenelig med anvendelsen af andre naturlige fjender til biologisk bekæmpelse. I praksis bør jordbæravlere tilpasse drivhusklimaet for at understøtte effekten af de anvendte mikrobielle bekæmpelsesmidler, f.eks. ved at opretholde relativ høj luftfugtighed om natten. Samtidig skal forhold, der fremmer udvikling af plantesygdomme, undgås. Effektiv udbringning af mikrobiologiske bekæmpelsesmidler ved brug af humlebier kræver desuden, at der holdes øje med aktivitet i stadet samt rettidig genopfyldning af dispensere for at sikre ensartet levering af det mikrobiologiske bekæmpelsesmiddel.

4. Introduction

Strawberries were the most produced berries in Denmark in 2020[i]. This crop is mainly cultivated in tunnels and greenhouses, but also in open fields. The use of ever-bearing cultivars in tunnels allows for production of strawberries throughout the season, but it requires the use of commercial pollinators such as bumblebees (*Bombus terrestris*) early in the season as few natural pollinators are present. Strawberry plants can be attacked by various arthropod pests and plant pathogens, which can cause significant yield loss. For instance, the most common disease in strawberry fruits is grey mold caused by the pathogen *Botrytis cinerea*, while arthropod pests infest leaves (*e.g.*, spider mites, aphids, whiteflies) or fruits (*e.g.*, tarnished plant bug, weevils) (Lahiri et al., 2022; Liburd & Rhodes, 2019). However, the flowers are also susceptible to insect pests, particularly thrips, which frequently occur at high densities in tunnels in May and June during the second flowering peak (Nauja Jensen, HortiAdvice A/S, pers.comm.). The thrips enter the semi-open tunnels, and recent efforts have been made to prevent the immigration of thrips and tarnished plant bugs by covering openings with insect nets, but this preventative strategy also reduces the entry of natural pollinators in May and June.
Select few chemical pesticides can be used for control of thrips and other pests in conventional strawberry production, but alternatives such as entomopathogenic fungi are available. These fungi, such as *Beauveria bassiana* (Mascarin & Jaronski, 2016; McKinnon et al., 2017), produce conidia that infect insects through the cuticle and develop a lethal mycosis that kills the host (Lacey et al., 2015). Entomopathogenic fungi can infect a wide range of insects and mites, and they can colonize plant roots and other organs as endophytes (Canassa, Esteca, et al., 2020; Quesada-Moraga et al., 2022). However, application of entomopathogenic fungi can also infect insect pollinators in laboratory experiments (Cappa et al., 2022; Erler et al., 2022) and can therefore be considered a risk when applied during pollination of the crop.

Bumblebees are highly adapted insects able to survive in diverse climates and habitats (Williams, 1998). About 260 bumblebees species are known, mainly distributed in the Holarctic, but some species are also found in the Oriental and Neotropical regions (Williams, 1998). Bumblebees' behavior and features make them important and efficient pollinators (Goulson, 2003). Bumblebees are generalist pollinators, indiscriminately pollinating flowers of wild plants and crops (Goulson, 2003), and a single bumblebee can visit several flowers per minute (Corbet et al., 1991). They are active in cold and rainy weather, making them attractive as pollinators in temperate climates (Corbet et al., 1991). In addition, their large and hairy body carry a substantial amount of pollen (Wahengbam et al., 2019), and their high-frequency sonication is essential for some self-fertilizing plants. These characteristics, as well as the decline in populations of wild pollinators (Colla & Packer, 2008; Kosior et al., 2007; Xie et al., 2008) have led to the domestication of *B. terrestris* in Europe and the commercialization of bumblebee rearing systems. Therefore, bumblebees are exploited for their pollination services, as they provide direct commercial benefits, such as high fruit quality and an increase in total crop yield (Velthuis & van Doorn, 2006).
In Denmark, commercial hives of bumblebees (*B. terrestris*) are regularly used for pollination of strawberry flowers in tunnels in March/April (Nauja Jensen, HortiAdvice, pers. comm). Recently, growers have also started to use bumblebees for combined pollination and vectoring of fungal biocontrol agents against grey mold (Nauja Jensen,

[i] https://www.fao.org/faostat/

HortiAdvice, pers. comm), which depends on specific commercial hives, 'Flying-Doctors', where a dispenser at the hive exit is loaded with the biocontrol product Prestop Mix WP, containing spores of the fungus *Clonostachys rosea* strain J1446 (previously *Gliocladium catenulatum* strain J1446) (Smagghe et al., 2020; Temmermans & Smagghe, 2022). During the past decade, several studies have shown the efficacy of entomo-vectoring of biocontrol agents by bumblebees, notably for control of grey mold in strawberries (Iqbal et al., 2022; Mommaerts et al., 2011; Van Delm et al., 2015). Other studies have demonstrated that also entomopathogenic fungi can be vectored by bumblebees to flowers to initiate infections in insect pests, such as the commercial product BotaniGard WP based on *B. bassiana* strain GHA against whiteflies and tarnished plant bugs in annual vegetable crops in greenhouses (Kapongo, Shipp, Kevan, & Broadbent, 2008; Kapongo, Shipp, Kevan, & Sutton, 2008). BotaniGard has been used successfully in controlling thrips (Mukawa et al., 2011) and it is authorized as minor use in Denmark as WP (wetable powder) formulation for use against thrips in ornamentals and against whiteflies in strawberries in open fields[ii]. According to the label, the product should not be used at the flowering stage to protect wild pollinators. However, if open ends of strawberry tunnels are closed with insect nets to prevent entry from insect pests and natural pollinators, commercial bumblebee hives such as Flying-Doctors may potentially be used both to pollinate strawberry flowers and to vector BotaniGard WP to the flowers for thrips control. The first aim of this project (Part 1) is therefore to determine whether bumblebee-vectored BotaniGard WP has biocontrol potential against thrips in strawberry flowers.

Bumblebees used as vectors of *B. bassiana* is a controversial idea as the bumblebees can become infected and suffer from lethal or sub-lethal effects (Karise et al., 2016). However, the demonstration of such effects is variable and depends on several factors such as the type and duration of exposure, the fungal strain or the formulation (Erler et al., 2022). For example, Leite et al. (2022) showed that *B. bassiana* affected bumblebees' survival when ingested but not when applied topically. Moreover, effects of biopesticides on pollinators are mostly assessed at the individual level (Cappa et al., 2022), while bumblebees live in a social colony context, which is characterised by communal hygienic behaviors against infections, encapsulated in the concept of social immunity (Cremer et al., 2018). Therefore, the assessment of a combination of traits (*e.g.*, survival, activity, weight) would provide an improved overview of the effects of *B. bassiana* on health status of bumblebees, at different scales (individual vs. colony level). The microbiota is also considered as a proxy for bumblebee health (Sauers & Sadd, 2019): it can reflect social interactions during development, which strongly drive gut microbial composition (Billiet et al., 2017); and it can play a role in the protection against parasite infection (Mockler et al., 2018). Additionally, the main contamination routes have been studied for some biopesticides, between habitats (*i.e.*, colony and flowers) or between species (Gradish et al., 2019; Ruiz-González et al., 2012), but not between individuals of the same colony. As a result, it remains unclear whether individuals exposed to biocontrol fungi can contaminate their nestmates through contact in the colony, how these interactions influence the survival, and to which extend the fungus is spread inside the hive. Therefore, the second aim of this project (Part 2) is to evaluate the health status of bumblebees when exposed to BotaniGard WP, at the individual and at the colony scale, by assessing a combination of traits (phenotype, transmission potential, gut microbiota).

[ii] https://middeldatabasen.dk/product.asp?productID=61184

Project overall objectives and hypotheses

The project addressed two main objectives, namely to:

1. Determine whether bumblebees can vector a commercial formulation of the entomopathogenic fungus *B. bassiana* to strawberry flowers in sufficient amounts and persistence to cause infections in thrips;
2. Assess the effects of the exposure of *B. bassiana* on bumblebee mortality and health both when evaluated at the individual, group and the colony level.

The project therefore tested the following overall hypotheses:

1. The entomopathogenic fungus *B. bassiana* can be vectored by bumblebees to strawberry flowers in sufficiently high densities to successfully infect thrips.
2. Exposure to *B. bassiana* will have both lethal and sub-lethal effects on bumblebees, but lethal effects will be highest at individual level and lowest at colony level; exposure to *B. bassiana* will modify the gut microbiota of bumblebees and fungal spores will be dispersed inside the colony with spatial segregation.

Therefore, this report is divided into two parts addressing each of the two main objectives: firstly focusing on api-vectoring efficacy and biocontrol potential, and secondly focusing on bumblebees' health status at different scales (**Figure 1**). Each objective includes separate aims, materials and methods, results and discussion sections.

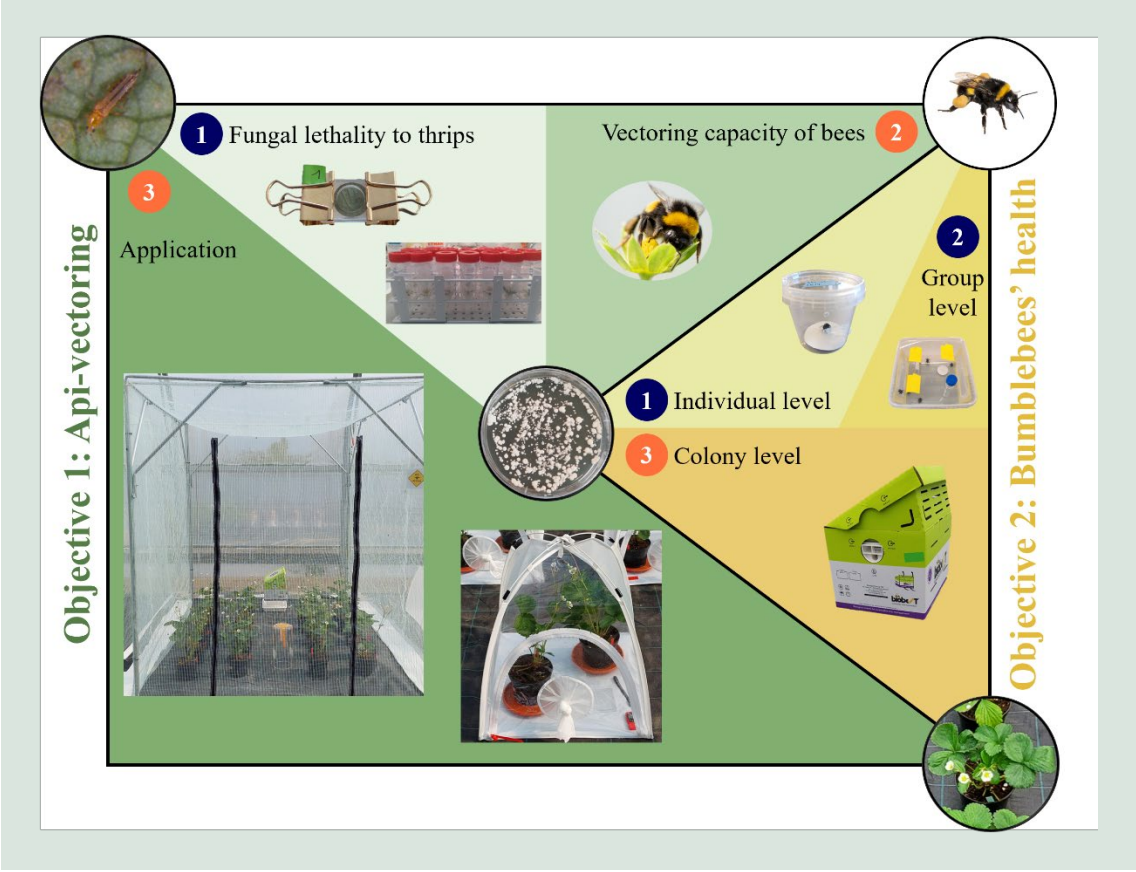

FIGURE 1. Graphical overview of the project structure and organization of the present report. The two main objectives are color-coded: green for Objective 1 (biocontrol efficacy through api-vectoring) and yellow for Objective 2 (health status of bumblebees exposed directly and indirectly to *B. bassiana*). Each objective comprised three experiments numbered 1–3. Dark-colored numbers indicate laboratory experiments and orange-colored numbers indicate greenhouse experiments. The photographs illustrate representative setups and organisms used across the experiments.

5. Materials and methods for organisms in experiments

5.1 Entomopathogenic fungus *Beauveria bassiana*

The commercial product BotaniGard®22WP (provided in Denmark by Borregaard Bioplant ApS), containing the active substance *B. bassiana* GHA, was used in all experiments. The product was prepared as a spore suspension or as a powder mixture. Two batches of the product were received, which were stored at 4°C.

5.1.1 Preparation of the inoculum

Spore suspensions were prepared by mixing 1 g of BotaniGard®22WP powder and 10 mL of sterile 0.05% Triton-X. The obtained suspension was then diluted to quantify spore concentration in a hemocytometer (Fuchs-Rosenthal) to prepare experimental spore suspensions with concentrations ranging from 10^4 to 10^9 spores/mL. Sterile 0.05% Triton-X served as the control treatment (*i.e.*, 0 spores/mL). Spore viability was tested for each suspension by plating 100 µL of concentration 10^5-10^6 spores/mL on Sabouraud Dextrose Agar (SDA) media, which were incubated in darkness at 23°C. Spore suspensions used in experiments displayed spore germination rates >95%.

Experimental BotaniGard powder was diluted by mixing the commercial product with heat-sterilized organic corn flour (Blogan A/S, Italy; heated at 120°C for 7 h in an oven) in batches of 100 g at different ratios: D0, D15, D25, D50, D100 for use in laboratory experiments. For experiments with detached flowers and for greenhouse experiments, inactive D15 and active D15 were used (**Table 1**). To obtain the inactive D15, a batch of D15 was heat-sterilized at 120°C for 7 h. All prepared doses were stored at 4°C until further use. Before an experiment, 1 g of each dose was suspended (as above) to assess the concentration of spores using a hemocytometer, and the spore viability was evaluated by plating the suspension on SDA. Additionally, 1 g of the inactive D15 mixture was suspended and plated to confirm that no spores germinated, ensuring that the *B. bassiana* spores had been effectively killed by the heating.

TABLE 1. Experimental BotaniGard powder mixtures at different proportions of sterilized corn flour. Concentration refers to the number of spores per gram, determined using a hemocytometer during the individual setting experiment (see section 11.1). N = 1 per dose.

Doses (description)	BotaniGard (%)	Corn flour (%)	Concentration (spores/g)	Characteristics
D0	0	100	0	
D15 (Inactive D15 or Control)	15	85	_	Heat-sterilized
D15 (Active D15 or BotaniGard)	15	85	4.05×10^9	
D25	25	75	7.13×10^9	
D50	50	50	1.30×10^{10}	
D100	100	0	3.36×10^{10}	

5.1.2 Cultivation-dependent quantification of *Beauveria bassiana*

Quantification of *B. bassiana* inoculum levels during and after experiments was performed following the protocol described by Iqbal *et al.* (2022). Collected samples (*i.e.*, inoculating loop, bumblebees, flowers, swabs of hive interior and cells) were placed in 50 mL Falcon tubes, each containing 10 mL of 0.05% Triton X. The suspensions were vortexed for 10 sec at maximum speed before agitation for 30 minutes at 250 rpm. A 10-fold dilution series was prepared and a volume of 100 µL was plated in duplicate from three dilutions on selective media composed of (per 500 mL): agar 6 g, glucose 10 g, peptone 5 g, dodine 0.2 mL (0.08 g/mL), streptomycin 0.5 mL (0.6 g/mL), tetracycline 0.5 mL (0.05 g/mL) and cyclohexamide 1 mL (0.05 g/mL), adjusted to pH 6.3–6.5. For swab samples from experimental hives samples, no duplicate plating was done. The plates were incubated at 23°C in darkness. Emerging Colony Forming Units (CFUs) of *B. bassiana* were counted daily during the first week and every two days during the second week until no more CFU appeared. The final number of CFUs was recorded after 13 days of incubation from the most readable dilution (*i.e.*, plates with 25 to 250 CFUs). The total number of CFUs per sample was calculated using the following formula:

$$Number\ of\ CFU_{sample} = Mean\ CFU_{plate} \times \frac{1}{dilution} \times \frac{1}{V_{plate}} \times V_{suspension}$$

- "Mean CFU_{plate}", the average number of CFUs from the duplicated plates of the same dilution;
- "dilution", the dilution used to count and record the final number of CFUs;
- "V_{plate}", the volume pipetted into the plate (*i.e.*, 100 µL, so 0.1 mL);
- "$V_{suspension}$", the volume of Triton X 0.05% used to suspend the bee (*i.e.*, 10 mL)

5.2 Bumblebees and Flying Doctors® hives

Commercial hives of *B. terrestris* were obtained from BioBest (Westerlo, Belgium; distributed in Denmark Borregaard Bioplant ApS), in which the bumblebees had continuous access to Biogluc® sugar solution.

For laboratory experiments, standard hives were maintained in darkness at 23°C upon arrival. Worker bees were retrieved from these hives to be used in experiments. Newly emerged bees were avoided.

For greenhouse experiments, standard Flying Doctors® hives (**Figure 2**) were either used as supplied and placed in the greenhouse enclosure upon arrival (Objective 1) or standardized immediately upon arrival (Objective 2). Standardization involved removing all bumblebees from the hive and introducing 85 workers and the queen into a clean Flying Doctors® hive, which had been cleaned with RODALON and ethanol. Both types of hives had dispenser at the exit, which could be loaded with a microbial control agent in powder formulation. During the greenhouse experiments, 10 g of the BotaniGard-corn flour mixture was placed manually in the dispenser with a spoon, and the surface was smoothed out.
Greenhouse experiments were conducted in a large tunnel, covered by 2 layers of clear plastic, measuring 16 × 10 × 2 m (length × width × height) and oriented east-west. In the tunnel, we placed custom made walk-in mesh cages (Ornata plus 27 mesh; Garditec ApS, Denmark) measuring 2.5 × 1.8 × 2.0 m (depth × width × height). The cages were designed to prevent bees from escaping. Inside each tent, a Flying Doctors® hive was installed 15 cm above the ground at the back of the cage. A container filled with water was placed under each hive to prevent potential ant infestation, attracted to the sugar solution inside the hives. Bumblebees were provided with plants and grinded pollen (Borregaard Bioplant ApS) in a Petri dish.

FIGURE 2. Flying Doctors® hives from BioBest. The dispenser can contain 10 g of powder.

5.3 Thrips rearing, maintenance and cohorts

Western flower thrips (*Frankliniella occidentalis*) were continuously reared on mini cucumbers (*Cucumis sativus*, snack cucumber or "skole agurker" in Danish) at 25°C, 60% relative humidity and a 16:8h light:dark cycle in an incubator. Thrips were kept in rectangular transparent plastic containers (18 × 11.5 × 7 cm) with ventilated mesh-covered lids (10 × 8 cm opening). Each container contained two cucumbers, which were replaced every three days to ensure a fresh food supply. Before use, cucumbers were gently washed with unscented dishwashing soap, disinfected with 70% ethanol, rinsed with distilled water, and dried with tissue paper.

Thrips cohorts for experiments were established by placing 50–60 females in a new container with one fresh cucumber for 48 h. Then, the females were removed by an aspirator. Cohorts were maintained under the same conditions as the main rearing and used, when thrips reached the 2nd instar larval stage or the adult stage (**Figure 3**).

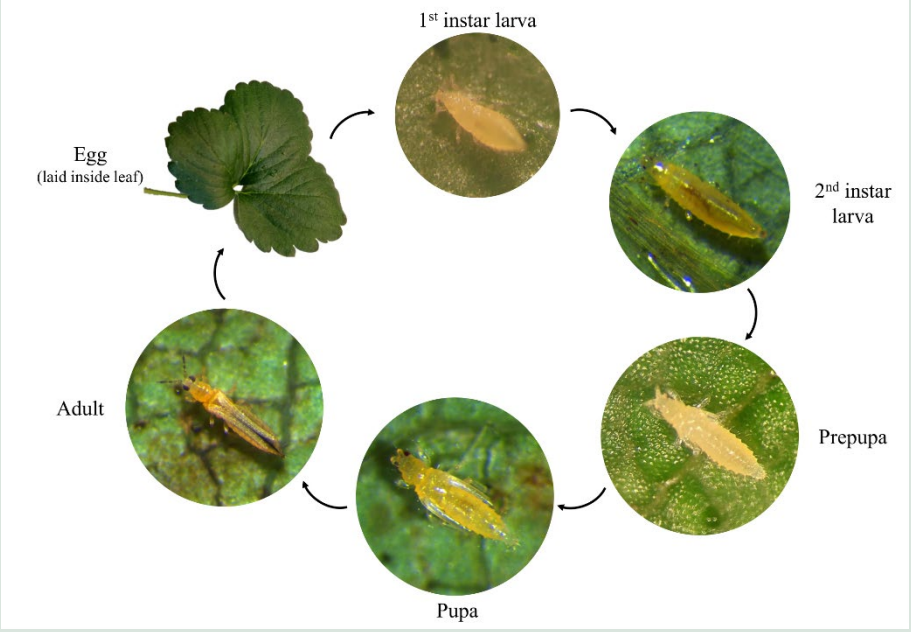

FIGURE 3. Life cycle of the western flower thrips, *F. occidentalis*. Photos are not to scale.

5.4 Strawberry plants

Strawberry plants (*Fragaria* × *ananassa*, everbearing “Favori” cultivar) were obtained as mini-trays from Goossens Flevoplant B.V. (The Netherlands) in March 2024 and as Frigo plants from Kraege Beerenpflanzen (Germany) in March 2025. Upon arrival, the plants were individually potted in 2 L pots filled with commercial coir substrate (Legro, type 02), previously moistened with Gnatrol (containing *Bacillus thuringiensis var. israelensis*, Borregaard BioPlant), to control sciarid larvae. Pots were placed in the greenhouse at an average temperature of 18°C, with supplemental lighting provided on a 16:8h light:dark cycle and ventilation activated when temperatures exceeded 22°C. Plants were watered 1-3 times per week with pure tap water and tap water with fertilizer. Dead leaves, fruits and weeds were regularly removed by hand to stimulate continued flowering.
For laboratory experiments, detached leaves and flowers were placed in plastic bags. For greenhouse experiments, pots with strawberry plants carrying at least one open flower were selected one day in advance of being introduced into the mesh tents; buds, apetalous flowers and fruits were trimmed by hand and pots were watered with tap water.

Objective 1: Api-vectoring of a fungal biopesticide to strawberry for thrips control

[Skillebladstekst]

6. Objective 1 – Research aims

This first objective focuses on the transmission of BotaniGard by api-vectoring by commercial bumblebees to control thrips in strawberry flowers.

The associated aims were to:

1. Assess the lethality of BotaniGard on thrips under laboratory conditions;
2. Evaluate bumblebees as vectors of BotaniGard, including deposition of fungal spores in strawberry flowers, persistence of fungal spores over time, and spore viability in the dispenser;
3. Validate the effectiveness of api-vectoring of BotaniGard against thrips in strawberry flowers under greenhouse conditions.

The hypotheses were that:

1. BotaniGard is lethal to thrips on leaves and in flowers of strawberry plants.
2. Bumblebees can carry and deposit fungal spores from BotaniGard to strawberry flowers, where the spores persist and remain viable for 1 week; the fungal spores of BotaniGard remain viable in the dispenser for 1 week.
3. Api-vectoring of BotaniGard to strawberry flowers reduces number of thrips under greenhouse conditions compared to control treatment without BotaniGard.

7. Objective 1 – Materials and methods

7.1 Lethality of BotaniGard towards thrips

7.1.1 Dose-response of two developmental stages on leaf discs

Green and healthy-looking strawberry leaflets were collected in the greenhouse between January and May 2025 on the day of each experiment, placed in a plastic bag and rinsed in the laboratory with distilled water. Leaf discs (22 mm diameter) were cut from random spots on the leaf surface and rinsed with sterile water. Each leaf disc was immersed for 5 seconds in a sterile glass Petri dish, containing one of the spore suspensions of BotaniGard, prepared as described in section 5.1.1, containing 0, 10^5, 10^6, 10^7, 10^8, or 10^9 spores/mL (larval experiment) and 0, 10^4, 10^5, 10^6, 10^7, or 10^8 spores/mL (adult experiment) using sterile tweezers. The inoculated leaf discs were then placed on sterile filter paper to air dry before being mounted in a clip cage (Xie et al., 2025; X. Zhang et al., 2015), into which a single *F. occidentalis* individual (either a 2nd instar larva or an adult, collected 7 or 17 days after female introduction, see section 5.3) (**Figure 4**). Larval and adult experiments were conducted separately, with 10 replicate clip-cages per concentration. Each experiment was repeated three times using independent spore suspensions, resulting in 30 replicates per concentration for each thrips developmental stage. Clip cages were covered by a glass slide and placed on moist cloth inside sealed plastic boxes to maintain leaf turgor, and the boxes were kept in an incubator at 25 °C and a 16:8h light:dark cycle. Survival and signs of mycosis (fungal growth on cadavers) were recorded daily for 8 days.

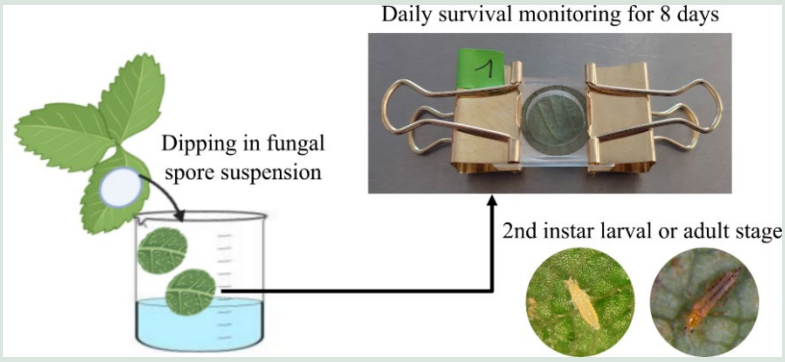

FIGURE 4. Experimental setup using an inoculated leaf disc enclosed with one thrips individual in a clip cage.

7.1.2 Manual vectoring of BotaniGard in detached flowers

Strawberry flowers were collected from greenhouse plants 1–2 hours before used in experimental setup and immediately placed with stalk in water. Only complete flowers with intact petals and 3–6 cm long stalks were used. Care was taken to remove any insects (*e.g.*, aphids or ants). Each flower stalk was inserted through a small hole in the lid of a 1.5 mL Eppendorf tube filled with distilled water and the lid was sealed with parafilm to prevent evaporation. The Eppendorf tube was then placed inside a 50 mL

Falcon tube to keep the flower upright and enclosed (**Figure 5A**). Falcon tubes were sealed with ventilated lids covered with 95 µm mesh for ventilation (**Figure 5E**).
Female thrips (*i.e.*, the largest and darkest adults) from the same cohort were introduced to each Falcon tube containing a flower. The experiments included two control and one fungus treatment: untreated and inoculation with inactive D15 ("Control") powder, and inoculation with active D15 ("BotaniGard") powder (**Table 1**). After introducing 5–10 females, "Control" and "BotaniGard" powders were applied to the centre of a flower using either a 1 µL or 10 µL inoculation loop (**Figure 5B–D**). The combination of thrips number and loop size resulted in a pilot and three independent experiments (**Table 2**). The flowers were then incubated in a climate cabinet at 23°C, 40-60% relative humidity, with a 16:8 light-dark cycle for 7–8 days. In experiment 3, high relative humidity was created by placing the tubes inside a sealed plastic box containing moist tissue paper.

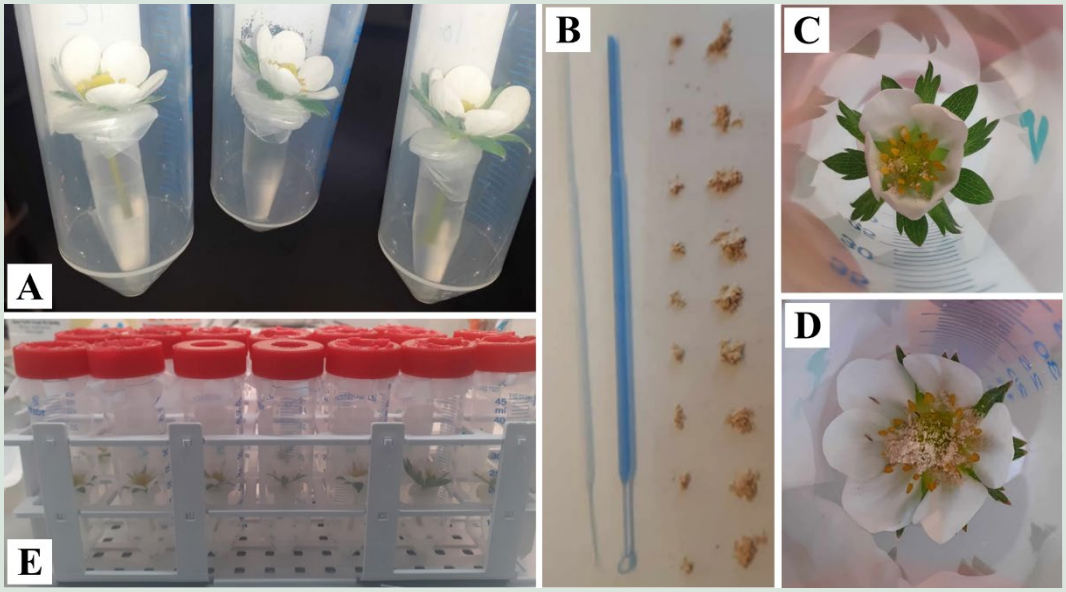

FIGURE 5. Detached flower setup. (A) Flowers with stalks placed in Eppendorf tubes with water, inside 50 mL Falcon tubes. (B) Amount of BotaniGard:corn flour powder collected by hand with inoculation loops (left: 1 µL; right: 10 µL). (C–D) Visual appearance of powder deposited on flowers using 1 and 10 µL loops respectively. (E) Experimental setup with ventilated Falcon tubes arranged on a rack for incubation.

TABLE 2. Overview of pilot and three independent experiments conducted using detached flower setups, varying in number of adult female thrips, inoculation loop size and relative humidity (RH). "U", "C", and "B" indicate Untreated, Control and BotaniGard treatments.

Experiment	Number of thrips	Repetition	Total number of replicates	Duration (days)	Application method	Incubation
Pilot	5/10	1	10 (U), 6 (B)	7/8	10 µL loop	23°C, low RH
Experiment 1	10	3	9 (U), 20 (C), 20 (B)	8	10 µL loop	23°C, low RH
Experiment 2	5	2	7 (U), 10 (C), 10 (B)	7	1 µL loop	23°C, low RH
Experiment 3	5	2	11 (U), 12 (C), 13 (B)	7	1 µL loop	23°C, high RH

Each Falcon tube was checked after 8 days to assess survival of the adult thrips and the offspring production. Live adults were initially assessed by external observation under normal light. Subsequently, 10 mL of 70% ethanol was added into the Falcon

tube to immobilize and preserve the thrips. The flower and Eppendorf tube were removed, and the ethanol suspension containing adults and larvae was transferred to a 6 cm Petri dish. Tubes were rinsed twice with ethanol to ensure recovery of all individuals. Samples were then examined under a stereomicroscope (10-100x), with millimeter paper placed beneath the dish, to count adults and larval offspring.

An additional experiment without thrips was conducted to quantify fungal inoculum in flowers using both inoculation loop sizes (1 µL or 10 µL) to manually transfer the powder. Loop and flower samples (N = 5) were collected and processed as described in section 5.1.2, and final fungal CFUs were assessed after 13 days.

7.2 Vectoring capacity of bumblebees

Greenhouse experiments with commercial Flying Doctors® hives and 20 strawberry plants inside mesh cages (section 7.3) were initiated with sampling of bumblebees and flowers for quantifying fungal inoculum. Hives had dispensers filled with one of two treatments (10 g of inactive or active D15 BotaniGard powder), and hives were opened at 09:00. Five bumblebees were collected individually in 50 mL Falcon tubes as they exited the hives ("hive departure"). Later the same day at 14:00, five bumblebees were caught individually in 150 mL cups while flying in the cages ("hive return") and then transferred to individual 50 mL Falcon tubes. Bumblebees were freeze-killed at −20°C for 30 min before being further processed. At 16:00, ten flowers were sampled per mesh cage and placed individually in 50 mL Falcon tubes (1 day post-inoculation, dpi). The strawberry plants were removed from the cages and kept next to them where flower sampling was repeated at 3 and 7 dpi. The experiment was repeated three times. All samples were processed immediately following the protocol described in section 5.1.2, and fungal CFUs were recorded.

7.3 Effects of api-vectored BotaniGard on thrips

Upon arrival, each commercial Flying Doctors® hive was placed open inside a mesh cage, provided with 10 flowering *Lobularia maritima* plants (EWH BioProduction ApS) and grinded pollen for one week for acclimation. After this period, the *L. maritima* plants were replaced with 20 flowering strawberry plants. The dispenser of the hive was loaded with 10 g of either inactive or active D15 powder (Control vs. BotaniGard treatment). Bumblebees were allowed to visit the strawberry flowers for 7h before the hives were closed.

The following day, 10 strawberry plants per treatment were transferred to five smaller insect tents (BugDorm, 60 × 60 × 60 cm), with two plants per tent, after labelling the stalks of all open flowers with a water-based paint (Uni Posca). In each tent, 20 adult thrips females from the same cohort were introduced (**Figure 6**). Plants were watered with tap water two to three times during one week. After 7 days, labelled flowers, and newly unlabeled flowers or buds were counted on each plant and then collected separately per plant in 150 mL plastic cups filled with 50 mL of 70% ethanol. The bottom of each tent was carefully inspected for presence of adult thrips, which were collected when observed.

In the laboratory, ethanol suspensions were vacuum-filtered through gridded filter paper, and thrips adults and larvae were counted under a stereomicroscope (10-100x). The experimental procedure was repeated twice for each hive. For each repetition, the dispenser was refilled with fresh powder, and new set of 20 flowering strawberry plants were introduced. Freshly ground pollen was provided into the mesh cage. Between strawberry batches, *L. maritima* plants were reintroduced into the mesh cages. Three

independent hives were used per treatment, generating total of six data set of thrips numbers. Temperature and relative humidity were monitored in one of the insect tents per treatment using a temperature logger (RS PRO RS-191A, Singapore).

For the first two sets of independent hives (2 x 2 x 5 tents per treatment), the insect tents with pairs of strawberry plants and thrips were placed adjacent to their associated mesh cage to minimize cross-contamination. For the third set of independent hives (1 x 2 x 5 tents per treatment), the insect tents with pairs of strawberry plants and thrips were placed in two rows of both treatments to avoid effect of a temperature/humidity gradient inside the main tunnel. The experiment was conducted from June to August 2025, with two repetitions per month using same hives; new hives were received once per month.

Fungal spore viability of inoculum was assessed on 1 g of powder both before loading into the dispenser (day 0) and on 1 g of powder from the dispenser after 7 days of bumblebee activity.

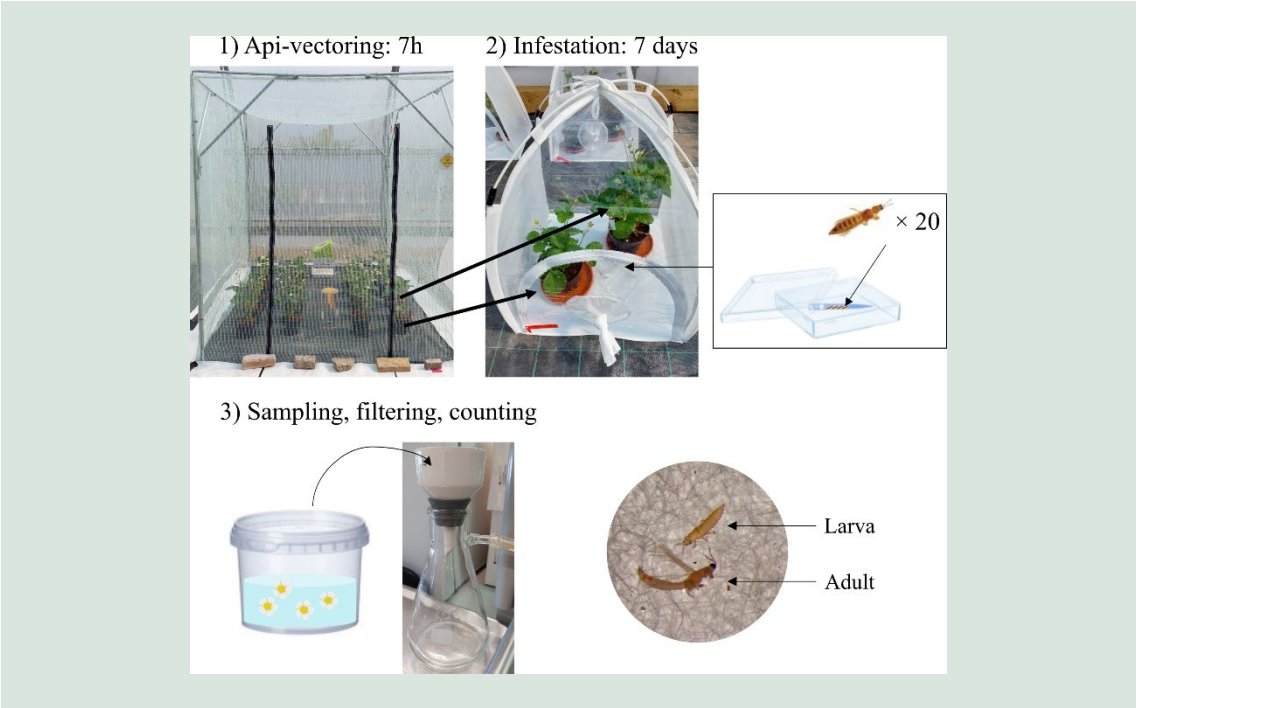

FIGURE 6. Api-vectoring setup to evaluated effect of vectored BotaniGard to strawberry flowers on thrips survival and reproduction. 1) Mesh cage setup with a commercial Flying Doctors® hive and 20 flowering strawberry plants, which were exposed for 7 h. 2) Pairs of strawberry plants of same treatment were incubated for 7 days inside insect tent with 20 adult female thrips. 3) After 7 days, flowers, buds, and developing fruits were placed in 70% ethanol, which was filtered and observed for number of adult and larval thrips.

7.4 Statistical analyses

Analyses were conducted in R version 4.5.1 (R Core Team, 2025) using “tidyverse” (Wickham et al., 2019) for data handling and “ggplot2” (Wickham, 2016) for visualization. Survival curves were estimated with a Cox Regression using Firth's Penalized Likelihood (“coxphf” package (Heinze et al., 2023)) to test the effects of *B. bassiana* (referred as to Concentration) on thrips. Dose-response models used a four-parameter Weibull function for the thrips larvae and a three-parameter log-logistic function for the thrips adults using the “drm” function and “drc” package (Ritz et al., 2015). Mycosis rate, number of larvae per adult, proportion of dead thrips, number of individuals

(larvae or adults) per flower were analyzed with linear models to test the effects of *B. bassiana* (referred as to Concentration or Treatment). All models considered Repetition and, when relevant, Age of the thrips cohort, Humidity, and Temperature. These factors were included in the models, where non-significant factors were excluded, while significant factors were treated as random effects and analyzed using a linear mixed model ("lmer" function, "lme4" package (Bates et al., 2015)).

The significance of each term of each model was assessed with Type II analyses of variance ("Anova" function, "car" package (Fox & Weisberg, 2019)) using F-test (linear model), Wald chi-square test (linear mixed models and Cox models).

Significant terms were followed by pairwise comparisons with estimated marginal means ("emmeans" function and package (Lenth, 2025)), adjusting p-values by false discovery rate ("cld" function of the "multcomp" package (Hothorn et al., 2008)). Pearson correlations were performed using ggplot2 (Wickham, 2016).

8. Objective 1 – Results

Statistical outputs are presented in **Appendix 1**.

8.1 Lethality of BotaniGard towards thrips

8.1.1 Dose-response of two developmental stages on leaf discs

The survival rates of thrips larvae and adults were significantly affected by the fungal spore concentration (**Appendix 1**), with 10^5 spores/mL being the lowest concentration that significantly reduced survival for both stages compared to the control (**Figure 7**). The highest concentrations of 10^9 spores/mL and of 10^8 spores/mL caused mortality to all larvae and adults within 5-6 days, respectively. The median lethal concentrations (LC_{50}) were 3.3 x 10^5 spores/mL for larvae and 4.0 x 10^5 spores/mL for adults.

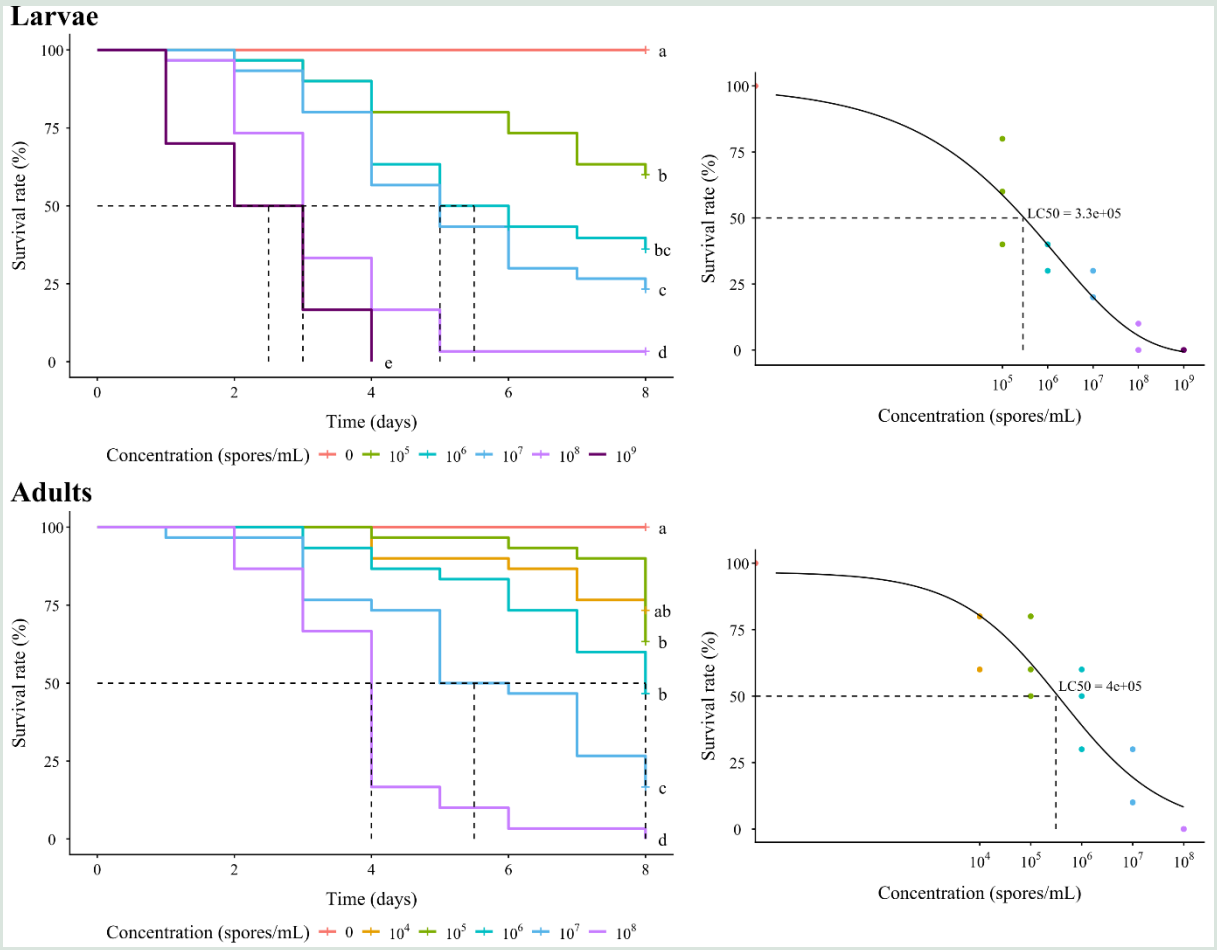

FIGURE 7. Survival of the thrips larval (top panel) and adult (bottom panel) stages in a clip cage setup. Left plots show survival curves, with dashed lines corresponding to median survival times and different letters to significant differences among concentrations. Right plots show dose-response curve, with indication of LC50 corresponding to the median lethal concentration.

Mycosis rate was also significantly affected by fungal spore concentration for both development stages (**Appendix 1**), with the concentrations of 10^5 spores/mL and higher

in larvae and of 10^6 spores/mL and higher in adults showing a significant mycosis rate compared to the control (**Figure 8**). The highest concentrations resulted in mycosis rates of 93–100% in adults and larvae, respectively.

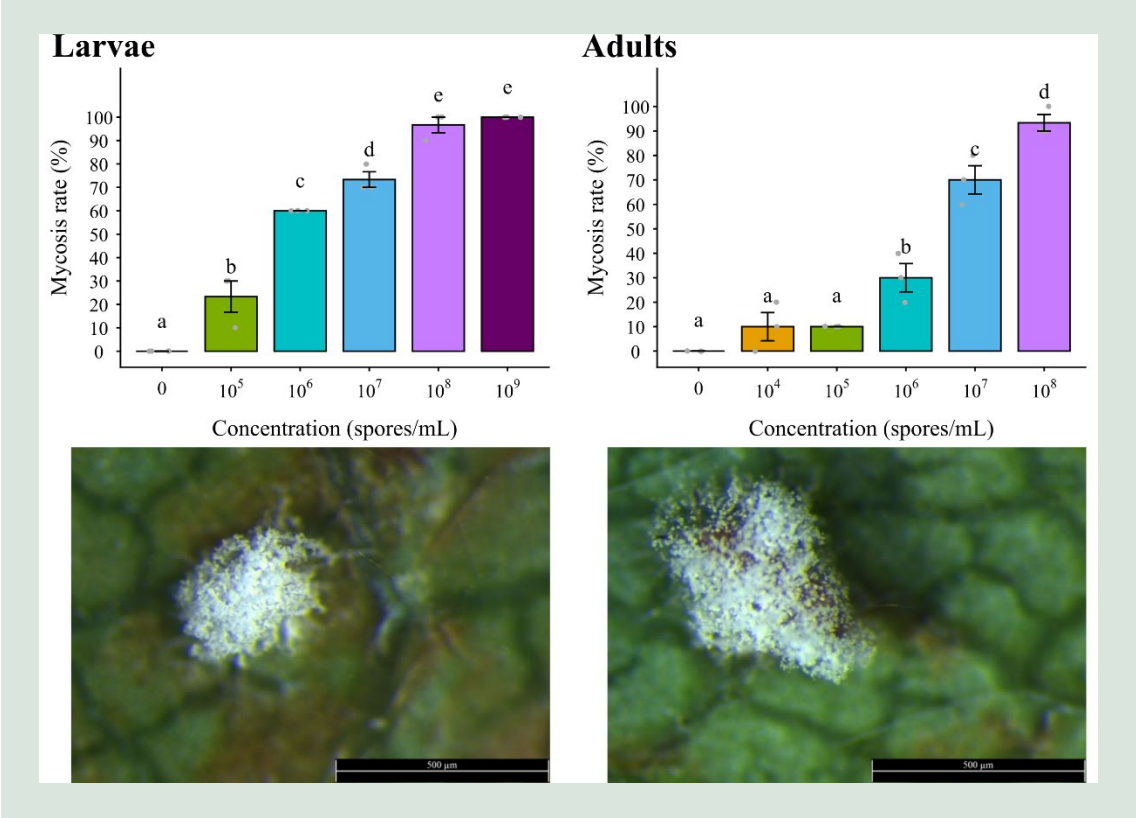

FIGURE 8. Mycosis of the thrips larval and adult stages in a clip cage setup. Top panel shows mycosis rate (mean ± standard error) at different concentrations; grey dots correspond to individual observations, while different letters above bars to significant differences. Bottom panel shows *B. bassiana* mycosis of a larva (left) and an adult thrips (right).

8.1.2 Manual vectoring of BotaniGard in detached flowers

After exposure of adult thrips in setup with detached strawberry flowers inoculated with BotaniGard:corn flour powder, the proportion of dead thrips was significantly influenced by the treatment for the pilot experiment and Experiment 1 and 3 (**Appendix 1**). However, pairwise comparisons of the treatments in Experiment 1 did not identify significant differences, while the BotaniGard treatment showed higher proportion of dead thrips in the pilot experiment and in Experiment 3 compared to the untreated and control treatment (**Figure 9A**).

In contrast, the number of larvae per thrips adult after 8 days in the flower setup was not significantly influenced by the treatment for any of the experiments (**Appendix 1**, **Figure 9B**).

Using the 1 and 10 µL inoculation loops yielded comparable amount of fungal spores, in the range of 1.37–3.51 × 10^7 CFUs (**Figure 9C**). However, flowers inoculated with the 1 µL loop showed about ten times fewer CFUs than if inoculated with the 1 µL loop.

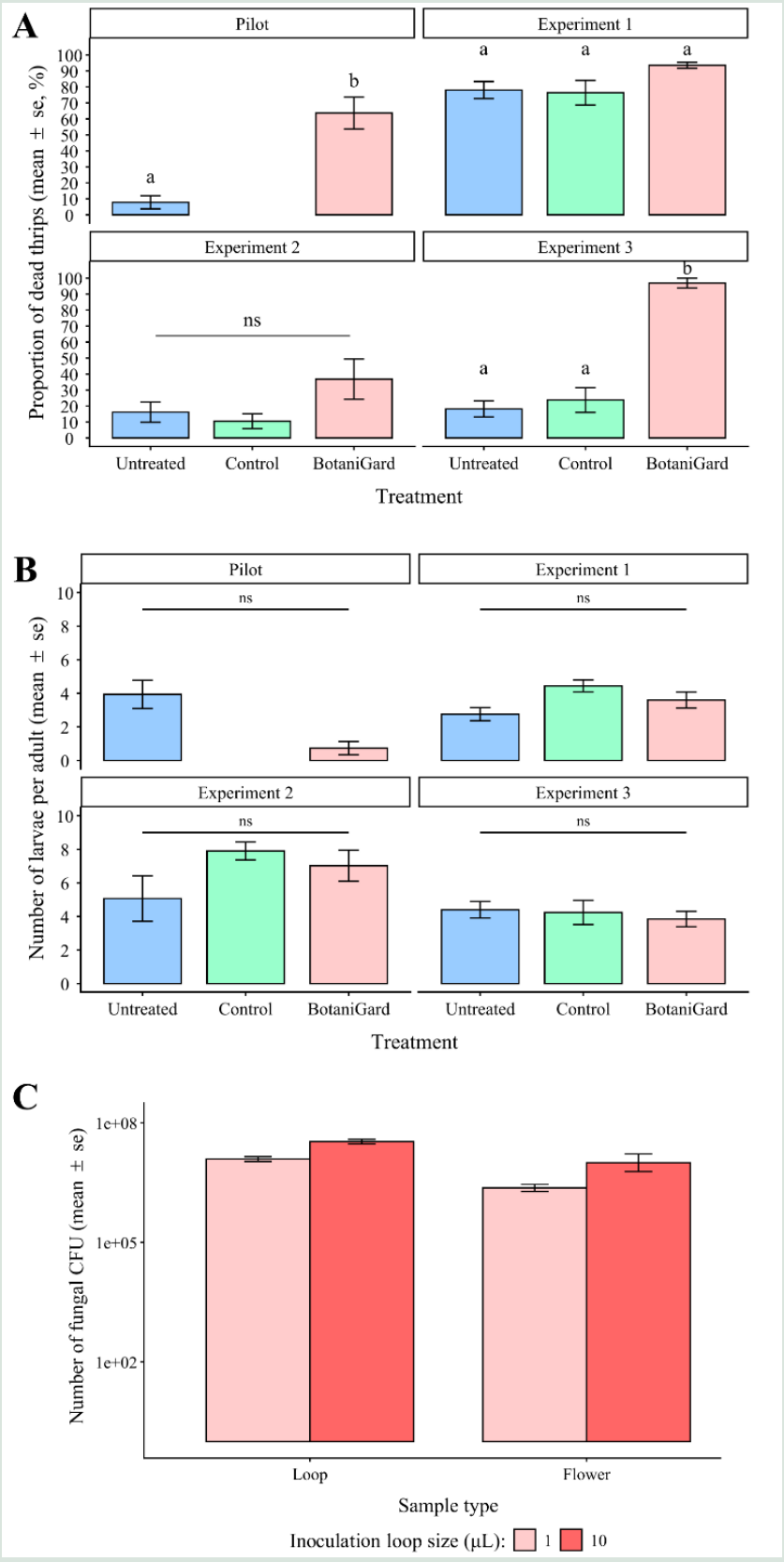

FIGURE 9. Survival (A) and reproduction (B) of adult thrips after 8 days in BotaniGard inoculated detached strawberry flowers. Plot in (C) show colony forming units of inoculation loop and flower. Different letters indicate significantly different treatments; "ns" is non-significant. Details of each experiment are provided in **Table 2**.

8.2 Vectoring capacity of bumblebees

According to the manufacturer, 1 gram of product of BotaniGard contains 4.4 × 10^{10} CFUs, while in this study 3.36 × 10^{10} spores per gram of product was recorded by observing dilutions under a hemocytometer (**Table 1**). In the greenhouse experiments, the dispenser of the Flying Doctors® hive was filled with 10 g of the D15 mixture, which corresponded to 5.99 × 10^{9} spores/g. Upon leaving the hives, bumblebees carried 1.64 × 10^{5} CFUs, while fewer CFUs were retrieved from bumblebees after flying inside the mesh cage. The deposition onto flowers corresponded to approximately one-fourth of the initial load of the bumblebee, and the inoculum in the flowers remained for 7 days although a slight decrease was observed over time (**Figure 10A**). CFUs were detected in all bumblebee and flowers samples analyzed.

Spore viability of the powder loaded in the dispenser was assessed before and 7–8 days after api-vectoring at greenhouse conditions. Germination rates remained high at 98.28% for both time points, indicating that spore viability was unaffected in the dispenser (**Figure 10B**).

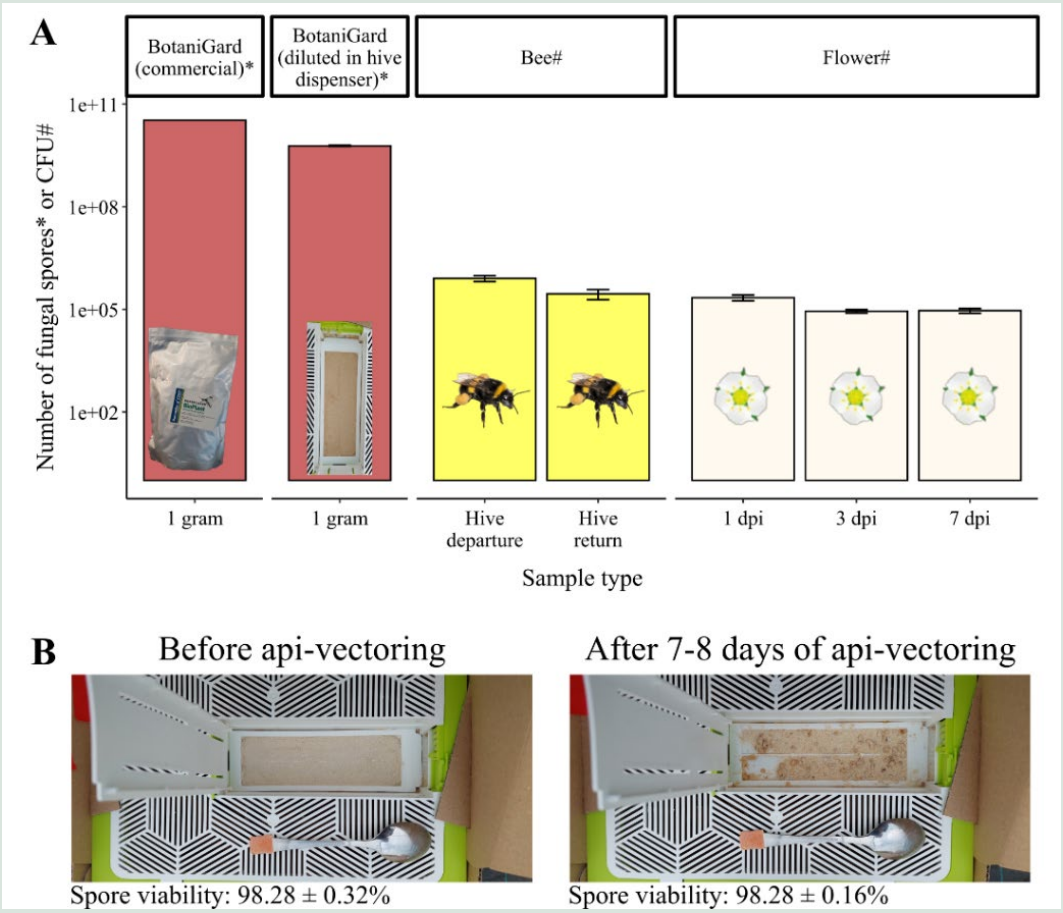

FIGURE 10. Vectoring of *B. bassiana* by bumblebees to strawberry flowers. (A) Fungal load detected in BotaniGard product (n=1) and when diluted with corn flour (N = 6), on bumblebees (N = 15 and 14) and flowers (N = 26, 20 and 26). (B) Spore viability when loading dispenser (left) and after 7-8 days of api-vectoring (right) (N = 6). Mean and standard error values are given as bar plot (A) and as numbers (B).

8.3 Effects of api-vectored BotaniGard on thrips

Two experimental repetitions with two sets of commercial Flying Doctors® hives were conducted in the greenhouse experiment where all insect tents containing pairs of strawberry plants of the same treatment were placed adjacent to the corresponding mesh cage to minimize cross-contamination (**Figure 11A**). Although the climatic conditions were comparable in the two mesh cages (**Figure 12A**), a microclimate gradient across the experimental area was detected during the incubation of the plants, with one side representing higher temperature and lower relative humidity than the other side (**Figure 12B**). While the adult thrips were unaffected by these variations in temperature and relative humidity, larval abundance showed a positive correlation with temperature and a negative correlation with relative humidity (**Figure 13**). To avoid the unintentional effect of these conditions, the insect tents were rearranged in two rows mixing the treatments for the third repetition during incubation (**Figure 11B**).

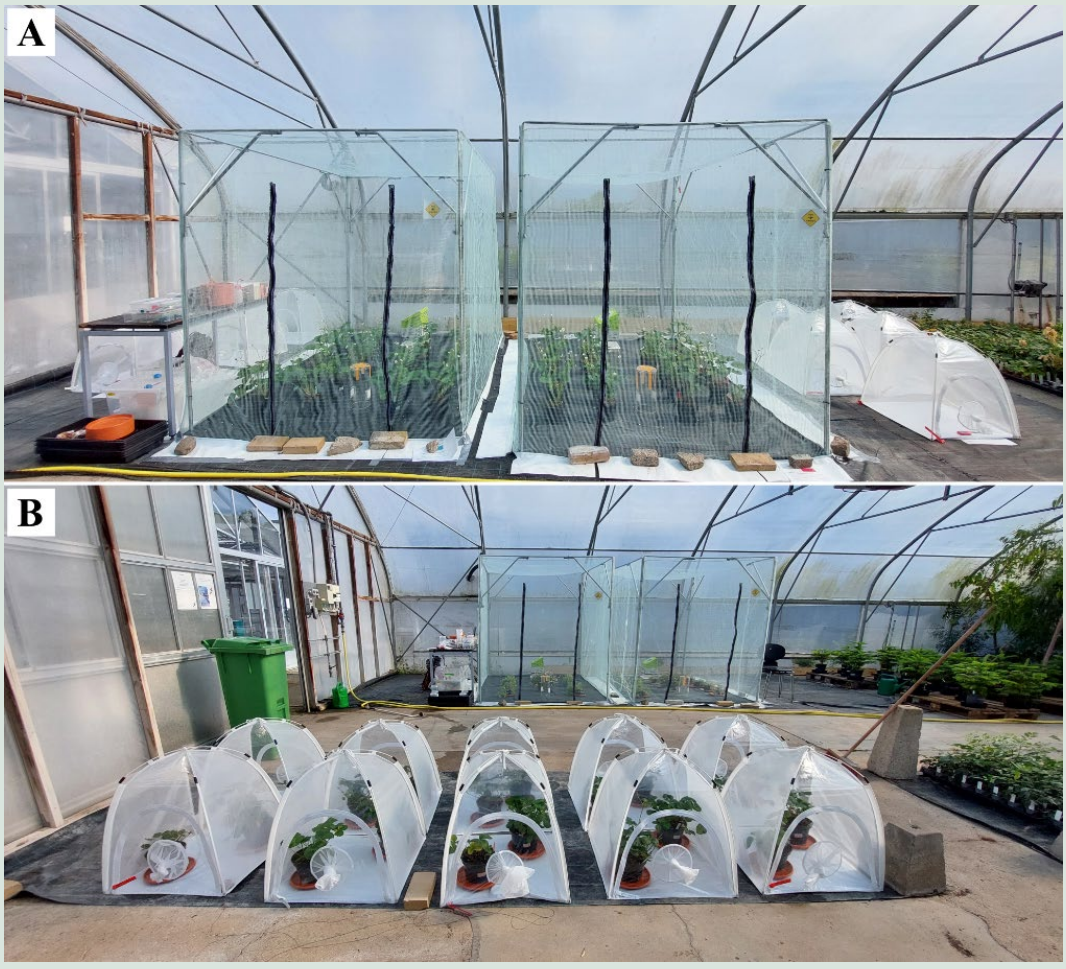

FIGURE 11. Experimental area with the mesh cages and insect tents. (A) Insect tents placed adjacent to the corresponding mesh cage for repetitions 1 and 2. (B) Insect tents mixed in two rows across treatment.

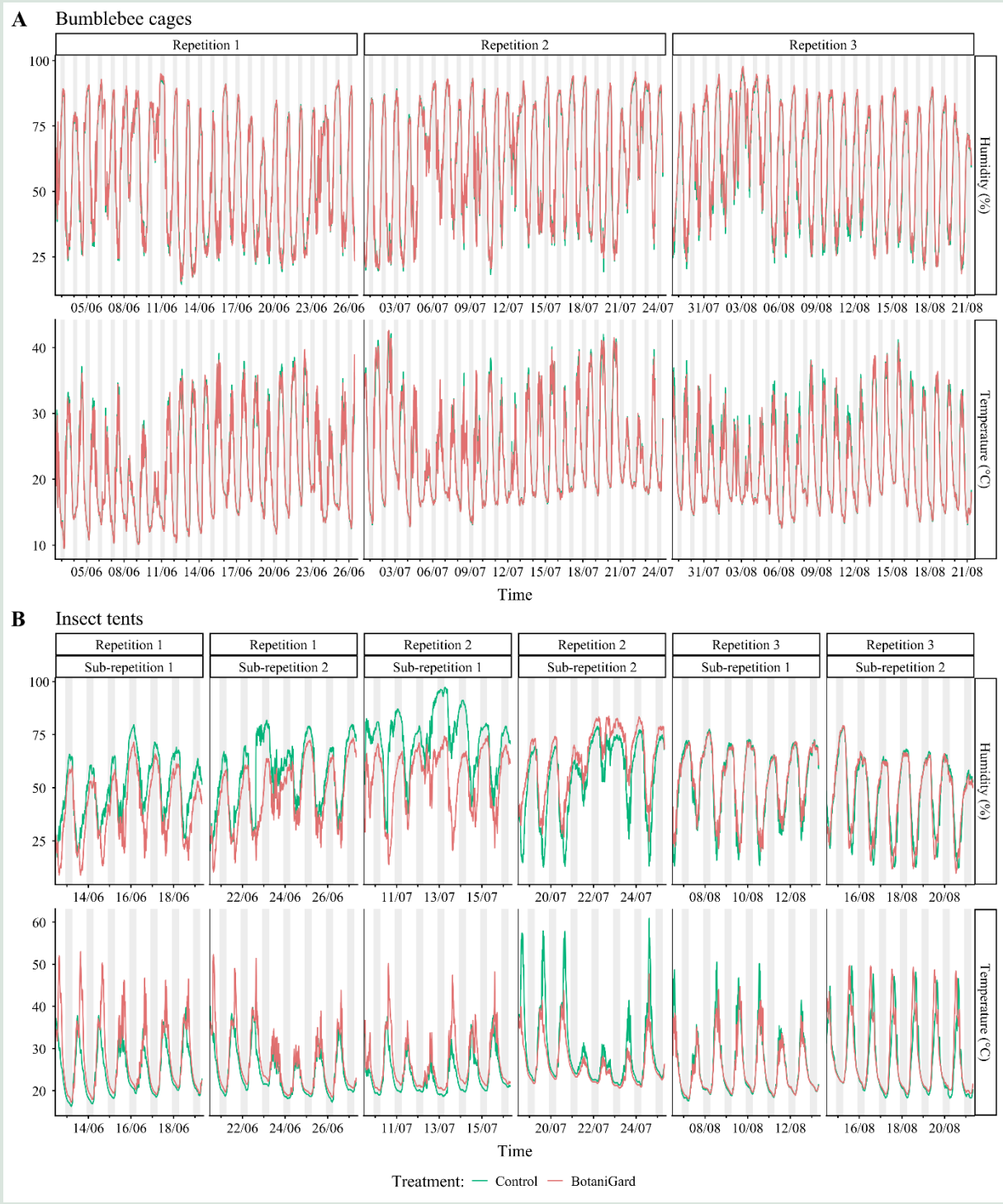

FIGURE 12. Climatic conditions measured in a bumblebee cage (A) and an insect cage (B) of each treatment during summer 2025. Dates are written as DD/MM. The grey shaded bars represent night, from 22:00 to 06:00. In panel B, insect tents of repetitions 1 and 2 were positioned as shown on **Figure 11A**, with tents of each treatment swapping position in sub-repetition 2 of repetition 2, while tents of repetition 3 were positioned as shown on **Figure 11B**. Descriptive statistics are provided in **Appendix 2**.

Overall, climatic conditions in the insect tents varied considerably across the experimental period, with relative humidity ranging from 8.8 to 97.3% and temperatures ranging from 16.3 to 60.9°C (**Appendix 2**).

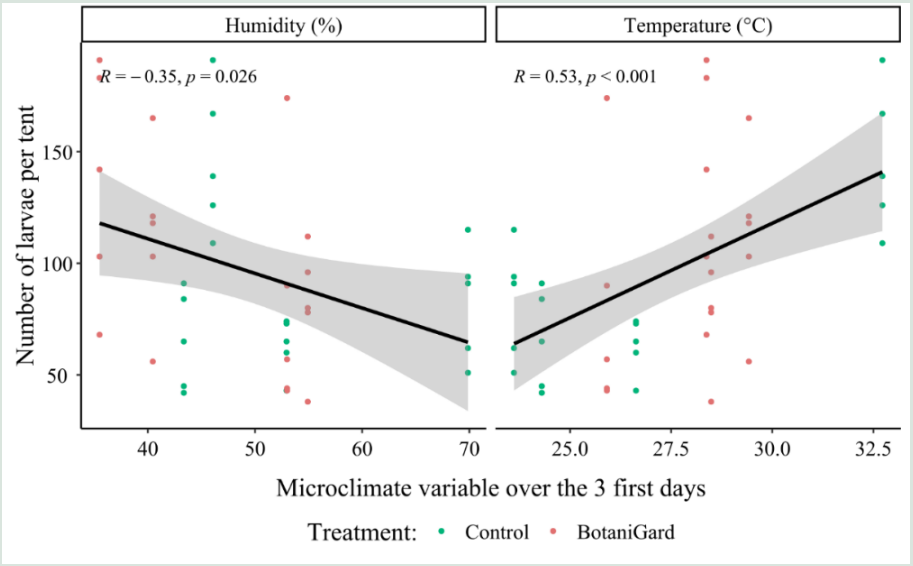

FIGURE 13. Pearson correlations between larval abundance and climate variables. The correlation values and p-values are given in each panel. The three first days correspond to fungal establishment in the flowers.

The strawberry plants used in the experiments exhibited variation in the number of flowers, which were positively correlated with both larval and adult thrips abundance (**Figure 14**). To account for this relationship, thrips abundances were expressed as the number of individuals (adults or larvae) per flower.

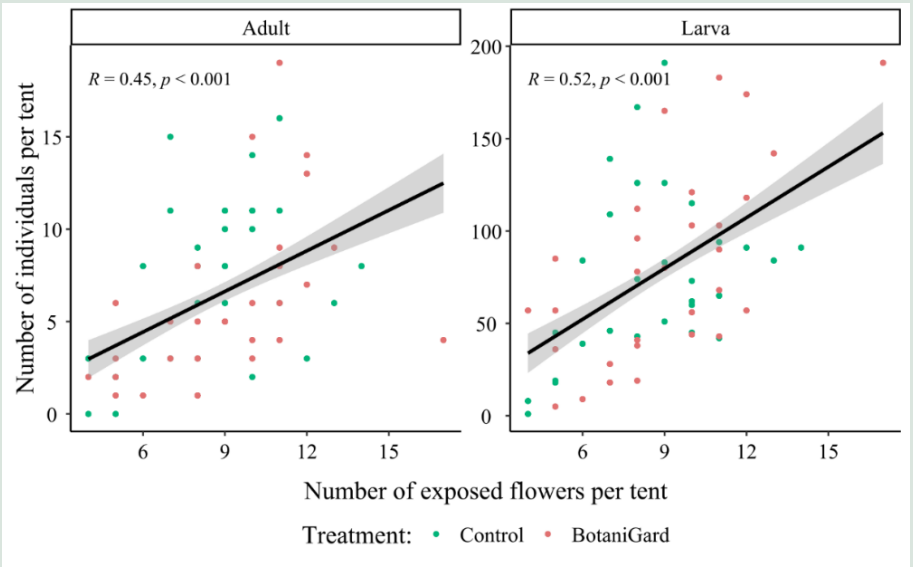

FIGURE 14. Peason correlations between flower and thrips abundance. The correlation and p-values are given in each panel.

Few thrips were found at the bottom of the insect tents after the incubation period and almost no thrips remained in the box used to transport the thrips for release into the tents. Across all experimental repetitions, the BotaniGard treatment had no significant effect on the thrips abundance (**Appendix 1**). This result was regardless of whether insect tents had been incubated separately with uneven microclimatic conditions

(repetitions 1 and 2) or mixed together in two rows (repetition 3). The adjusted abundance of recovered thrips under these conditions are presented in **Table 3**.

TABLE 3. Thrips abundance per flower recorded 7 days after the api-vectoring exposure of Control or BotaniGard treatments in three experimental repetitions; “ns” indicates no significant difference between the two treatments.

Treatment	Repetition	Mean number of individuals per flower (standard error)			
		Adult		Larva	
Control	1 & 2	0.92 (0.11)	ns	9.69 (1.28)	ns
BotaniGard		0.70 (0.09)		9.78 (0.92)	
Control	3	0.61 (0.15)	ns	6.89 (1.49)	ns
BotaniGard		0.47 (0.10)		6.64 (1.80)	

9. Objective 1 – Discussion

In the present study, the entomopathogenic fungus *B. bassiana* strain GHA, the active ingredient of the commercial biopesticide BotaniGard, induced lethality in *F. occidentalis* thrips at both the adult and larval stages when applied as a liquid suspension on strawberry leaf discs in a clip cage setup under laboratory conditions. Manual application of BotaniGard powder formulation on detached strawberry flowers showed variable results under laboratory conditions, which depended on different factors: thrips density, amount of powder applied and the relative humidity. Fungal spores of BotaniGard powder were successfully carried by *B. terrestris* bumblebees and deposited in strawberry flowers under greenhouse conditions, where the spores persisted for up to one week. However, api-vectoring of *B. bassiana* to the flowers by bumblebees in greenhouse conditions did not reduce the number of thrips compared to the control treatment, potentially due to variation in microclimatic conditions.

9.1 Efficacy of *Beauveria bassiana* against thrips in laboratory conditions

In the clip cage setup, significant mortality of larvae at 10^4 spores/mL and adults at 10^5 spores/mL were observed, each representing the lowest concentrations of BotaniGard (*i.e.*, *B. bassiana*) causing effects within 8 days. Complete (100%) mortality occurred at 10^8 spores/mL, and the median lethal concentration (LC_{50}) for both stages was 3-4 x 10^5 spores/mL.

These findings align with previous studies reporting thrips susceptibility to *B. bassiana* in laboratory bioassays. For example, Abe and Ikegami (2005) found that three *B. bassiana* strains significantly reduced the survival of five thrips species, including *F. occidentalis*, within 7 days at 10^6-10^7 conidia/mL. The higher mortality observed in the present experiment at comparable concentrations may reflect methodological differences (1 individual per clip cage vs. 10 individuals in an Eppendorf tube).

Efficacy of different formulations of *B. bassiana* has also been documented. Ugine, Wraight and Sanderson (2005) evaluated three formulations (*i.e.*, technical grade powder, wettable powder and emulsifiable oil) of *B. bassiana* strain GHA applied in water on leaf discs against 2nd instar larvae, with the highest doses inducing 84-98% mortality. The authors detected fungal conidia on all body parts (*e.g.*, mouthparts, head) but especially on the abdomen, legs and thorax of larvae, and on the legs, wings and thorax of adults.

In the detached flower setup, we observed that high thrips density (10 adults per flower) and relatively large amount of BotaniGard powder led to an increase in mortality across all treatments. In contrast, high relative humidity, associated with lower thrips density (5 adults per flower) and reduced powder amount, led to higher adult mortality only in the fungal treatment (Experiment 3).

To our knowledge, no previous study has examined *B. bassiana* efficacy directly on strawberry flowers, which represent the most fragile plant structure and deteriorate rapidly once detached. The combination of high thrips density and the large powder amount may have accelerated flower degradation, which in turn would create a suboptimal environment for the thrips and may contribute to their mortality regardless of whether viable *B. bassiana* spores were present. However, *B. bassiana* efficacy on floral tissues has been demonstrated in a related system; a study using detached rose branches at the bud stage, infested with 20 adult and larval *F. occidentalis* thrips per

branch, found that *B. bassiana* reduced survival in both stages without affecting flower longevity (Mousavi et al., 2017).
Application of *B. bassiana* may perform more consistently outside the above-ground environments. When *B. bassiana* granules were incorporated into soil, survival of *F. occidentalis* pre-pupae was significantly reduced (X. Zhang et al., 2019). Similarly, root-inoculated strawberry plants showed reduced adult thrips populations, coinciding with endophytic colonization of leaf tissues by the fungus (Mantzoukas et al., 2022). The present findings suggest that *B. bassiana* may also act efficiently within strawberry flowers when environmental conditions favor fungal establishment and colonization. Relative humidity, in particular, is a critical factor for *B. bassiana* efficacy as a biocontrol agent in both laboratory and field conditions (Jaronski, 2010; Mascarin & Jaronski, 2016). A study showed that long exposure of *Beauveria*-inoculated thrips to saturated air humidity (100% RH) induced high mortality, with a median exposure time of 20 h (Mukawa et al., 2011).
Although adult mortality was increased by high relative humidity in the detached flower setup, no effect of BotaniGard powder application was observed on the offspring production in the different experimental conditions. This observation contrasts with a recent study, testing different combinations of healthy and fungal-infected males and females of *F. occidentalis*, which showed that *B. bassiana* could affect several reproduction traits in the thrips, such as successful mating, offspring sex ratio, female fecundity, and oviduct length (Q. Zhang et al., 2025). Additionally, Zhang *et al.* (2015a) compared the life-history traits of *F. occidentalis* offsprings from control parents with those from *Beauveria*-treated parents, which had been exposed during the second larval instar. Offspring from *Beauveria*-treated parents exhibited longer egg, pre-pupal and male immature stages, while survivorship remained similar between treatments. Interestingly, the authors projected population growth based on the obtained life-history traits and showed that the population originating from *Beauveria*-treated parents would increase more slowly than the population originating from control-treated parents.

Finally, experiments conducted on detached plant organs (*e.g.*, leaf discs, excised flowers) remain debated in plant–insect or plant-pathogen research. Detached organs offer practical advantages, such as reduced space and time requirements and suitability for rapid screening bioassays, but effects detected on detached plant tissues do not always translate to whole-plant conditions. This discrepancy has been noted by Bellec *et al.* (2024), who compared potted plants with detached buds of white mustard for pollen beetle resistance, and by Miller-Butler *et al.* (2018), who compared whole strawberry plants with detached leaves for anthracnose resistance screening. A sound approach is therefore to use detached-organ assays for preliminary experimental testing to confirm the potential of treatments, followed by experiments on whole plants under more realistic conditions to test promising treatments.

9.2 Vectoring and persistence of BotaniGard

In the mesh-cage studies in the greenhouse tunnel, we observed that 10^5 *B. bassiana* CFUs were carried by *B. terrestris* bumblebees at exit through the dispenser loaded with BotaniGard powder. The bumblebees deposited the powder on strawberry flowers in the mesh-cage and the CFUs persisted there up to 7 days under ambient conditions. Temmermans *et al.* (2023) provided an overview across multiple entomo-vectoring studies of CFU loads of the initial powders, on bee vectors and on target flowers using different pollinator vectors and biocontrol agents. Comparable CFU loads on bumblebees to those observed here have been reported in previous work, including by Iqbal et al. (2022) with *B. terrestris* vectoring a yeast using Flying Doctors® hives, and by Kapongo, Shipp, Kevan, & Sutton (2008) with *Bombus impatiens* vectoring *B.*

bassiana. However, both studies reported lower CFU deposition in flowers compared to our findings: 10^2–10^3 CFUs per strawberry flower (Iqbal et al., 2022) and 10^4 CFUs per tomato and pepper flowers (Kapongo, Shipp, Kevan, & Sutton, 2008). A recent study evaluating *B. terrestris* vectoring the bacteria *Bacillus subtilis* to tomato flowers using a custom-made 3D dispenser also reported lower CFU load on flowers (10^2–10^3) but substantially higher loads on bumblebees (10^8–10^9 CFUs) (Y. Liu et al., 2025). This latter difference could be due to the generally smaller size of bacterial spores compared to fungal conidia, which may allow individual bumblebees to carry larger quantities of bacteria than fungi.

The persistence of *B. bassiana* on strawberry flowers for up to 7 days observed in our study is consistent with persistence previously reported of this fungus in other plant organs and habitats following inoculation. For example, *B. bassiana* has been shown to persist for up to two weeks in the bark and leaves of ash trees (Castrillo et al., 2010), and for up to three weeks in the leaves of several crop species (Gatarayiha et al., 2010). Longer persistence has been reported in soil, where *B. bassiana* GHA remained detectable for up to 55 weeks, although at low concentrations (Świergiel et al., 2016). These findings collectively indicate that the persistence recorded on strawberry flowers with limited reduction in density provides environmental durability of viable *B. bassiana* spores to potentially initiate infections in insects located in the flower and developing fruit.

According to Borregaard Bioplant ApS, the provider in Denmark of the Flying Doctors® hives, it is recommended to refill the dispenser every 3 to 4 days[iii]. In most previous studies, hive dispensers were refilled twice per week (Iqbal et al., 2022) or after 10 days (Kapongo, Shipp, Kevan, & Sutton, 2008). Beyond re-filling due to depletion, authors also cited the need to maintain spore viability as a reason for frequent re-filling. However, these studies did not directly assess the viability of the fungal spores in the remaining powder. Our results demonstrate that the BotaniGard powder remained viable within the dispenser at similar high rate as at loading for at least 7 days, but that the dispenser typically required re-filling after one week, aligning with previously reported intervals for refilling.

9.3 Api-vectoring of BotaniGard to control thrips in greenhouse conditions

Our study showed that api-vectoring of BotaniGard powder by *B. terrestris* bumblebees did not significantly reduce the abundance of either adults or larvae of *F. occidentalis* during a week on strawberry flowers under greenhouse conditions. During the experiment, we identified a microclimatic gradient within the tunnel, which may have influenced the results, as thrips larval abundance per flower was positively correlated with temperature and negatively correlated with relative humidity.

The experiment was conducted at ambient conditions in a greenhouse tunnel and consisted of several sequential steps: bumblebee acclimation within mesh-cage; BotaniGard vectoring to flowers by bumblebees for 7h inside the mesh-cages; transfer of inoculated plants to insect tents and controlled thrips infestation; final flower sampling and thrips quantification after one week of incubation.

A one-week acclimation period was necessary upon hive arrival, as a previous experiment demonstrated that a shorter two-day acclimation resulted in highly variable bumblebee activity among hives during experimentation (see section 12.3.1).

A 7h api-vectoring period was selected in the present experiments to allow for introduction of thrips in controlled numbers the following day. Other studies reported of different api-vectoring periods of longer durations, e.g., 1 hour, two to three times per

[iii] Prestop Mix WP: http://uk.bioplant.dk/media/43370/Prestop%20Mix%20med%20bier_2017.PDF

week for three weeks (Iqbal et al., 2022), three consecutive days (Kapongo, Shipp, Kevan, & Sutton, 2008), or an entire week (Coates et al., 2023). In the current study, fungal CFUs were detected in 100% of flower samples, and the fungal load per flower exceeded that reported in these studies, which indicates that the 7 h exposure was sufficient.
The use of insect tents allowed for control of initial thrips population sizes while preventing escape, which facilitated sampling and made it possible to include true replicates by assigning one tent per replicate. However, transferring plants from the mesh cages to the insect tents may have resulted in the loss of fungal powder from the flowers, which was a concern in the study by Iqbal *et al.* (2022), where plants were moved between greenhouses between api-vectoring session. This potential unintended reduction in inoculum may have impacted effects on thrips in the study.

The Flying Doctors® hive dispenser was filled with 10 g of powder composed of 15% of BotaniGard and 85% of sterilized corn flour (D15), corresponding to a content of 5.99×10^9 spores/g powder. This dilution ratio was previously tested for its potential mortality effect on individual bumblebees under laboratory conditions and resulted in 100% mortality (see section 12.1). Despite this, we still chose this dilution ratio in the greenhouse experiment as further reducing the BotaniGard proportion would have lowered the fungal load below levels needed to achieve effective control of thrips. In addition, bumblebee survival in the mesh-cage was not affected by BotaniGard exposure in the greenhouse api-vectoring setup (see section 12.3.1). Thus, it is possible that a higher proportion of BotaniGard could be more effective in reducing thrips abundance while still remaining safe for the bumblebees in this setting.
A Canadian study with comparable aims to the present study (Coates et al., 2023) used a higher BotaniGard-corn flour ratio (30:70) in the Flying Doctors® dispenser, which was selected based on information from the literature that such amounts of entomopathogenic fungi would not adversely affect bumblebee survival. In the Canadian experiment, api-vectoring of BotaniGard powder to strawberry plants was carried out with *B. impatiens* to evaluate effects on natural thrips infestations in a commercial greenhouse setting (Coates et al., 2023). In that study, a mesh-exclusion zone served as the control area (vs. BotaniGard api-vectored area), preventing bumblebee access, but allowing thrips to pass through. The authors affirmed that the survival of bumblebees was not affected by BotaniGard exposure, despite the absence of a control hive for comparison. During the experiment, more thrips were collected from the exposed and from the mesh exclusion area, and a limited number of live thrips (up to 20) were incubated for survival in the laboratory for one week. Fungal infections were observed in thrips from both exposed and exclusion areas. Although the authors stated that api-vectoring of BotaniGard reduced thrips survival, their data showed comparable survival rates in thrips from both areas, and instances of low survival in thrips from the BotaniGard exposed area coincided with comparable low survival in thrips from the exclusion area. Notably, no statistical analyses were conducted on data of the survival rates, limiting the strength of their conclusions.
Consistent with our findings, Coates *et al.* (2023) found that vectoring by *B. impatiens* of BotaniGard powder did not reduce the abundance of thrips on strawberry plants, and they recovered lower amounts of fungal CFUs from bumblebees and flowers compared to the present experiment. Still, the authors observed infections in thrips after incubation, thus the inoculum level on plants may have been sufficient to initiate infections.
We did not observe fungal infection in dead adult thrips, which were collected from the insect tent after one week of exposure during experimental repetitions 1 and 2. In contrast, infections were evident in all laboratory experiments (section 8.1). Previous studies have shown that entomopathogenic fungal conidia can adhere to several thrips body parts (Ugine et al., 2005), germinate and penetrate the insect cuticle, as

confirmed through scanning electron microscopy (Z. Zhang et al., 2021). The lack of infection in the greenhouse tunnel setup may be attributable to sub-optimal environmental conditions, particularly insufficient relative humidity, which is known to be critical for successful *B. bassiana* establishment and infection (Jaronski, 2010; Mascarin & Jaronski, 2016).
In another study focusing on api-vectoring of *B. bassiana* by bumblebees in in greenhouse grown sweet pepper plants, the survival of the tarnished plant bug (*Lygus lineolaris*) was reduced (Al-mazra'awi et al., 2006). Thus, the strategy may have effects on larger pest insects, of which the European tarnished plant bug (*Lygus rugulipennis*) is a pest in strawberry tunnels.

Thus, conducive microclimatic conditions, mainly high relative humidity, are necessary for significant infections to occur. The microclimate gradient detected in the greenhouse tunnel may correlated with the limited BotaniGard efficacy. One side of the area with mesh-cages was warmer and less humid than the other side, reflecting a negative correlation between temperature and relative humidity (**Appendix 3**). Thrips larval abundance followed this pattern, increasing with higher temperatures and decreasing with lower relative humidity. Because *B. bassiana* requires high relative humidity for infections to occur, these warmer and drier conditions may be suboptimal, thereby reducing the impact of BotaniGard. In laboratory conditions, Fiedler and Sosnowska (2009) showed that *B. bassiana*-induced mortality of *F. occidentalis* 1st and 2nd instar larvae and adults was highest at 25°C, but declined at both 20°C and 30°C, which is consistent with *B. bassiana* having an optimum growth temperature of 25–28°C (Fargues et al., 1997). Previous greenhouse studies have likewise demonstrated the importance of relative humidity for achieving high efficacy of *B. bassiana* against insect pests. Fargues *et al.* (2003) reported increased mortality in the more humid compartment of a glasshouse (with minimum relative humidity of 50% by day and 75% by night) compared with a drier compartment, although effects were not always statistically significant. Similarly, in greenhouse experiments conducted at constant temperature, Shipp *et al.* (2003) found that the infection rate of adult (and to a lesser extent larval) *F. occidentalis* by BotaniGard increased with higher relative humidity (70–92%). Optimal germination of *B. bassiana* occurs at or near 100% relative humidity, with progressively reduced and delayed germination at lower humidity levels like 93% (Luz & Fargues, 1997)

Another important aspect to consider is the effect of api-vectored *B. bassiana* on strawberry yield. Studies on root-inoculated *B. bassiana* have reported no negative, and in most cases positive, effects on strawberry yield in pot experiments (Canassa, D'Alessandro, et al., 2020; Zinan et al., 2025) or in commercial plantations (Khoury et al., 2020). However, most of the api-vectoring studies, including ours, have not assessed crop yield (Al-mazra'awi et al., 2006; Coates et al., 2023; Kapongo, Shipp, Kevan, & Sutton, 2008). One exception is the study by Kapongo, Shipp, Kevan, & Broadbent (2008), who reported personal observations indicating that tomato fruit yield and quality remained "within acceptable commercial standards". Further studies should evaluate this dimension to verify whether api-vectored *B. bassiana* has any impact on crop agronomic parameters.

Overall, the lack of documented efficacy of api-vectored BotaniGard in the greenhouse experiment on flowering strawberry plants is likely explained by a combination of insufficient inoculum levels in flowers and suboptimal relative humidity for successfully stimulating *B. bassiana* infection process on insect cuticles. Increasing relative humidity in the greenhouse during exposure in flowers would meet requirements of

BotaniGard for effective performance as indicated on the label[iv] and such conditions may thus improve the efficacy of api-vectored *B. bassiana* against thrips, but may also stimulate grey mold development in the fruits. Although *B. bassiana* may have additional control potential against plant pathogens, an increased relative humidity during api-vectoring of BotaniGard for thrips control must be done without negatively affecting crop yield and quality.

[iv] BotaniGard WP label: http://uk.bioplant.dk/media/40082/Botanigard%20WP%20etiket%202015.pdf

Objective 2: Bumblebees' health

[Skillebladstekst]

10. Objective 2 – Research aims

This second objective focuses on bumblebees' health when exposed to BotaniGard, evaluated through different parameters at different experimental scales.

The associated aims were to:

1. Assess bumblebee survival when exposed to BotaniGard in three experimental settings: individual incubation, group incubation, and complete colony.
2. Evaluate how BotaniGard exposure affect different health related traits, flight activity, weight of individual bumblebees and the hive, and quantify presence of *B. bassiana* within the hive.
3. Examine the effect of BotaniGard exposure on bumblebee gut microbiota of workers over time, including both bacterial and fungal communities.

The related hypotheses were that:

1. Bumblebee survival of exposed individuals is lowest during individual incubation, increases in a group setting, and is highest in the colony setting.
2. BotaniGard exposure reduces flight activity, decreases bumblebee and hive weight, and leads to presence of *B. bassiana* inside the hive.
3. BotaniGard exposure reduces gut microbial diversity of bumblebee workers over time by altering the structure of microbial communities.

11. Objective 2 – Materials and methods

11.1 Individual and group experiments

Bumblebee exposure — Each individual was removed from the hive and placed at one end of a detached dispenser (**Figure 15**), which was loaded with BotaniGard powder representing one of four treatments (D0, D15, D25, D50; **Table 1**). Individuals were allowed one minute to exit; those that did not were gently removed with forceps. This simulated natural conditions where bumblebees normally pass through quickly. Manual removals were recorded for later analysis.

Individual incubation experiment — Each exposed bumblebee was kept individually in a ventilated cup (95 mm diameter × 80 mm height) lined with filter paper (**Figure 15**). A 35% sugar solution (organic sugar, sterile water) was provided *ad libitum* in an Eppendorf tube pierced at the base and inserted horizontally. The experiment was repeated at three different occasions, with a total of 50 individuals for D0 and 40 individuals for each of D15, D25, and D50 treatments.

Group incubation experiment — Before exposure, some of the bumblebees underwent a tagging procedure: after a 5-second CO_2 anesthesia, a colored tag was glued to the dorsal thorax. After a 30-minute recovery, these bumblebees went individually through the dispenser for exposure. Groups of five non-exposed and one tagged and exposed bumblebees originating from the same colony were kept in cubic containers (195 x 195 x 115 mm, **Figure 15**). Ventilation was achieved through two rectangular mesh-covered sections on the sides. The lid had four small holes covered with tape, for removal of dead individuals when necessary. A 50% sugar solution was provided in a 15 mL Falcon tube, pierced at the base and inserted through the lid. The experiment was repeated twice, each time with 20 boxes, resulting in 10 container replicates per treatment (*i.e.*, D0, D15, D25, D50; **Table 1**).

Monitoring — Cups and cubic containers from both experiments (*i.e.*, individual and group settings) were maintained in darkness at 28°C to mimic hive conditions. Survival was monitored daily for 14 days, and dead individuals were collected to be surface-sterilized by immersion in 70% ethanol for 10 sec, distilled water for 10 sec, 3–5% NaClO for 1 min, twice in sterile water for 10 sec, before being dried on filter paper (Jüds et al., 2023; Lacey & Solter, 2012). They were then placed individually in sealed 30 mL medicine cups at 23°C with moist filter paper for observation for *B. bassiana* growth, indicating fungal infection.

Fungal load — In a separate experiment, bumblebees were exposed in the dispenser to one of five BotaniGard treatments (D0, D15, D25, D50, D100; **Table 1**) as described above. After exposure, each individual was placed in a 50 mL Falcon tubes, then freeze-killed at −20°C for 30 min. The tubes were processed as described in section 5.1.2 to determine the number of CFUs carried by each individual. This was performed on 20 individuals for D0 and 15 individuals for each of the other treatments.

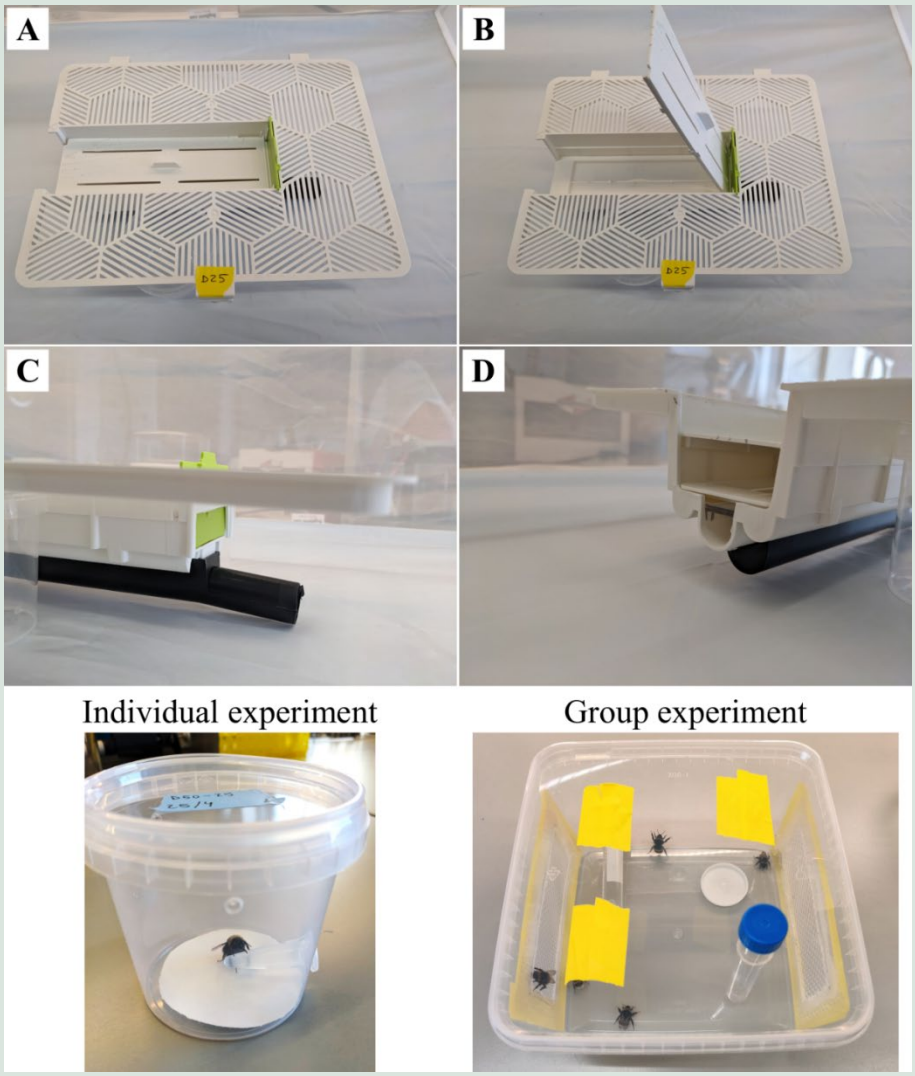

FIGURE 15. Laboratory setup for exposing bumblebees to BotaniGard using a detached Flying Doctors® lid, and for monitoring their survival in both individual and group experiments. (A) Top view of the lid with the tunnel closed with green barrier; dispenser not visible. (B) Top view of the lid with the tunnel open; dispenser is visible. (C) Dispenser entry point: the green barrier can be removed for bumblebees to enter the dispenser. (D) Dispenser exit point: for the experiment, a cup was placed immediately at the exit for the bumblebee to walk into.

11.2 Colony experiment

Experimental setup and bumblebee exposure — On the day of hive standardization (section 5.2), two closed hives (each containing 65 workers and 1 queen) were placed in separate mesh cages for acclimation. During the two-day acclimation period, bumblebees had access to artificial flowers (40 per cage) for 7 hours each day, followed by both artificial flowers and flowering plants (20 per cage) for another 7 hours (**Figure 16**). After acclimation, the experiment started by introducing 20 fresh flowering strawberry plants into each mesh-cage, alongside the same artificial flowers. Each hive's dispenser was filled with 10 g of powder: inactive D15 (control treatment) and active D15 (BotaniGard treatment). The hives were then opened for 7 hours for workers to exit through the dispenser. Fresh flowering strawberry plants were introduced after 1 and 7 days of treatment (12 per cage). Pollen was added and replaced accordingly, and the dispenser was refilled with 10 g after 7 days. On day 13, the hives were closed

and brought back to the laboratory the following day. The experiment was conducted during summer (July–September 2024) and repeated three times using new hives and artificial flowers.
During the experiment, temperature and relative humidity inside the mesh cages were recorded using a temperature logger (RS PRO RS-191A, Singapore). Light intensity inside the cages was measured and corrected by subtracting 30% to account for absorption by the tunnel plastic above the cages. Atmospheric pressure was obtained from World Weather Online for the nearest station to the greenhouse (Frederiksberg, Denmark).

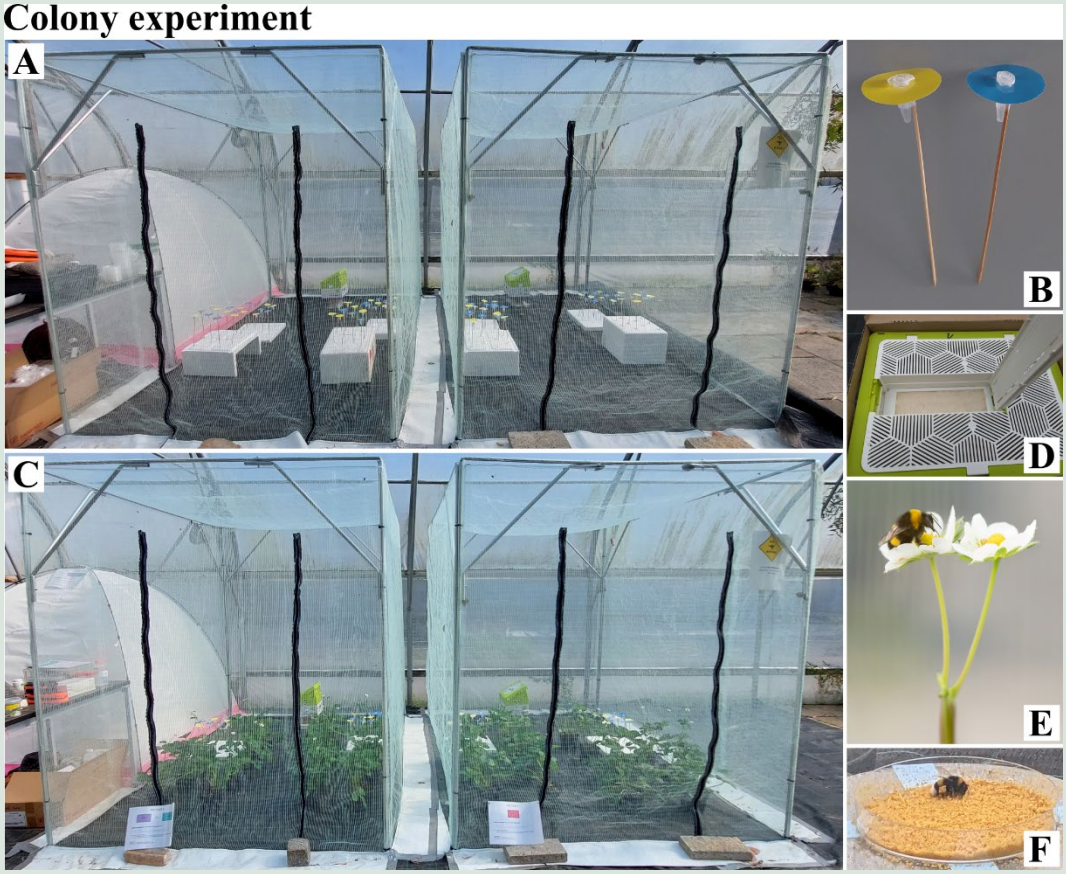

FIGURE 16. Greenhouse setup for assessing colony health during api-vectoring. (A) Mesh cages inside the greenhouse tunnel. On day 1, bumblebees had access only to artificial flowers. (B) Artificial flowers contained a 50% sugar-water solution. (C) On day 2, bees had access to both artificial flowers and flowering strawberry plants. (D) Treatments were introduced by filling the dispenser with either inactive or active D15 powder. (E) Bumblebees deposited the fungal powder on flowers while pollinating. (F) Bumblebees also had access to pollen.

Fungal load — On the day the dispenser was loaded, five individuals exiting each hive were sampled in a 50 mL Falcon tube (“hive departure”). In the afternoon of the same day, five individuals were sampled while flying (“hive return”) using a honey cup and then transferred to individual 50 mL Falcon tubes. All individuals were processed as described in section 5.1.2 to determine the number of CFUs per bumblebee. Sampling was always carried out after activity monitoring (see below). Due to hive variability across the three repetitions, a total of 7 and 5 control individuals were sampled at hive departure and hive return, respectively, and 15 and 14 BotaniGard-treated individuals at hive departure and hive return, respectively. To maintain the same number of

individuals in each hive, bumblebees that could not be collected during departure or return due to variability among hives, were manually removed from the hives and discarded.

Survival — Bumblebee survival was checked daily throughout the week. Dead individuals inside the mesh cage were collected and surface-sterilized as previously described to observe fungal growth. Individuals that were missing at the end of the experiment (*i.e.*, not found in the hives and not recorded as dead or removed) were excluded from the analysis. These missing bumblebees were evenly distributed across treatments and assumed to have escaped from the cages during the experiment.

Activity — The activity of bumblebees was recorded as the number of individuals entering and exiting the hive in a 15-minute period. A baseline measurement was taken on day 2 of the acclimation period at 12:00. After treatments were added, activity was measured twice daily (09:15 and 12:00) on days 0, 1, 6, 8, 11, and 13.

Microbiota — At standardization (*i.e.*, baseline) and at 1, 6, 13 days after starting the treatment (*i.e.*, days post inoculation, dpi), 5 worker individuals from each hive was collected individually in honey cup after the 12:00 bumblebee activity monitoring while the queen was retrieved at 14 days. A total of 15 workers per time and per treatment and of 3 queens per treatment were collected. Each bumblebee was chilled for 10 min at –20°C, then individually exposed to CO_2 for 20 sec at 4 bar. The gut was extracted from each individual by pressing the insect with a finger on the thorax to make the stinger emerge, then by pulling the stinger with sterile tweezers to extract the whole gut system and by cutting the cuticle edge and stinger out with a sterile scalpel (**Figure 17**). The gut was then transferred to a 2 mL Eppendorf and stored at –70°C until further processing.

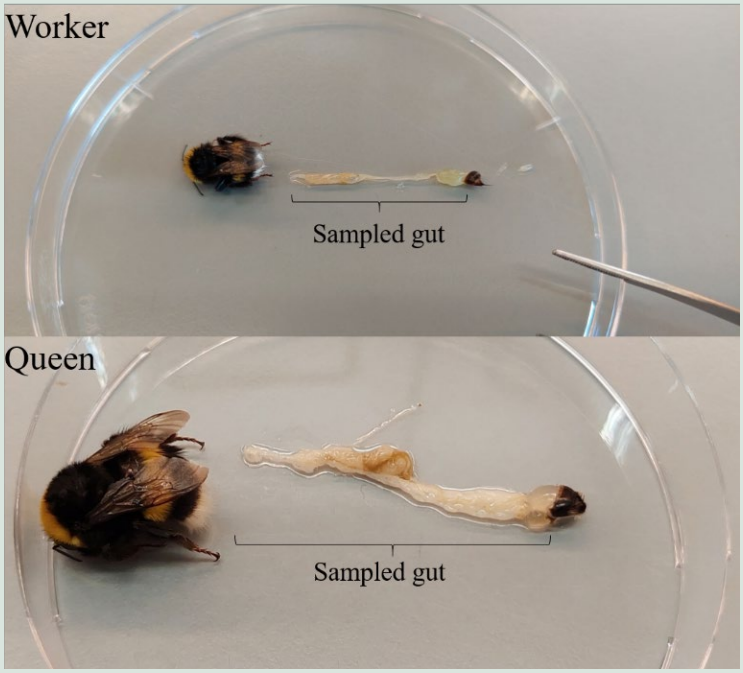

FIGURE 17. Extraction of the gut from a worker and a queen of *B. terrestris*.

DNA was extracted from each gut using the DNeasy® Blood & Tissue kit (Qiagen, Germany) according to the manufacturer instructions and with several modifications.

Briefly, 3 glass beads (diameter of 3 mm) were added to each worker gut sample and grinded for 30 sec in a tissue lyser (30 beats/s). The incubation at 56°C was performed in a water bath for 3h, during which tubes were vortexed (5 sec, maximum speed) every 15 min. The addition of Buffer AL and of 100% ethanol was done in two steps, while the elution step was performed twice, with 75 µL of Buffer AE each time to obtain an eluted volume of 150 µL. Additionally, each queen gut sample required two extraction preps: the double amount of glass beads and volume of buffers were used in order to split the final mixture (approx. 600 µL each) in two DNeasy Mini spin columns, which had to be centrifuged several times (6000-8000 g, 1 min) for all the suspension to go through; each column was eluted once and they were then pooled to obtain 150 µL. Lastly, DNA was also extracted from negative and positive controls, respectively corresponding to an empty tube and to 70 µL of a microbial community standard (ZymoBIOMICS). The quality and quantity of DNA were assessed using the Nanodrop (DeNovix, DS-11, USA) and the Qubit (Thermo Fisher Scientific, USA) respectively.
The bacterial communities were analyzed by amplifying a 440 bp fragment of the V3-V4 region of the bacterial 16S rRNA gene using the primers 341F (5´-CCTACGGGNGGCWGCAG-3´) and 806R (5´-GACTACNVGGGTWTCTAATCC-3') while the fungal communities were analyzed by amplifying a 380 bp fragment of the ITS2 region using the primers ITS3F (5´-GCATCGATGAAGAACGCAGC-3´) and ITS4R (5´-TCCTCCGCTTATTGATATGC-3´). PCR amplification, purification, library preparation, Illumina MiSeq sequencing (2×300 bases paired-end version), demultiplexing and barcode suppression were performed by StarSeq (Mainz, Germany). Raw 16S and ITS sequences will be uploaded in the National Center for Biotechnology Information (NCBI).
Bioinformatic analyses were conducted in R (R Core Team, 2025) using the dada2 pipeline for processing 16S and ITS sequences (B. J. Callahan et al., 2016), along with Cutadapt (Martin, 2011) for primer and primer complement removal. For 16S reads, forward and reverse sequences were trimmed to 260 bp and 205 bp with maximum expected errors ("maxEE" argument) of 2 and 5, and only sequences between 404 and 447 bp were retained, whereas ITS sequences were trimmed to 260 and 210 bp with maximum expected errors of 5 and 10. Amplicon Sequence Variants (ASV) were obtained and taxonomic assignation was performed using the Silva database (v.138.2) for bacterial sequences (B. Callahan, 2024) and the UNITE database for fungal sequences (Abarenkov et al., 2025).

Hive assessment — At the end of the experiment on the 14th day after treatment, leftover bumblebees were freeze-killed, removed from the hives, dried 24 h at 70°C in an oven, and weighed in bulk. The average weight per bumblebee was calculated. Then, the number of cells built by the bumblebees in the hive was counted while the hive was weighed to compare the final weight to the initial weight at the start of the experiment. Lastly, samples were collected from inside each hive: eight swabs were performed with separate 10 µL inoculating loops, which were individually placed in a 50 mL Falcon tube. Two cell samples were taken, each composed of two cells built by the bumblebees during the experiment and also placed in a Falcon tube (**Figure 18**). All samples were processed using the protocol described in section 5.1.2 to determine the number of CFUs detectable in the hive.

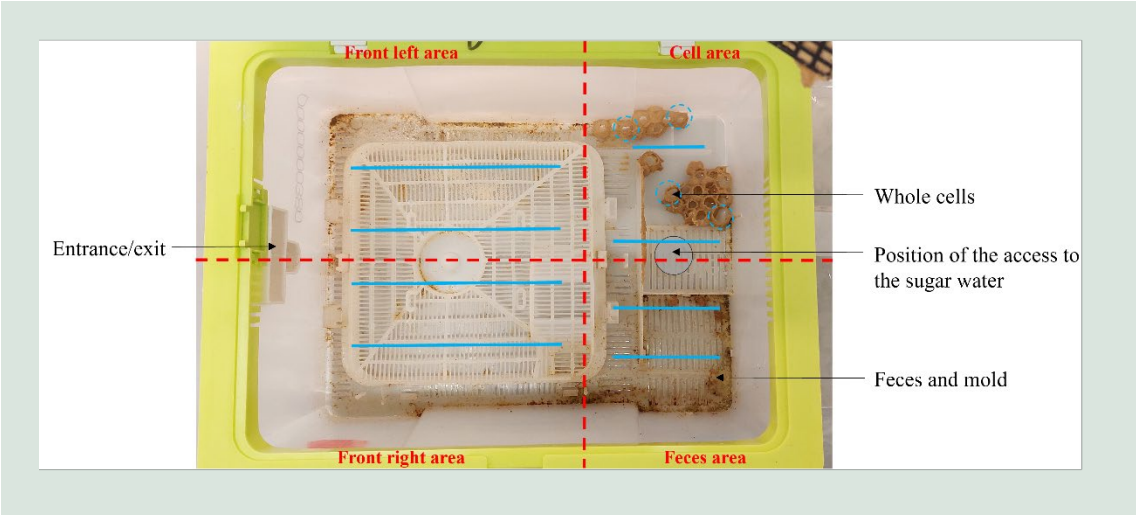

FIGURE 18. Visual plan of the swab sampling performed in the hives. The opened hive was divided into 4 areas (red dashed lines), separating functional areas. Two swabs were performed per area (blue lines). Two cells were sampled twice and correspond to the dashed blue circles.

11.3 Statistical analyses

Analyses were conducted in R version 4.5.1 (R Core Team, 2025) using “tidyverse” (Wickham et al., 2019) for data handling and “ggplot2” (Wickham, 2016) for visualization.

Survival curves were obtained from a mixed effects Cox model for the individual setting experiment (“coxme” function and package (Therneau, 2024b)); from a parametric survival regression model with the Weibull distribution for the group setting experiment, and from a Cox Proportional Hazards Regression Model for the colony setting experiment (“survival” package (Therneau, 2024a)). Mycosis rate was analyzed using linear models and the fungal load (number of CFU per bumblebee) with a generalized linear mixed models via Penalized Quasi-Likelihood (“glmmPQL” function, “MASS” package (Venables & Ripley, 2002)). The effects of *B. bassiana* (referred as to Dose or Treatment), and when applicable of EPF-exposure and of their interaction were tested, along with the Repetition, which were removed from the model if non-significant or treated as a random factor if significant.
Bee activity was analyzed using a generalized linear mixed model with Template Model Builder (“glmmTB” function and package (Brooks et al., 2017)). The model included the treatment, the day, their interaction, the time of the day, relative humidity, temperature, light, atmospheric pressure, bee bulk weight and the baseline activities as fixed-factors and covariates, while the Repetition was treated as a random factor.

The significance of each term of each model was assessed with Type II analyses of variance (“Anova” function, “car” package (Fox & Weisberg, 2019)) using F-test (linear model), Wald chi-square test (linear mixed models, generalized linear mixed models and Cox models), Likelihood Ratio Chi-Square (for survival regression).
Significant terms were followed by pairwise comparisons with estimated marginal means (“emmeans” function and package (Lenth, 2025)), adjusting p-values by false discovery rate (“cld” function of the “multcomp” package (Hothorn et al., 2008)). When applicable, predictor effects of covariates were computed using the “effects” package (Fox & Weisberg, 2018) and plotted.

Microbiota data were handled using the “phyloseq” package (McMurdie & Holmes, 2013) and processed as follows: Mitochondrial and Chloroplast taxa were removed to keep only bacterial and fungal taxa, contaminants were filtered out using the prevalence method of the “decontam” package (Davis et al., 2017), as well as negative and positive controls and singletons, before rarefying to 18,400 reads for bacteria and to 2,000 reads for fungi. ASV 1 of the fungal dataset, corresponding to *Beauveria* genus, was re-assigned based on NCBI results to the species *B. bassiana*.

Alpha diversity indices were calculated separately for bacteria and fungi: observed ASVs (richness), Shannon, Pielou (evenness). Two linear mixed models were performed on each index using the “lme4” package (Bates et al., 2015) with the repetition as a random factor: i) only workers (*i.e.*, both treatments and all time points) to test the effects of the treatment, the time and their interaction, ii) workers and queens of the end-time-point (13–14 dpi), to test the effects of the treatment, the bee type (*i.e.*, worker *vs.* queen) and their interaction. The steps related to testing the significance, performing pairwise comparisons and correcting p values were carried out as previously described.

Beta diversity (*i.e.*, community structure) was assessed separately for bacteria and fungi by performing a permutational multivariate analysis of variance using Bray Curtis matrices using “adonis2” function from the “vegan” package (Oksanen et al., 2025). As previously described, two models (i and ii) were made with the repetition as random factor. Whenever a term was significant, pairwise comparisons were carried out using the “pairwise.adonis” function of the “pairwiseAdonis” package (Martinez Arbizu, 2017). A Principal Coordinate Analysis (PCoA) was used to visualize differences.

12. Objective 2 – Results

Statistical outputs are presented in **Appendix 4** and **Appendix 5**.

12.1 Individual level effects

Each bumblebee picked up 1×10^7 - 8.8×10^7 CFUs from the D15–D100 mixture when passing through the dispenser, with significantly most CFUs on bees from D100, then D50, while D25 and D15 gave similar amounts of CFUs (**Figure 19A**, **Appendix 4**). Complete mortality was observed irrespective of dose, while 86% survival in controls, with a median survival time of 7–8 days (**Figure 19B**, **Appendix 4**). Nearly all dead individuals (97.5–100%) exhibited signs of mycosis (**Figure 19C**, **Appendix 4**).

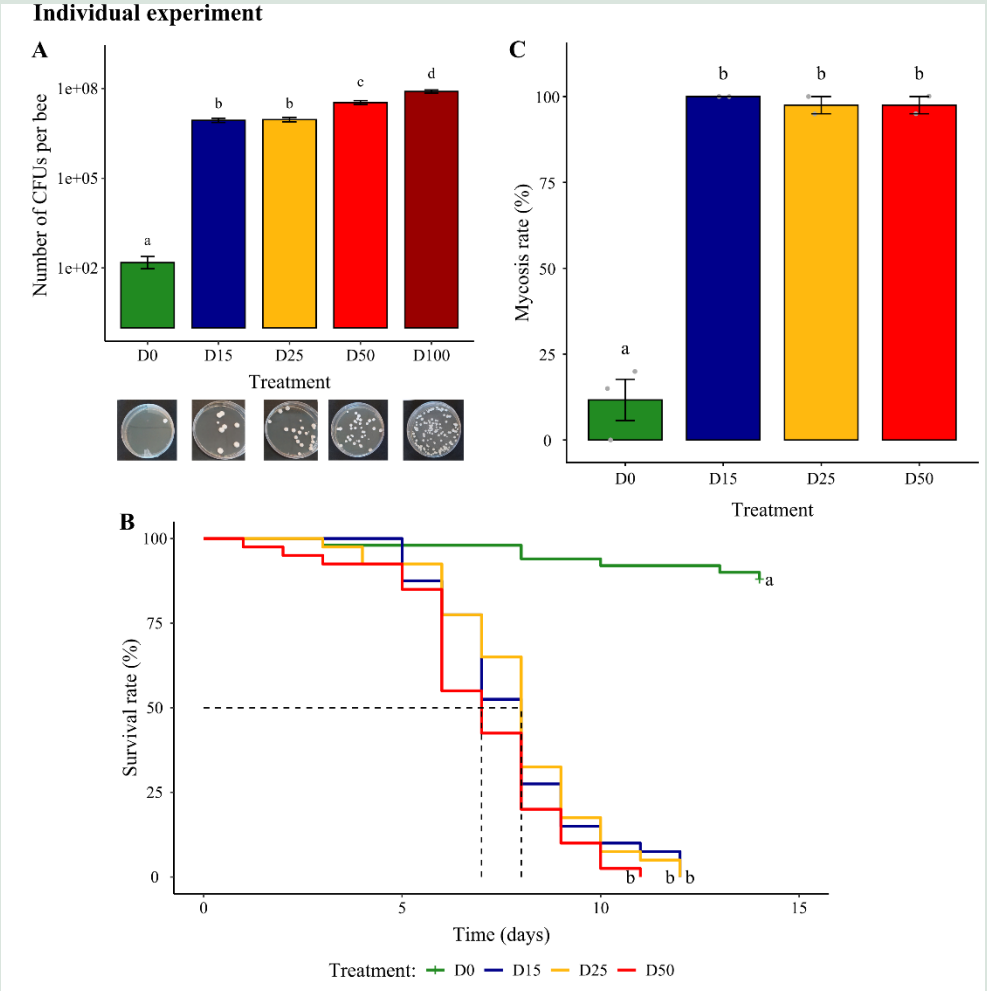

FIGURE 19. Survival of bumblebees in individual incubation experiment in laboratory conditions. (A) Fungal load carried by bumblebees when exiting the dispenser. Images below the plot show examples of culture plates: undiluted sample D0, and samples diluted to a factor of 10^{-4} for D15 through D100. (B) Survival curves, with dashed lines corresponding to median survival time. (C) Mycosis rate. Barplots show means and standard errors, with grey dots corresponding to observations. Different letters in each sub-figure indicate significant differences.

12.2 Group level effects

The exposed bumblebees had no survival across all BotaniGard doses, D15, D25, D50 (compared to 90% survival in the control group, D0), with a median survival time of 6–6.5 days, when kept with five non-infected nestmates (**Figure 20**, **Appendix 4**). The survival of the nestmates was significantly lower than at D0, at 60–70%, but higher than the exposed nestmate. Nearly all exposed bumblebees (90–100%) exhibited signs of mycosis, whereas only 12–36% of the non-exposed nestmates that died showed such symptoms (**Figure 20**, **Appendix 4**).

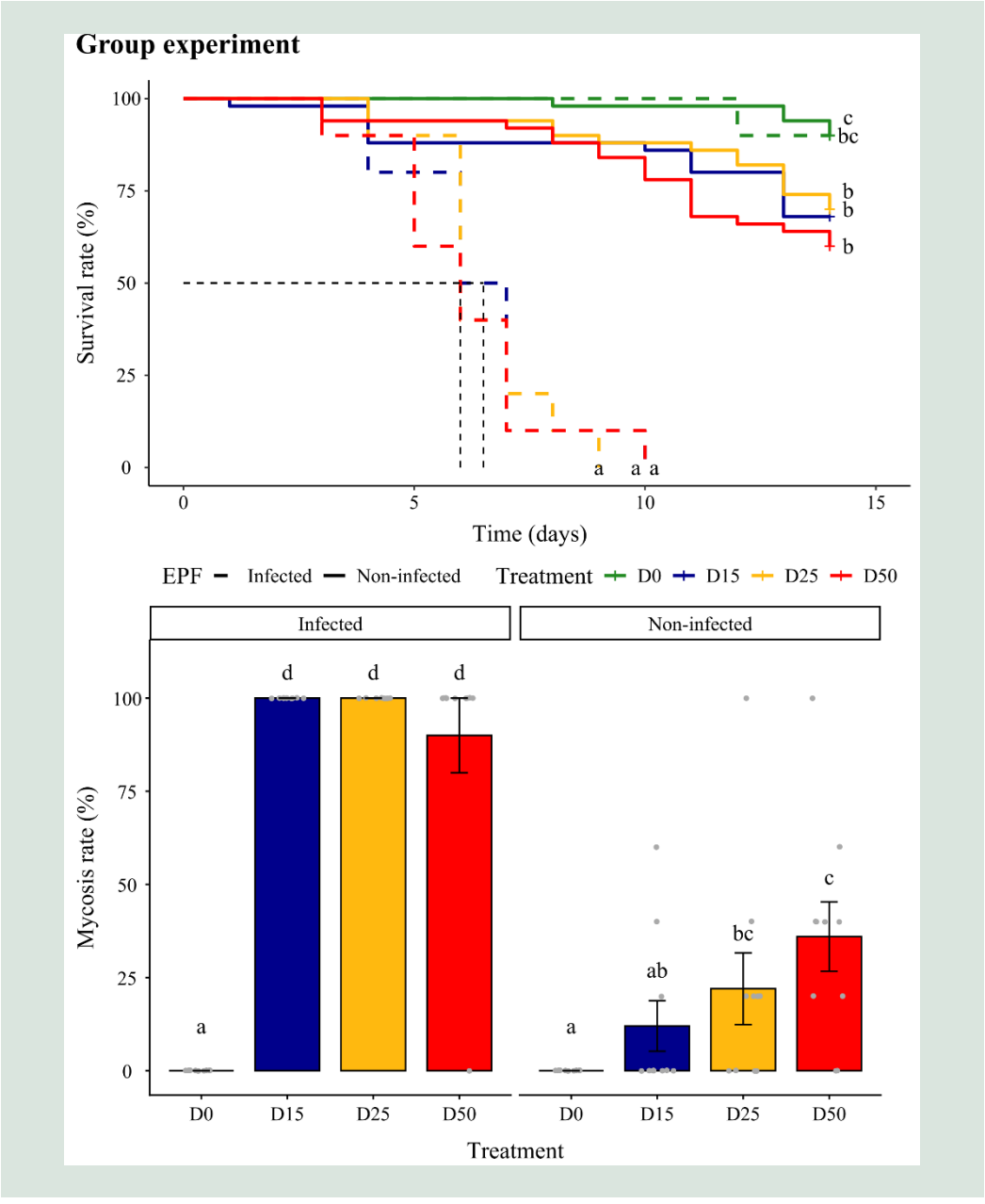

FIGURE 20. Bumblebees' survival in a group experiment carried out in laboratory conditions. Top: survival curves, with dashed black lines corresponding to median survival time. EPF refers to whether a bumblebee had been exposed to *B. bassiana* (*i.e.*, "infected" or "non-infected") at the start of the experiment. Bottom: mycosis rates of dead bumblebees, with bars showing mean and standard error values and grey dots corresponding to individual observations. Different letters indicate significant differences within each sub-figure.

12.3 Colony level effects

12.3.1 Survival and activity of bumblebees

Among the 12 control bumblebees, 3 developed fungal CFUs (5×10^{1}–1.65×10^{3}, **Figure 21A**). BotaniGard-treated bumblebees carried slightly more CFUs on average at hive departure (8.04×10^{5} per bee) than at hive return (2.84×10^{5} per bee). Surprisingly, bumblebees exposed to the control and BotaniGard mixture (*i.e.*, corresponding to inactive and active D15) showed similar survival rates over 14 days, ranging from 87 to 92% (**Figure 21B**, **Appendix 4**). However, among the few bumblebees that died following BotaniGard exposure, 60% exhibited signs of mycosis, compared to only 3% in the control group (**Figure 21C**).

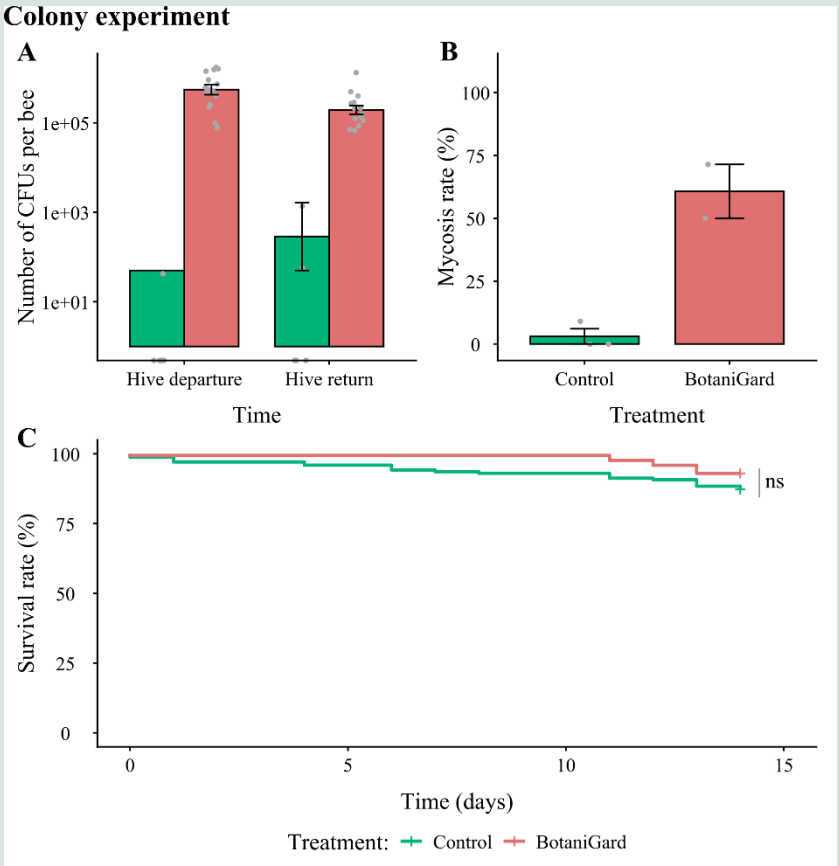

FIGURE 21. Fungal load and mortality effects on bumblebees in the colony experiment in greenhouse conditions. (A) Fungal load carried by bumblebees when exiting the dispenser and after returning to the hive. (B) Mycosis rate, with bars showing mean and standard error values and grey dots corresponding to raw data. (C) Survival curves, with dashed lines corresponding to median survival time. “ns” stands for non-significant.

The total activity of the bumblebees in the mesh-cages, measured as the number of individuals entering and exiting the hives, was significantly affected by the treatment, day, the interaction, relative humidity, temperature, atmospheric pressure and baseline total activity (**Appendix 4**). Over time, bumblebees exposed to BotaniGard exhibited higher activity compared to those exposed to the control mixture (**Figure 22, top panel**). Increases in humidity, temperature and atmospheric pressure was associated with reduced activity, whereas higher baseline activity was associated with greater activity during the experiment (**Figure 22, bottom panel**). Notably, hive-level activity

patterns varied across repetitions: during the first repetition, control and BotaniGard hives differed substantially, while in the second and third repetitions, their activity levels were remarkably similar (**Figure 23**).

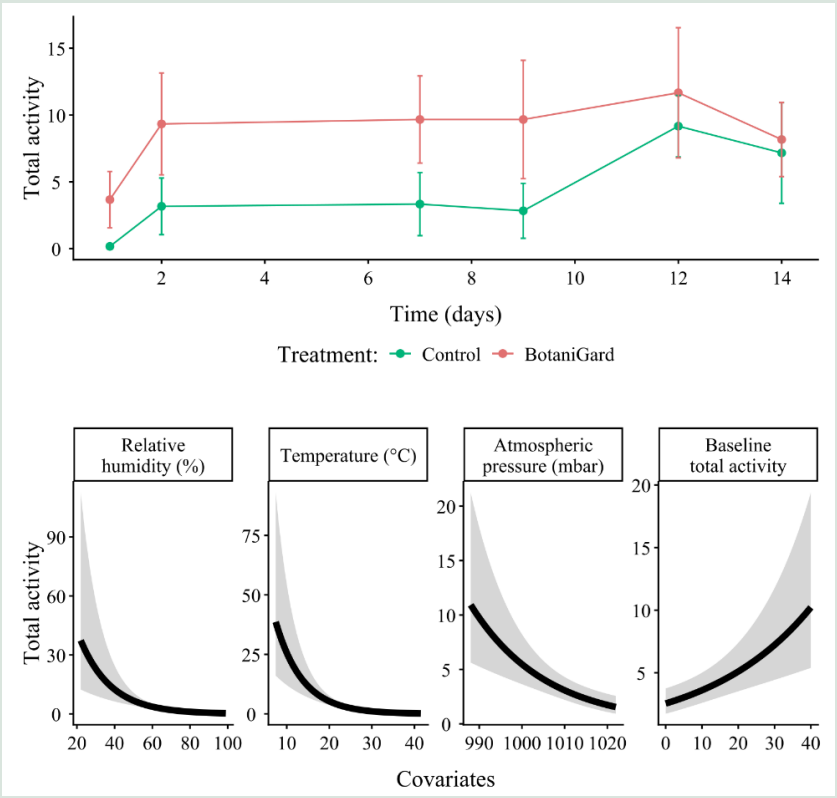

FIGURE 22. Activity of bumblebees in a colony experiment in greenhouse conditions. Total activity corresponds to the daily average (mean ± standard error of the 9:15 and 12:00 measurements). Top panel shows the average of the three repetitions. Bottom panel shows the significant effects of covariates on the bumblebees' activities, with the grey color corresponding to confidence intervals.

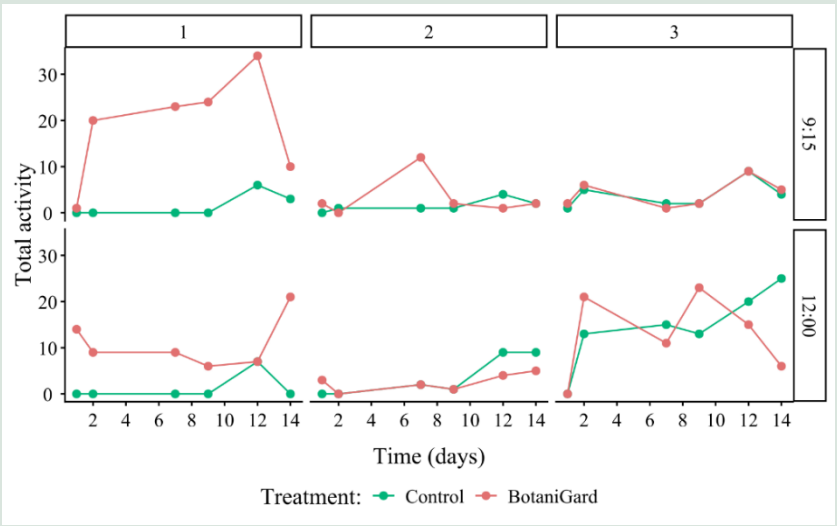

FIGURE 23. Bumblebees' activities in a colony experiment in greenhouse conditions over three repetitions (1, 2, 3) at two different timepoints of the day.

12.3.2 Microbiota

After removal of non-bacterial and non-fungal taxa, contaminants and singletons, and after rarefying, a total of 2,318,400 bacterial reads for 363 ASVs and of 244,000 fungal reads for 692 ASVs were retained for the analyses. Four control samples were removed from the fungal dataset as they did not pass the rarefying threshold.

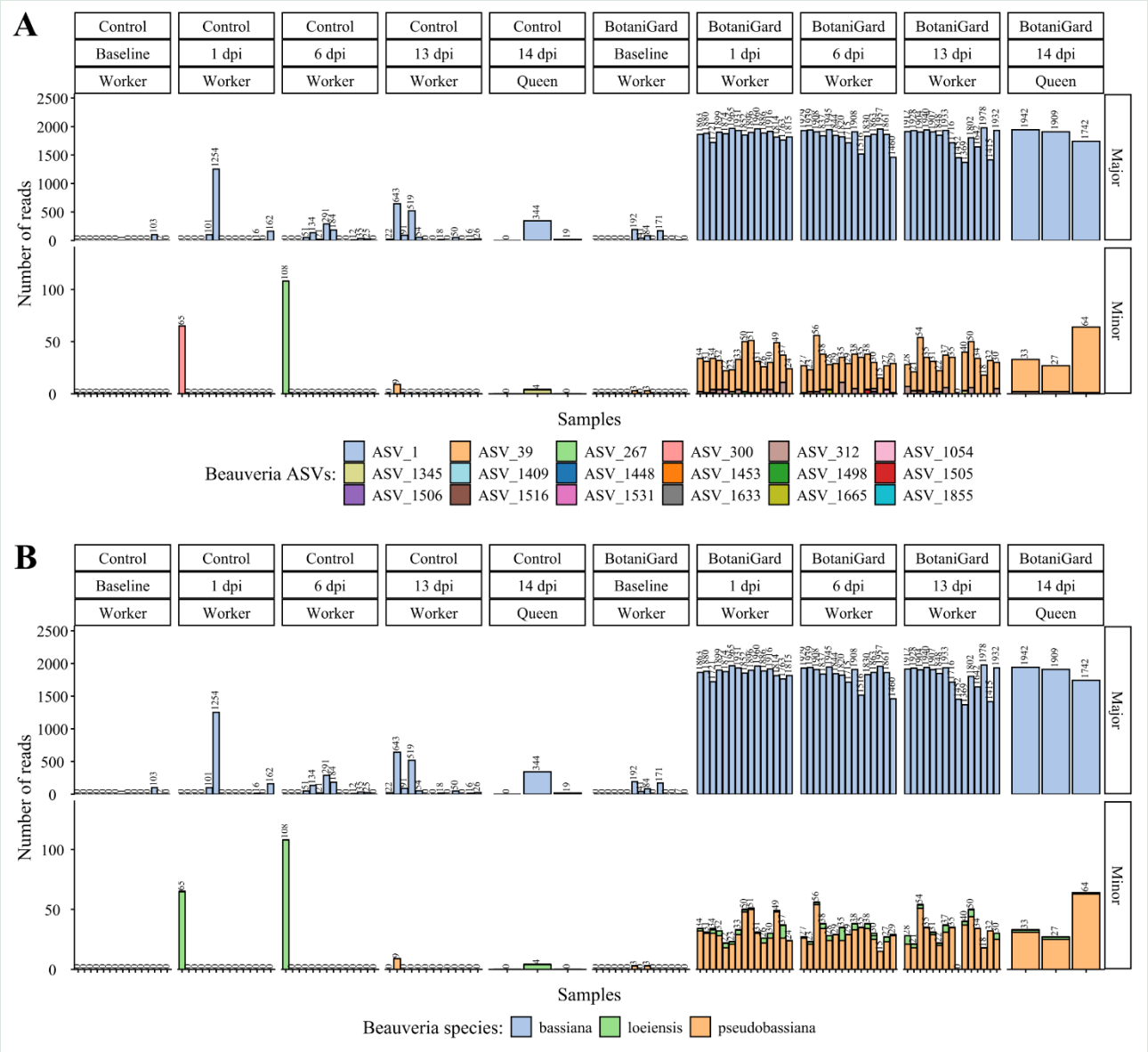

FIGURE 24. *Beauveria* abundance in the bumblebee gut at the ASV (A) and at the species (B) levels. Numbers above the bars correspond to the total number of reads. ASVs are categorized into major (ASV 1, top panel) and minor (all other ASVs, bottom panel). "dpi" stands for days post inoculation. N = 15 workers per time and per treatment, 3 queens per treatment.

Amplicon sequencing showed that 28 control-treated and 53 BotaniGard-treated bumblebees contained *Beauveria* reads. Eighteen ASVs were assigned to the *Beauveria* genus (**Figure 24A**) and to different *Beauveria* species (**Figure 24B**), with *B. bassiana* being the most abundant species, followed by *B. pseudobassiana*, and *B. loeiensis*. ASV 1 was the most abundant ASV, corresponding to *B. bassiana*, and ranging from 0 to 1,254 reads per control-treated samples and from 1,369 to 1,978 reads per

BotaniGard-treated sample (*i.e.*, 1–14 dpi). It was one of the two ASVs detected in all BotaniGard-treated samples, along with ASV 39 corresponding to *B. pseudobassiana*.

Bacterial alpha diversity indices were differentially affected by the experimental factors (**Appendix 5**, **Figure 25A**). The number of observed ASVs was influenced only by the interaction between treatment and bee type in the end-time-point model: control queens exhibited a higher number of ASVs than BotaniGard-treated queens, whereas no treatment-related differences were detected in workers at 13 dpi. In contrast, Shannon and Pielou indices were both significantly affected by treatment, time and their interactions in the worker models. Both indices were lower in BotaniGard-treated workers compared to control workers at baseline and 1 dpi for the Shannon index and at baseline only for the Pielou index. In control workers, both indices remained stable over time, while they increased over time for the BotaniGard treated workers. No significant effects of the treatment were observed in queens for either index.

Fungal alpha diversity indices were significantly affected by treatment, time and their interactions in the statistical model for worker gut microbiota and by treatment only in the end-time-point models (**Appendix 5**, **Figure 25B**). The number of observed ASVs was lower in BotaniGard-treated workers at time-points after the baseline and in the queens compared to the control individuals, whereas values in control workers increased over time. Both Shannon and Pielou indices were consistently lower in the BotaniGard-treated workers across all time points and in queens, except at baseline. In contrast, control bumblebees showed a high increase after the baseline in both Shannon and Pielou indices.

Bacterial beta diversity (*i.e.*, community structure), was significantly affected by treatment and time for the worker model, which explained 23.8% of the variance, and by treatment and bee type in the end-time-point model, which explained 22.4% of the variance (**Appendix 5**, **Figure 25C**). The PCoA revealed two distinct clusters corresponding to control and BotaniGard-treated bumblebees. In addition, baseline and 1 dpi workers showed a tendency to cluster together, while samples from 6 and 13 dpi were more closely associated with each other. Pairwise comparisons indicated that workers sampled at baseline differed significantly from those collected at 6 and 13 dpi.

Fungal beta diversity was significantly influenced by treatment, time and their interaction for the worker model, which explained 67% of the variance, and only by treatment in the end-time-point model, which explained 52% of the variance (**Appendix 5**, **Figure 25D**). The PCoA showed clearer separation than that observed for bacteria, with control bumblebees forming their own cluster and BotaniGard-treated individuals another one. Baseline workers from both treatment groups clustered together and differed significantly from workers sampled at 1, 6 and 13 dpi.

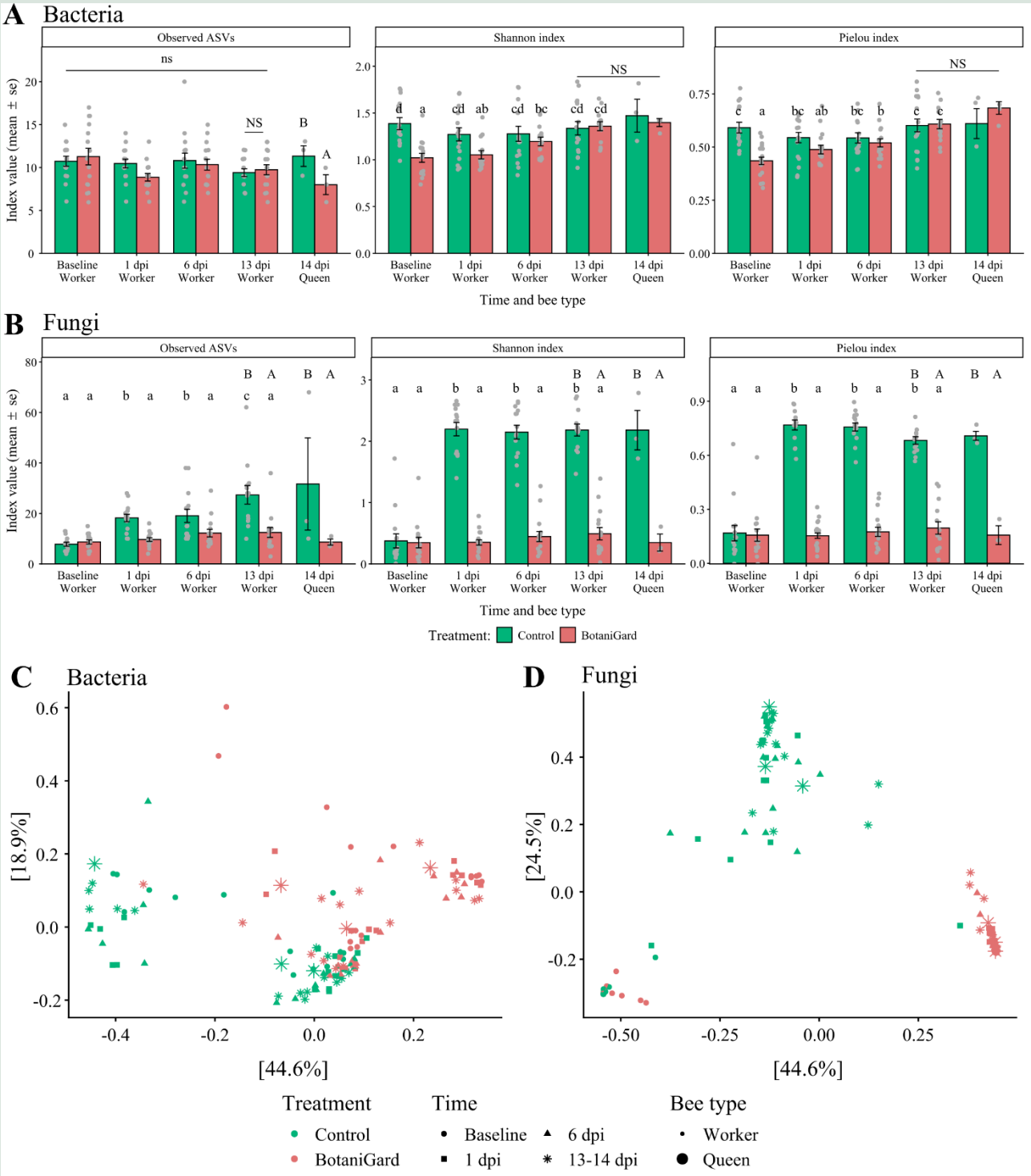

FIGURE 25. Bumblebee gut microbial diversity. Alpha diversity is represented by three indices (A, B) while beta diversity is displayed on a PCoA (C, D), for bacteria (A, C) and fungi (B, D). "dpi" stands for days post inoculation. N = 15 workers per time and per treatment, 3 queens per treatment. For panels A and B, different lowercase letters indicate significant differences between the two-way interaction 'Treatment' and 'Time' of the model with workers only, uppercase letters indicate significant differences between the two-way interaction 'Treatment' and 'Bee type (bacteria) and between 'Treatment' (fungi) of the model with the end-time-point (13–14 dpi), while "ns" and "NS" stand for not significant. Grey dots correspond to individual data points.

Bacterial communities were mostly composed of taxa from the Pseudomonadota phylum, followed by the Bacillota and the Actinomycetota phyla. A total of 48 genera was

identified but the dominant ones (identified species) were *Snodgrassella* (*S. alvi*), *Gilliamella* (*G. apicola*), *Lactobacillus* (*L. bombicola* and *L. panisapium*), *Bombiscardovia* in decreasing order (**Figure 26A**). However, no clear difference was observed between the treatments, times and bee types when looking at genus relative abundance.
When examining number of reads for the most abundant bacterial genera, we observed patterns associated with the experimental factors (**Figure 26B**). The abundance of *Snodgrassella* genus (*i.e.*, corresponding to 92 ASVs) increased in BotaniGard-treated bumblebees compared to controls, mainly at 1 dpi, and was lower in the queens compared to the workers. Conversely, the abundance of *Gilliamella* genus (86 ASVs) decreased in BotaniGard-treated workers and queens relative to controls. *Lactobacillus* (51 ASVs) did not appear to be affected by the treatment and showed higher abundance in queens than in workers. The abundance of *Bombiscardovia* genus (12 ASVs) was the only one affected by the interaction between time and treatment, where it was higher in control bumblebees at 1 dpi and tended to become higher in BotaniGard-treated bumblebees in later time points.

Fungal communities were mostly composed of taxa from the Ascomycota phylum, followed by the Basidiomycota phylum and unknown fungi, and a total of 178 genera was identified. Bumblebees collected at baseline from the control and BotaniGard hives had similar fungal community composition, dominated by *Saccharomyces* genus (**Figure 27A**). Exposure to the control and BotaniGard powders substantially changed the fungal communities, which composition seemed to be diverse with *Cladosporium* and different unknown genera (*i.e.*, Ascomycota Phylum, Fungi Kingdom) in the control-treated bumblebees while it was more homogenous and dominated by *Beauveria* genus in the BotaniGard-treated bumblebees.
When examining number of reads for the most abundant fungal genera (aside from *Beauveria*), we observed patterns associated with the experimental factors (**Figure 27B**). The abundance of *Saccharomyces* genus (28 ASVs) was high exclusively at baseline, when the hives were received from the manufacturer and thus before exposure to the treatment. In contrast, the abundance of *Cladosporium* (16 ASVs), *Penicillium* (27 ASVs), *Vishniacozyma* (8 ASVs) genera tended to be lower in BotaniGard-treated bumblebees.

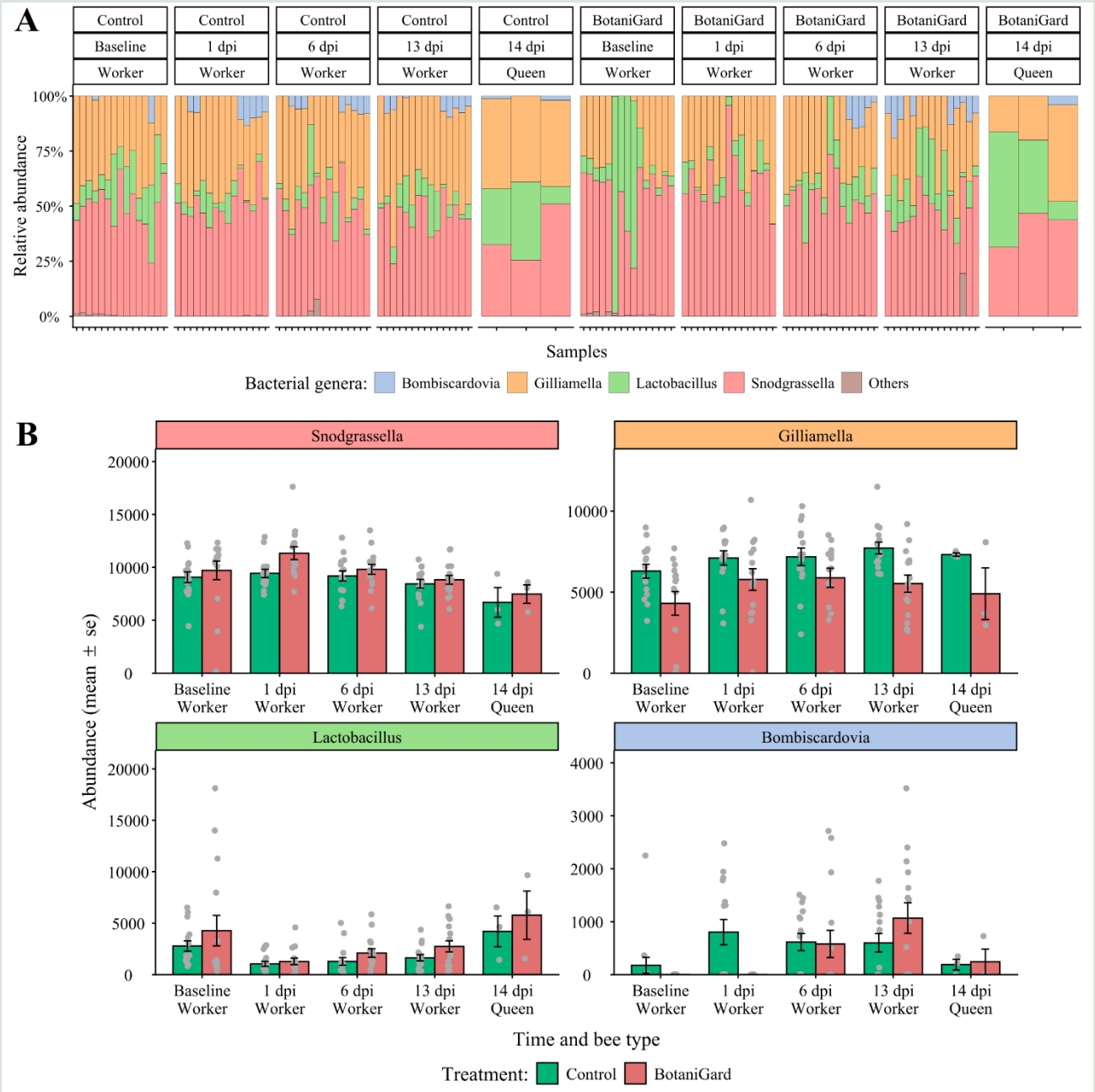

FIGURE 26. Composition of bumblebee gut bacterial communities at the genus level. (A) Whole community across all samples. (B) Most abundant genera, based on the total number of reads across the dataset; abundance is expressed as the number of reads, with grey dots corresponding to raw data. Colors are consistent between panels A and B, with each genus represented by the same color in both plots. “dpi” stands for days post inoculation. N = 15 workers per time and per treatment, 3 queens per treatment. “Other” refers to taxa which average abundance across the dataset was less than 1%.

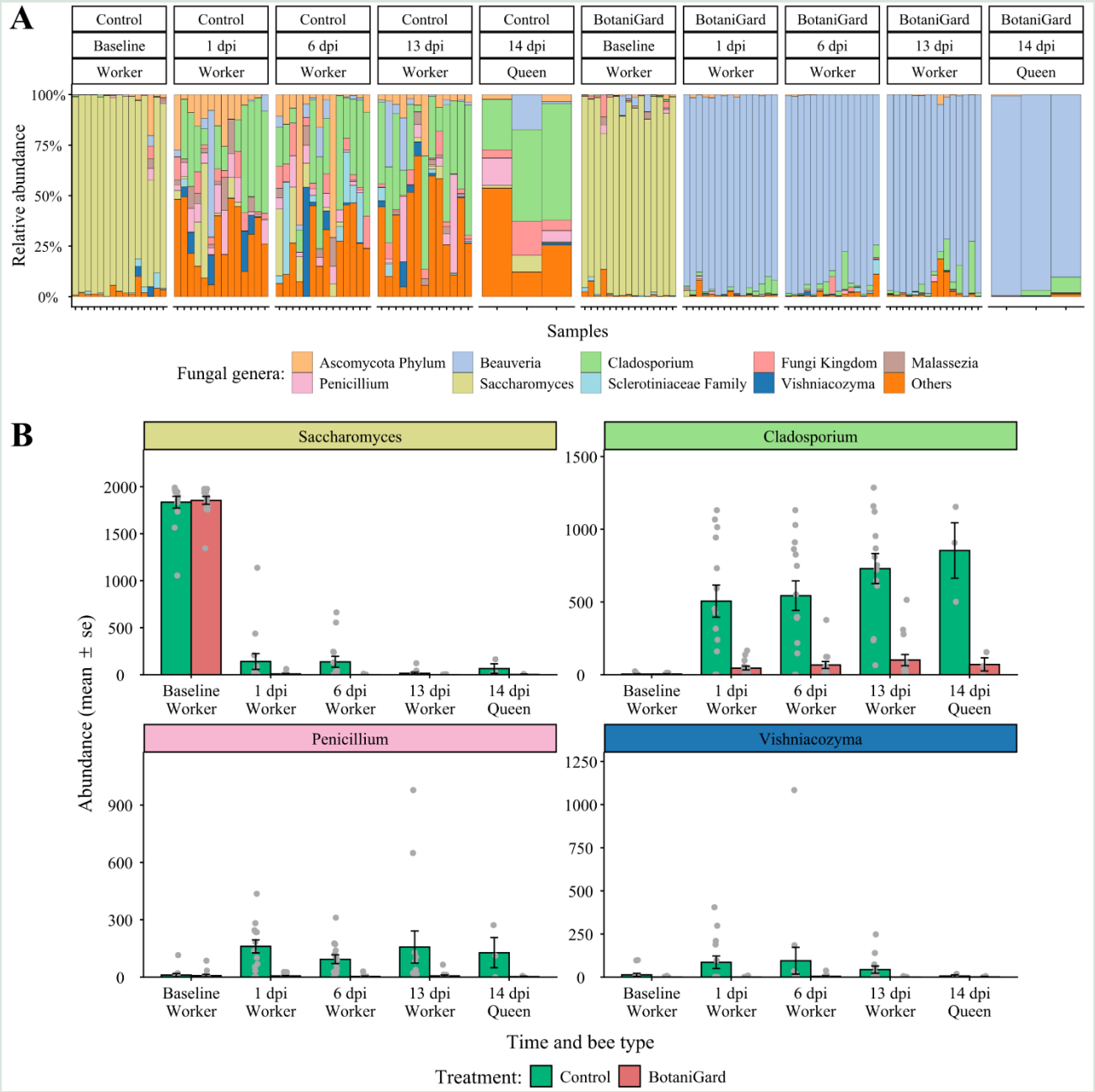

FIGURE 27. Composition of bumblebee gut fungal communities at the genus level. (A) Whole community across all samples. (B) Most abundant genera, based on the total number of reads across the dataset; abundance is expressed as the number of reads, with grey dots corresponding to raw data. Colors are consistent between panels A and B, with each genus represented by the same color in both plots. "dpi" stands for days post inoculation. N = 15 workers per time and per treatment, 3 queens per treatment. "Other" refers to taxa which average abundance across the dataset was less than 1%.

12.3.3 Hive parameters

At the end of the experiment, various parameters were measured on the hives once they have been emptied from the remaining bumblebees; a trend of higher weight in the BotaniGard-treated hives was seen (**Table 4**). On average, each colony gained 17–24 g in weight by building 16–24 cells. The remaining bumblebees each had an average individual weight of 70–74 mg.

TABLE 4. Hive parameters measured at the end of the colony setting experiment. Mean and standard error values are presented. N = 3 per treatment.

Hive parameters	Control	BotaniGard
Starting hive weight (g)	444.12 ± 1.38	443.99 ± 1.26
Ending hive weight (g)	461.72 ± 7.15	468.71 ± 4.48
Colony weight gain (g)	17.60 ± 7.90	24.72 ± 4.81
Number of built cells	16.33 ± 4.48	24.33 ± 8.11
Cell weight (g)	1.01 ± 0.20	1.19 ± 0.33
Average bumblebee weight (mg)	74.10 ± 8.27	70.39 ± 7.70

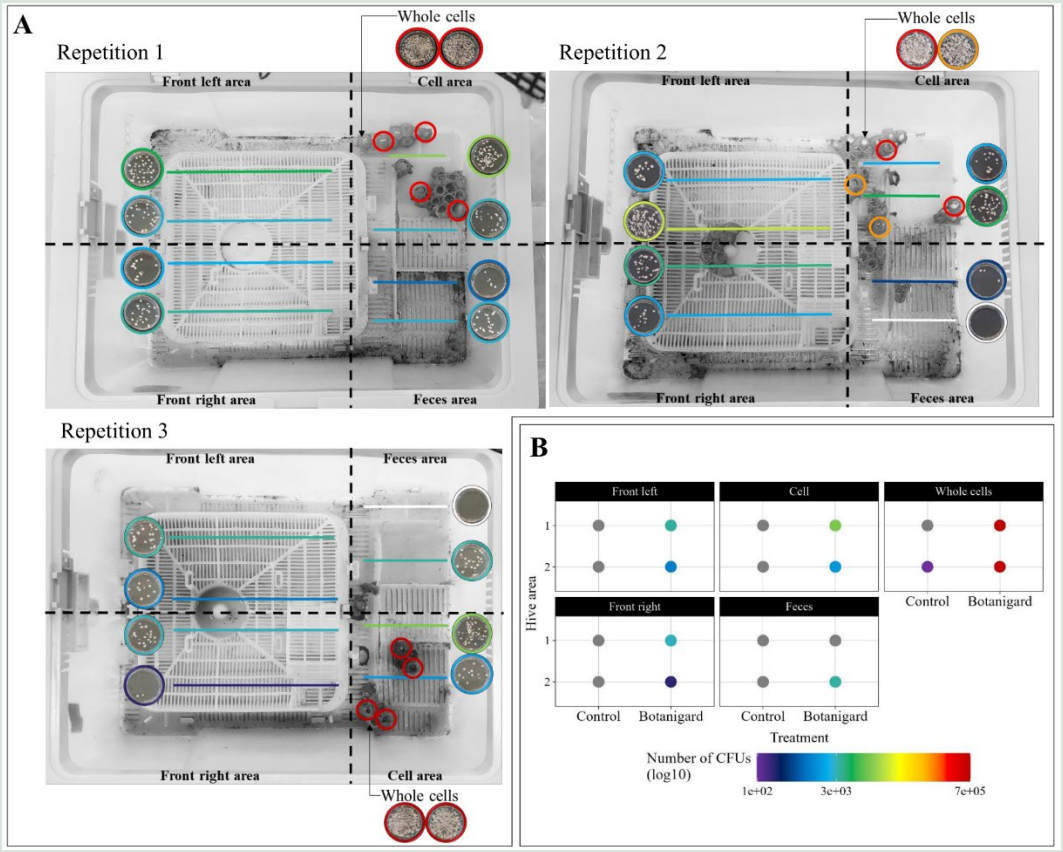

FIGURE 28. Detection of *B. bassiana* in the hives. Panel A shows the picture of the three "BotaniGard" hives used in the experiment. Each sample analyzed (*i.e.*, lines for swabs and circles for cells) from each hive is associated to the picture of the plate (dilution 0) and to a color corresponding to a number of CFUs indicated by the color key gradient of panel B. A white color in panel A corresponds to the absence of CFUs. Panel B shows the mean number of CFUs from the three hives for both control and BotaniGard treatments (N = 3). A grey color corresponds to the absence of CFUs.

The internal structure of each hive varied across repetitions. In repetition 1, distinct functional areas were clearly identifiable (*i.e.*, cell building and defecation areas), whereas in repetition 2, the cells built by the bumblebees were more dispersed. In repetition 3, a liquid had accumulated in the center of the hive (**Figure 28A**). A few CFUs were detected in 3 out of 6 cells samples from the control treatment. In contrast, *B. bassiana* was consistently detected and quantified in all BotaniGard-treated hives, ranging from 10^2 to 10^3 CFUs per swab line across the four identified areas of the hive and reaching up to 7×10^5 CFUs in the cell samples (**Figure 28B**). Interestingly, the

hive from repetition 1 exhibited higher fungal accumulation on swabs taken from the outermost swab line of the hive for each area, whereas the opposite trend was observed in repetitions 2 and 3.

13. Objective 2 – Discussion

In this study, direct exposure to BotaniGard (*B. bassiana* strain GHA) was lethal to bumblebees when isolated, while non-exposed bumblebees in a group suffered limited mortality in the laboratory. In contrast, significant mortality effects could not be detected in full hive setting over two weeks. When applied in the Flying Doctors® hive setting, BotaniGard was associated with modified bumblebee flight activity, gut microbial diversity and composition, while no clear effects on colony weight or architecture were observed despite high *B. bassiana* load inside the hive.

13.1 Lethal effects of *Beauveria bassiana*

Across the three experiments at different scales, the lethal effect of *B. bassiana* (BotaniGard) on *B. terrestris* workers depended strongly on levels of group complexity. In both the individual and group experiments, all tested doses of BotaniGard mixed with corn flour caused 100% mortality when bumblebee workers were directly exposed. In the group experiment, only the treated individuals all died, while untreated nestmates showed only a minor decline in survival. By contrast, exposure in the whole colony experiment had no detectable impact on hive survival.

Under laboratory conditions, Mommaerts et al. (2009) reported high mortality among *B. terrestris* workers following topical application of *B. bassiana* spore suspension, moderate and low mortality when ingested through sugar water and pollen, respectively. However, other studies showed contradictory results, where *B. bassiana* ingestion reduced *B. terrestris* survival, whereas topical application at the same concentration had no effect (Leite et al., 2022). A surprising contrasting result was reported by Karise *et al.* (2016), who covered the bodies of *B. terrestris* workers with 50 mg of pure BotaniGard powder (approx. 10^9 CFU) and observed reduced longevity at 18°C but not at 28°C, although mycelial growth occurred only at 28°C. In our individual and group experiments, conducted at 28°C, direct exposure of individual bumblebees carried around 10^7 CFU and all experienced complete mortality. A possible explanation for this discrepancy is variation in spore viability among BotaniGard batches. In the present experiments, spore viability exceeded 95% before each experimental application. Notably, one batch from another provider displayed lower viability (87%) and was therefore excluded from experiments (personal observation).

In the colony experiment in mesh cages under greenhouse tunnel conditions, two exposure pathways occurred: subjacent exposure, where foragers contacted BotaniGard powder as they exited the hive (approx. 10^5 CFU/ bumblebee), and secondary exposure, where individuals inside the hive contacted residues brought back by returning foragers (10^2-10^3 up to 10^5 in the cells) during two weeks. However, neither exposures had any measurable impact on bumblebees' survival. Our results differ from those of Demirozer et al. (2022), who used a liquid suspension of *B. bassiana* (Nostalgist, commercial product) and found that both topical application and 15-minute residue exposure induced mortality in *B. terrestris*, with residue exposure having the stronger effect. However, our results also partially resemble studies by Kapongo, Shipp, Kevan, & Sutton (2008) who reported lack of mortality in *B. impatiens* on tomato crops when bumblebees vectored *B. bassiana* and *C. rosea*, and some mortality on pepper crops, though it was unclear whether this effect was due to one fungus or their combination. In a related study, Kapongo, Shipp, Kevan, & Broadbent (2008) showed that *B.*

bassiana did not reduce *B. impatiens* survival at low powder doses (10^9 spores/g, comparable to the present D15 powder), but mortality increased at medium and high doses (10^{10}–10^{11} spores/g) on tomatoes, and at all doses on peppers. Thus, impact of *B. bassiana* may be dependent on interaction with crop species.

In all three experiments, bumblebees encountered BotaniGard powder by passing through a dispenser, detached in the laboratory trials and mounted in the Flying Doctors® hive in the greenhouse tunnel experiment. Two main factors likely explain the difference of survival between laboratory and greenhouse outcomes: first, the estimated dose per bumblebee was considerably higher in the laboratory (approx. 10^7 CFU/ bumblebee) compared with the greenhouse experiment (approx. 10^5 CFU/ bumblebee), and second, bumblebees inside the hive may have benefitted from social immunity (Cremer et al., 2018). This phenomenon includes collective behaviors and physiological responses that limit pathogen spread among nestmates despite congregations of closely related individuals. Bumblebees show hygienic behavior within the hive, such as removing dead individuals from the hive (Munday & Brown, 2018). Group size also influences infection dynamics: infection intensity was found to be lower in larger groups, although infections with *Crithidia bombi* (*i.e.*, a gut parasite) reduced bumblebee survival more strongly in large groups (Valdes et al., 2025). Physiologically, social living enhances immune activation. Bumblebees kept in groups upregulated antimicrobial peptide (AMP) and lysozyme gene expression compared with isolated individuals (Richter et al., 2012). Similarly, exposure to *B. bassiana* increased expression of the AMP abaecin in *Bombus lantschouensis* (L. Wang et al., 2021), indicating that immune responses within the colony may help mitigate pathogen effects.

13.2 Sub-lethal effects of *Beauveria bassiana* on activity and development within the hive

In the greenhouse tunnel experiment in mesh cages, we monitored bumblebee flight activity at 9:15 (15 minutes after loading the dispenser) and again at 12:00, two to three times per week for two weeks. This schedule aligns with natural daily foraging patterns: bumblebee abundance in the field peaked around midday and afternoon, while hive return rates were highest in the morning and again later in the day (Karbassioon & Stanley, 2023).

Flight activity in the BotaniGard hive was overall higher than in the control hive, although this difference was driven primarily by data of the first repetition, where the BotaniGard hive was already more active prior to powder application. Repetitions 2 and 3 showed, respectively, relatively low and medium activity in both treatments, resulting in substantial variability across hives and experimental runs. Previous studies using BotaniGard either reported no detectable effects on bumblebee flight behavior, although these were conducted without a control hive for comparison (Coates et al., 2023), or did not assess flight activity (Kapongo, Shipp, Kevan, & Broadbent, 2008; Kapongo, Shipp, Kevan, & Sutton, 2008). Our findings are consistent with research using other microbial control agents, such as *C. rosea* and *Aureobasidium pullulans*, which were similarly reported to have no negative effects on *B. terrestris* flight activity in greenhouse settings (Iqbal et al., 2022; Mommaerts et al., 2011). In our study, bumblebee activity was negatively associated with humidity, temperature, and atmospheric pressure, and positively associated with baseline hive activity, reflecting the previously mentioned variability among hives. These patterns partially align with H. Wang et al. (2024), who also observed a negative correlation with humidity but a positive correlation with temperature, likely due to methodological differences: discrete monitoring (morning and midday observations over two weeks) of the present study contrasts with their continuous monitoring over 26 days.

Limited physiological effects of exposure to *B. bassiana* has been shown in bees, while some behavioral changes are reported. Karise et al. (2016) found that BotaniGard had no impact on the metabolic rate or water-loss components in *B. terrestris*. In contrast, Dos Santos et al. (2025) reported that another commercial *B. bassiana* formulation (Boveril) reduced the duration of aggressive interactions toward both nestmates and non-nestmates in the stingless bee *Scaptotrigona depilis*.

In the present study, BotaniGard had no clear effect on hive development during two weeks of exposure, including hive weight gain, cell construction, and worker weight, although the limited number of hives per treatment (N = 3) restricts the strength of this conclusion. Comparably, Iqbal et al. (2022) found no effect of the yeast *A. pullulans* on weight of Flying Doctors® hives over time. Results from other studies using commercial *B. bassiana* products vary. Mommaerts et al. (2009) reported a reduction in drone production following topical application of BotaniGard. Conversely, Demirozer et al. (2022) found no impact of *B. bassiana* (Nostalgist formulation) on emergence timing of males and queens or the total number of workers produced. In our experiment, the absence of detectable effects on hive growth may be related to variability in hive activity, particularly repetition 2, where both hives showed minimal activity. However, the fungus was consistently detected inside all BotaniGard hives at comparable levels. We also observed that the bumblebees compartmentalized hive space for different functions (*e.g.*, cells vs. waste), consistent with the patterns described by Valdes & Scofield (2023). Finally, the duration of the colony experiment of two weeks in greenhouse conditions may have been too short to capture potential effects on hive development, especially when compared with earlier studies that monitored colonies for substantially longer periods: from 11 weeks (Mommaerts et al., 2009) to the end of the colony life cycle (Demirozer et al., 2022).

13.3 Sub-lethal effects of *Beauveria bassiana* on gut microbiota

The data suggest that BotaniGard was ingested by bumblebees, as *B. bassiana* sequences were detected in gut samples. Although no previous studies have examined the effects of entomopathogenic fungi on gut microbiota of social bees, investigations of specific bee parasites and pathogens, including *Crithidia bombi*, *Nosema ceranae*, *Paenibacillus larvae* (American foulbrood disease), and *Ascosphaera apis* (chalkbrood disease) have been carried out as further detailed below.

Although alpha diversity indices (Shannon and Pielou) were initially different between the BotaniGard and control treatment workers at baseline, reflecting variation among hives, exposure to BotaniGard increased these indices over time. Fungal richness and diversity of both workers and queens increase from baseline in the control group, but it remained comparably low in the BotaniGard treatment. Recovery of gut bacterial diversity over time is consistent with findings that *N. ceranae* infection decreased bacterial alpha diversity in honeybees, *Apis mellifera* (L. Liu et al., 2024), that bacterial alpha diversity in *B. impatiens* was negatively correlated with *C. bombi* load (Mockler et al., 2018), and that both bacterial and fungal diversity were reduced in *A. mellifera* larvae infected with *P. larvae* and with *A. apis* (Ye et al., 2021). In contrast, Straw et al. (2023) reported no effect of *C. bombi* infection on bacterial alpha diversity in *B. terrestris*, indicating that responses may vary across host-pathogen combinations.
BotaniGard exposure also affected microbial beta diversity by altering the structure of both bacterial and fungal communities. This pattern aligns with studies showing that bacterial gut community structure shifts in response to *C. bombi* infection in *B. terrestris* (Blasco-Lavilla et al., 2024), *N. ceranae* infection in honeybees (L. Liu et al., 2024), and changes in both bacterial and fungal community structures following *P. larvae* and *A. apis* infection (Ye et al., 2021).

The relative composition of bacterial communities in our study remained stable across treatments (control vs. BotaniGard) and bee types (workers vs. queens), with the gut microbiota consistently dominated by the genera *Snodgrassella*, *Gilliamella*, *Lactobacillus*, and to some extend of *Bombiscardovia*. This pattern is consistent with Billiet et al. (2017) and with Su et al. (2021), both of whom reported the same four dominant bacterial taxa in adult workers, with Su et al. (2021) also noting similar compositions between workers and queens. Furthermore, the stable composition observed here aligns with Blasco-Lavilla et al., (2024), who found that *C. bombi* infection did not seem to alter bacterial community composition in bumblebees with a restored microbiota.
Although the number of reads of one dominant gut bacteria, *Gilliamella*, was lower in the BotaniGard treatment group at baseline, the pattern remained consistent over time. Earlier work showed that *N. ceranae* infection decreased *Gilliamella* abundance in honeybees (L. Liu et al., 2024) and that *Gilliamella* abundance in *B. impatiens* was negatively correlated with *C. bombi* load, while *Snodgrassella* was not correlated (Mockler et al., 2018). This matches our observation that *Snodgrassella* appeared unaffected by *B. bassiana*. Both bacterial genera have important roles in the bumblebee gut: *Snodgrassella* and *Gilliamella* contribute to nutrient metabolism, by degrading carboxylates and carbohydrates respectively, and formation of biofilm coating the ileum wall (Hammer et al., 2021; Kwong et al., 2014). In contrast, abundance of *Lactobacillus* was unchanged following BotaniGard exposure. Changes in this genus might have been expected under some pathogen pressures, as *C. bombi* infection increased *Lactobacillus* abundance in a previous study (Blasco-Lavilla et al., 2024), and several *Lactobacillus* species isolated from bumblebee guts show antimicrobial activity against pathogens such as *P. larvae* (Praet et al., 2018).

The relative composition of the gut fungal communities in our study differed clearly between treatments, with *B. bassiana* dominating the gut microbiota of BotaniGard-treated bumblebees, while control individuals exhibited a higher abundance of *Cladosporium* and a variable assemblage of unidentified fungal taxa. Bumblebees originating from the commercial rearing facility (contained indoor environment) initially harbored high levels of *Saccharomyces* in their gut communities, but the dominance of yeast disappeared once the experiment began in the greenhouse. The presence of yeasts is consistent with Brysch-Herzberg (2004), who detected several yeast species in the guts of *Bombus* workers. The shift observed after moving indoor-reared bees into greenhouse conditions also aligns with Parmentier et al. (2016), who reported differences in bacterial community composition between commercially reared colonies and those placed outdoors. The observed transition from *Saccharomyces* dominance to a diverse gut fungal community in the control treatment may reflect exposure to the environment although no untreated control hives were analyzed. Similarly, the queens of the control treatment hives did not show high abundance of *Saccharomyces*, but reflection of worker gut communities.
In our study, *Beauveria* was the most abundant fungal genus in the bumblebee gut communities, because of the exposure to BotaniGard, which consequently led to a decrease of fungal diversity. Surprisingly, ASV_39, resembling *B. pseudobassiana*, was also relatively abundant in the gut of BotaniGard treated workers and queens. This species is naturally occurring in Europe and infects insect hosts, mainly in the above-ground environment (Pedrazzini et al., 2024), including in Denmark (Meyling et al., 2009). The consistent occurrence of ASV_39 in the BotaniGard treatment could indicate presence of a fungal contaminant during the preparation of the experimental powder, but this would require further detailed analyses. In addition, the species *B. loeiensis*, only described from Thailand (Ariyawansa et al., 2015), was detected in both BotaniGard and occasionally in control treatments.

Dominance of applied entomopathogenic fungi in microbial communities of insects have been documented in other systems. Yang et al. (2025) reported that the inoculated entomopathogenic fungus *Metarhizium anisopliae* constituted the predominant fungal taxon in the gut of the predatory dock leaf bug (*Arma custos*), independent of the inoculation dose. Ye et al. (2021) further documented that pathogens can also dominate microbial communities of *A. mellifera* under natural infection conditions. Further, *B. bassiana* can produce toxic secondary metabolites with antimicrobial properties (Ávila-Hernández et al., 2020; H. Wang et al., 2021), which may affect the abundance of microbial taxa in the gut.
Our study showed a decrease in the number of reads of the fungal genera *Cladosporium*, *Penicillium*, and *Vishniacozyma* after exposure to BotaniGard. All three genera have been reported as often detected from honey (Luca et al., 2024). Ye et al. (2021) also detected *Cladosporium* and *Penicillium* at low abundance in healthy *A. mellifera* larvae but not in diseased larvae. *Cladosporium* includes species with diverse roles: some are strawberry pathogens (Ayoubi et al., 2018), while others act as biocontrol agents against the plant pathogen *Botrytis cinerea* (Pan et al., 2023). *Penicillium* species are ubiquitous opportunistic fungi, which have been isolated from commercial bumblebee colonies, more specifically from honey cup walls (Chow et al., 2024). The genus *Vishniacozyma* represent yeast species with antagonistic abilities; two species were shown to control fungal pathogens on apples, including *B. cinerea* (Sánchez et al., 2026). In our study, all *B. terrestris* colonies underwent a standardization procedure that included thorough cleaning of the plastic hive; although residual molds may have persisted or re-established, their abundance was lowest in the bumblebees sampled upon hive receipt (*i.e.*, baseline). Additional sources of these fungi could include the pollen or strawberry flowers accessible to the bumblebees from the start of the greenhouse experiment.

Plant-derived sequences, including those from the strawberry genus *Fragaria*, were detected in the bumblebee gut (**Appendix 6**). This suggests that the ingestion of pollen and nectar may introduce microorganisms originating from floral tissues, which may help explain the increase in fungal diversity observed in control bumblebees at 1 dpi. According to Olimi et al. (2022), the microbiota of strawberry flowers is dominated by the bacterial genera *Sphingomonas*, *Pseudomonas*, *Pantoea*, *Acinetobacter*, and *Massilia*, as well as the fungal genera *Mycosphaerella*, *Alternaria*, and *Cryptococcus*. In our dataset, there was limited overlap between the bumblebee gut microbiota and the flower-associated taxa previously reported, with only a few genera detected at low abundance: *Pseudomonas* (1 ASV), *Acinetobacter* (1 ASV), *Alternaria* (13 ASVs), and *Cryptococcus* (6 ASVs). Temmermans *et al.* (2025) similarly reported, based on empirical data, that only a subset of bacterial ASV taxa is shared between floral and bumblebee microbiota, which also differ between open field and tunnel experiment (*i.e.*, the genera *Prevotella*, *Leuconostoc*, *Schwartzia*, *Staphylococcus* and *Pseudomonas* in open field vs. the genera *Bacillus*, *Staphylococcus* and *Pseudomonas* in tunnel environments).

Overall, the colony context appeared to buffer the effects of exposure to *B. bassiana*, maintaining individual survival, flight activity and colony growth during two weeks of experiments. Future studies should extend experimental time over the expected colony life span for commercial hives (6-8 weeks) to ensure that the unaffected colony viability and activity was not due to limited observation period. Applying entomopathogens by api-vectoring should include risk assessment for the pollinator vector.

14. Conclusions

Api-vectoring of the biopesticide BotaniGard WP (active ingredient *B. bassiana* strain GHA) by *B. terrestris* in greenhouse experiments showed potential in targeted vectoring of entomopathogenic fungal spores to strawberry flowers with at least one week of persistence under fluctuating ambient environmental conditions. However, achieving effective suppression of *F. occidentalis* thrips populations in strawberry flowers under such greenhouse conditions appears challenging, likely because suboptimal environmental conditions hampered biocontrol efficacy. Although the biopesticide is effective in killing different developmental stages of *F. occidentalis* in laboratory settings, the findings highlight the importance of appropriate microclimatic conditions when evaluating the feasibility of applying fungal biocontrol agents through pollinator-mediated delivery.

Although the results indicate that BotaniGard poses substantial risks to individual *B. terrestris* workers exposed under laboratory conditions, repeated exposure appears far less detrimental at the colony scale in ambient greenhouse conditions using Flying Doctors® hives. Despite considerable fungal occurrence in hives and bumblebee guts, flight activity and hive weight gain remained unaffected, suggesting that *B. terrestris* colonies may tolerate the exposure to entomopathogenic fungi without health impairment, at least during the experimental period of two weeks.

Research perspectives

A key challenge for improving the efficacy of api-vectored *B. bassiana* in strawberry flowers would be alignment of greenhouse climatic conditions to meet the requirements of temperature and particularly relative humidity conducive for successful fungal infections. However, increased relative humidity may stimulate plant pathogens, such as *B. cinerea*, which would compromise the biocontrol strategy. Potentially, co-vectoring of BotaniGard with *C. rosea* (commercial product PreStop Mix WP) to target both insect pests and plant pathogens in the flowers simultaneously could be possible with commercial Flying Doctors® hives. Combination of the commercial formulation of *C. rosea* against grey mold with BotaniGard against greenhouse whitefly and tarnished plant bug was tested by Kapongo, Shipp, Kevan, & Sutton (2008), while the challenge of appropriate timing of elevated humidity, *e.g.*, by mist generation, must be addressed.

So far, research on the potential of entomo-vectoring has mainly focused on bees (Temmermans & Smagghe, 2022). Other pollinators such as hoverflies (Syrphidae), of which many species are aphid predators in the larval stage, have shown potential for vectoring biocontrol agents, with hoverfly-mediated delivery reducing grey mold and improving fruit quality of strawberries (Rehermann et al., 2026).

Practical implications

Climatic conditions in the strawberry production system should be adjusted to match the requirements for efficacy of microbial formulation used in api-vectoring while remaining compatible with natural enemies, crop growth, and disease management. Flying pests such as thrips and whiteflies could be reduced using yellow and blue sticky traps[v] before introducing Flying Doctors® hives, as bumblebees are sensitive to ultraviolet wavelengths (Balamurali et al., 2018; Gumbert, 2000; Raine & Chittka, 2007). During flowering, 1-2 Flying Doctors® hives should be installed per 1000 m^2 tunnel[vi]

[v] Sticky traps: http://bioplant.dk/produkter/fangplader/fangplader.aspx

[vi] Overview of hive types: http://bioplant.dk/media/43502/Humlebier%20til%20best%C3%B8vning_oversigt%202018.pdf

for 4–8 weeks (*i.e.*, hive lifespan) depending on the hive size, and the hive dispenser filled with BotaniGard mixed powder. For *B. bassiana*, maintaining >75% relative humidity for 48 h would meet labelled requirements of BotaniGard[iv] to support fungal infection, though humidity must be controlled carefully, as high levels also favor development of plant pathogens (Morandi et al., 2006). Approaches to increase humidity without raising disease pressure could include short, frequent misting events; *e.g.*, 1-min pulses of water misting four times daily were equally effective as chemical fungicide applications against powdery mildew (Asalf et al., 2021). Compatible biocontrol agents can further support disease suppression under higher humidity. Prestop WP[vii], for instance, is recommended during flowering against grey mold at 600–1200 L/ha of a 0.5% solution (3–6 kg/ha), providing *B. cinerea* control while allowing humidity levels that support microbial establishment. Prestop Mix WP[iii] is also targeting *B. cinerea*, but is designed for delivery by api-vectoring in the hive dispenser for Flying Doctors® hives. Natural enemies tolerating high relative humidity should also be considered. Predatory mites show species-specific humidity preferences (Ferrero et al., 2010) with *Amblyseius swirskii*[viii] responding well under high humidity conditions, and some Syrphidae (hoverflies) species[ix] maintaining activity at night. *Lobularia maritima*[x], a plant species of Brassicaceae with extended flowering, could supply pollen to pollinators and predators (*e.g.*, *Orius* spp. for thrips control) when floral resources in the main crop become limited, and it may function as a trap plant (Koller et al., 2024); 10–15 plants/ha are recommended.

Effective api-vectoring further depends on managing both bumblebee hive activity and maintain enough microbial control product inoculum in the dispenser. Dispenser should be refilled twice weekly, as recommended for Prestop Mix WP[iii] for Flying Doctors® hives, since the bumblebees readily empty them. Because bumblebee foraging activity can fluctuate, an acclimation period before flowering may ensure consistent delivery. Addressing some of these approaches could facilitate the practical implementation of api-vectoring for wider use within integrated pest and disease management in future strawberry tunnel production.[iii]

[vii] Prestop WP label: http://uk.bioplant.dk/media/54453/prestop_wp_880-4_godkendt_etikette_20220525.pdf
[viii] Information on *A. swirskii*: http://bioplant.dk/media/44787/Swirskii.pdf
[ix] Information on Syrphidae: https://www.agrobio.es/products/pest-control/sirficontrol-sphaerophoria-rueppellii-aphid-control/?lang=en
[x] Information on *L. maritima*: https://www.agrobio.es/products/pest-control/planta-lobularia/?lang=en

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16. Outreach

16.1 Educational

A video (English with Danish subtitles) was made in 2024 for broad audience and posted by the University of Copenhagen on LinkedIn: link to video.

The project was used as **teaching material** in the "Applied Insect Ecology and Biological Control" course (https://kurser.ku.dk/course/nplk18001u). Students gained the ability to understand the principles and challenges of using entomo-vectoring for biological control in crop protection and to analyze ecological and health implications through risk assessment of introducing biocontrol agents on non-target organisms. They also developed the ability to design and plan biological experiments by considering organism interactions, environmental influences, and research transitions, while applying collaborative and problem-solving approaches by identifying challenges, proposing solutions, and valuing teamwork in research.

16.2 Scientific

Two scientific manuscripts are currently in preparation, one for each objective of this report, and will be submitted to peer-reviewed journals.

2024 conferences:

- **Fungal Research Symposium**; May in Copenhagen (Denmark). 50 people attended. Talk given by Annette Bruun Jensen
- **SIP 2024**, International Congress on Invertebrate Pathology and Microbial Control and 56th Annual Meeting of the Society for Invertebrate Pathology; July-August in Vienna (Austria). 400 people attended. Talk given by Nicolai Vitt Meyling
- **EurBee 10**, 10th European Conference of Apidology; September in Tallinn (Estonia). 350 people attended. Talk given by Marta Montoro

2025 conferences:

- 49th **APIMONDIA** Congress; September in Copenhagen. 8000 people attended. Poster presented by Morgane Ourry (**Figure 29**)
- **Plant Biologicals Network Symposium**; November in Copenhagen. 150 people attended. Fash talk and poster presented by Morgane Ourry

2026 conference:

- XIII **European Congress of Entomology** (ECE 2026), June-July in Tours (France). Talk given by Morgane Ourry

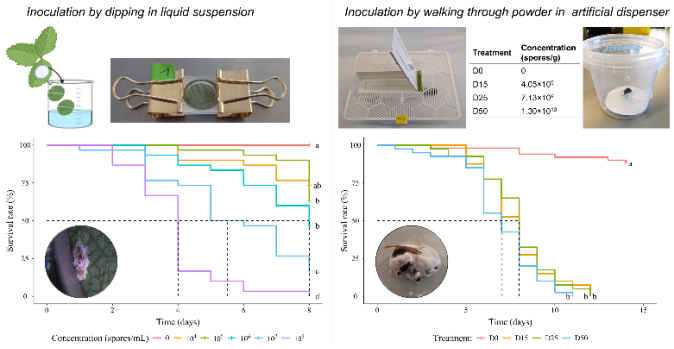
Api-vectoring potential of the entomopathogenic fungus *Beauveria bassiana* by *Bombus terrestris* for thrips control in strawberry production

Morgane Ourry, Marta Montoro, Søren S. Henriksen, Anastasia Karatza, Katja K. Nielsen, Paulo Stipanic, Zhicong Wu, Shuhan Zhang, Antoine Lecocq, Annette B. Jensen, Nicolai V. Meyling

Background and objective

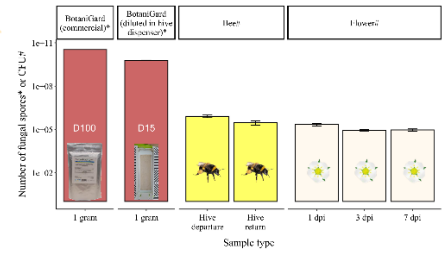
Thrips are an increasing problem in early-season tunnel strawberry production, with limited control options available. The fungus *Beauveria bassiana* shows potential as a microbial control agent, though direct targeting of thrips is difficult. While api-vectoring using the 'Flying Doctors' hive (BioBest, Belgium) is used against grey mold (Iqbal *et al.*, 2022), its application for insect pest control remains largely unexplored (Temmermans & Smagghe, 2022). **Project BUMBLEBIO aimed to evaluate the possibility of api-vectoring BotaniGard by bumblebees for thrips (*Frankliniella occidentalis*) control in strawberry crops.**

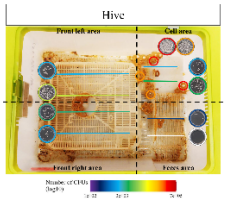
How lethal is BotaniGard to thrips and to bumblebees in lab conditions?



- *Beauveria bassiana* suspensions **(≥10^5 spores/mL)** applied to leaf discs significantly **reduced survival in adult thrips, but also in larvae**.
- The biopesticide BotaniGard (*B. bassiana*), diluted with corn flour, caused **0% survival in individually exposed bumblebees to the tested doses** (D15, D25, D50).
- **Observation of fungal growth** on cadavers confirmed that death was caused by *B. bassiana* in both thrips and bumblebees bioassays.

How much BotaniGard can be vectored by bees?





- Bumblebees **carried and deposited 10^5 CFUs** of fungus on flowers.
- The same fungal load remained **detectable for 7 days** in flowers.
- Fungal residues were also **traced back to the hive**, with:
  - 10^2–10^3 CFUs in hive areas
  - Up to 10^5 CFUs in cells

Is api-vectoring of BotaniGard efficient for thrips control in greenhouse conditions?

Scan here—this bee's got a video to show you!

Heterogenous hives and bee activity

Standardized flying hives

Treatment	Average bee survival rate (SE)	
Control	87.36% (3.27)	ns
BotaniGard	92.71% (4.36)	

Treatment	Repetition	Average number of individuals per flower (SE) Larva		Adult	
Control	R1-R2	9.69 (1.28)	ns	0.92 (0.11)	ns
BotaniGard		9.78 (0.92)		0.70 (0.09)	
Control	R3	6.89 (1.49)	ns	0.61 (0.15)	ns
BotaniGard		6.64 (1.80)		0.47 (0.10)	

- Api-vectored BotaniGard had **no effect on the number of thrips (adults or larvae)** and very few individuals were infected by *B. bassiana*.
- A **microclimate gradient** (Δ_{RH} = 7 to 18%, Δ_{temp} = 2 to 4°C) was observed in the greenhouse: the **number of larvae was correlated**, negatively to air relative **humidity** and positively to air **temperature**.
- Surprisingly, **no impact of BotaniGard on bee survival** was observed in standardized hives (*i.e.*, contained the same number of bees).

Conclusions

BotaniGard was successfully vectored to flowers by bumblebees, despite variability in hive activity, and persisted over time. However, its lethality against both thrips and bumblebees appeared reduced under greenhouse conditions—less harmful to the bees, but less effective for pest control. This reduced efficacy may result from variable climate in the greenhouse that favor thrips reproduction while hindering fungal pathogenicity.

References: Iqbal *et al.* (2022), doi: 10.1094/PHYTO-05-21-0205-R; Temmermans & Smagghe, 2022. doi: 10.20506/rst.41.1.3308

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Ministry of Environment and Gender Equality
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FIGURE 29. Poster presented at conferences in 2025.

Appendixes

APPENDIX 1. Statistical output of univariate analyses performed on activities related to objective 1. For each analysis, the model, formula, test value, degree of freedom (df) and p-value are given. Significant terms and their associated p-value are written in bold.

Experiment	Response (y)	Model	Formula	Term	Test value#	df	p-value	
Toxicity to thrips on disc leaf								
Dose Response of adults	Survival curves	coxphf	y ~ Concentration	**Concentration**	113.46	5	**<0.001**	***
	Mycosis rate	lm	y ~ Concentration	**Concentration**	76.96	5	**<0.001**	***
				Residuals	_	12	_	
Dose Response of larvae	Survival curves	coxphf	y ~ Concentration	**Concentration**	128.99	5	**<0.001**	***
	Mycosis rate	lm	y ~ Concentration	**Concentration**	145.07	5	**<0.001**	***
				Residuals	_	12	_	
Toxicity to thrips in artificial flower setup								
Pilot experiment	Proportion of dead thrips	lm	y ~ Treatment	**Treatment**	36.23	3	**<0.001**	***
				Residuals	_	14	_	
	Number of larvae per adult	lm	y ~ Treatment	**Treatment**	7.87	1	**0.014**	*
				Residuals	_	14	_	
Experiment 1	Proportion of dead thrips	lmer	y ~ Treatment + (1|Repetition)	Treatment	6.01	2	0.050	*
	Number of larvae per adult	lm	y ~ Treatment	Treatment	2.97	2	0.061	.
				Residuals	_	46	_	
Experiment 2	Proportion of dead thrips	lm	y ~ Treatment	Treatment	2.52	2	0.101	
				Residuals	_	24	_	
	Number of larvae per adult	lm	y ~ Treatment	Treatment	2.18	2	0.135	
				Residuals	_	24	_	
Experiment 3	sqrt(Proportion of dead thrips)	lm	y ~ Treatment	**Treatment**	30.03	2	**<0.001**	***

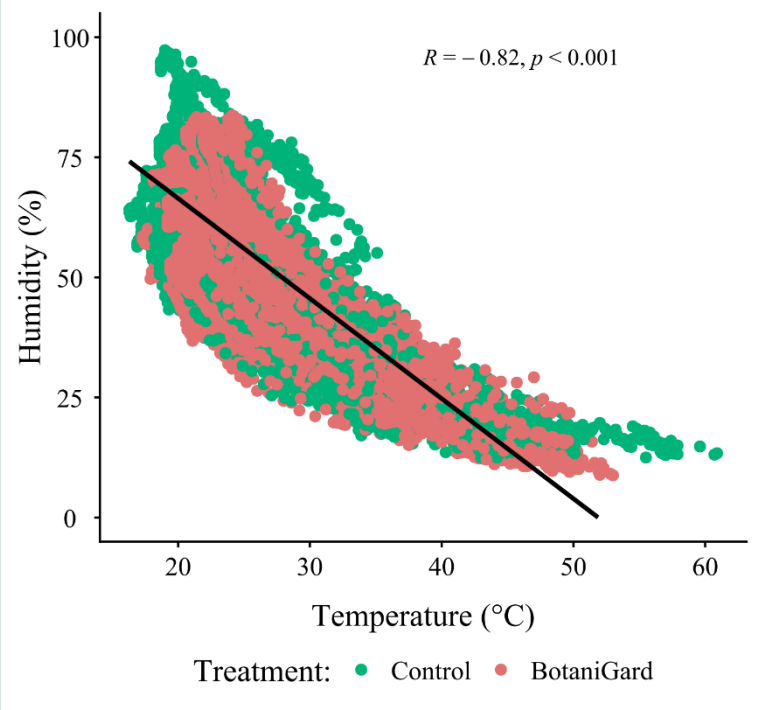
Experiment	Response (y)	Model	Formula	Term	Test value#	df	p-value
				Residuals	_	33	_
	Number of larvae per adult	lm	y ~ Treatment	Treatment	0.65	2	0.530
				Residuals	_	33	_
Application of api-vectoring							
Repetitions 1 and 2	Number of larvae per flower	lmer	y ~ Treatment + (1|ThripsCohortAge) + (1|Temperature)	Treatment	0.001	1	0.973
	Number of adults per flower	lm	y ~ Treatment	Treatment	2.33	1	0.135
				Residuals	_	38	_
Repetition 3	Number of larvae per flower	lm	y ~ Treatment	Treatment	0.01	1	0.915
				Residuals	_	18	_
	Number of adults per flower	lm	y ~ Treatment	Treatment	0.63	1	0.439
				Residuals	_	18	_

#Type II analysis of variance differs based on the model used: Wald chi-square test for Cox Regression with Firth's Penalized Likelihood (coxphf) and for linear mixed model (lmer); F-test for linear model (lm).
.: P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001

APPENDIX 2. Descriptive statistics of the climatic variables measured during summer 2025 in Denmark in bumblebee mesh cages and in insect tents. Recordings were performed every 10 minutes from the moment hives were placed into the cages until they were removed, or thrips were introduced into the tents until sampling was performed. SE stands for standard errors. Day and Night correspond to daytime (06:00-22:00) and nighttime (22:00-06:00). The warmer and less humid side of the first insect tent setup (repetitions 1 and 2, **Figure 11A**) is indicated with #.

Variable	Metrics	Time	Repetition 1		Repetition 2		Repetition 3	
Bumblebee cages – 3 weeks								
			Control	BotaniGard	Control	BotaniGard	Control	BotaniGard
Temperature (°C)	Mean ± SE	Day	25.03 ± 0.13	25.15 ± 0.14	27.16 ± 0.14	27.16 ± 0.14	25.63 ± 0.13	25.03 ± 0.13
		Night	14.38 ± 0.07	14.36 ± 0.07	17.70 ± 0.06	17.68 ± 0.06	16.53 ± 0.05	16.54 ± 0.05
	Minimum	Day	11.20	10.60	14.00	14.30	13.00	13.00
		Night	9.50	9.50	12.80	12.80	12.60	12.60
	Median	Day	25.00	25.20	26.10	25.90	25.50	24.80
		Night	14.20	14.10	17.70	17.70	16.60	16.60
	Maximum	Day	39.10	39.70	42.50	42.60	41.20	40.70
		Night	21.90	22.10	23.00	22.90	22.40	22.40
Humidity (%)	Mean ± SE	Day	45.85 ± 0.37	45.67 ± 0.38	52.07 ± 0.39	52.27 ± 0.40	50.38 ± 0.39	52.08 ± 0.39
		Night	79.13 ± 0.28	79.11 ± 0.29	83.52 ± 0.21	83.84 ± 0.22	80.47 ± 0.24	80.91 ± 0.25
	Minimum	Day	14.50	15.60	18.20	19.90	19.70	18.60
		Night	48.50	48.70	53.00	54.50	57.70	58.10
	Median	Day	41.30	41.30	50.60	51.05	47.35	49.80
		Night	80.95	80.70	84.90	85.00	81.50	81.90
	Maximum	Day	92.60	93.00	92.00	92.60	93.00	94.00
		Night	94.00	95.00	94.50	95.70	96.60	97.80
Number of recordings		Day	2299	2299	2290	2290	2284	2284
		Night	1152	1152	1152	1152	1152	1152

Variable	Metrics	Time	Repetition 1				Repetition 2				Repetition 3			
Insect tents – 1 week														
			Sub-repetition 1		Sub-repetition 2		Sub-repetition 1		Sub-repetition 2		Sub-repetition 1		Sub-repetition 2	
			Control	BotaniGard#	Control	BotaniGard#	Control	BotaniGard#	Control#	BotaniGard	Control	BotaniGard	Control	BotaniGard
Temperature (°C)	Mean ± SE	Day	27.52 ± 0.20	31.69 ± 0.31	26.99 ± 0.20	29.39 ± 0.26	25.74 ± 0.17	28.78 ± 0.23	32.56 ± 0.36	28.25 ± 0.20	28.73 ± 0.29	27.67 ± 0.24	30.28 ± 0.32	31.52 ± 0.35
		Night	19.31 ± 0.08	20.45 ± 0.08	19.88 ± 0.08	21.02 ± 0.09	20.14 ± 0.05	21.66 ± 0.05	23.46 ± 0.08	22.68 ± 0.07	19.97 ± 0.06	20.30 ± 0.06	20.67 ± 0.09	21.21 ± 0.07
	Minimum	Day	17.10	18.10	17.80	18.90	19.00	20.20	21.30	20.70	17.80	18.30	18.20	19.20
		Night	16.30	17.30	17.30	18.50	18.70	20.00	21.30	20.60	17.60	18.10	18.30	19.20
	Median	Day	27.00	31.20	26.50	28.40	24.70	27.30	29.20	26.70	27.50	26.30	28.90	29.00
		Night	19.20	20.40	19.80	20.80	20.00	21.60	23.30	22.60	20.00	20.30	20.60	21.10
	Maximum	Day	39.70	53.00	40.00	52.30	37.80	50.20	60.90	47.70	50.50	44.70	49.60	49.70
		Night	22.70	24.20	23.90	25.70	23.00	24.20	27.30	25.80	22.30	22.50	24.50	24.20
Humidity (%)	Mean ± SE	Day	43.09 ± 0.48	33.22 ± 0.52	49.52 ± 0.58	40.24 ± 0.52	67.93 ± 0.56	48.84 ± 0.55	48.06 ± 0.70	58.40 ± 0.58	44.45 ± 0.62	47.68 ± 0.57	38.96 ± 0.64	38.61 ± 0.65
		Night	62.78 ± 0.44	54.89 ± 0.44	69.25 ± 0.43	59.92 ± 0.45	82.37 ± 0.40	65.89 ± 0.22	68.55 ± 0.37	74.11 ± 0.36	67.93 ± 0.26	66.91 ± 0.26	61.97 ± 0.34	61.64 ± 0.37
	Minimum	Day	17.10	8.80	20.30	10.30	27.80	13.80	12.50	24.70	13.10	19.60	12.10	9.70
		Night	43.30	36.80	50.00	41.00	68.90	53.20	51.90	57.70	53.00	52.40	48.70	46.80
	Median	Day	42.00	31.60	47.70	38.20	70.30	49.80	51.00	58.80	42.90	47.70	37.60	37.80
		Night	63.25	55.30	67.90	59.50	79.40	66.20	69.80	74.90	69.20	68.10	62.35	62.20
	Maximum	Day	72.60	63.90	79.60	71.20	97.30	74.40	78.10	83.70	76.30	74.20	78.90	79.10
		Night	79.70	71.40	81.80	73.40	96.30	74.30	78.90	83.40	77.60	76.50	78.40	79.20
Number of recordings		Day	655	655	655	655	655	655	655	655	655	655	655	655
		Night	336	336	336	336	336	336	336	336	336	336	336	336



APPENDIX 3. Pearson correlations between air temperature and relative humidity measured during summer 2025 in the insect tents. The correlation and p-values are given.

APPENDIX 4. Statistical output of univariate analyses performed on activities related to objective 2. For each analysis, the model, formula, test value, degree of freedom (df) and p-value are given. Significant terms and their associated p-value are written in bold.

Experiment	Response (y)	Model	Formula	Term	Test value#	df	p-value	
Individual setting								
Survival	Survival curves	coxme	y ~ Treatment + (1 | Repetition)	**Treatment**	63.81	3	**<0.001**	***
	Mycosis rate	lm	y ~ Treatment	**Treatment**	103.66	3	**<0.001**	***
				Residuals	_	5	_	
Fungal load	Number of CFUs	glmmPQL	y ~ Treatment + (1 | Sample ID)	**Treatment**	1160.3	4	**<0.001**	***
Group setting								
Survival	Survival curves	survreg (weibull)	y ~ Treatment * EPF	**Treatment**	62.09	3	**<0.001**	***
				EPF	80.51	1	**<0.001**	***
				Treatment:EPF	10.16	3	**0.017**	*
	Mycosis rate^(1/4)	lm	y ~ Treatment * EPF	**Treatment**	38.9	3	**<0.001**	***
				EPF	47.4	1	**<0.001**	***
				Treatment:EPF	8.53	3	**<0.001**	***
				Residuals	_	72	_	
Colony setting								
Survival	Survival curves	coxph	y ~ Treatment	Treatment	3.41	1	0.065	.
Activity	Total activity	glmmTMB	y ~ Treatment * Day + Time + Relative Humidity + Temperature + Light + Atmospheric Pressure + Bee Bulk Weight + Baseline Total Activity + (1 | Repetition)	**Treatment**	4.98	1	**0.026**	*
				Day	24.74	1	**<0.001**	***
				Time	0.36	1	0.55	
				Relative Humidity	22.57	1	**<0.001**	***
				Temperature	35.47	1	**<0.001**	***

Experiment	Response (y)	Model	Formula	Term	Test value#	df	p-value	
				Light	0.21	1	0.645	
				Atmospheric Pressure	16.72	1	**<0.001**	***
				Bee Bulk Weight	3.66	1	0.056	.
				Baseline Total Activity	13.64	1	**<0.001**	***
				Treatment:Day	3.92	1	**0.048**	*

#Type II analysis of variance differs based on the model used: Wald chi-square test for Cox models (coxph and coxme) and for linear mixed model (glmmPQL and glmmTMB); F-test for linear model (lm); Likelihood Ratio Chi-Square (survreg)
.: P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001

APPENDIX 5. Statistical output of univariate analyses performed on the microbial diversity variables (objective 2). For each analysis, the model, formula, test value, degree of freedom (df) and p-value are given. The variance of each term are given for multivariate analyses. Significant terms and their associated p-value are written in bold.

Diversity	Response (y)	Data	Model	Formula	Term	Variance	Test value#	df	p-value	
Bacteria										
Alpha	Observed	Workers	lmer	y ~ Treatment * Time + (1 | Repetition)	Treatment	_	0.46	1	0.497	
					Time	_	7.47	3	0.058	.
					Treatment:Time	_	3.60	3	0.308	
		13-14 dpi	lmer	y ~ Treatment * Bee type + (1 | Repetition)	Treatment	_	0.24	1	0.628	
					Bee type	_	0.02	1	0.896	
					Treatment:Bee type	_	5.69	1	**0.017**	*
	Shannon	Workers	lmer	y ~ Treatment * Time + (1 | Repetition)	**Treatment**	_	20.09	1	**<0.001**	***
					Time	_	14.79	3	**0.002**	**
					Treatment:Time	_	16.58	3	**0.001**	***
		13-14 dpi	lmer	y ~ Treatment * Bee type + (1 | Repetition)	Treatment	_	0.01	1	0.915	
					Bee type	_	1.02	1	0.313	
					Treatment:Bee type	_	0.30	1	0.581	
	Pielou	Workers	lmer	y ~ Treatment * Time + (1 | Repetition)	**Treatment**	_	15.37	1	**<0.001**	***
					Time	_	25.95	3	**<0.001**	***
					Treatment:Time	_	17.57	3	**0.001**	***
		13-14 dpi	lmer	y ~ Treatment * Bee type + (1 | Repetition)	Treatment	_	0.50	1	0.479	
					Bee type	_	1.60	1	0.206	
					Treatment:Bee type	_	1.02	1	0.312	
Beta	Bray-Curtis, count data	Workers	adonis2	y ~ Treatment * Time + (1|Repetition)	**Treatment**	0.183	27.45	1	**0.005**	**
					Time	0.055	2.74	3	**0.005**	**

Diversity	Response (y)	Data	Model	Formula	Term	Variance	Test value#	df	p-value	
					Treatment:Time	0.016	0.78	3	0.440	
					Residual	0.747	_	112	_	
					Total	1	_	119	_	
		13-14 dpi	adonis2	y ~ Treatment * Bee type + (1|Repetition)	**Treatment**	0.179	7.39	1	**0.005**	**
					Bee type	0.045	1.84	1	**0.035**	*
					Treatment:Bee type	0	0.02	1	0.980	
					Residual	0.776	_	32	_	
					Total	1	_	35	_	
Fungi										
Alpha	Observed	Workers	lmer	y ~ Treatment * Time + (1 | Repetition)	**Treatment**	_	31.44	1	**<0.001**	***
					Time	_	40.60	3	**<0.001**	***
					Treatment:Time	_	19.43	3	**<0.001**	***
		13-14 dpi	lmer	y ~ Treatment * Bee type + (1 | Repetition)	**Treatment**	_	13.81	1	**<0.001**	***
					Bee type	_	0.00	1	0.974	
					Treatment:Bee type	_	0.49	1	0.483	
	Shannon	Workers	lmer	y ~ Treatment * Time + (1 | Repetition)	**Treatment**	_	396.72	1	**<0.001**	***
					Time	_	156.26	3	**<0.001**	***
					Treatment:Time	_	133.67	3	**<0.001**	***
		13-14 dpi	lmer	y ~ Treatment * Bee type + (1 | Repetition)	**Treatment**	_	176.56	1	**<0.001**	***
					Bee type	_	0.19	1	0.662	
					Treatment:Bee type	_	0.18	1	0.669	
	Pielou	Workers	lmer	y ~ Treatment * Time + (1 | Repetition)	**Treatment**	_	409.01	1	**<0.001**	***
					Time	_	151.58	3	**<0.001**	***

Diversity	Response (y)	Data	Model	Formula	Term	Variance	Test value#	df	p-value	
					Treatment:Time	_	140.23	3	**<0.001**	***
		13-14 dpi	lmer	y ~ Treatment * Bee type + (1 | Repetition)	**Treatment**	_	213.05	1	**<0.001**	***
					Bee type	_	0.02	1	0.895	
					Treatment:Bee type	_	0.59	1	0.444	
Beta	Bray-Curtis, count data	Workers	adonis2	y ~ Treatment * Time + (1|Repetition)	**Treatment**	0.221	72.63	1	**0.005**	**
					Time	0.366	40.07	3	**0.005**	**
					Treatment:Time	0.083	9.10	3	**0.005**	**
					Residual	0.329	_	108	_	
					Total	1.000	_	115	_	
		13-14 dpi	adonis2	y ~ Treatment * Bee type + (1|Repetition)	**Treatment**	0.520	33.89	1	**0.005**	**
					Bee type	0.009	0.57	1	0.685	
					Treatment:Bee type	0.011	0.70	1	0.575	
					Residual	0.460	_	30	_	
					Total	1.000	_	33	_	

#Type II analysis of variance differs based on the model used: Wald chi-square test for linear mixed model (lmer); permutation test with pseudo-F ratios for distance matrix (adonis 2).

.: P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001

Appendix 6. Composition of plant communities from the Viridiplantae kingdom, detected in bumblebee gut, at the family (A) and the genus (B) levels. “dpi” stands for days post inoculation. “Other” refers to taxa which average abundance across the dataset was less than 1%. After removing the singletons and after rarefying (*i.e.*, 2,000 reads as threshold), a total of 200,000 reads for 1,014 ASVs and for 162 genera was retained, with 26 samples failing the filtering and rarefying steps.

BumbleBio: Bumblebees as vectors of fungi for biocontrol of insect pests in strawberry flowers

Strawberries are among the most important fruit crops in Denmark and are commonly grown in tunnels to enable season-long production using ever-bearing cultivars. These systems depend heavily on commercial bumblebees (*Bombus terrestris*) for pollination, especially early in the season when natural pollinators are scarce. Tunnel-grown strawberries are affected by arthropod pests and diseases, with Western Flower Thrips (*Frankliniella occidentalis*) representing a major challenge during flowering. Control options for thrips are limited, and microbial agents such as the entomopathogenic fungus *Beauveria bassiana* are considered alternatives, although their use during flowering raises concerns for pollinator safety.

This study investigated whether commercial bumblebee hives can vector *B. bassiana* (BotaniGard WP, strain GHA) to strawberry flowers to control thrips, and assessed potential effects on bumblebee health.

Laboratory bioassays showed dose-dependent mortality of thrips exposed to *B. bassiana*. Bumblebees successfully transferred viable fungal spores to flowers in greenhouse experiments, where spores persisted for up to one week. However, api-vectoring did not significantly reduce thrips infestations under greenhouse conditions.

Direct laboratory exposure to *B. bassiana* was lethal to individual bumblebee workers, but colony-level exposure in greenhouse settings showed no detectable effects over two weeks. Api-vectoring resulted in fungal accumulation within hives and changes in gut microbiota, without observable impacts on mortality, activity, or colony growth. The results demonstrate that api-vectoring of *B. bassiana* to strawberry flowers is feasible, but improved environmental conditions and longer-term studies are needed to evaluate practical efficacy and pollinator safety under commercial production.

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